José M. Alvarez-Suarez *Editor*

Bee Products - Chemical and Biological Properties

Springer

Bee Products - Chemical and Biological Properties

José M. Alvarez-Suarez
Editor

Bee Products - Chemical and Biological Properties

Springer

Editor
José M. Alvarez-Suarez
Facultad de Ciencias de la Salud
Universidad de Las Américas
Quito
Ecuador

ISBN 978-3-319-59688-4 ISBN 978-3-319-59689-1 (eBook)
DOI 10.1007/978-3-319-59689-1

Library of Congress Control Number: 2017953371

Printed on acid-free paper

This Springer imprint is published by Springer Nature
The registered company is Springer International Publishing AG
The registered company address is: Gewerbestrasse 11, 6330 Cham, Switzerland

Contents

Part I
Honey

Chapter 1
Botanical Classification

Estefanía Sánchez Reyes and José Sánchez Sanchéz

Contents

1.1 Melissopalynological Analysis

1.1.1 Palynology: A Young Science—Applications

In 1944, Hyde and Williams coined the term *palynology* to name the science responsible for the study of pollen grains and fungal spores. It was from the middle of the twentieth century onwards that the study of these particles progressed considerably, thanks to the appearance of electron microscope (Knoll and Ruska 1932) which improved the resolution and magnification reached with the optical microscope at the beginning of the seventeenth century.

Size or type, number or position of apertures or even sporoderm ornamentation of pollen grains and fungal spores can be used as taxonomic characters for the identification, differentiation and classification of plant species or fungi they are

E.S. Reyes (✉) • J.S. Sanchéz
Hispano-Luso Institute for Agricultural Research (CIALE), University of Salamanca, Villamayor Campus, C/Río Duero N°12, 37185 Villamayor (Salamanca), Spain
e-mail: fani_sanchez@usal.es

J.M. Alvarez-Suarez (ed.), *Bee Products - Chemical and Biological Properties*,
DOI 10.1007/978-3-319-59689-1_1

coming from. For that reason, the study of pollen grains and fungal spores has its application in disciplines apparently as disparate as vegetal taxonomy, paleopalynology (study of palynomorphs present in different sediments for paleoclimatic and paleoecological reconstructions), aeropalynology (airborne pollen and fungal spores content), medicine (related to allergies) or even in forensic palynology, agronomy (control of crop pollination or phytopathogen detection, among others), biodeterioration, etc. and of course, the subject that concerns us in this book, melissopalynology (study of pollen content and fungal spores in honeys and other hive products).

In honey, pollen grains come mainly from the plant species foraged by honey bees (Ohe et al. 2004), so that palynological studies provide a good fingerprint of the plant species where the honeys come from (botanical origin).

However, we could find pollen grains in the nectar as a consequence of secondary (pollen from the inside of the hive), tertiary (inclusions of pollen grains during the extraction process) and quaternary (aerial contamination) enrichment (Corvucci et al. 2015). As such it appears exactly in Directive 2014/63/EU "*additional pollen in honey can come from pollen on bees' hair, from pollen in the air inside the hive and from pollen that was packed in cells by bees and released as a result of the accidental opening of those cells during the extraction of honey by food business operators*".

On the other hand, the entire pollen spectrum is consistent with the flora of a particular region depending thus on the agricultural and forest conditions where a honey was produced, giving an idea of the geographical origin (Ohe et al. 2004). In that sense, markers of geographical origin are generally pollen grains found in lower percentage as Conceição Silva and Ribeiro Dos Santos (2014) and Corvucci et al. (2015) reported.

Quantitative and qualitative analysis are carried out in order to determine the botanical and geographical origin of honeys, royal jelly, bee pollen and propolis, which contribute to the quality control of hive products (together with sensory analyses). In addition, they allow the identification of possible fraudulent adulteration or contamination and provide important information on honey extraction and/or filtration.

1.1.2 The Pollen Grain

The *pollen grain* or *microgametophyte* is the male germ element responsible for the fertilization in flowers and subsequent seed formation. It is the male gametophyte of seed plants (spermatophytes) and it is formed in the androecium of the flower, composed of *stamens* (*microsporophylls*) consisting of a *filament* and the *anther*. Anthers are usually two-*lobed*. Each anther lobe develops two *pollen sacs* or *microsporangia*.

Pollen sacs are composed of sporogenous tissue formed by a mass of cells called *pollen mother cells* or *arquesporium*, which undergo reduction division (meiosis). The four haploid cells generated form a tetrad within the mother cell until their maturation.

Inside each of these microspores, an uneven mitotic division occurs, generating two morphologically and functionally different nuclei: the *vegetative nucleus*, larger in size, with vacuoles and reserves, is responsible for the formation of the *pollen tube*

and it performs a nutritional function for the *generative nucleus* (which could be placed in an eccentric position), smaller and fusiform, which will generate (through mitosis) two *sperm nuclei* or *male gametes*.

Simultaneously to the formation of the generative and vegetative nuclei, they will make their own wall, so that the mother cell wall degenerates. Subsequently, the wall is thickened and it is possible to differentiate between two layers: an external or *exine* and an internal or *intine* (that is generated after the aforementioned), which give pollen grain a great resistance against the adverse atmospheric conditions to which it will be exposed, thus avoiding possible dehydration.

Once the pollen grains reach this stage of development, they will be disseminated thanks to dehiscence of the anther, which will occur in response to certain environmental conditions. In general, pollen grains are released in isolation (monad), although sometimes, in some species, they are released in groups of 4 (tetrad), as in the case of the family Ericaceae and even in groups of 16 grains (poliads) in the genus *Acacia* Mill., or in pollinia as occurs in Orquidaceae and Asclepiadaceae.

1.1.3 Pollen Morphology

A specific terminology, accepted internationally by palynologists, is used for the determination and description of the microscopic characteristics of pollen grains. Below is a summary of the main pollen characteristics that form the basis for the identification of pollen grains: polarity and symmetry, size and color, shape, sporoderm, ornamentation and apertural system.

1.1.3.1 Polarity and Symmetry

As indicated above, pollen grains are three-dimensional structures (with volume), so they may have polarity and symmetry. If within a tetrad, we analyze a monad individually (Fig. 1.1) we can define the zone that is closest to the center of the tetrad as the *proximal pole*, and the *distal pole* as that one furthest from the tetrad. The line connecting the two poles, passing through the center of the monad and the tetrad, coinciding with the axis of rotation of an imaginary ellipsoid, will be called the *polar axis*. The *equatorial diameter* corresponds to the perpendicular axis to this polar axis that crosses the grain by its middle part.

We say a pollen grain is in polar view, when the polar axis coincides with the optical axis of the observer. However, an equatorial view of a pollen grain is one in which the equatorial plane is directed towards the observer.

Erdtman (1952) defined as *apolar* those grains in which no pole is seen once liberated from the anther, whereas the *polar* ones present these zones more or less differentiated. Within the latter, we differentiate the *isopolar* grains, when the equatorial plane determines two polar zones more or less similar, and the *heteropolar* ones, in which the poles are clearly different either in shape, ornamentation or apertural system.

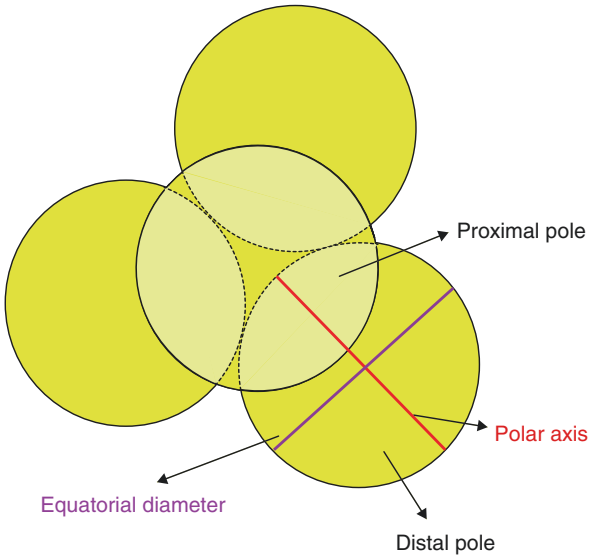


Fig. 1.1 Pollen tetrad (http://www.pollen.mtu.edu/glos-gtx/glos-int.htm)

With respect to symmetry (always defined in polar view), we can differentiate the *asymmetric* pollen grains (when they do not present any plane of symmetry) and the *symmetrical* ones (which do have planes of symmetry). These are divided into two types: those with *bilateral* symmetry (if they only have two planes of symmetry, but of different length) or with *radial* symmetry, if they have more than two planes of symmetry or only two but of equal length (Erdtman 1969).

Therefore, taking into account the polarity and the symmetry, several combinations arise between these concepts (Fernández and Díez 1990) which can considerably complicate the description of pollen grains.

1.1.3.2 Size and Colour

In order to establish the average size of a pollen type it is necessary to perform multiple measurements and a subsequent statistical treatment of the obtained values. However, it is a very useful characteristic from the taxonomic point of view, since it is kept constant between species, which allows to separate those next to each other in some cases. Nevertheless it is possible to appreciate important variations according to the different varieties, geographic areas or the physiological state of the plant (cultivated plants that grow in optimal conditions usually develop larger grains). It can even be influenced by the pretreatment of the pollen grains, which are able to adapt to changes in volume since the openings also have a harmomegathic function (Wodehouse 1935), remaining practically constant in the fossilized and acetolyzed pollen grains.

It is usually defined by its diameter when the pollen grain is spherical and by the lengths of their polar and equatorial axes if it is ellipsoid, regardless of the size of the ornamentation they possess (about 0.5 μm in length in general).

Erdtman (1952) defined the size of the pollen grains as a function of the mean length of their major axis (either polar or equatorial): *very small* (<10 μm), *small* (10–25), *medium* (25–50), *large* (50–100), *very large* (100–200), *gigantic* (>200).

We can find grains from 5 μm as in the case of some forget-me-not (genus *Myosotis* L.) to some of more than 200 μm produced by the four o'clock flower (*Mirabilis jalapa* L.), most of them being between 25 and 50 μm (Sánchez Sánchez and Baldi Coronel 2010).

This could be a very interesting parameter to consider in relation to filtration processes since the larger pollen grains would be removed depending on the filters used.

Colour is of relative importance as it is imperceptible in microscopic studies. However, it can be seen when the grains are grouped in mass. In fact, the bee pollen loads have very variable colors, which fundamentally depend on the chemical composition of the pollen grain and ultimately on its botanical origin. For this reason, it is possible to find bee pollen loads even black or bluish-violet, although commercial bee pollen usually has a brownish-yellow colour hue.

1.1.3.3 Shape

Pollen grains are three-dimensional structures, so they can have different shapes depending on the view we have (polar or equatorial), being one of the most important characteristic for its identification. It is a variable property because it is subject to changes due to the physical discontinuity of the wall that covers them (which causes them to deform) or because of the volume variations that the mechanism of harmomegathy cannot neutralize. For this reason, the palynologists have based morphological descriptions mainly on acetolyzed grains.

In order to achieve a better description of pollen grains, Reitsma (1970) classified them according to the contour of both equatorial and polar views, which may be either non-angular (circular or elliptical) or angular, corresponding to some rhomboid, rectangular, triangular, quadrangular, pentagonal or hexagonal polygons, with sharp or obtuse edges and with flat, concave or convex sides.

Likewise, the shape of a pollen grain can be defined according to the relationship between the length of the polar axis and the equatorial diameter. In this way, both Erdtman (1943, 1952) and Reitsma (1970) separated nine morphological types which they named using different terminology (Table 1.1).

1.1.3.4 Sporoderm

This name is given to the layers surrounding the live content of the pollen grain that will act as a protective cover against drying, abrasion and the mutagenic effect of ultraviolet rays. The *intine* corresponds to the innermost cellulosic layer, homogeneous, the thickness and position of which are the main characteristics to take into account. It is the only stratum that develops to originate the pollen tube. The *exine* is the most external stratum and the one that really protects the pollen grain because it is very resistant, being able to withstand the action of strong bases and acids, as

Table 1.1 Pollen terminology regarding to morphology proposed by Erdtman (1943, 1952) and Reitsma (1970) based on polar axis/equatorial diameter ratio (P/E)

P/E	Erdtman	Reitsma
<0.50	Peroblate	Pertransverse
0.50–0.75	Oblate	Transverse
0.75–0.88	Suboblate	Semitransverse
0.88–1.00	Oblate-spheroidal	Subtransverse
1.00	Spheroidal	Adequate
1.00–1.14	Prolate-spheroidal	Suberect
1.14–1.33	Subprolate	Semi-erect
1.33–2.00	Prolate	Erect
<2.00	Perprolate	Pererect

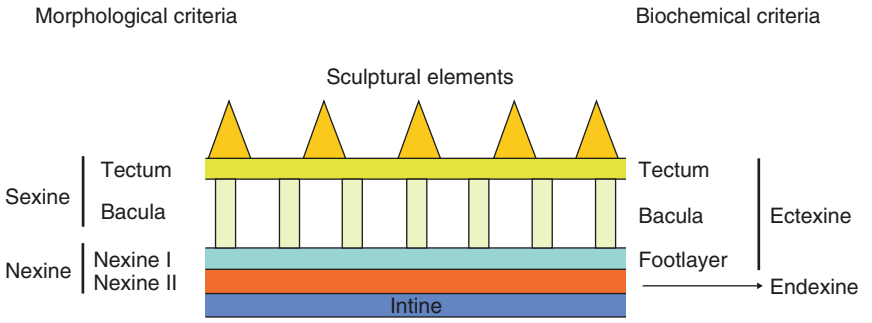


Fig. 1.2 Sporoderm structure according to morphological or biochemical criteria (http://www.pollen.mtu.edu/glos-gtx/glos-int.htm)

well as the heating up to 300 °C. Its degradation occurs only after the action of some microorganisms or certain very strong oxidants.

According to biochemical criteria (Fægri and Iversen 1950; Fægri 1956), it is possible to differentiate the *ectexine*, with a strong coloration after staining with fuchsin, from the weakly stained *endexine* (Fig. 1.2). The ectexine is formed by a set of radial elements called *bacula*, *columellae* or *infratectum*. Generally the expansion and welding of its upper ends gives rise to a structure that receives the name of *tectum*, whereas its inferior ends fuse, giving origin to a common support or *footlayer*. The *endexine* is located below the footlayer.

From a morphological point of view (Fig. 1.2) the exine is composed of two layers: the *sexine*, outer structured layer, constituted by the *tectum* and the *bacula*, and the *nexine* or unstructured layer, in which we differentiate the *nexine I* and *nexine II* which correspond to the footlayer and the endexine, respectively (Erdtman 1952, 1966).

1.1.3.5 Sculpturing

As has been described above, tectum is the outermost layer of the pollen grain on the surface of which the sculptural elements, if any, are to be found, being both structures of vital importance in the identification of the different pollen types.

Tectum is not always present though and pollen grains without tectum are named *intectates*, unlike those that present it, either partially (*semitectates*) or complete (*tectates*).

Likewise, pollen grains may have ornamentation without sculptural elements (Fig. 1.3).

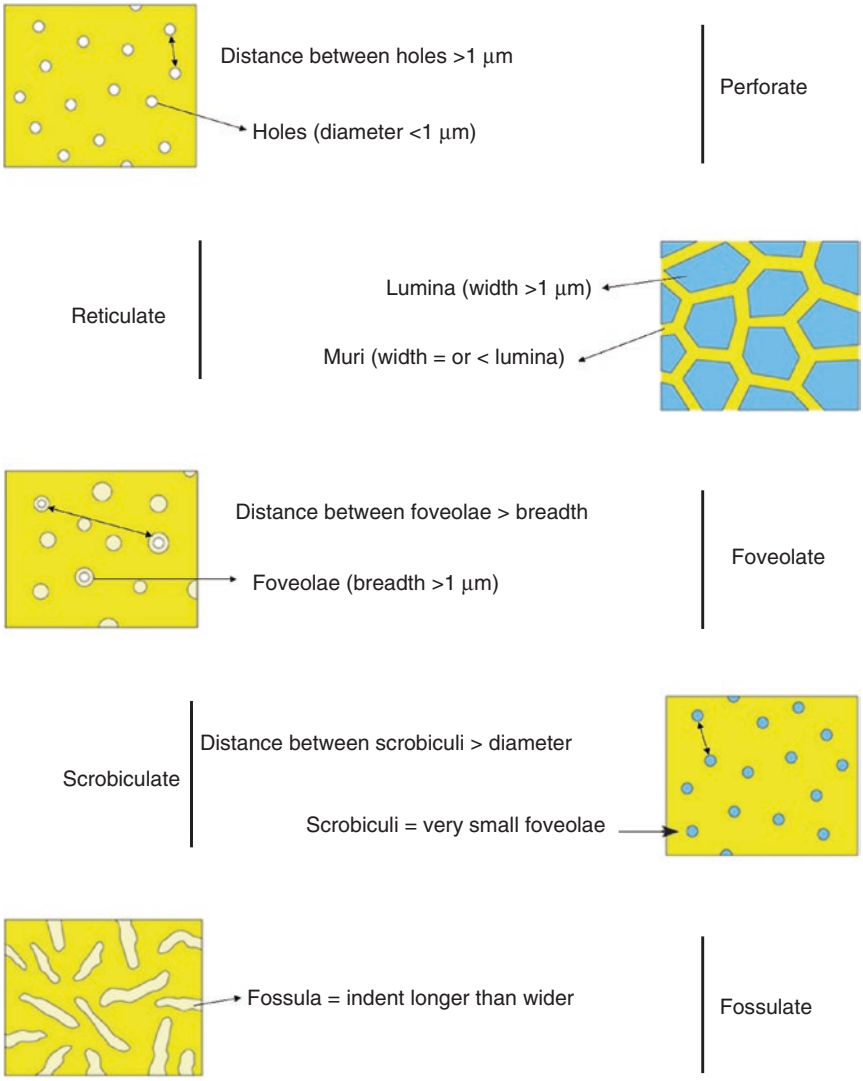


Fig. 1.3 Sculpturing types (http://www.pollen.mtu.edu/glos-gtx/glos-int.htm)

When sculptural elements do exist, they are located on the tectum or on the footlayer when there is no tectum. They remain constant in the same species and they are described below (Fig. 1.4).

Spine, a pointed element >3 μm in length with a height greater than the width (*equinate* or *spinate* pollen e.g. *Helianthus annus* L.); *spinule*, small spines, <3 μm in length (*equinulate* or *spinulate* pollen e.g. Campanulaceae); *baculum*, a not pointed element >1 μm in length and less than this in diameter (*baculate* pollen e.g. Nymphaea alba type-not written in italics since it is a pollen type not a species's name); *pilum*, a no pointed element >1 μm in length, with a height greater than the width, sharply swollen in the apical part (*pilate* pollen e.g. *Jatropha* L.); *clava*, a club-shaped element that is higher than 1 μm, with diameter smaller than height and thicker at the apical part than the base (*clavate* pollen e.g. Ilex aquifolium type); *verruca*, a wart-like element >1 μm wide, that is broader than it is high. It is not constricted at the base (*verrucate* pollen e.g. Plantago coronopus type); *gemma*, a non pointed element, higher than 1 μm that has approximately the same width as its height. It is constricted at its base (*gemmate* pollen e.g. Cupressaceae) and *granulum*, a rounded element that is less than 1 μm in all directions (*granulate* pollen e.g. Alnus glutinosa type).

In contrast to the *psilate* pollen (Fig. 1.5) which corresponds to the one that presents a smooth surface, we can find the sculptural elements distributed in a more or less elongated form. In that sense, they define the *striate* pollen when distributed parallel and separated by grooves, and *rugulate* if they are arranged in an irregular pattern. It is *insulate* when the elements are grouped in circular or polygonal areas separated by grooves.

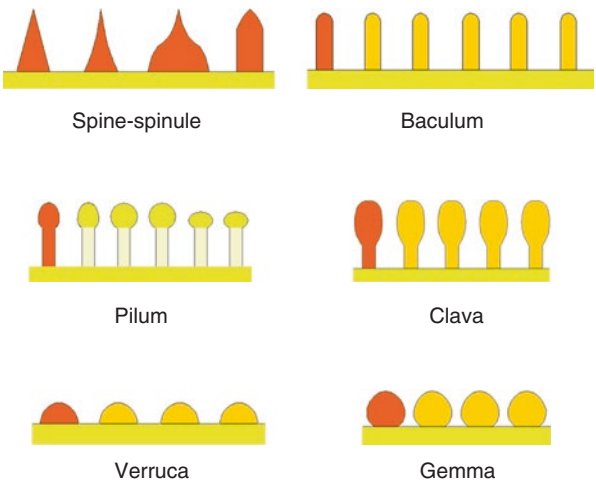


Fig. 1.4 Sculptural elements (http://www.pollen.mtu.edu/glos-gtx/glos-int.htm)

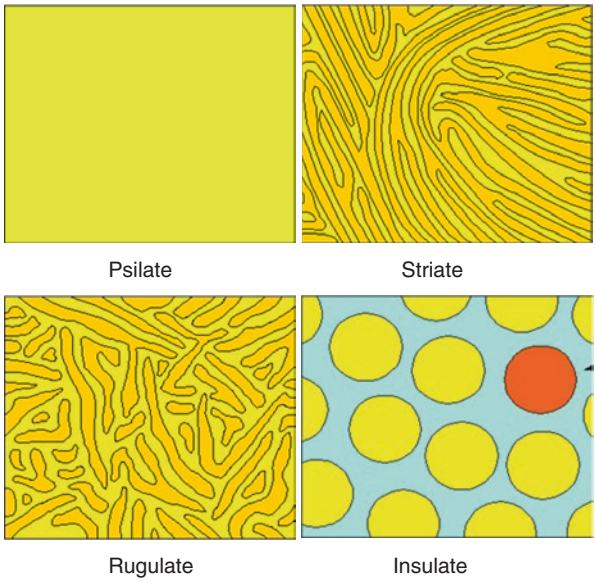


Fig. 1.5 Ornamentation types according to arrangement of elements (http://www.pollen.mtu.edu/glos-gtx/glos-int.htm)

1.1.3.6 Apertures

Other chief criteria in the identification of pollen grains are the type, number and position of the apertures they can present. Apertures are areas on the walls of a pollen grain, where the wall is thinner and/or softer, that will function as sites of germination (Erdtman 1969). They also act as regulators of pollen grain volume changes, produced as a consequence of hygroscopic variations, thus presenting harmomegathy function (Wodehouse 1935).

It is possible to distinguish two main types according to their morphology: *colpus* or *furrow*, when it is at least twice as long as wide, and *pore*, a circular or elliptic aperture with a length/breadth ratio less than 2, which will define *colpate* and *porate* grains, respectively (Fig. 1.6). They are described as *simple* if they are present in only one wall layer of the exine, or *compound* if they affect more than one layer of the wall. In compound apertures the shape of apertures may differ between layers giving rise to *colporate* grains when there is a colpus-type of ectoapertura and *pororate* if it is pore-type. However, the designation of simple aperture is maintained if both apertures coincide in shape and/or size.

Those pollen grains that do not have any apertures will be designed as *inaperturates*; as their number increases, they are described using the following prefixes before the terms that define the type (Erdtman 1969): *mono-*, *di-*, … *poly-* (more than six apertures).

Considering the arrangement of the apertures in the surface of the grain, different particles are added between the terms that define number and type: *-cata-*, if they appear in the proximal pole; *-anacata-*, if they are located in the two poles; *-ana-*, if

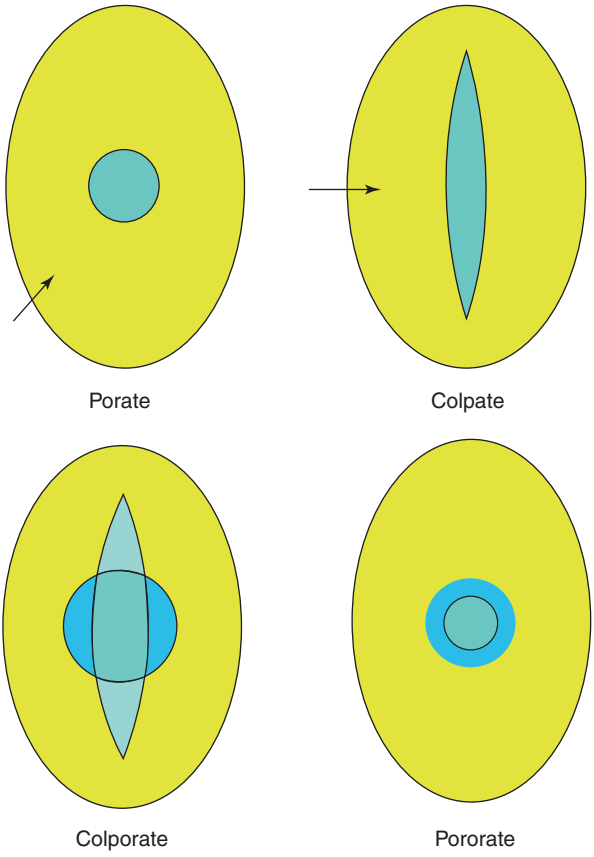


Fig. 1.6 Pollen types according to aperture types (http://www.pollen.mtu.edu/glos-gtx/glos-int.htm)

they appear in the distal pole; *-zono-*, if they are at the equator; *-dizono-* if they are in two more or less parallel zones of the equator and *-panto-*, if the apertures occur throughout the surface.

1.1.4 Methodology

In order to determine botanical and geographical origin of honeys, both quantitative and qualitative analysis of pollen sediment are carried out, and are of great importance for quality control processes.

1.1.4.1 Quantitative Analysis

Together with the technique we normally use in our laboratory (Sánchez Sánchez and Baldi Coronel 2010) there are many methodologies used up to date (Louveaux et al. 1978; Ohe et al. 2004, among others), although they are all based on the

number of pollen grains and honeydew elements per unit of weight, usually 10 g of honey. In this way, honeys are classified according to different classes established by Maurizio in 1939.

Class I (less than 20,000 elements/10 g honey) includes monofloral honeys with under-represented pollen; *class II* (20,000–100,000) includes most of multifloral honeys, honeydew honeys and mixture of flower and honeydew honeys; *class III* (100,000–500,000) includes monofloral honeys with over-represented pollen and honeydew honeys; *class IV* (500,000–1,000,000) includes monofloral honeys with strongly over-represented pollen and some pressed honeys and *class V* (more than 1,000,000) includes almost only pressed honey.

In those honeys where it is important to reflect the honeydew elements content, counts are customarily expressed by a formula such as 37/36/0.5-II, which means that 10 g of honey contained 37,000 pollen grains, 36,000 fungal spores and 500 algae cells. The 73,500 plant constituents placed the honey in group II (Louveaux et al. 1978).

Although the quantitative study is not decisive, we believe that a high amount of sediment could indicate a pressed honey whereas a low amount of sediment may indicate honey naturally poor in pollen (e.g. *Citrus* spp.), excessive filtering or falsification (e.g. by feeding sugar to the bees) as Louveaux et al. already pointed out in 1978.

The mean of the pollen absolute number for the 15 main European monofloral honeys can be found in Persano Oddo and Piro (2004), with values concordant in any case with the results obtained after an extensive bibliographical review of previous European works (Piazza and Persano Oddo 2004), which would indicate a good characterization of these honeys at this level.

The results showed that citrus honey-*Citrus* spp., sunflower honey-*Helianthus annuus* L., rhododendron honey-*Rhododendron* spp., black locust honey-*Robinia pseudoacacia* L., rosemary honey-*Rosmarinus officinalis* L. and lime honey-*Tilia* spp. belong to class I; rape honey-*Brassica* spp., calluna heather honey-*Calluna vulgaris* L. (Hull), dandelion honey-*Taraxacum officinale* group and thyme honey-*Thymus* spp. belong to class II whereas chestnut honey-*Castanea sativa* Miller, eucalyptus honey-*Eucalyptus* spp. and honeydew honeys to class III (except for metcalfa honeydew honey that belongs to class II).

In any case, ranges between classes are very high and, in fact, the information obtained from their classification is not very relevant.

1.1.4.2 Qualitative Analysis

The acetolysis method was first introduced by Erdtman in 1960 but it is still followed throughout the world for the qualitative analysis, as most pollen descriptions in palynological literature are based on acetolysed material (Louveaux et al. 1978; Simeão et al. 2015). It is based on the treatment of pollen grains with the acetolysis mixture (consisting of nine parts of acetic anhydride and one of sulfuric acid), which destroys the living content but preserves the external part (exine) in which the apertures and the ornamentation are located, allowing to observe and identify the

different pollen types. It also prevents the deformation of the grains as a result of the changes in humidity, allowing to standardize size values through biometric studies.

This methodology presents certain disadvantages though, as acetolysis destroys fungal spores and hyphae, algae, etc., the identification and quantification of which are associated to honeydew honeys (Louveaux et al. 1978), so that these structures should be observed during the performance of the quantitative analysis.

Subsequent modifications were incorporated and accepted due to the complexity of the method, as proposed by Hideux (1972) or Louveaux et al. (1978), the bases of which are still used today (La Sena-Ramos et al. 2002; Soria et al. 2004; Rodríguez de la Cruz et al. 2013; Conceição Silva and Ribeiro Dos Santos 2014; Simeão et al. 2015; Atanassova et al. 2016).

Considering the proposals made by Louveaux et al. in 1978, the International Honey Commission-IHC elaborated and proposed another method well established nowadays in most European laboratories involved in routine honey analyses (Ohe et al. 2004), for the practical purpose of verifying if the pollen spectrum complies with the declared botanical (and even geographical) origin. Moreover, this method was used to produce the data for the European monofloral honeys descriptions (Persano Oddo and Piro 2004) and has been used in many studies worldwide (Ponnuchamy et al. 2014; Belay et al. 2015; Corvucci et al. 2015; Juan-Borrás et al. 2015; Kadri et al. 2016). Pollen grains are extracted from a solution of honey and distilled water by centrifugation, however, the use of sulfuric acid is still contemplated even in current legislation of some countries like Spain (Orden de 12 de junio de 1986).

At European level it is established that "*Member States shall, whenever possible, use internationally recognized validated methods of analysis such as those approved by the Codex Alimentarius to verify compliance with the provisions of this Directive*" (Directive 2014/63/EU). However, the revised Codex Alimentarius Standard for Honey (Codex Alimentarius Commission 2001) only specifies that "*samples should be prepared in accordance with AOAC 920.180*" in relation to the methods of sampling and analysis (7.1 point), but there is no specification regarding the melissopalynological analysis itself.

On the other hand, a collection of reference slides is an indispensable supplement to literature on melissopalynology, in order to identify different pollen types. Different keys and pollen atlases must be also used, both of native and foreign flora, always considering defined pollen types and taking into account that these can present different taxonomic range (at the species, genus or even family level). Indeed, the main critical point of melissopalynological analysis remains the correctness of pollen identification and the subsequent interpretation of the results (Ohe et al. 2004) depending greatly on the performance and the experience of the operator (Louveaux et al. 1978) being necessarily specialized personnel (Persano Oddo and Bogdanov 2004; Schievano et al. 2015).

We believe that a minimum of 1200 pollen grains per sample should be counted and identified for the certification of both the botanical and geographical origin of a honey (Louveaux et al. 1978; Orden de 12 de Junio de 1986; Sánchez Sánchez and Baldi Coronel 2010), which will make it necessary to perform and analyze more than one microscopic slide in the case of under-represented honeys. In honeydew honeys,

poor in pollen grains, counts of only 600 grains would be necessary as Louveaux et al. (1978) pointed out. However, there are frequent papers in which the analyzed number of pollen grains was lower (e.g. 500 pollen grains in Belay et al. (2015), Corvucci et al. (2015), Atanassova et al. (2016) and Kadri et al. (2016), among others).

The number of pollen grains to be counted might be more important if we are trying to certify the geographical origin of a honey since it should be tried not to ignore any pollen type that was a geographical marker.

1.1.4.3 Development of Results Report: Quality Control

The sample must be perfectly identified in the final results report, including the date of receipt of the sample, data included in the label, as well as any other additional information considered relevant.

According to the quantitative analysis, the total number of plant constituents in 10 g of honey should be expressed, thus indicate into which Maurizio's class it is placed.

Regarding the qualitative study, a list with the complete pollen spectrum must be included, which will determine the geographic origin of the sample. It should be accompanied by the relative frequency as the percentage with respect to the total number of pollen grains counted/identified.

However, for the determination of the botanical origin, it is necessary to recalculate the relative frequencies including only pollen types from nectariferous species since they actually contribute to the elaboration of a certain honey. For that reason, these are the percentages that have to be taken into account to determine the monoflorality of a honey. That is, pollen types from nectarless (*Plantago* spp., Chenopodiaceae, Cistaceae, etc.) and/or wind-pollinated plants (Poaceae, Cyperaceae, pollens of conifers, etc.), have to be removed from the final spectrum.

Abortive and misshapen pollen grains should be counted as far as they can be identified (Louveaux et al. 1978). Non-identifiable or non-identified grains should be noted (spoiled, folded, etc.) but never exceed a significant % of the sample.

Lastly, a final evaluation must be made taking into account all stated above.

It may be the case that the calculated percentages indicate the possible monofloralities of two honey types simultaneously, e.g. a sample that presents 20% of *Citrus* spp. and 50% of *Helianthus annus* L., which would indicate that complementary sensory analysis will be essential to confirm monofloralities in one direction or another.

1.2 Monofloral, Polyfloral and Honeydew Honey

It is necessary to perform physicochemical, palynological and sensory analyses for the classification and, therefore, the correct labeling of monofloral honeys (Persano Oddo et al. 1995), although the last two types of studies are the most relevant in order to decide on their monofloralities.

The palynological study of a honey, as already mentioned, can help in its botanical characterization, since it will allow us to define a honey as monofloral, multifloral (when it is not possible to define the honey as monofloral because the sample does not come either mainly from a single nectar source or from honeydew) or honeydew (if it presents honeydew elements such as mold hyphae, fungal spores, etc.).

In general, a honey is considered as coming predominately from a given botanical origin (monofloral) if the relative frequency of the pollen type of that taxon exceeds 45% (Maurizio 1975) but each honey type has its own pollen spectrum which has to be taken into account before issuing a conclusion about its botanical origin. In that sense, all the botanical species giving monofloral honeys in Europe are listed in Persano Oddo et al. (2004).

There are countries like Argentina, where the legislation contemplates 45% as the minimum percentage of a certain pollen type that a honey must contain in order to be considered as monofloral (Resolution 274/95 SAGPyA 1998). Some exceptions are contemplated though, such as *Citrus* spp. honey (10–20%) and *Eucalyptus* spp. (70%), among others.

Other countries, without enforced legislation, set the threshold values for their monofloral honeys according to recommendations made by specialists (such as the national associations of palynologists).

Such is the case of Spain, whose quality standard for honey states that "honey will have its normal content of pollen that cannot be removed in the filtering process" (Orden de 5 de agosto de 1983; Real Decreto 1049/2003) but it does not define what must be considered as "normal".

At this point, we consider, for example, that eucalyptus honey must contain more than 80% of pollen from these plants (Rodríguez de la Cruz et al. 2013), since it is an over-represented pollen type (there are too many pollen grains per unit of nectar) being determined even greater for Italian honeys (Persano Oddo and Piro 2004).

Castanea sativa Miller is also likely to be over-represented, and only honeys containing 90% or more of this pollen type can be regarded as *Castanea* honey in many European laboratories (Louveaux et al. 1978; Persano Oddo et al. 1995), as our expertise confirms. Conversely, other nectar sources can be under-represented, which leads us to consider as monofloral those honeys with percentages around 15–20%, and even lower such as *Citrus* spp., Labiatae, *Tilia* spp., among others (Piazza and Persano Oddo 2004).

Levels higher than 10% are acceptable in our laboratory for *Citrus* spp. honeys because of the increment in the cultivation of sterile hybrid varieties, which are characterized by their small amounts of pollen (Juan-Borrás et al. 2015).

We would like to highlight the case of sunflower honey, since there are varieties that produce little pollen and nectar, so the threshold is set at 25% and even with levels above 15%, even if morphology of flower and pollen grains would not justify any under-representation (Persano Oddo and Piro 2004).

At the European level (2001/110/EC, EU Council 2002), regarding pollen content of honeys it is only mentioned that "*no pollen or constituent particular to honey may be removed except where this is unavoidable in the removal of foreign inorganic or organic matter*" (Annex II) or "*the product names may be supplemented by*

information referring to floral or vegetable origin, if the product comes wholly or mainly from the indicated source and possesses the organoleptic, physic-chemical and microscopic characteristics of the source" (article 2b), but these properties are not defined through the prescription of given limits or any legal criteria that guarantee an efficient control of the denominations (Persano Oddo and Bogdanov 2004).

In that sense, in 2004 Persano Oddo and Piro characterized the 15 most important monofloral European honey types (in terms of abundance of production or commercial interest), determining mean % of specific pollen obtained in 21 countries of the European geographical area.

Values obtained were as follows: lavandula honey-*Lavandula* spp. (8.2%), dandelion honey-*Taraxacum officinale* group (17.2%), citrus honey-*Citrus* spp. (18.6%), lime honey-*Tilia* spp. (22.9%), black locust honey-*Robinia pseudoacacia* L. (28.1%), rosemary honey-*Rosmarinus officinalis* L. (28.7%), thyme honey-*Thymus* spp. (36%), calluna heather honey-*Calluna vulgaris* L. (Hull) (37%), rhododendron honey-*Rhododendron* spp. (38.6%), sunflower honey-*Helianthus annuus* L. (56.7%), rape honey-*Brassica* spp. (82.8%), chestnut honey-*Castanea sativa* Miller (94.5%) and eucalyptus honey-*Eucalyptus* spp. (94.8%). Honeydew honeys obtained a HDE/P ratio of 1.5 except for metcalfa honeydew honey (4.8).

Moreover, the results obtained in IHC work were compared to other European studies, being fairly consistent with literature available (Piazza and Persano Oddo 2004). However, results have not been reflected in European legislation up to date, as it was mentioned before.

Different criteria are accepted at international and national levels (Atanassova et al. 2016) which may affect the trade of monofloral honeys (Persano Oddo and Bogdanov 2004).

1.2.1 Honeydew Honey

It is necessary to consider honeydew honeys apart from what has been discussed so far, since they are clearly different from nectar honeys.

They are produced by a wide range of different sucking insects on a big number of trees, Coniferae (fir, spruce and pine trees) and Latifoliae (produced in most of European countries, mainly from different *Quercus* species) mainly (Persano Oddo et al. 2004).

On the other hand, according to our expertise in the laboratory, in many European countries a mixture of nectar and honeydew honey is still marketed, labeled as *forest honey*, which may have abundant honeydew elements but without reaching the minimum value of electrical conductivity (0.8 mS/cm) established in European legislation (2001/110/EC, EU Council 2002) for honeydew honeys.

They do not have a floral origin, so they practically lack pollen grains, although they do have those named honeydew elements (HDE) consisting of fungal spores, mold hyphae, microscopic algae, tricomas, etc. and pollen from nectarless plants including anemophilous pollen (by air pollution).

Honeydew index is determined according to the HDE/P ratio (Louveaux et al. 1978), where HDE is the total number of honeydew elements counted and P is the total number of pollen grains counted. It is considered to be predominately honeydew if the ratio exceeds 3 (Louveaux et al. 1978), although Persano Oddo and Piro (2004), often found a HDE/P ratio below that limit for European monofloral honeys.

As a conclusion, it could be said that the botanical and geographical classification of honeys substantially determines their value in the market, the processes of quality control and certification being of utmost importance for both consumers and the honey industry.

References

Atanassova J, Pavlova D, Lazarova M, Yurukova L (2016) Characteristics of honey from Serpentine area in the Eastern Rhodopes Mt., Bulgaria. Biol Trace Elem Res 173:247–258

Belay A, Solomon WK, Bultossa G, Adgaba N, Melaku S (2015) Botanical origin, color, granulation, and sensory properties of the Harenna forest honey, Bale, Ethiopia. Food Chem 167:213–219

Codex Alimentarius Commission (2001) Revised Codex Standard for Honey, Codex STAN 12–1981, Rev. 1 (1987), Rev. 2 (2001)

Conceição Silva AP, Ribeiro Dos Santos FA (2014) Pollen diversity in honey from Sergipe, Brazil. Grana 53(2):159–170

Corvucci F, Nobili L, Melucci D, Grillenzoni FV (2015) The discrimination of honey using melissopalynology and Raman spectroscopy techniques coupled with multivariate analysis. Food Chem 169:297–304

Directive 2014/63/EU of the European Parliament and of the Council of 15 May 2014 amending Council Directive 2001/110/EC relating to honey. OJEU L164:1–5.

Erdtman G (1943) An introduction to pollen analysis. The Ronald Press Co, New York, pp 1–239

Erdtman G (1952) Pollen morphology and plant taxonomy. Angiosperms. Almqvist and Wiksell, Stockholm, pp 1–539

Erdtman G (1960) The acetolysis method. Sven Bot Tidskr 54:561–564

Erdtman G (1966) A propos de la stratification de l'exine. Pollen Spores 8:5–7

Erdtman G (1969) Handbook of palynology—an introduction to the study of pollen grains and spores. Hafner Publishing Co, Copenhague, pp 1–486

EU Council (2002) 2001/110/EC of 20 December 2001 relating to honey. OJEC L10:47–52

Fægri K (1956) Recent trends in palynology. Bot Rev 22:639–664

Fægri K, Iversen J (1950) Textbook of modern pollen analysis. Munksgaard, Copenhagen, pp 1–169

Fernández I, Díez M (1990) Algunas consideraciones sobre terminología palonológica. I, Polaridad y simetría. Lagascalia 16(1):51–60

Hideux M (1972) Techniques d'étude du pollen an MED: effects comparés des différents traitements physicochimiques. Micron 3:1–31

Hyde HA, Williams DA (1944) The right word. In: Sears PB (ed) Pollen analysis circular no. 8. Department of Botany, Oberlin College, Oberlin, Ohio

Juan-Borrás M, Periche A, Domenech E, Escriche I (2015) Correlation between methyl antranilate level and percentage of pollen in Spanish citrus honey. Int J Food Sci Technol 50:1690–1696

Kadri SM, Zaluski R, Pereira Lima GP, Mazzafera P, de Oliveira Orsi R (2016) Characterization of *Coffea arabica* monofloral honey from Espírito Santo, Brazil. Food Chem 203:252–257

Knoll M, Ruska E (1932) Das Elektronenmikroskop. Z Phys 78:318

La Sena-Ramos I, Méndez Pérez B, Gómez Ferreras C (2002) Pollen spectra of different unifloral honeys from La Palma (Canary Islands, Spain). Grana 41:48–57

Louveaux J, Maurizio A, Vorwhol G (1978) Methods of melissopalynology. Bee World 59(4):139–157
Maurizio A (1939) Untersuchungen zur quantitativen pollenlyse des honing. Mitt Lebensmittelunters Hyg 30:27–69
Maurizio A (1975) Microscopy of honey. In: Crane E (ed) Honey: a comprehensive survey. Heinemann in cooperation with the International Bee Research Association, London, pp 240–257
Ohe WVD, Persano Oddo L, Piana ML, Morlot M, Martin P (2004) Harmonized methods of melissopalynology. Apidologie 35:S18–S25
Orden de 12 de junio de (1986) por la que se aprueban los métodos oficiales de análisis para la miel. BOE 145:22195–22202
Orden de 5 de agosto de (1983) por la que se aprueba la norma de calidad para la miel destinada al mercado interior. BOE 193:22384–22386
Persano Oddo L, Bogdanov S (2004) Determination of honey botanical origin: problems and issues. Apidologie 35:S2–S3
Persano Oddo L, Piro R (2004) Main European unifloral honeys: descriptive sheets. Apidologie 35(Suppl. 1):S38–S81
Persano Oddo L, Piazza MG, Sabatini AG, Accorti M (1995) Characterization of unifloral honeys. Apidologie 26:453–465
Persano Oddo L, Piana L, Bogdanov S, Bentabol A, Gotsiou P, Kerkvliet J, Martin P, Morlot M, Ortiz Valbuena A, Ruoff K, Ohe KVD (2004) Botanical species giving unifloral honey in Europe. Apidologie 35:S82–S93
Piazza MG, Persano Oddo L (2004) Bibliographical review of the main European unifloral honeys. Apidologie 35:S94–S111
Ponnuchamy R, Bonhomme V, Prasad S, Das L, Patel P, Gaucherel C, Pragasam A, Anupama K (2014) Honey pollen: using melissopalynology to understand foraging preferences of bees in Tropical South India. PLoS One 9(7):e101618
Real Decreto 1049/2003, de 1 de agosto, por el que se aprueba la Norma de calidad relativa a la miel. BOE 186:30181–30183.
Reitsma TJ (1970) Suggestions towards unification of descriptive terminology of Angiosperm pollen grains. Rev Palaeobot Palynol 10:39–60
Rodríguez de la Cruz D, Sánchez Reyes E, Sánchez Durán S, Sánchez Sánchez J (2013) Análisis palinológico de mieles comerciales monoflorales. Botanica Complutensis 37:171–180
SAGPyA (Secretaría de Agricultura, Ganadería, Pesca y Alimentación) (1998) Miel, Buenas prácticas de manufactura. Guía de aplicación. Normas y legislación vigentes. Resolución 274(95):72–73
Sánchez Sánchez J, Baldi Coronel B (2010) Características microscópicas y origen botánico. In: Baldi B (ed) La miel. Una mirada científica. EDUNER. Universidad Nacional de Entre Ríos, Córdoba, pp 1–221
Schievano E, Finotello C, Mammi S, Belci AI, Colomban S, Navarini L (2015) Preliminary characterization of monofloral *Coffea* spp. honey: correlation between potential biomarkers and pollen content. J Agric Food Chem 63:5858–5863
Simeão CMG, Silveira FA, Sampaio IBM, Bastos EMAF (2015) Pollen analysis of honey and pollen collected by *Apis mellifera linnaeus*, 1758 (Hymenoptera, Apidae), in a mixed environment of *Eucalyptus* plantation and native cerrado in Southeastern Brazil. Braz J Biol 75(4):821–829
Soria AC, González M, de Lorenzo C, Martínez-Castro I, Sanz J (2004) Characterization of artisanal honeys from Madrid (Central Spain) on the basis of their melissopalynological, physicochemical and volatile composition data. Food Chem 85:121–130
Wodehouse RP (1935) Pollen grains. Their structure, identification and significance in science and medicine. McGraw-Hill, New York, pp 1–574

Chapter 2
Sensory Studies

Silvia Sánchez Durán and José Sánchez Sánchez

Contents

2.1 Introduction

Some decades ago the shortage of food was the biggest problem of the population. All efforts were focused on production (quantity over quality). But when quantity was not a problem quality started to be considered. Nowadays consumers not only look for food quality, but also consider other issues of food products such as

S.S. Durán (✉) • J.S. Sánchez
Hispano-Luso Institute for Agricultural Research (CIALE), University of Salamanca, Villamayor Campus, C/Río Duero N° 12, Villamayor (Salamanca) 37185, Spain
e-mail: sylvias8@usal.es

J.M. Alvarez-Suarez (ed.), *Bee Products - Chemical and Biological Properties*,
DOI 10.1007/978-3-319-59689-1_2

healthiness. It is not only about getting food but getting health benefits. In this sense, honey has been known world-wide as a healthy food for centuries and it is culturally recognized as a beneficial product. This is how we go from looking for quantity to quality and then to healthy or even functional.

To appraise honey quality, physical, chemical, palynological and, certainly, organoleptic analysis is needed. These sensory analysis processes consist in measuring and quantifying its valuables characteristics by human senses. The sensory evaluation is an integrated discipline that allows to establish honey quality, origin, appearance, etc., based on the organoleptic attributes smell, taste, and texture (Salamanca 2007). This discipline started in 1979 in France (Gonnet and Vache 1979, 1985, 1998). The process, properly done, can help to differentiate, for example, the botanical honey origin, or which honey is better considered (closest to the typical attributes) to be a unifloral honey type. Besides, it is important that the results obtained in a sensory analysis can recognize the handling the beekeeper gives to that honey (harvest conditions, extraction, conservation, packing, promotion, etc.).

The sensory analysis process must be applicable to any honey, independent of its botanical or geographic origin, its production method, treatment or physical condition. Otherwise, we would not be able to establish comparisons between different honey samples of the same type, condition, etc.

An international common normative (International Organization for Standardization, ISO) about standard creation (in this case for honey sensory analysis) exists, but not all countries are members of this organization, so each author bases his results on specific scales. In some world areas or countries, such as Chile, they are based on the method used by Piana et al. (2004) and Galán-Soldevilla et al. (2005); Avilés and Matos (2009) use the honey technical data sheet from the Technical Advisory Society (S.A.T.), 2528-2007 quality certificate own by Peru; Ciappini et al. (2009) and Gaggiotti et al. (2014) followed the IRAM rules (Argentine Institute for Standardization and Certification) although they also used ISO normative. Pires et al. (2005) used criteria established by Gonnet and Vache (1985) to sensory analysis of Portuguese honey from Tras-Os-Montes and Alto Douro. The use of reference parameters for Colombian honey characterization was established as NTC 1273 (Colombian Technical Normative) by Aguas et al. (2010).

In order to make a descriptive honey analysis, it is important to select appropriate descriptors and, therefore, apply normalized vocabulary (Bentabol 2002). In this sense, for some countries, such as Spain, there is no specific normative.

The sensory analysis must be carried out with a reliable and controllable methodology to minimize the variability of different senses´ appreciations. Therefore, this chapter, besides the honey testing process, also includes other important issues such as the panelists and their sense training, tasting room characteristics or required material.

It is understood that everything said before referred to honey collected by *Apis mellifera* bees, although some existing sensory studies compare organoleptically with the melipona honey from *Melipona beecheii* (Fonte et al. 2013).

2.2 The Senses

The senses used in the honey sensory analysis are sight, smell, taste and touch (in the mouth).

In order to realize a sensory analysis, these senses must be sharpened to get a reliable evaluation. Furthermore, the panelists must be trained as we will explain later.

2.2.1 Sight

One of the most important aspects of human sight is the color appreciation. The honey testing process not only considers the color (tone, intensity, and brightness), but other characteristics like appearance (homogeneous or not, fragments of bees or wax, etc.) and its physical condition (crystallization, separation in two phases (Fig. 2.1), possible fermentation process (Fig. 2.2), etc.). The sight describes the product appearance and helps us to detect some anomalies and defects (Abdullah et al. 2004).

Because humans have a great capacity to distinguish colors but a low visual memory, it is good to provide pattern scales to point honey color and make more realistic comparisons, or even help us with laboratory methods to measure the color, which is frequent in commercial transactions of this product. The most used devices are colorimeters from several brands that compare the honey with seven color tones (water: 0–8 mm, extra white: 9–17 mm, white: 18–34 mm, extra light amber: 35–48 mm, light amber: 49–83 mm, amber: 84–114 mm, dark amber: >114 mm) corresponding to the Pfund scale. The fact is that a low quantity of honey is employed which makes this method not as reliable as it would be wanted (Mendoza and Aguilera 2004), aggravated by the fact that honey is translucent (Lomas et al. 1997).

It is important to clarify that color could be a sign of quality, although it depends on consumer preferences. For example, talking on big scale, North

Fig. 2.1 Honey with separation into two phases, and their corresponding points of the two scales, both liquid and crystallized

Fig. 2.2 Arbutus honey, with separation into two phases and fermentation start

American consumers prefer light honey (water, extra white, white) while those in some European areas prefer the darker ones (light amber, amber and dark amber). The same happens on a lower scale, some areas of a country choose light honey and other areas the dark ones, and because of that, it is very important to relate the color to the product marketing target (Delmoro et al. 2010). A consumer who wants light honey does not consider dark ones as quality honey, and vice versa.

2.2.2 Smell

The olfactory sensation is the consequence of a volatile molecule entering into the nasal mucus, with further processing by the brain. These volatile components are present in the honey, derived from its botanical origin and the physiology and bee collecting habits (De María and Moreira 2002; Montenegro et al. 2003; Ramírez and Montenegro 2004; Muñoz et al. 2007). For example, certain components are typical for some honey: methyl anthranilate in citrus honey (*Citrus* L.) (Serra Bonvehi 1988; Ferreres et al. 1994), formaldehyde and acetaldehyde in rape and clover honey, dehydrovomifolol in heather honey and diketone in eucalyptus honey (Bouseta and Collin 1995). These molecules can arrive straight toward the olfactory pathways (more volatile molecules) where the smell is appreciated, or post nasal pathways (rhinopharynx) where the honey aroma is appreciated when this is already in the mouth (heavier molecules).

It is important that, although this sense is very important to describe or differentiate honey, it is hard to assess. Because of that, there are many different ways to classify smells and aromas, by assessing descriptive attributes: acid, bitter, smoked, animal, sour, balsamic, sweet, floral, fruity, herbaceous, resinous, etc. or the generic attributes: defined, delicate, cloying, pleasant, velvety, etc.

Trying to mitigate this subjectivity and establishing a universal identification way there is the so-called Aroma Wheel (International honey commission of Apimondia

IHC 2001, with some later upgrades) that helps to unify criteria to define perceived colors and aromas, which is very useful for the training and maintenance of the honey panelists test.

This wheel distinguishes some smells, for example floral (subtle or heavy), aromatic (spicy, wood, resinous, balsamic), fruity (fresh, citric, sugared), animal (perspiration, barn), etc.

Attributes like floral or fruity aromas are not really useful to differentiate honey samples, although they are indicative of the panelists´ acceptance, unlike the waxy tastes, according to Anupama et al. (2003). However, attributes like vanilla, caramel, smoked, propolis and spicy are more useful, and help to recognize the honey obtaining process, as propolis or smoked smells are related to Maillard reactions due to high temperature used during the honey extracting process.

2.2.3 Taste

We can describe the taste as the sense that allows us to perceive chemical substances dissolved in saliva. Human beings have a big number of taste bulbs that clusters taste receptors placed over the tongue surface, as well as palate mucous, epiglottis, and pharynx.

The taste is linked quite well to the smell because of influences on flavor appreciation (a clear example is the tastelessness feeling when we have a cold). This sense, together with the aromas, confers the sense of flavor.

The primary flavor could be split in sweet, acid, bitter and salty. Nowadays some theorists recognize the existence of the fifth flavor, umami.

Umami is a subtle flavor but with a prolonged aftertaste and complicated to define. It induces salivation and a velvety feeling on the tongue, so that it gives more flavor to food; for example, substances such as monosodium glutamate have this characteristic (Yamaguchi 1998; Yamaguchi and Ninomiya 1998).

In the panelist training sessions realized, we could not get criteria unanimity among them and therefore umami is not included in our scales, or in specialist training (judges).

Sweet: is mainly appreciated at the tip of the tongue and it is very intense in honey due to the fructose that has a big sweetening power, one and a half times more than sucrose, and two and a half times more than glucose.

Acid: this flavor is essentially appreciated at the sides of the tongue. We could say that all honeys are sweet but they are all acidic too. In general light honey has more intense acidity than the dark ones. On the other hand, it must be pointed out that there is no direct relation between the perceived acidity in a tasting session and the pH or the product acidity.

Bitter: it is mainly localized at the back of the tongue. We can appreciate it when food is swallowed. By experience, it is a flavor that is not easily appreciated by non-professional tasters, likely due to the trend of taking bitter products such as coffee,

tea, mate, etc. In honey, it is appreciated thanks to some substances from the polyphenol family, in a really low quantity, usually in dark honey such as chestnut or arbutus honey.

Salty: this flavor is localized primarily in some areas close to the tip of the tongue, toward both sides. Is perceived in just a few honeys that are weakly mineralized. At initiation sessions for honey sensory analysis, we do not consider this sense as an essential learning, although some panelists have detected a slight salty note.

Also associated to taste, there are some chemical sensations as astringency, that is a rough sensation as a passing rugosity of the mouth mucous and commonly a bitter taste is perceived (that is the consequence of the tannins in some substances). Examples for astringent food are some fruits like apples (the more immature the more astringent), khaki, quince, etc. Another chemical sensation found in honey is a "smoked taste" that is characteristic by giving a bitter taste, noticed at the end of the mouth and the pharynx. All these aspects must be considered in the honey panelists training.

2.2.4 Touch

Thanks to the touch we can define the texture by differentiating three interesting properties:

Viscosity: it is the mechanical property related to the flow resistance. It could be considered a synonym of density, but, in nature, there are materials such as mercury, which are dense but not viscous. To easily check the viscosity (that it is related with a right percentage of humidity) you must let the honey drop in a container and if it makes overlapping layers the honey has a well-considered viscosity. As a curiosity, the calluna heather honey (*Calluna vulgaris* (L.) Hull.) is considered thixotropic.

Adhesiveness it is the strength needed to overcome the existing attraction between the surfaces of two materials (due to molecule mutual attraction).

Crystallization is formation of crystals, that is the external expression of the internal structure of atoms, molecules, and ions in a three-dimensional net persistently repeated in a substance. In this case, crystallized honey implies the presence of sugar crystals at the honey flow matrix.

We consider that the viscosity and the adhesiveness are important to describe honey, although consumers do not appreciate them to a large extent but crystallization they like a lot.

2.3 Testing Panel: Tasters

According to normative UNE 87-024-1 (1995); UNE 87-024-2 (1996), the testing panel must consist of a minimum of ten panelists and the panel chief. At least two or three times the number of panelists must be recruited on the final testing panel, but this number will be reduced after the selection test, training and control of candidates to

check their threshold perception (less amount of stimulus needed for a sensation to emerge), threshold recognition (minimum quantity of stimulus required to differentiate a perceived sensation) and differential (minimum perceptive change value that can experience the intensity of a stimulus), detailed at the normative UNE 87-003 (1995). It is important that these panelists are representative of the population and be aged between 18 and 65 years.

Participants of the final testing panel must observe and analyze through their senses and describe the perceived sensations in the most objective way (they must not take into account their likes and aversions), they must be in good health conditions, so they must take care of themselves physically (no breathing issues, sight issues, etc.) and mentally (avoiding distractions to have a good perception).

Before the panel session, smoking is forbidden and also certain kinds of food (coffee, alcohol, etc.), or using perfumes, soaps, skin creams, toothpaste, etc. that can leave residual smells and interfere with the diagnosis. Besides, drugs or medicaments or any substance that could reduce the professionality of the panelist must not be consumed. They must be rested, calm and attentive.

A professional panelist must have an exhaustive training, first detecting and recognizing smells, flavors and textures, besides, have a training on using normalized scales (further detailed) and learning on developing and using descriptors (agreed by the panelists).

After this general training, the coaching of the future panelists continues with blind tests for different flavors (quiz). These samples will have specific values of different primary flavors scales and panelists must identify them. The session will continue in case of answering right, if not, the right values will be given as a reminder. All, right and wrong answers, will be taken into account in the evaluation of the tasters.

Later, the duo-trio test will be prepared (ISO 5495 2005, UNE 87-010 (1993)). Three samples will be given to the tasters, the reference one and two coded ones, where one of them is the same as the reference one. First of all, the reference sample is tested and then the others. Tasters must know which coded one is perceived in the same way as the reference one. Other tests, used before the specific analysis, are, on one hand, the triangular proof (ISO 4120 2004, UNE 87-006 (1992)) that allows to distinguish which ones are the same honey among three samples simultaneously presented and, on the other hand, the ordering classification test (ISO 8587 1998, UNE 87-023 (1995)) that consists in introducing to the tester, simultaneously, several samples that must be ordered with a specific criteria (for example, the intensity of a specific attribute).

After these tests, panelists can work with specific honey, multi-flower or unifloral (specific training), in order to give a sensitive memory to the tasters and make a right comparative among scales and be able to make a reasoned judgment. This training must be realized in a constant and methodical way to raise the retention capacity.

The testing panel is led by a panel chief, who is in charge of training session and testers performance. He will select the panelists, check the different scales and tests previously mentioned, as well as honey samples and the order in which they are going to be analyzed at the panel session. Besides being a honey and sensory analysis expert, he needs to create a good working ambiance during the organoleptic analysis sessions without interfering in the decision taking time in order not to influ-

ence other panelists (unless he is required in the panel session as another panelist, in this case, he must not have prepared or know anything about the honey samples). This expert judge will be also in charge of teaching theory about honey, and everything related to them and sensory analysis, as well as moderate sessions and analyze the obtained results statistically. Finally, he must identify and correct if the panelists deviate their punctuations or the disparity of criteria among them, by remembering the needed scales to make an accurate evaluation (scale memorization sessions).

This implies that it is the panel chief's responsibility to ensure the complete analysis session, from the sensory analysis to the statistics of the obtained data.

For each evaluated attributes the arithmetic average and standard deviation will be obtained, which will help to decide how tasters are trained or how the samples must go on being assessed, when existing deviation must be valued (if there is no deviation, there will be more sessions, if there is deviation, more memorization sessions must be made).

2.4 The Tasting Room

Our research group at the Hispano-Luso Institute for Agricultural Research (CIALE) in the University of Salamanca does not organize professional sensory analysis sessions but extraordinary courses, conference days, etc. in which we organize practical sensory analysis of honey in order to sensitize the population to the organoleptic knowledge of different honeys. Thus, our tasting room (Fig. 2.3) usually is a well-ventilated room, well illuminated with nice thermal conditions and tables to receive groups of about 4 or 6 people (there must not be more than 24 people per session). This number is set because the aim of these sessions is the basic knowledge and training on honey sensory analysis, so that our preference is the communication, at some point of the session, among honey panel members.

Fig. 2.3 Tasting room in the Hispano-Luso Institute for Agricultural Research (CIALE). University of Salamanca

A professional tasting room must accomplish some specifications defined by the normative (ISO 8589 1988, UNE 87-004 (1979)), for example: it must have a uniform lighting, adjustable and with diffused light, if possible daylight, not to interfere with color scale samples and mislead panelists. Temperature should be around 20–22 °C (panelists must be focused and that is not possible when they are cold or hot). We must also take into account relative humidity that must be around 60–70% because smells are perceived worse in a dry ambience, the sample must be at ambient temperature, the furniture and walls must be in a light, white or pastel tone color pattern; of course there must not be outdoor noise, or secondary smells (cooked food, smoke, paint, etc.). Enough time must be left between consecutive sessions to refresh the room air conditions.

Having individual cabins is important for the isolation of each panelist, with both sides and frontal walls and high enough not to get distracted (the measures are specified in the normative UNE 87-004 (1979)); they must be wide enough, with individual lighting and a comfortable and adjustable chair. The samples to be analyzed are given through a frontal trapdoor by the session chief in the appropriate order. It may be advantageous to have two rooms, one for the panel session and another one to get the samples ready or to give instructions before and after the panel session.

2.5 The Material

For the scale set up is needed glass lab material (beaker, pipettes, glass stirring rod, etc.), a precision balance, reference foodstuffs to elaborate the scales (detailed in the next paragraph), glass containers (they must be opaque because their look cannot interfere in the panel participants analysis).

About the required material for the panel session, the glass cups must be balloon glasses (the best ones for accuracy smelling phase are 6 cm rim diameter and 12 cm high, Fig. 2.4). The amount of honey that must be introduced in the glass is 35 g and soup spoons or similar can be used in case it is a crystallized honey. Aluminum foil is

Fig. 2.4 Honey cups prepared for a sensory analysis session

also required to cover them (the smells cannot get lost and mask the tasting room). The glasses will be labeled, with glass marker pens, to be coded with three digits that are only known by the panel chief to not mislead the panelists during the sensory analysis.

All glasses must contain the same amount of honey since different amounts of honey will show some color variations. It is also important that the honey is at room temperature and has not undergone any modification by the organization (e.g. homogenization of the sample). Some authors like Aguas et al. (2010) or Montenegro et al. (2008) prefer to modify its physical appearance by liquefying honey to standardize its physical status; nevertheless, we consider it better to avoid modifications of the samples, it is essential if we are talking about a unifloral honey competition.

During the panel session, coffee spoons and disposable odorless plastic spatulas will be used to taste the scales and honey; water glasses, dishes, napkins, knives, pens, document paper and the record of data. On the table, panelists can be provided with low mineralization water (the same used to make the dilutions for primary flavors scales, UNE 87-003 1995)), low salt bread and Granny Smith apples, all this is for cleaning the mouth palate between scale points or between different honey not to get influenced by previous tastes.

2.6 The Honey Tasting Process

Before the honey sensory analysis process, the samples are studied both physicochemically (humidity, conductivity, etc.) and palynologically (following the methodology proposed by Louveaux et al. (1978) and others) as detailed in the corresponding chapter of this book. This helps to identify the botanical and geographical origin in order to know if honey samples meet the quality standards and if the labeling, from the botanical point of view, is correct.

Anyway, knowing the amount of pollen of a specific pollen type could corroborate some appreciations of the tasting panel.

In the first place, it is necessary to differentiate types of sensory analysis, such as the qualitative ones, in which the organoleptic preferences of honey are studied without carrying out a study in detail (using hedonic scales to measure preferences), such as Avilés and Matos in 2009; and, on the other hand, the quantitative ones, where it is important to complete the description of the honey in the panelists´ own words or using the method of quantitative descriptive sensory analysis (QDA™), following Anupama et al. (2003) for Indian honey or Montenegro et al. (2008) for Chilean honey. Both analyses can be combined, using the technique of Profiles with Consumers, where these consumers (not trained people) make a descriptive analysis also including how much they like the attributes described (Arrabal and Ciappini 2000).

The number of samples to be examined depends on the kind of study to be performed, although the tasting session should not have more than 15–20 honey

samples. If it is a descriptive analysis (which takes much longer than an analysis of a single attribute, for example) there should be no more than 5–7 samples per session.

2.6.1 Phases of the Tasting (Valuation of Each Stage)

These phases of the honey sensory analysis are carried out in order to give the tasters some previous knowledge of issues such as the main characteristics of the product, possible adulterations, methods of collection and packaging, etc. So, before the session, there will be a talk about these aspects.

It is important to emphasize that, we have done the training of the tasters based on the scales we have considered the best fit for our activities (González and De Lorenzo 2002a, b; González et al. 2008) with certain considered variations to improve the appreciation of the panelists, or that have been derived from the lack of stock of some products or changes in their organoleptic qualities and which we will see in detail below.

VISUAL PHASE: among other characteristics, the color and the physical state (liquid honey, crystallized, separation into two phases, type of crystallization, etc.), and appearance (cleaning, turbidity, homogeneity, more or less translucent, etc.) are evaluated. It is important to keep in mind that when a liquid honey crystallizes its color becomes clearer.

This phase is very important because it is the first impression perceived from a sample, and therefore is remembered for the rest of the analysis. In this sense, although it is evaluated in a specific glass, it could be also interesting to observe the presentation in its original container (if we are judging appearance).

Our research group modified the nine-point color scale for liquid honey by González and De Lorenzo (2002a), establishing a ten-point one (photograph 5) and created another scale for crystallized honey also with ten points (photo 6), as we think that the tonality, brightness and other characteristics are not the same as for liquid honey (photograph 1), which meant that there were many problems to assess the color in this type of honey. The dilutions to make the points of these two scales are detailed in Tables 2.1 and 2.2 respectively.

There are middle-term conservation problems in the color scale for liquid honey due to the growth of fungi at points 1–3 of the scale, which have been reduced by adding phenol crystals. Another problem with this scale, and other ones, is the availability of standard foods, since not all the liquors of the scale maintain their properties over time.

The two scales should begin with a colorless (liquid) or white (crystallized honey) and end with a black (liquid honey) or a dark brown (since crystallized honeys are lighter than liquid honey, they would never get a black tone) (Figs. 2.5 and 2.6). The color is valued by comparing the honey with the corresponding scale, that stays on the table throughout the analysis session, as it does not interfere with the development of the rest of the session.

Table 2.1 Color scale for liquid honey

Liquid honey		
N° scale	N° solution	Solutions[a]
10	1	Liquid caramel (Royal)
9	2	250 ml water: 35 ml liquid caramel
8	3	Cocoa liqueur (Bardinet)
7	4	Cocoa liqueur: water (1:1)
6	5	Solution 4: water (1:1)
5	6	Almond liqueur: cocoa liqueur (1:16)
4	7	Almond liqueur: banana liqueur (1:1)
3	8	330 ml banana + 33 ml cocoa + 637 ml water
2	9	Solution 8: water (1:1)
1	10	Solution 9: water (1:1)

[a]**Trademarks**: liquid caramel (Royal), cocoa liqueur (Bardinet), almond liqueur (Teichenné, S.A.), banana liqueur (Frutaysol)

Table 2.2 Color scale for crystallized honey

Crystallized honey		
N° scale	N° solution	Solutions[a]
10	1	5 g soluble coffee + 10 ml almond liqueur
9	2	6 g soluble coffee + 20 ml distilled water
8	3	Solution 2: distilled water (1:1)
7	4	Solution 3: distilled water (1:1)
6	5	Solution 4: distilled water (65:35)
5	6	Solution 4: distilled water (1:1)
4	7	Solution 5: distilled water (1:1)
3	8	Solution 6: distilled water (1:1)
2	9	Solution 7: distilled water (1:1)
1	10	Solution 8: distilled water (1:2)

[a]**Trademarks**: almond liqueur (Teichenné, S.A.) and soluble coffee (Hacendado)

Fig. 2.5 Color scale for liquid honey

Fig. 2.6 Color scale for crystallized honey

OLFACTORY PHASE: the correct way to smell is to inhale the air that contains the volatile particles of the honey for a few seconds at a discontinuous beat, bringing the nose to the glass or introducing it slightly. We must detect the primary odour and then we will appreciate other smells. It is convenient to leave a few seconds between one inspiration and another. We must appreciate the type of smell, the intensity and persistence over time, as well as the saturation ability.

Because our research group makes initiation honey tastings to introduce the technique to nonprofessional people, to train these olfactory sensations, only a four-point intensity scale is used, as shown in Table 2.3 (even so, it is not objective because volatile molecules are released from the containers of the scale so fast, thus polluting the environment, creating perceptions that are not easy to value correctly). In this sense, it would be better to work with perfectly known chemical products.

After appreciating the primary smell (the most intense) and becoming familiar with that smell, we can perceive other secondary odors, intensity, persistence, defects (such as smoke or fermentation), etc. The important thing is the ability to translate the perceived smell stimulus into words, which may be practiced with common smells of our daily life.

It is important that the trainee tasters write, in the corresponding tab, the perceived sensation of the primary odour, as well as the other attributes, to be commented on together at the end of the session. During the initial sessions, there will not be any comments by the future tasters, as these may influence the rest of the group. Therefore, comments to define any of the odours during the analysis process are totally forbidden.

To release the honey volatile molecules in a better way, we can lightly warm the glass with our hands or remove the honey with a spatula or spoon (it is necessary especially in crystallized or low aroma honey).

TASTING PHASE: first, a low amount of honey is introduced into the mouth, equally distributed with the tongue to be able to describe the flavors by comparing with the scales detailed below.

As has already been mentioned, other sensations may appear besides from intensity and persistence, such as astringency (training with tannic acid), freshness (practice with menthol, UNE 87-013 (1996)), itch, etc. or any defect (undesirable aroma).

Table 2.3 Olfactory intensity scale

Smell intensity		
N° scale	Sensory descriptor	Solutions[a]
1	Not intense	Distilled water
2	Slightly intense	Banana liqueur: water (1:6)
3	Moderate intense	Banana liqueur: water (1:1)
4	Extremely intense	Banana liqueur

[a]**Trademark:** banana liqueur (Frutaysol)

Table 2.4 Primary taste range: sweet

Sweetness		
N° scale	Sensory descriptor	Solutions
1	Not sweet	Mineral water (Bezoya)
2	Slightly sweet	25 g saccharose/liter
3	Moderate sweet	50 g saccharose/liter
4	Very sweet	100 g saccharose/liter
5	Extremely sweet	200 g saccharose/liter

Table 2.5 Primary taste range: acid

Acidity		
N° scale	Sensory descriptor	Solutions
1	Not acid	Mineral water (Bezoya)
2	Slightly acid	0.500 g tartaric acid/liter
3	Moderate acid	0.750 g tartaric acid/liter
4	Extremely acid	1 g tartaric acid/liter

Table 2.6 Primary taste range: Bitter

Bitterness		
N° scale	Sensory descriptor	Solutions
1	Not bitter	Mineral water (Bezoya)
2	Moderate bitter	0.200 g caffeine/liter
3	Extremely bitter	0.400 g caffeine/liter

Sweetness: the scale for the training of this primary flavor has five points and it is performed with sucrose dilutions (Table 2.4).

Acidity: the scale used to train the tasters is made of four points with the dilutions of tartaric acid (Table 2.5) or citric acid in the same proportions.

Bitterness: this scale is made of three points and it is performed with dilutions of crystallized caffeine monohydrate (Table 2.6) or quinine in similar proportions.

Salty: a scale with two points, not salted and salted, is used which is made by dilution of common table salt.

TACTILE PHASE: in order to assess these attributes, a small amount of honey must be introduced in the mouth again, trying to check viscosity, adhesiveness and crystallization in the same process. In appendix I, there are the products and trademarks to elaborate the scales that are currently used in Spain. For other countries, it may be necessary to modify these scales again, because the brands and/or products can be very different. The scales we provide are just for guidance but we must be very careful because, for example, in the viscosity scale there are two products "dulce de leche" in two consecutive points, corresponding to two different brands.

Viscosity: this refers to the necessary strength to transfer the honey from the spoon to the mouth, to swallow it or to spread it (the more force you must exert with

Table 2.7 Touch scale: Viscosity

Viscosity		
Nº scale	Sensory descriptor	Reference sample (Trademark)
1	Not viscous	Mineral water (Bezoya)
2	Very slightly viscous	Extra-virgin olive oil (Koipe)
3	Slightly viscous	Hot chocolate (Ram)
4	Moderated viscous	Chocolate syrup (Royal)
5	Viscous	Liquid caramel (Royal)
6	Very viscous	Dulce de leche (La Lechera)
7	Extremely viscous	Dulce de leche (Mardel)

Table 2.8 Touch scale: Adhesiveness

Adhesiveness		
Nº scale	Sensory descriptor	Reference sample (Trademark)
1	Not adhesive	Mineral water (Bezoya)
2	Very slightly adhesive	Butter (Flora)
3	Slightly adhesive	Cheese (El Caserío)
4	Moderate adhesive	Nocilla (Nutrexpa)
5	Very adhesive	Peanut butter (Capitán Maní)
6	Extremely adhesive	Caramel (Sugus)

Table 2.9 Touch scale: Crystallization

Crystallization		
Nº scale	Sensory descriptor	Reference sample (Trademark)
1	Not crystallization	Mineral water (Bezoya)
2	Very fine crystallization	Icing sugar (Azucarera española)
3	Fine crystallization	Brown sugar <700 μm (Azucarera española)
4	Moderate crystallization	Brown sugar between 700 and 900 μm
5	Slightly coarse crystallization	Brown sugar between 900 and 1.12 μm
6	Coarse crystallization	Brown sugar >1.12 mm

the lips to drag all the honey, the more viscous will be that sample). The scale used is shown in Table 2.7.

Adhesiveness: this refers to the force needed to remove a honey that is adhered to the mouth (palate and denture). The scale used is shown in Table 2.8.

Crystallization: to analyze this attribute, we carry the honey to the palate and by making pressure with the tongue and performing zigzag movements, we appraise the crystals´ characteristics (not only the size, also the shape, angularity, if they are more or less homogeneous, etc.) also appreciating the time it takes to dissolve in the mouth. The scale has six points; number one corresponds to liquid honey and number six to hard crystallization honey. For the realization of this scale (except points one and two, water and glacé sugar respectively), sieved brown sugar is used, using

a sieves set of various mesh sizes (700, 900 and 1120 μm), each point of the scale corresponds to the sugar that is kept in each sieve except the point three, which becomes the equivalent to the sugar passing through the 700 μm sieve (see Table 2.9). In this way, the scale is standardized.

In relation to the order in which the honey samples should be evaluated, it is important to start the organoleptic analysis with the clearest samples and the smoother and more delicate odour and flavor ones, after that, the halfway color honey and more pronounced smell and flavor will be tasted and finally the darkest and those with more intense smells and flavors.

The results of all the phases are written down on a data record sheet, in our case, for a descriptive honey session (Fig. 2.7). In this tab we can indicate, on the left, the points of the different scales that correspond with the honey sample and on the right we can describe in each box the perceived sensations of each attribute, "what does it remind us of?". Regarding the color scale, the values of the corresponding scale shall be noted, taking into account if it is a liquid or crystallized honey. About the smell, the scale at the record card is the one corresponding to the intensity that is perceived. Taste and touch will be annotated first as values of the scales, after which the perceived sensations are written down in the appropriate boxes. The appreciation of the whole of flavors and the final note reflected on the data sheet are merely informative, to have a global and subjective appreciation of the honey they like most and least. This registration tab will be given to the panel chief for statistical analysis. The record sheets are different according to the purpose of the analysis.

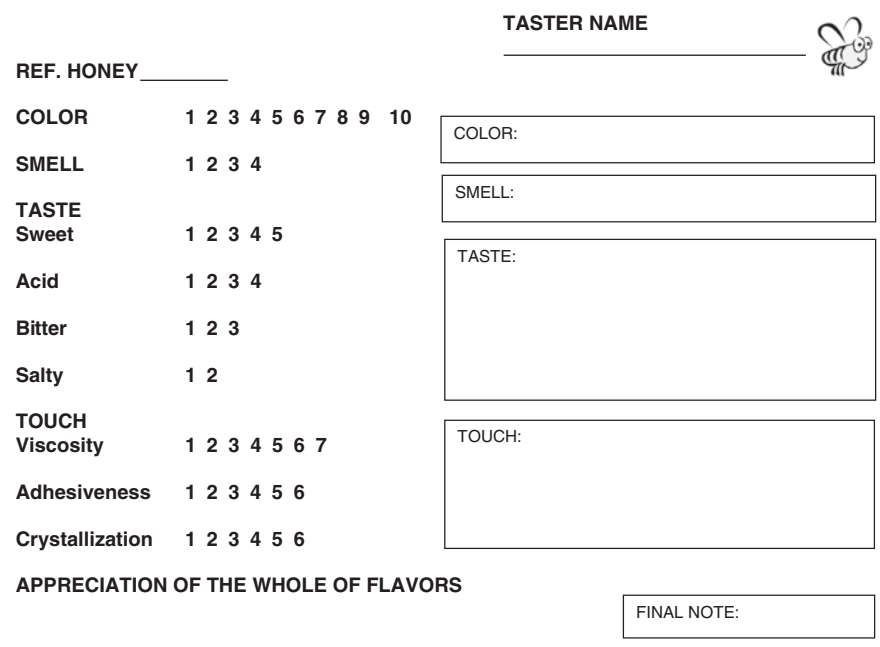


Fig. 2.7 Data record sheet

To perform the final statistical analysis, each attribute may have more or less weight. For example, it may be less important to the color if you are working with multi-flower honey and more if you are working with unifloral honey (since these honeys usually have a certain color with narrow variations, so if the color is lighter or darker than the typical one, should be penalized). If it is the color that a specific brand wants to obtain for their honey, it could have much more weight in the final evaluation.

To describe unknown honey it is proceeded in a different way and also to decide the quality within a sensory well-known type by the tasting panel. For example, penalizing honey for not achieving exactly the typical characteristics of that particular type (so, a rosemary honey that is dark, although it complies with all other attributes, will have a very low score).

2.7 Utility of a Honey Sensory Analysis

After gathering the answer tabs, all the members of the tasting panel make a collective assessment of important data such as the honey's state of conservation, the overall appearance, its characteristics, etc. (Sánchez Sánchez 2010).

It is very useful to evaluate the quality of different honeys with similar physical and pollen analysis to be able to judge which is better. These analyses are very important in the regulations in force for "quality denomination" honey (for example "Denomination Origin"), so, in addition to other analysis, a tasting panel may be responsible for performing the organoleptic studies and deciding whether a honey can be marketed as a procedure for that name, or not.

It is frequent that in honey competitions or fairs, the procedures of analysis are different from those discussed here. Often honey is presented in the same contest at two different awards, one for clear honey and the other for dark honey. At other times the competition may be for a particular type of unifloral honey. In all these cases, in addition to other quality tests, sensory analysis is performed; the contest cards in different countries by Gómez Pajuelo (2004) may have some variations from what is presented here. For example, certain attributes may be given different priority in that specific country area.

In short, the organoleptic characteristics are the result of the preparation, manipulation and conservation of honey, among other characteristics and honeys that, although legally marketable, do not have an acceptable sensory quality could be eliminated from the market. After all, it is the consumer who decides, in some way, which product is a quality honey and which one is not.

2.8 Attributes of Some Honeys

In this section, we collect the organoleptic description of different unifloral honeys, based on the results of our own sessions of sensory analysis made during years and also based on the available bibliography; for example, in Europe there is a great data

bank (Persano Oddo et al. 1995; Persano Oddo and Piro 2004; Piazza and Persano Oddo 2004; Pires et al. 2005):

- The black locust honey or incorrectly called acacia honey (*Robinia pseudoacacia* L.) is one of the clearest; it has a weak intensity of odour and floral mild taste of a medium intensity, strong sweetness, vanilla, with little tendency to crystallize.
- The rosemary honey (*Rosmarinus officinalis* L.) has a short but persistent scent with floral, fresh fruit and vegetal aroma. The more pollen of Rosmarinus the more camphorated aroma.
- The citrus honey (*Citrus* spp.) has a characteristic citric aroma (methyl anthranilate), orange blossom, regarding to flavor; this is a honey with great acidity, fresh fruit (floral) and anise.
- The eucalyptus honey (*Eucalyptus* spp.) has light amber color with a green tone, with high intensity and persistent wet wood or wet dog aroma, it also has sweet flavor with slight acidity, soft candy.
- The lime honey (*Tilia* spp.) has light amber color with a green tone, soft floral odour that resembles menthol, the taste is like the smell but sometimes a bit bitter.
- The thyme honey (*Thymus* spp.) has an amber color with a red tone, has a very intense woody aroma, fresh fruit with a marked appreciation of phenol, sweet flavor with slight acidity.
- The sunflower honey (*Helianthus annuus* L.) has light amber color, an herbaceous and floral scent with oleaginous notes, sweet, bit fruity and floral flavor that swallowing causes a certain irritation in the mucosa.
- The lavender honey (*Lavandula* spp.) has a floral and fruity scent and taste, unequivocal and intense flavor of lavender with a marked appreciation of phenol, sweet with slight acidity.
- The cantueso honey (*Lavandula stoechas* Lam.) has an intense floral odour, the taste is sweet but sometimes slightly bitter, leaving a sensation of glycerin in the mouth.
- The dandelion honey (*Taraxacum* Weber) has a typically intense and persistent ammonia scent (this reminds of "cat pee" smell).
- The rape honey (*Brassica* spp.) has a strong odour and the taste reminds of cabbage and turnip.
- The avocado honey (*Persea americana* Mill.) has dark color (which is less characteristic of floral honey), fruit and floral appreciations, sweet taste, sometimes slightly salted
- The heather honey (*Erica* spp) has an amber color with a red tone, with varied intense aromas as wet forest, mushrooms, humus, floral, wood, fresh fruit, caramel and with bitter components in the mouth.
- The chestnut honey (*Castanea sativa* Mill.) has amber-dark amber color, an intense and persistent odour that reminds of dry wood, malted, with balsamic notes, bitter flavor and astringent.
- The honeydew honey of holm oak (*Quercus ilex* L.) or pyrenean oak (*Quercus pyre*naica Willd.) is very dark, almost black, has a malted woody aroma, some astringency, some tasters appreciate salty components.

Table 2.10 Arithmetic means of the results obtained in sensory analysis performed by our group at the University of Salamanca

Results of sensory analysis of honey in Salamanca							
		Primary tastes			Touch		
Honey type	Color	Sweet	Acid	Better	Viscosity	Adhesiveness	Crystallization
Black locust	3	3–4	2	1	3	2–3	1
Citrus	4(3)	3(4)	2	1(2)	3	2(3)	1 or 5[a]
Rosemary	4(5)	4	2(3)	1(2)	3	2–3	1
Sunflower	5	4	3(4)	1	4–5	3–4	1
Eucalyptus	6(7)	4	2–3	1(2)	3–4	3	1 or 4[a]
Chestnut	9(10)	3(2,4)	2	2(3)	3	2–3	1
Honeydew	9(10)	4	2–3	1(2)	3–4	3	1

[a]Value for a crystallized honey

- The arbutus honey (*Arbutus unedo* L.) is very dark too, scent as heather honey but much more floral, characteristic bitter taste for the arbutin. This honey has more tendency than other honey to separate in two phases and to ferment.
- The Chilean honey like Ulmo (*Eucryphia cordifolia* Cav.), provide aromatic appreciations to anise and jasmine; the Quillay honey (*Quillaja saponaria* Molina) has smoked, raisins and propolis attributes and the Corontillo honey (*Escallonia pulverulenta* Ruiz and Pav.) smells like caramel and vanilla.

Probably there are organoleptic differences if two unifloral honeys of the same kind have different percentage of pollen types. For example, Gaggiotti et al. (2014) were able to appreciate that clover Argentinian honey (*Trifolium* spp. L.) has a warm, subtle (beeswax) and caramelized odour, however, Ciappini et al. (2009) considered that this type of honey has a fruity and floral smell, with aromatic appreciations, sometimes citrus and vegetables, but if the percentage of *Medicago sativa* is majority, more warm notes emerge.

With the intention of evaluating more in detail some of these honeys studied by our group in Salamanca over the years, Table 2.10 shows some scales data that were taken from the set of cards. It is necessary to take into account that these sensory analyses involved students, teachers, researchers from different centres, not university people, etc. The data in this table are informative, average data, without being a detailed statistic. The majority data is the number that follows the valued attribute. In parentheses is the data provided by some tasters for that attribute. If two data are included without parentheses, they correspond to approximately 50% for each point on the scale. As a comment, acacia honeys were analyzed without crystallization, but some crystallized through time.

Finally, we want to emphasize that it is important to describe all sensory properties of honeys to know them better. We started this chapter saying that honey is considered a quality and healthy food, but after this chapter, we know that there is a great variety of colors, smells, flavors, kinds of crystallization, etc. We must try many different honeys to decide which one has the highest quality for us.

References

Abdullah MZ, Guan LC, Lim KC, Karim AA (2004) The applications of computer vision and tomographic radar imaging for assessing physical properties of food. J Food Eng 61:125–135

Aguas Y, Olivero R, Cury K (2010) Determination of adulteration and honeys acceptability (*Apis mellifera*) market in Cartagena, Bolivar Colombia. RECIA 2:349–355

Anupama D, Bhat KK, Sapna VK (2003) Sensory and physico-chemical properties of comercial samples of honey. Food Res Int 36:183–191

Arrabal MV, Ciappini MC (2000) Prueba de aceptabilidad en miel. Invenio 3:141–147

Avilés HA, Matos A (2009) Análisis comparativo de la Calidad Físicoquímica, Microbiológica y Organoléptica de la Miel de abeja (*Apis mellifera*) producida en diferentes regiones de Perú. Rev Investig Univ 1:5–11

Bentabol A (2002) Aportaciones metodológicas al análisis sensorial descriptivo de las mieles. Descripción de olores y aromas. Alimentaria 339:49–52

Bouseta A, Collin S (1995) Optimized Likens-Nickerson methodology for quantifying honey flavors. J Agric Food Chem 43:1890–1897

Ciappini MC, Gattuso SJ, Gatti MB, Di Vito MV, Gómez G (2009) Mieles de la provincia de Santa Fe (Argentina). Determinación palinológica, sensorial y fisicoquímica, según provincias fitogeográficas. Invenio 12(22):109–120

De María CAB, Moreira RF (2002) Compostos voláteis em méis fl orais. Quím Nova 26:90–96

Delmoro J, Muñoz D, Nadal V, Clementz A, Pranzetti V (2010) El color en los alimentos: determinación de color en mieles. Invenio 13(25):145–152

Ferreres F, Giuer JM, Tomás-Barberán FA (1994) A comparation study of hesperetin and methyl anthranilate as markers of the floral origin of citrus honey. J Sci Food Agr 65:371–372

Fonte L, Díaz M, Machado R, Demedio J, García A, Blanco D (2013) Caracterización físicoquímica y organoléptica de miel de *Melipona beecheii* obtenida en sistemas agroforestales. Pastos y Forrajes 36(3):345–349

Gaggiotti MC, Signorini M, Sabbag N, Wanzenried RA, Cuatrín A (2014) Miel de abeja producida en un sistema lechero en base a pastura de alfalfa (composición fisicoquímica, palinológica y sensorial). Arch Latinoam Prod Anim 22(1/2):15–20

Galán-Soldevilla H, Ruiz-Pérez-Cacho M, Serrano S, Jodral M, Bentabol A (2005) Development of a preliminary sensory lexicon for floral honey. Food Qual Prefer 16:71–77

Gómez Pajuelo AG (2004) Mieles de España y Portugal. Conocimiento y cata. Montagud Editores, Barcelona, p 151

Gonnet M, Vache G (1979) Technique de dégustation des miels et recherché d'un système de notation et de classification objectif pour appécier leur qualité para l'analyse sensorielle. 27th Apimondia Int. Apic. Congr. Athènes, p 499–506

Gonnet M, Vache G (1985) Le goûte du miel. Edition U.N.A.F., París, France, p 146

Gonnet M, Vache G (1998) Analyse sensorielle descriptive de quelques miels monofloraux de France et d'Europe. Ed. Abeille de France, Paris, France, p 86

González M, De Lorenzo C (2002a) Calidad sensorial de las mieles de Madrid (I). Configuración de un grupo de cata y obtención de escalas normalizadas. Alimentaria 97:97–102

González M, De Lorenzo C (2002b) Calidad sensorial de las mieles de Madrid (II). Correlación con el análisis instrumental. Alimentaria 103:103–109

González M, De Lorenzo C, Pérez RA (2008) Sensory attributes and antioxidant capacity of Spanish honeys. J Sens Stud 23:293–302

IHC International Honey Commission of Apimondia (2001) Minutes of the IHC meetings (Louvain-la-Neuve)

ISO 4120 (2004) Sensory analysis: Methodology. Triangle test. International Organization for Standardization, Geneva

ISO 5495 (2005) Sensory analysis. Methodology. Paired comparison test. International Organization for Standardization, Geneva

ISO 8587 (1998) Sensory analysis. Methodology. Ranking. International Organization for Standardization, Geneva
ISO 8589 (1988) Sensory analysis. General guidance for the design of test rooms. International Organization for Standardization, Geneva
Lomas AM, Echávarri JF, Negueruela AI, Ayala F (1997) Aplicación de la teoría de Kubelka-Munk a la miel. IV Congreso Nacional de Color. Universidad de Extremadura, Extremadura, Jarandilla de la Vera
Louveaux J, Maurizio A, Vorwohl G (1978) Methods of melissopalynology. Bee World 59:139–157
Mendoza F, Aguilera JM (2004) Application of image analysis for classification of ripening bananas. J Food Sci 69:471–477
Montenegro G, Pizarro R, Avila G, Castro R, Ríos C, Muñoz O, Bas F, Gómez M (2003) Origen botánico y propiedades químicas de las mieles de la región mediterránea árida de Chile. Cien Inv Agr 30:161–174
Montenegro G, Gómez M, Pizarro R, Casaubon G, Peña RC (2008) Implementación de un panel sensorial para mieles chilenas. Cien Inv Agr 35(1):51–58
Muñoz O, Copaja S, Speisky H, Peña RC, Montenegro G (2007) Contenido de flavonoides y compuestos fenólicos de mieles chilenas e índice antioxidante. Quím Nova 30:848–851
Persano Oddo L, Piro R (2004) Main European unifloral honeys: descriptive sheets. Apidologie 35(1):38–81
Persano Oddo L, Piazza MG, Sabatini AG, Accorti M (1995) Characterization of unifloral honeys. Alimentaria 26:453–465
Piana ML, Persano Oddo L, Bentabol A, Bruneau E, Bogdanovd S, Declerck C (2004) Sensory analysis applied to honey: State of the Art. Apidologie 35:26–37
Piazza MG, Persano Oddo L (2004) Bibliographical review of the main European unifloral honeys. Apidologie 35(1):94–111
Pires SMA, Gómez Pajuelo AG, Pereira JOB, Carvalho MFM (2005) Tras-Os-Montes y Alto Douro. Contribución al estudio del análisis sensorial de mieles de Portugal. Vida Apícola 134:35–39
Ramírez R, Montenegro G (2004) Certificación del origen botánico de miel y polen corbicular pertenecientes a la comuna de Litueche, VI región de Chile. Cien Inv Agr 31:197–211
Salamanca GG (2007) Diez criterios técnicos para el reconocimiento de la calidad de la miel. APITEC Revista Mexicana de Apicultura 1:10–13
Sánchez Sánchez J (2010) Análisis sensorial. In: Baldi B (ed) La Miel. Una mirada científica. EDUNER (Editorial de la Universidad Nacional de Entre Ríos), Entre Ríos, p 232
Serra Bonvehi J (1988) Determinación de antranilato de metilo en la miel de cítricos (Citrus sp.) del Levante Español, y su influencia en la actividad diastásica de la miel. Alimentaria 197:37–40
UNE 87-003 (1995) Análisis sensorial. Metodología. Método de investigación de la sensibilidad gustativa. Norma española. AENOR, Madrid
UNE 87-004 (1979) Análisis sensorial. Guía para la instalación de una sala de cata. Norma española. AENOR, Madrid
UNE 87-006 (1992) Análisis sensorial. Metodología. Prueba triangular. Norma española. AENOR, Madrid
UNE 87-010 (1993) Análisis sensorial. Metodología. Prueba dúo-trío. Norma española. AENOR, Madrid
UNE 87-013 (1996) Análisis sensorial. Metodología. Iniciación y entrenamiento de jueces en la detección y reconocimiento de olores. Norma española. AENOR, Madrid
UNE 87-023 (1995) Análisis sensorial. Metodología. Ensayo de clasificación por ordenación. Norma española. AENOR, Madrid
UNE 87-024-1 (1995) Análisis sensorial. Guía general para la selección, entrenamiento y control de jueces. Parte 1: Catadores. Norma española. AENOR, Madrid
UNE 87-024-2 (1996) Análisis sensorial. Guía general para la selección, entrenamiento y control de jueces. Parte 2: Expertos. Norma española. AENOR, Madrid
Yamaguchi S (1998) Basic properties of umami and its effects on food flavor. Food Rev Int 14(2):139–176
Yamaguchi S, Ninomiya K (1998) What is umami? Food Rev Int 14(2):123–138

Chapter 3
Chemical Composition of Honey

Celestino Santos-Buelga and Ana M. González-Paramás

Contents

3.1 Introduction

Honey is the best known primary product of beekeeping and the history of its use is parallel to the history of mankind; in every culture evidence can be found of the use of honey as a food source but also as a symbol employed in religious, magic and therapeutic ceremonies (Krell 1996).

Nowadays, honey is the most important primary product of beekeeping both from a quantitative and an economic point of view. Most recently published statistics estimate an annual world production greater than 1.5 million tonnes, worth about 4000 million US dollars on the world market. Following the production trends, China dominates the global honey sales; it is the number one honey producer and exporter in the world, with more than 300,000 tonnes that represented around

C. Santos-Buelga • A.M. González-Paramás (✉)
Grupo de Investigación en Polifenoles (GIP-USAL), Facultad de Farmacia, Universidad de Salamanca, Campus Miguel de Unamuno, 37007 Salamanca, Spain
e-mail: paramas@usal.es

J.M. Alvarez-Suarez (ed.), *Bee Products - Chemical and Biological Properties*,
DOI 10.1007/978-3-319-59689-1_3

250 million dollars worth in 2013. Mexico is the second largest exporter followed by Germany (Faostat 2016).

According to Codex Alimentarius (2001), "honey is the natural sweet substance, produced by honeybees from the nectar of plants or from secretions of living parts of plants, or excretions of plant-sucking insects on the living parts of plants, which the bees collect, transform by combining with specific substances of their own, deposit, dehydrate, store and leave in honeycombs to ripen and mature". Although there are other honeybee species that make honey, and other bees and even wasps that store different kinds of honeys as their food reserves, in the EU, according to the legal definition, honey can only be referred to as such when it is produced by *Apis mellifera* honeybees (EU Council 2002).

Generally, honey is classified according to its provenance in blossom and honeydew honeys. Blossom honey is obtained predominantly from the nectar of flowers, while honeydew honey is produced by bees after collecting the secretions of aphids or other plant sap-sucking insects, which pierce plant cells, ingest plant sap and then secrete it again (Bradbear 2009). Among floral honeys, the most commercially available ones are multifloral honeys, meaning that they come from several botanical sources none of which is predominant. However, it is also possible to find monofloral honeys made primarily from the nectar of one type of flower and they are named according to that plant.

From a chemical point of view, honey could be defined as a natural food mainly composed of sugars and water together with minor constituent such as minerals, vitamins, amino acids, organic acids, flavonoids and other phenolic compounds and aromatic substances (Table 3.1). This composition, and especially minority components, is related to the botanical and geographical origin and can undergo significant changes depending on the storage time and conditions.

Although because of its unique composition and chemical properties, honey is suitable for long-term storage, some compositional variations usually occur due to different chemical and biochemical processes, such as fermentation, oxidation or dehydration of sugars, leading to changes in acidic content and the formation of

Table 3.1 Chemical composition of honey (adapted from Solayman et al. 2016 and Bradbear 2009)

Majority constituents (%)			
	Mean	SD	Range
Water	17.90	3.16	13.21–26.50
Fructose	39.44	2.11	37.07–42.65
Glucose	28.15	5.74	18.20–32.10
Sucrose	3.19	3.81	0.36–16.57
Other sugars	8.5	–	0.1–16.0
Minority constituents (%)			
Minerals	0.36	0.18	0.11–0.72
Total protein	1.13	1.22	0.22–2.93
Acids (as gluconic acid)			0.17–1.17
Vitamins, enzymes, aromas	<0.1		
Phenolic compounds	0.1		0.02–0.2

compounds like 5-hydroxymethylfurfural (5-HMF), which give rise to modification in sensorial properties and reduce the quality of honey.

3.2 Carbohydrates

Honey is mostly a concentrated aqueous solution composed of a mixture of fructose and glucose, but it also contains some amounts of near 30 other complex sugars (Table 3.2). Due to its high sugar content, on average 80–83%, honey is an excellent energy source (304 kcal/100 g). The production of this supersaturated sugar solution is only possible at hive temperature (30 °C) and by the addition of the enzyme invertase, secreted from hypopharyngeal glands of bees, which inverts sucrose, from the nectar or honeydew, into glucose and fructose (Crane 1990). Monosaccharides represent about 75% of the sugars found in honey, along with 10–15% disaccharides and small amounts of other sugars (da Silva et al. 2016).

Table 3.2 Main sugars described in honeys (adapted from Alves-Moreira and Bastos-de Maria 2001; Alvarez-Suarez et al. 2010b; Ruiz-Matute et al. 2010).

Monosaccharides		
Glucose Fructose (Galactose)[a]		
Disaccharides		
Majority	*Minority*	*Traces*
Isomaltose	Cellobiose	Isomaltulose
Kojibiose	Gentiobiose	Laminaribiose
Maltose	Maltulose	Leucrose
Sucrose	Nigerose	Melibiose
Turanose	Palatinose	Trehalose
Trisaccharides		
Majority	*Minority*	*Traces*
Erlose	Isomaltotriose	Centose
Theanderose	Isopanose	1-Kestose
Panose	Melezitose	Laminaritriose
Maltotriose	Raffinose	Planteose
		α-3′-Glucosyl-isomaltose
Higher oligosaccharides		
		Traces
		Isomaltotetraose
		Maltotetraose
		Isomaltopentaose
		Nystose

[a]Occassionally cited in trace levels (Goldschmidt and Burkert 1955; Val et al. 1998)

Different analytical approaches, especially using chromatographic techniques, have been employed to characterize the sugar profile of honeys. However, the structural similarity of sugars present, derived mainly from the combination between glucose and fructose linked through different glycosidic bonds, makes the chromatographic resolution of many of the compounds difficult. Methods based on gas chromatography with flame-ionization detection (GC-FID) have been extensively used since derivatization to obtain the trimethylsilyl (TMS) ethers of sugars was firstly reported in 1963 (Sweeley et al. 1963). Subsequent modification of the method by reaction of the sugars with hydroxylamine to give the corresponding oximes, before trimethylsilylation, was proposed, since reducing sugars give rise to only two different derivatives corresponding to the *syn* (E) and *anti* (Z) isomers, thus simplifying the chromatograms (Mason and Slaver 1971). This has been the method most widely employed (Battaglini and Bossi 1972; Low and Sporns 1988; Serra and Ventura 1995; Gómez-Bárez et al. 1999; Cotte et al. 2004a, b). GC-FID methods are quite sensitive and have capacity to separate and quantify a great number of oligosaccharides, but they have disadvantages like the need for derivatization to make sugars volatile and the appearance of various peaks for a single compound. GC-MS has also been used to characterize tri- and tetrasaccharides in honey after their derivatization into trimethylsilyloxime derivatives (Sanz et al. 2004a). Based on retention data and mass spectra, Ruiz-Matute et al. (2010) were able to detect up to 25 trisaccharides, although only 12 could be unequivocally identified using standards, 2 of them (planteose and α-3′-glucosyl-isomaltose) detected for the first time in honey. The same research group proposed a two dimensional GC-TOF-MS method to overcome the coelution problem encountered with anomeric structures (Brokl et al. 2010).

HPLC coupled to refraction index or pulsed amperometric detectors can also be used for the analysis of sugars (Shaw 1988; Cotte et al. 2003; Senyuva et al. 2009; Primorac et al. 2011). In particular, high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) has been employed for the determination of mono- and oligosaccharides (i.e., fructose, glucose, sucrose, melezitose, maltose, isomaltose, turanose, erlose, raffinose, trehalose) in honeys (Ouchemoukh et al. 2010; Escuredo et al. 2014). HPLC methods require little sample preparation, but they offer poor resolution of structurally similar carbohydrates and their detection limits are two to three orders of magnitude greater than those reached with GC-FID.

Other analytical techniques such as nuclear magnetic resonance (NMR) or Raman and Infrared spectroscopy have also been applied for characterization of honey sugars. Mazzoni et al. (1997) published the first ^{13}C NMR study for the analysis of carbohydrates in honey, quantifying ten different saccharides. Consonni et al. (2012) were able to identify up to 19 saccharides in Italian honey samples by ^{1}H and ^{1}H-^{13}C NMR spectroscopy, on the basis of their anomeric proton. A similar approach was employed for the analysis of honeys produced by Sicilian black honeybees (*Apis mellifera* ssp. *sicula*), allowing to describe the presence of high contents of turanose in all tested honeys and raffinose in dill honey (Mannina et al. 2015). Özbalci et al. (2013) quantified glucose, fructose, sucrose and maltose in honey by

coupling Raman spectroscopy with chemometrics methods such as PCA (Principal Component Analysis). Near-infrared spectroscopy has been applied to the accurate determination of the contents of fructose, glucose, sucrose, and maltose in commercial honeys, and shown to be also useful to detect adulterants in honey (Qiu et al. 1999). More recently, a Fourier transform infrared spectroscopic method with attenuated total reflectance (FTIR-ATR) has been used to quantify the main sugar components in honeys (Anjos et al. 2015). Recent findings in honey characterization by using emerging new techniques, not only for sugars but also for different chemical parameters, have been reviewed by Consonni and Cagliani (2015).

Fructose is, in general, the dominant sugar in most honeys (from 42% of the sugar fraction in some unifloral honeys such as Robinia or *Thymus* to around 32% in honeydew honey), but in some particular unifloral honeys, such as rape (*Brassica napus*) or dandelion (*Taraxacum officinale*), the glucose contribution is greater than that of fructose (Crane 1990; Persano-Oddo and Piro 2004). Honey disaccharides are mainly constituted by regioisomers of α-glucosyl-glucose and α-glucosyl-fructose; disaccharides with β-glycosidic linkages are present in minor amounts while fructosyl-fructoses are very scarce. Among disaccharides, maltose, isomaltose, kojibiose and turanose are the most abundant ones in nectar honeys and represented between 8 and 10% of total sugars. In blossom honeys, sucrose is also important, although due to the invertase enzyme action its content represents less than 3% of total sugars. The most abundant trisaccharides are derivatives of sucrose (de la Fuente et al. 2011). Relatively high amounts of the trisaccharides melezitose, erlose and raffinose have been reported in honeydew honey (Doner 1977; Bogdanov et al. 2008).

The sugar composition can be influenced the botanical/geographical origin of honeys. Thus, the concentrations of fructose (F) and glucose (G), in addition to the ratio between them have been proposed as indicators to distinguish honeydew honey from nectar honeys (Terrab et al. 2001; Sanz et al. 2005; Escuredo et al. 2014). In general, honeydew honeys have the lowest mean concentration of both glucose and fructose but present high F/G ratio. However, there are many exceptions, as some nectar honeys also present relatively high F/G ratios, like heather honey (*Calluna vulgaris* L.) (Waś et al. 2011) or *Robinia* and *Thymus* honeys (Persano-Oddo and Piro 2004). Multivariate analysis on physicochemical parameters and sugar composition has also been applied to differentiate blossom and honeydew honeys (Bentabol-Manzanares et al. 2011), as well as attempts to establish correlations between the carbohydrate profiles and the nectar sources (Sanz et al. 2004b; Nozal-Nalda et al. 2005a, b; Senyuva et al. 2009; Ouchemoukh et al. 2010). Using linear Spearman rank correlation, Escuredo et al. (2014) found positive correlations between the contents of maltose with *Rubus* honey and of melezitose with both *C. sativa* and *Rubus* honeys. Different carbohydrate combinations have been suggested to be characteristic of some important honey types in Spain (i.e., *Citrus*, heather, *Eucalyptus*, rosemary, *Echium* and Rosaceae), although they did not always allow an unambiguous classification of the main unifloral sources, possibly as some honey samples assigned as unifloral by pollen analysis might include contributions from other floral sources (de la Fuente et al. 2011). According to Kaškonienė and

Venskutonis (2010), sugar composition would be only a reliable indicator for honey classification in the case of unifloral honeys with high amount of a dominating sugar(s). Other authors conclude that sugar composition alone is not enough to identify the botanical or geographical origin of honeys (Bogdanov and Baumann 1988; Földházi 1994; Kaškonienė et al. 2010). Indeed, references related to geographical discrimination of honey using sugar composition are scarce and not conclusive (Sancho et al. 1991; Sanz et al. 1995; Gómez Bárez et al. 2000; Consonni et al. 2013).

During storage, sugar composition of honey continues to change due to the action of enzymes and temperature. When the honey is stored at temperatures below those in the hive, some of the sugars, especially glucose and fructose, may crystallize. This phenomenon is largely influenced by the relative proportions of the main sugars in honey. Honeys with high F/G ratio, greater than 1.33, do not crystallize for long periods, while honeys with an F/G ratio less than 1.11 crystallize quickly (Smanalieva and Senge 2009). In addition, the ratio between glucose and water contents (G/W) was also proposed in 1975 to predict the granulating behaviour of honey (White 1975), and it has largely been used by other groups all around the world with different satisfaction (Zamora and Chirife 2006). In general, slow crystallization of honey should occur when the G/W ratio is less than 1.7, whereas when the ratio is greater than 2.0 the phenomenon is fast and complete (Dobre et al. 2012). More recently, through the analysis of 136 honeys (including bramble, chestnut, eucalyptus, heather, acacia, lime, rape, sunflower and honeydew), Escuredo et al. (2014) have confirmed that fructose, glucose and moisture contents, and F/G and G/W ratios are the main value indicators to predict the crystallization phenomenon in honey.

3.3 Proteins, Enzymes and Amino Acids

The number of studies on **proteins** in honey samples is relatively low, mainly because of their presence in small amounts, the difficulty in extracting them from the sugar-rich environment, and the complexity of their characterization by conventional approaches (Chua et al. 2013). Most of the studies determine protein contents by general methods such as Bradford's or Kjeldahl's, although only 40–80% of the total nitrogen is in the protein fraction and the rest might be attributed to free amino acids (Azeredo et al. 2003; Escuredo et al. 2013). Gel electrophoresis has been the technique most usually employed to estimate the molecular size of proteins, although it is unable to identify them. A first study carried out by Marshall and Williams (1987) obtained at least 19 protein bands from honey samples on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using silver staining. The same technique was used to separate proteins from Spanish honeys of the Galicia region, observing 12 different bands, some of which were attributed to α- and β-glucosidase, amylase and glucose oxidase (Rodríguez-Otero et al. 1990). A review on protein extraction, separation and characterization by MS spectrometry

was published by Chua et al. (2013). More recently, the same authors compared different extraction/precipitation methods (dialysis, ammonium sulfate and sodium tungstate precipitation) to separate proteins from honey. They found that membrane dialysis provided the highest yield of proteins and also the clearest and highest number of protein bands on a 12% polyacrylamide gel compared with the other two precipitation methods, which might lead to some protein degradation owing to the use of strong chemicals and losses after repeated transfers (Chua et al. 2015).

Honey proteins can originate from nectar and pollen of flowers, and occasionally also from the sap of plants, but more often they derive from secretions of cephalic glands of honeybees that are responsible for the enzymatic breakdown of pollen and nectar. The mean protein content of honey is around 0.2–0.7% with molecular weights ranging from 22 to 75 kDa, although this content is quite variable depending upon different factors, such as the honeybee species, the honeydew-producing insects and the vegetal source of nectar and pollen. Protein concentration ranges 0.2–1.6% in the honey from *Apis mellifera*, and 0.1–3.3% in that from *Apis cerana* (Lee et al. 1998). Regarding the vegetal origin, European honeydew and chestnut honeys have been shown to possess higher protein content (around 1 g of proteins per 100 g of honey) than others, like eucalyptus (0.6%), blackberry or polyfloral honeys (0.7%) (Escuredo et al. 2013). Some types of honey, such as manuka and heather honeys, may present unusually high contents of protein, near to 1.5%, which cause an elevated viscosity that makes their extraction by centrifugation difficult (Crane 1990). Quantitative and qualitative changes in protein composition may occur during the storage of honey by the formation of protein-polyphenolic complexes (Brudzynski et al. 2013).

The main protein identified in honey is the so-called major royal jelly protein 1 (MRJP1), which is similar to apalbumin-1. The average amount of MRJP1 measured by ELISA relating to the total protein content was estimated to be 23.4% (Bilikova and Simuth 2010). This protein shows distinct molecular weights and surface structures depending on the honeybee species, due to differences in the sugar chain length and glycosylation pattern, and this feature can be applied for the discrimination of honey according to the honeybee species (Won et al. 2009). Moreover, up to nine major royal jelly proteins have been detected in honey samples from different botanical and geographical origins (Simuth et al. 2004; Won et al. 2009; Bilikova and Simuth 2010; Rossano et al. 2012).

Trace proteins have been used to discriminate the botanical or geographical origin of honey (Cimpoiu et al. 2013). Baroni et al. (2002) applied immunoblot assays for the analysis of proteins from sunflower and eucalyptus honey, concluding that the pollen proteins could be used as a marker for authentication of floral origin. A method based on rapid protein extraction, MALDI/TOF/MS analysis and transformation of protein mass spectra into barcodes with MALDI Biotyper 1.1 software, was applied to obtain the protein fingerprints and determine the geographical origin in Hawaiian honeys (Wang et al. 2009). SDS-PAGE for protein separation and MS for identification allowed Di Girolamo et al. (2012) to identify up to eight proteins in honey samples of different botanical origins (chestnut, acacia, eucalyptus and orange). The authors concluded that only one

protein was of plant origin (glyceraldehyde-3-phosphate dehydrogenase) whereas the rest belonged to the *Apis mellifera* proteome, five of which were assigned to the MRJP class and the other two as α-glucosidase and defensin-1. Similar observations were made by Rossano et al. (2012) regarding the protein profile of commercial unifloral honeys, i.e., orange, chestnut, eucalyptus and Italian sainfoin (*Hedysarium coronarium* L., commonly known in Italy as "Sulla"). Proteins of plant origin were not detected by 2-D gel electrophoresis, probably because they were mostly degraded by honeybee proteases. However, using bidimensional zymography, a very sensitive technique that allows the detection of the activity of enzymes present in the order of ng, those authors were able to detect proteolytic activities in raw honey samples, suggesting that proteolytic enzymes can significantly modify the honey protein profile. More interestingly, the resulting zymograms were specific for each type of the four unifloral honeys under study. According to the authors, the discovered honey proteases may influence honey properties and quality, and bidimensional zymograms might be useful to distinguish between different honey types, as well as to establish their age and floral origin, thus allowing honey certification (Rossano et al. 2012). Very recently, Valachová et al. (2016) have developed and evaluated a new polyclonal antibody-based competitive ELISA method for the quantification of defensin-1 in honey as a promising approach to verify the authenticity of honey.

Honey protein that originated from bees mostly consists of **enzymes** secreted from the salivary and hypopharyngeal glands of worker bees (Table 3.3). The presence of various enzymes in honey has been known for years (White 1978). The most prominent one is α-glucosidase (invertase or saccharase), present in the hypopharyngeal gland of the forager bee, reaching about 50% of the total protein of the gland, whereas amylase and glucose oxidase were each estimated to account for 2–3% (Ohashi et al. 1999).

Table 3.3 Main honey enzymes and their activities (Belitz et al. 2009)

Enzymes	Activity	
α-Glucosidase (invertase)	Converts sucrose to glucose and fructose (inverts sugar)	7.5–10 g saccharose hydrolyzed by 100 g honey per hour at 40 °C
α- and β-amylase (diastase)	Transform starch to other carbohydrates (dextrins, oligo-, di- and monosaccharides)	16–24 g starch degraded by 100 g honey per hour at 40 °C
Glucose oxidase	Converts glucose to gluconolactone, which in turn yields gluconic acid and hydrogen peroxide	80.8–210 μg H_2O_2 formed per g honey/h
Catalase	Converts hydrogen peroxide to water and oxygen	0–86.8 catalytic activity/ g honey
Acid phosphatase	Removes phosphate from organic phosphates	5.07–13.4 mg P/100 g honey released in 24 h
Proteases	Hydrolyze proteins and polypeptides to yield peptides of lower molecular weight	
Esterases	Break down ester bonds	

Enzyme activities are related with the intensity of the nectar flow, i.e., amount of nectar that flows from the flowers, concentration and composition of the nectar. Honeys from rich nectar sources, such as acacia, often contain low natural enzyme activities (Wehling et al. 2006). Enzyme content can be considerably diminished by honey processing, heating and prolonged storage, so that the measurement of their activities can provide information about the intensity of the heat treatment carried out and the degree of aging of the product (White 1978; Sánchez et al. 2001; Serrano et al. 2007).

α-Glucosidase is an exo-carbohydrase that hydrolyzes the non-reducing terminal α-glucosidic bond and releases α-glucose from the substrate. In the presence of high concentrations of substrate, the enzyme also catalyzes the transferring reaction to form oligosaccharides (Chiba and The Amylase Research Society of Japan 1998). Up to 18 isoenzymes have been described for α-glucosidase, most of them (13 out of 18) have a constant ratio of hydrolysis activity upon disaccharides such as sucrose and maltose (White and Kushnir 1967). α-Glucosidases from both Japanese honeybee (*Apis cerana japonica*) and Western honeybees (*Apis mellifera*) have been purified and characterized showing that diverse isoenzymes are different in molecular size, pH- and temperature-stability, substrate specificity and organ localization (Wongchawalit et al. 2006). α-Glucosidase activity is believed to be responsible for most of the chemical changes taking place during the conversion of nectar to honey by providing the required supersaturated sugar solution (Babacan and Rand 2005).

One of the best known enzymes in honey are amylases or diastase (mixture of amylases). Although their presence in honey has been sometimes associated to pollen or nectar, based on the observation of amylase activity in honey produced by sugar-fed bees, the most accepted theory assumes that honey diastase derives from salivary secretions of bees (Stadelmeier and Bergner 1986; Persano-Oddo et al. 1990). Despite its bee origin, the activity of diastase may vary depending on the honey type. Some unifloral honey (e.g. black locust and citrus honeys) and honeys from tropical regions have a naturally low diastase activity (Flanjak et al. 2016). As for invertase, diastase activity is usually employed as an indicator of freshness and storage duration, and the minimum values for fresh, unprocessed honey are regulated in international and national regulations (Codex Alimentarius 2001; EU Council 2002).

The estimation of the molecular weights of amylases has been matter of discrepancies, possibly due to the ionic interaction between the enzyme and plant proteins present in honey that leads to the formation of multiple forms (Ohashi et al. 1999). A molecular weight of 57,000 Da was estimated by Babacan and Rand (2005) for the major α-amylase in honey following its isolation and purification using a combination of ultrafiltration, ultracentrifugation and ion-exchange chromatography.

Glucose oxidase is another important enzyme incorporated into honey by bees that was already partially purified and characterized in the 1960s (Schepartz and Subers 1964; White and Kushnir 1967). Like invertase and diastase, it plays a role in the formation of honey in the hive through the oxidation of glucose to gluconolactone, which is further transformed to gluconic acid and hydrogen peroxide (H_2O_2), thus contributing to the acidity and the antibiotic activity of honey (White

et al. 1963). Honey also contains catalase that decomposes hydrogen peroxide to water and oxygen, and therefore has an opposite role of glucose oxidase. Unlike this latter, catalase originates from flower pollen (Dustman 1971); therefore, the level of hydrogen peroxide in a honey will depend on how much pollen is collected by bees, the floral source of the pollen and the catalase activity of that pollen (Weston 2000).

The presence of acid phosphatase in honey was first described by Giri (1938). This enzyme is a hydrolase that catalyzes the formation of inorganic phosphates from organic phosphates, whose activity is related to the fermentation processes of honey. Acid phosphatase originates mainly from nectar and pollen and can be used as a parameter for honey characterisation (Alonso-Torre et al. 2006).

Although the enzymes in honey mainly originate from the bee, some relationships have been established between the enzyme activity observed in different honey types and their botanical origin. Most likely the enzyme content is a result of several factors including floral source, abundance of nectar flow, nectar sugar content, temperature, diet and physiological stage of the bee, and strength of the colony (Persano-Oddo et al. 1999). Enzyme activities have been sometimes used to indicate botanical origin of honey, but this is only possible for fresh honeys since the activity decreases after processing and storing honey (Kenjerić et al. 2006; Primorac et al. 2009). Recently, four of the most abundant honey types produced in Croatia (black locust (*Robinia pseudoaccacia* L.), sage (*Salvia officinalis* L.), chestnut (*Castanea sativa* Mill.), and honeydew honey) have been characterised according to the protein and proline content and enzyme activities. The results showed higher proline content and diastase, invertase and glucose-oxidase activities in dark honey types (honeydew and chestnut honeys) than in lighter ones. Although black locust honey showed naturally low enzyme activity, it exhibited the highest acid phosphatase activity among the analysed honey types, while honeydew honey, otherwise known to possess high proline content and enzyme activity, had low total protein content comparable to black locust honey (Flanjak et al. 2016).

Honey also contains free **amino acids** at a level of around 1% (W/W) of their components (Table 3.4). Three possible sources have been considered that may contribute to the amino acid composition in honey: nectar (Baker and Baker 1986; Wunnachit et al. 1992), pollen (Marshall and Williams 1987; Sing and Sing 1996) and bees themselves, especially for proline (Petrov 1974; Ball 2007; Truzzi et al. 2014).

A total of 26 free amino acids have been described in honey. In addition to those listed in Table 3.4, serine (Ser), threonine (Thr), homoserine (Hser), taurine (Tau) and α-aminoadipic acid have also been reported in some honey types (González-Paramás et al. 2006). Proline is the prevalent one, representing between 50–85% of this fraction, followed by phenylalanine (Belitz et al. 2009). Proline content has been used as a criterion for the evaluation of the maturation of honey and, in some cases, of the adulteration with sugar. A minimum value of 180 mg/kg is accepted as limit value for genuine honey (Bogdanov et al. 1999).

During storage some changes in free amino acid contents can occur; Iglesias et al. (2006) indicated that the contents of most of the free amino acids decrease with storage time, with the greatest reduction observed in the first 9 months. In

Table 3.4 Average contents of free amino acids in honey (Belitz et al. 2009)

Amino acid	mg/100 g honey (dry weight basis)	Amino acid	mg/100 g honey (dry weight basis)
Asp (aspartic acid)	3.44	Tyr (tyrosine)	2.58
Asn + Gln (asparagine + glutamine)	11.64	Phe (phenylalanine)	14.75
Glu (glutamic acid)	2.94	β-Ala (β-alanine)	1.06
Pro (proline)	59.65	γ-Abu (γ-aminobutyric acid)	2.15
Gly (glycine)	0.68	Lys (lysine)	0.99
α-Ala (α-alanine)	2.07	Orn (ornithine)	0.26
Cys (cysteine)	0.47	His (histidine)	3.84
Val (valine)	2.00	Trp (tryptophan)	3.84
Met (methionine)	0.33	Arg (arginine)	1.72
Ile (isoleucine)	1.12	Others	24.53
Leu (leucine)	1.03	Total	118.77

contrast, the contents of the amino acids aspartic acid, α-alanine, and proline increased in the first few months of storage, reaching maximum values at 6 months, possibly due to the activity of proteolytic enzymes. A reason for the amino acid decrease could be their involvement in Maillard reactions; actually, the presence of Amadori compounds derived from the amino acids lysine, proline, γ-aminobutyric acid, and arginine has been described in honey (Sanz et al. 2003).

Amino acid analysis involves sample preparation (extraction), derivatization and chromatographic analysis. Although some authors proposed the simple dissolution of honey in water or buffer previous to the analysis (Cotte et al. 2004a, b; Bernal et al. 2005), most of the analytical methods described in the literature point out the isolation of amino acids from honey sugar as an essential step. GC (Gilbert et al. 1981; Pirini et al. 1992) and HPLC (Pawlowska and Armstrong 1994; Bouseta et al. 1996) are the most usual techniques to determine the amino acid profile of honey; in both cases the derivatization step is the main bottleneck of the process. Different reagents such as *o*-phthaldialdehyde (OPA), dansyl chloride (DANS-Cl), 9-fluorenylmethylchloroformate (9-FMOC) and phenylisothiocyanate (PTIC) have been proposed for derivatization before chromatographic analysis. DANS-Cl, PTIC and FMOC react both with primary and secondary amines, but high temperature and long time are required in the case of DANS-Cl; furthermore, its derivatives are rather unstable and each amino acid yields more than one, which complicates the chromatograms. PTIC yields stable and UV-absorbing derivatives, without interfering by-products and detection limits of picomol, however, successive drying steps are required, making the method time consuming and poorly reproducible, especially when the operator is not trained. Reaction with both OPA and FMOC gives rise to highly fluorescent derivatives and, thus, they are advocated when high sensitivity is needed. Nevertheless, the production of fluorenylmethylchloroformate, an alcoholic derivative, is generally reported as a major drawback when this reagent is used. On the other hand, secondary amino groups, such as those of proline and

hydroxyproline, do not react with OPA in the presence of 2-mercaptoethanol (MCE), so that an appropriate control of both time of reaction and injection is crucial for the reproducibility (González-Paramás et al. 2006). To overcome these drawbacks, Rebane and Herodes (2010) developed a method consisting of solid phase extraction, derivatization with diethyl ethoxymethylenemalonate (DEEMM) and liquid chromatography with ultraviolet and mass spectrometric detection that applied to the analysis of amino acids in Estonian honeys. More recently, other techniques like NMR have been used for the analysis of amino acids in honey (Boffo et al. 2012; Mannina et al. 2015).

Since pollen is one of the most important sources of amino acids in honey, different approaches based on the use of free amino acid profiles have been suggested for the discrimination of honeys according to their botanical and/or geographical origin. Early contributions in this respect were made by Davies (1975, 1976) who, following analysis of the amino acid profile in 98 honeys from different botanical sources coming from six geographical origins, proposed the ratios aspartic acid/proline and amides/phenylalanine to distinguish samples from different countries. Conte et al. (1998) analysed the amino acid composition of 92 honey samples from 17 botanical and 4 different geographical origins; although some trends could be observed for thyme (high levels of Ser, Tyr and Lys) and rosemary honeys (Tyr was the most abundant), they concluded that the application of linear discriminate analysis including other parameters than amino acids was required for distinguishing honeys. In contrast, the application of statistical methods to the contents of 13 amino acids determined in 56 honey samples from three different regions in Argentina, allowed Cometto et al. (2003) to cluster the samples into nine groups related to the collecting regions and more strictly to the flora around apiaries. They concluded that between five and seven amino acids, selected by principal component analysis (PCA), could be sufficient to verify both the botanical or geographic origin of honey. Canonical variate analysis (CVA) was also used to differentiate samples from different countries (Argentina, Australia, UK and Canada) based on their amino acid profile (Gilbert et al. 1981). Senyuva et al. (2009) suggested that phenylalanine and tyrosine contents might contribute to distinguishing honeys, with valine, leucine, and isoleucine being other important amino acids for recognizing botanical and geographic origins.

Regarding the botanical origin, in a study carried out by Pirini et al. (1992) on different types of honeys (acacia, citrus, chestnut, rhododendron, rosemary and lime), the presence of some amino acids like arginine, tryptophan or cystine was found to be characteristic for some honeys, although a single amino acid or a group of amino acids could not be selected as suitable for the recognition of a particular kind of honey. Hermosín et al. (2003) described that lavender honey from Spain contains a high concentration of tyrosine, while Cotte et al. (2004a, b), analyzing samples from France, pointed to high amounts of threonine and phenylalanine as a characteristic of sunflower and lavender honeys. Amino acids profiles, especially glutamic acid and tryptophan, have been suggested useful for the discrimination of honeydew honey and floral honey (Iglesias et al. 2004). In another study, 40 monovarietal honeydew (ilex and oak) or nectar (heather and chestnut-tree) samples were analyzed for their free amino acid profiles. Proline was always the principal

amino acid, followed by γ-aminobutyric acid in chestnut-tree (22.4%) and oak (7.2%), asparagine in ilex, and phenylalanine in heather honeys. The discrimination between ilex and oak honey is of particular interest since neither the melissopalynological analysis—absence of pollen—nor the physico-chemical one enables their differentiation (González-Paramás et al. 2006). More recently, lysine, arginine and histidine were reported as majority amino acids in honeydew honey, in contrast with rhododendron honeys where the main detected one was aspartic acid (Silici and Karaman 2014). The free amino acids profile of 192 samples of seven different floral types of Serbian honey (acacia, linden, sunflower, rape, basil, giant goldenrod and buckwheat) from six different regions was analysed in order to distinguish honeys by their botanical origin. Principal component analysis revealed that basil honey samples form a well-defined cluster imposed with phenylalanine content. Furthermore, the model obtained by linear discriminant analysis could be used to distinguish basil honey from the rest of the samples, and also showed moderate predictive power to separate genuine acacia, linden, sunflower and rape honeys (Ješckeš et al. 2013). In honeys from Poland, high concentrations of aspartic acid and asparagine were suggested as an indicator of raspberry and buckwheat honeys (Janiszewska et al. 2012). The application of combined NMR and chemometric data analysis approach allowed the characterization of wildflower honeys based on their higher concentration of phenylalanine and tyrosine (Boffo et al. 2012).

In summary, amino acid profiles might on occasion be useful for the determination of the botanical and/or geographical origin and more suitable than protein composition. However, the methods must be employed in conjunction with other techniques, namely statistical analysis, in order to obtain a reliable characterization (Anklam 1998; Kaškonienė and Venskutonis 2010).

3.4 Organic Acids

Organic acids are present in honey at levels around 0.5–0.6% and contribute to its organoleptic and physico-chemical properties (acidity, pH and electrical conductivity) (Mato et al. 2006). These acids are obtained directly from nectar or derived from sugars by the activity of enzymes secreted by honeybees when transforming nectar into honey. Many authors have reported the increase of acidity over time, as well as during fermentation as a result of the transformation of honey sugars and alcohols into acids by the action of honey yeasts (Cavia et al. 2007).

Up to 32 organic acids have been identified in honey including acetic, butyric, citric, formic, fumaric, glyoxylic, propionic, lactic, maleic, malic, oxalic and succinic acids (Wilkins et al. 1995). Although formic and citric acids were originally described as predominant, it is now clear that gluconic acid (produced by the action of glucose oxidase on glucose) is the majority acid in honey, with some exceptions like fir honey, where galacturonic acid has been indicated as the main acid (Daniele et al. 2012).

Organic acids have been traditionally analyzed in honey by enzymatic methods (Tourn et al. 1980; Mato et al. 1998) but also techniques like gas chromatography

(Wilkins et al. 1995; Pilz-Güther and Speer 2004) and liquid chromatography (Cherchi et al. 1994; Del Nozal et al. 1998; Suárez-Luque et al. 2002). The advantages of enzymatic methods are sensitivity, specificity and simple instrumentation; however, only one by one acid can be determined. In order to analyse organic acid profiles, chromatographic techniques have to be used, although they also present drawbacks. Thus, gas chromatography requires a derivatization process, because most organic acids are non-volatile, whereas sugar interferences occur in liquid chromatography, so that honey pre-treatment techniques (solid-phase extraction, columns connected in series) are needed to eliminate matrix interferences and improve organic acid separation (Mato et al. 2007). Methods based on capillary zone electrophoresis (CZE) have also been applied for the separation of low molecular weight organic acids, having the advantages of good resolution, simplicity, short analysis times, low consumption of chemicals and minimal sample preparation (Navarrete et al. 2015; Mato et al. 2007).

The interest of organic acids in honey is diverse and includes their antibacterial and antioxidant activities, their utility as possible indicators of incipient fermentation and the potential use for treatment of Varroa infestation. Organic acids have been deployed to discriminate honeys according to their botanical and/or geographical origins. Daniele et al. (2012) proposed a qualitative and quantitative method, based on ion chromatography with electrochemical detection followed by isotope ratio mass spectrometry (IRMS) determination of $^{13}C/^{12}C$, to analyze organic acids in honeys. The principal components analysis of the results obtained regarding the content of 14 organic acids enabled good discrimination among monofloral honeys from seven botanical origins (acacia, chestnut, rapeseed, lavender, fir, linden and sunflower). Even though galacturonic acid has been indicated to be the main acid in fir honeys, through the PCA analysis these honeys could be easily distinguished from the other varieties by their higher contents of pyruvic and citric acids, whereas pyroglutamic acid allowed to distinguish chestnut honey (Daniele et al. 2012). The authors also suggested that the organic acids could be used as internal standards instead of proteins in order to detect honey adulteration, especially when ultrafiltration is employed in honey processing.

^{1}H-NMR analysis of Polish multifloral and monofloral honeys (heather, buckwheat, lime, rape and acacia) allowed their differentiation according to the botanical origin with the only exception of acacia's. Based on the results, the authors proposed phenylacetic acid and dehydrovomifoliol as markers for heather honey, formic acid and tyrosine for buckwheat honey, and 4-(hydroxy-1-methylethyl) cyclohexane-1,3-dienecarboxylic acid for lime honey (Zieliński et al. 2014).

3.5 Mineral and Trace Elements

Minerals originate in the soil, are transported into plants through the roots and get into honey via the nectar or the honeydew (Anklam 1998), although they may also come from anthropogenic sources, such as environmental pollution, or be

Table 3.5 Principal mineral elements described in honey (based on Crane 1990; Solayman et al. 2016)

Majority minerals	Average contents in light to dark honey (ppm)	Trace elements
Potassium (K)	40–1350	Copper (Cu)
Chlorine (Cl)	52–427	Chromium (Cr)
Sulphur (S)	15–100	Lithium (Li)
Sodium (Na)	3–237	Nickel (Ni)
Calcium (Ca)	5–218	Lead (Pb)
Phosphorus (P)	29–119	Tin (Sn)
Magnesium (Mg)	2–564	Osmium (Os)
Silicon (as SiO_2)	9–41	Beryllium (Be)
Iron (Fe)	0.4–224	Vanadium (V)
Zinc (Zn)	0.2–74	Zirconium (Zr)
Manganese (Mn)	0.3–4	Silver (Ag)
		Barium (Ba)
		Gallium (Ga)
		Bismuth (Bi)
		Gold (Au)
		Germanium (Ge)
		Strontium (Sr)

influenced by beekeeping practices and honey processing (Bogdanov et al. 2007; Pohl 2009; Silici et al. 2016). Table 3.5 compiles the main mineral elements described in honey; most of them are metals, some of which are present only in minute amounts (i.e., trace elements).

Different techniques can be used to analyze minerals, metals and non-metals, in honey samples. The usual analytical approach includes an acid digestion of the sample and the use of diverse spectrometry techniques, such as flame emission (FES) and flame atomic absorption (FAAS), graphite furnace atomic absorption (GF-AAS), electrothermal atomic absorption (ET-AAS), hydride generation-atomic fluorescence (HG-AAS), inductively coupled plasma optical emission (ICP-OES) and inductively coupled plasma mass spectrometry (ICP-MS). FAAS seems to be the preferred technique used routinely in the analysis of major minerals and some minor and trace elements. This is because its operational costs are low and the technique offers a reasonably good analytical performance, including instrumental detection limits. However, to eliminate possible chemical and physical interferences and especially those related to the high sugar concentration, samples of honey must be mineralized before the analysis, which can be made by either acid digestion or ignition. In addition, the calibration is only possible in a narrow concentration range and frequently sequential dilutions of samples before measurements are necessary (Pohl et al. 2012). Different aspects of the analysis of minerals in honey, including sample preparation, calibration and the quality assurance of results have been recently reviewed (Pohl et al. 2012; Solayman et al. 2016). In the last years ICP-MS has gained large consensus because of its advantages over other techniques, like multi-elemental measurements endowed with very low detection limits (Consonni

and Cagliani 2015). This technique has been applied to investigate the mineral profile of honeys from different countries like Brazil (Batista et al. 2012), China (Chen et al. 2014) or Poland (Chudzinska and Baralkiewicz 2011), and also for the quantification of Cd and Pb in honey under an environmental point of view (Frazzoli et al. 2007). Total Reflection X-Ray Fluorescence (TXRF) was used for mineral analysis in Brazilian honeys, allowing the simultaneous detection of up to 12 elements (Ribeiro et al. 2014). Capillary electrophoresis has also been applied to determine the content of cations (K^+, Na^+, Ca^{2+}, Mg^{2+} and Mn^{2+}) in honeys produced in different regions of Brazil (Rizelio et al. 2012).

The total weight of the mineral elements in honey (total ash) varies within a wide range of values (0.02–1%), the largest of any honey component (Silva et al. 2009), and has been systematically investigated in honey with the aim to certify their botanical or geographical origin. Up to 54 minerals have been reported in honey; the research presented by Batista et al. (2012) possibly being the most complete one, describing the presence of 42 chemical elements in Brazilian honeys. Almost all macrominerals were commonly found in honeys from all countries, which the exception of Cl, which was only detected in honey samples from Spain (Solayman et al. 2016). Among them, K was the predominant one representing one-half to three-quarters of total mineral content (Abu-Tarboush et al. 1993; Terrab et al. 2004; Kadri et al. 2017), followed by Ca, Na or Mg depending on the honey types (González-Paramás et al. 2000; Atanassova et al. 2012; Mondragón-Cortez et al. 2013). Other minerals such as Fe, Si and S are present in intermediate amounts (around 0.05 mg/g). Trace and ultratrace elements are present at levels below 1 μg/g and apparently when some of them (e.g., Cd, Cu, Co, Ni or Pb) are not detected in honey, it is an indication that no relevant contamination has happened during processing, shipping and storage due to its contact with steel or galvanized containers (Baroni et al. 2009). Updated tables with bibliographic data of mineral composition of honeys from different countries have been compiled by Solayman et al. (2016).

Due to differences in soils, floral source, and nectar and pollen composition, some minerals cannot be detected in some types of honey collected from certain areas whereas they are common in others. Therefore, the mineral composition of honey can actually be used to identify its geographical origin. One of the first works in this sense was done by Morse and Lisk (1980) who studied the elemental composition of honeys from different countries (USA, Mexico, El Salvador and China) and found significant differences among them. Feller-Demalsy et al. (1989) undertook an ambitious study to differentiate honeys from the ten Canadian provinces by application of principal component analysis to the data of 20 mineral elements, achieving 92% correct classification; they found that these differences were independent of the floral origin of the honeys and seemed to be very complex, probably since they were related to maritime influence coupled with the extent of rainfall rather than being dependent upon soil composition. The provenance of honey samples from northern and southern Argentina was also successfully evaluated by Baroni et al. (2009) by the application of SDA and κ-nearest-neighbor (κ-NN) to their mineral profiles and chemical properties. Similarly, multi-element analysis

was applied to the classification of Brazilian honey samples according to the region based on their trace element composition, using chemometric tools (Batista et al. 2012). The mineral composition has also been used to typify honeys from different regions of Spain. The contents of Na, K, Ca, Mg, Cu, Fe, Mn, P, Cl, Si and S were determined in samples of raw honeys from Galicia, concluding that they showed, in general, higher mineral contents than those reported in the literature for other Spanish regions (Rodriguez-Otero et al. 1994). In another study, 60 honey samples from six different production zones in western Spain (provinces of Salamanca, Zamora and Caceres) were analysed for 13 common legal physicochemical parameters and 17 mineral elements (13 cations and 4 anions); application of linear stepwise discriminant analysis allowed discrimination of honeys from all six zones with a result of 91.38% of correctly classified samples (González-Paramás et al. 2000). Similar approaches were used to characterize honeys from the Canary Islands (Hernández et al. 2005) and Andalusia under the protected designation of origin "Miel de Granada" (de Alda-Garcilope et al. 2012). The levels of 14 minerals were investigated in 23 varieties of honey from Saudi Arabia and six other countries, although in this case no correlation between geographical origin and composition was tried (Alqarni et al. 2014).

Although the contents of macro- and microelements reflect rather the geographical provenance and the environmental contamination in the respective locations than the botanical origin (Atanassova et al. 2012), some attempts have also been made to use minerals to classify honeys according to the plant origin. Thus, Nozal-Nalda et al. (2005a, b) achieved a global success rate close to 90% in the classification of ling (*Calluna vulgaris*), heather (*Erica* sp.), rosemary (*Rosmarinus officinalis*), thyme (*Thymus vulgaris*), lavender (*Lavandula* sp.), and oak (*Quercus* sp.) honeydew honeys by applying discriminant analysis to their metal content data (15 minerals) and other common physicochemical parameters. Similarly, the concentrations of Zn, Mn, Mg and Na were reported to be strongly dependent on the botanical origin in eucalyptus, heather, orange blossom and rosemary honeys from Spain (Fernandez-Torres et al. 2005). Based on their trace element profile, honeydew, buckwheat and rape honeys from Poland were correctly classified as honeydew or nectar samples (Chudzinska and Baralkiewicz 2010). Twelve mineral elements were determined by ICP-MS in 163 Chinese honey samples from different plant sources (linden, vitex, rape and acacia), allowing their classification according to the botanical origin by application of principal component analysis, which reduced the variables to four principal components and could explain 93.06% of the total variance (Chen et al. 2014). Other authors, however, could not find statistically significant differences among honey samples of different botanical origin (tualang, gelam, acacia and forest honeys) based on two-factor ANOVA and cluster analysis, even though the mineral and trace element composition could vary depending on the type of honey (Chua et al. 2012).

Mineral content is frequently correlated with colour of honey. Dark and amber honeys contain higher amounts of certain major, minor and trace elements when compared to pale-coloured honeys (González-Miret et al. 2005; Pisani et al. 2008). Positive correlation between the mineral content of honey and its electrical conduc-

tivity has also been demonstrated (Bogdanov et al. 2007; Silva et al. 2009; Kropf et al. 2008; Vanhanen et al. 2011), as well as their contribution to the intracellular redox balance (Alvarez-Suarez et al. 2012). Interestingly, Perna et al. (2012) found a statistically significant correlation ($P < 0.05$) between metal content and both phenolic content and antioxidant activity.

In summary, the analysis of trace element profiles in combination with modern statistical data evaluation techniques could be suitable for the characterization of geographical and/or botanical origin of honey (Anklam 1998). However, some risk should be carefully considered when using microelements for the authentication of honey. Thus, whereas most minerals are of floral and soil origin, some metals, particularly Pb and Cd, may be present in honey as a result of the environmental contamination and, therefore, their concentration may be more dependent on contamination than on the floral or geographical origin of the honey (Kaškonienė and Venskutonis 2010).

3.6 Vitamins

Honey contains small but detectable quantities of vitamins, mostly water-soluble owing to the aqueous nature of the matrix, although it should not be considered a good source of vitamins at all, because their concentrations are in the parts per million (ppm) ranges. Most of the vitamins are from the pollen grains present in suspension, so that commercial filtration of honeys dramatically reduces their contents due to the almost complete removal of pollen (Ciulu et al. 2011).

Ascorbic acid is the predominant vitamin and it is usually found in almost all types of honey; mean contents have been indicated to be around 2 mg/100 g (Alvarez-Suarez et al. 2010a). Vitamin C analysis is one of the indicators often used to evaluate the nutritional quality of a food, since it is very vulnerable to chemical and enzymatic oxidation, further accelerated by factors such as light, oxygen or heat (León-Ruiz et al. 2013). Different B complex vitamins are also present in honey, including thiamine (B1), riboflavin (B2), nicotinic acid (B3), pantothenic acid (B5), biotin (B8 or H) and folic acid (B9).

In general, the analytical methods are addressed to the determination of only one specific vitamin or a few of them, whereas the simultaneous analysis of all water-soluble vitamins in honey is infrequent. Viñas et al. (2004) determined vitamins B6 and B2 in different types of honey. Vitamin B6, as pyridoxine, was found in one eucalyptus honey and in a multifloral honey at levels lower than 0.021 mg/100 g, whereas it was not detected in rosemary or orange blossom honey. Riboflavin vitamers were detected in chestnut tree, eucalyptus, cane and multifloral honeys at levels up to 0.074 mg/100 g. Ciulu et al. (2011) optimised a gradient RP-HPLC method to analyse vitamins C, B2, B3, B5 and B9 in 28 honey samples (mainly from Sardinia, Italy). The presence of vitamin B3 was detected in all the studied samples, although in some of them the concentration was below the limit of quantification. Vitamins B2 and B9 were quantified in about half of the samples, whereas vitamin

B5 was much less common than the others. Neither vitamin B1 nor B6, which often appear in honey, were determined by the proposed method. Interestingly, although the concentration of water-soluble vitamins in honey was very low, there might be a potential correlation with the origin of honeys, in particular vitamin B3 that seemed to be strongly dependent on the botanical origin. An isocratic RP-HPLC method was developed by León-Ruiz et al. (2013) for the quantification of seven water-soluble vitamins (B1, B2, B3H, B3N, B5, B6 and C) that was applied to their analysis in different monofloral honeys (rosemary, thyme, lavender, chestnut, echium) and a honeydew honey. The results confirmed the presence of vitamin C in all samples, highlighting the thyme honeys with a content ten times higher than the other ones. Vitamin B1 was also found in all honey samples with the exception of rosemary. In samples belonging to the Protected Denomination of Origin "La Alcarria Honey" (Spain), only vitamin C was detected in rosemary's, and B1, B3H and C in lavender's honeys (León-Ruiz et al. 2011).

3.7 Aroma Compounds

Volatile and semivolatile compounds are present in honeys and are responsible for aroma and flavour qualities. The honey flavour is produced by complex mixtures of volatile compounds, which may differ depending on the nectar or honeydew, conversion by honeybees of plant constituents into other compounds with volatile properties, processing conditions, especially heating, and storage (da Silva et al. 2016). The changes in the volatile fraction occurring in honey during the storage have been attributed to two principal causes: compounds that are labile and may be destroyed, and volatile compounds produced by non-enzymatic browning (Maillard reaction) (Wootton et al. 1978; Castro-Vázquez et al. 2008; Moreira et al. 2010)

The isolation of volatile components from honey in order to obtain representative extracts is challenging, as it contains many volatile components with different chemical structures, at a low concentration and in a sugar matrix where polar substances are the major components (Castro-Várquez et al. 2003). Initially, one of the most popular techniques to isolate volatile compounds from complex matrices was the simultaneous distillation-extraction (SDE) system developed by Likens and Nickerson (1964) and its modified version (Godefroot et al. 1981). However, the protocol of this technique includes heat treatment of the sample, which can lead to a cooked honey flavour (furfural and hydroxymethylfurfural are formed). For that reason, an under vacuum SDE protocol providing furfural free extracts with a fresh honey note was developed (Maignial et al. 1992) and further optimized (Bouseta and Collin 1995). Another approach used for the isolation of volatiles is ultrasonic solvent extraction (USE) that significantly reduces the extraction time and improves extraction efficiency, allowing the isolation of both low and high molecular weight compounds. However, the solvents usually employed (n-hexane, acetone, chloroform, pentane…) are toxic and/or expensive and also solubilise non-volatile compounds which frequently contaminate the Gas Chromatography port (Alissandrakis

et al. 2005; Cuevas-Glory et al. 2007). An improved protocol for the isolation of the volatile fraction from honey based on the extraction with chloroform and n-hexane solvents and subsequent fractionation and purification of extracts by silica gel chromatography was proposed by Manyi-Loh et al. (2010).

In order to avoid the disadvantages of SDE and USE solvent extraction other techniques, like headspace (HS) and solid phase extraction and micro-extraction (SPE, SPME), have been developed. Among them, SPME is the preferred one because it allows the extraction of a large number of molecules, with little or no preparation of samples, and can be easily coupled to analytical instruments like GC, GC-MS, HPLC, LC-MS. SPME sampling can be performed by direct extraction, extraction with membrane protection and headspace extraction, which looks like the most appropriate one since it protects the fiber from adverse compounds (namely sugars) (Pontes et al. 2007). Nevertheless, the efficiency of SPME technique is affected by some factors such as fiber coating, sample amount, matrix modification by water and sodium chloride addition, agitation and temperature, extraction time and analyte desorption (Peña et al. 2004). Different fiber supports (polydimethylsiloxane (PDMS)-coated fiber, polyacrylate (PAC)-coated fiber, carboxen-coated fiber, PDMS/divinlybenzene) have been used in the extraction of honey volatiles, divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) being proposed as the best option when HS-SPME is used (Plutowska et al. 2011). Selected ion flow tube mass spectrometric (SIFTMS) is another technique designed for rapid identification of volatile compounds with minimal sample preparation. This technique performs real-time analysis of complex volatile mixtures by utilizing three positive precursor ions H_3O^+, NO^+, and O_2^+ to ionize volatile compounds. In association with chemometric tools (e.g., soft independent modelling of class analogy, SIMCA), SIFTMS was used to differentiate diverse unifloral honeys from Ohio and Indiana based on their volatile composition (Agila and Barringer 2012). Methods based on electronic noses have also been employed as simple and sensitive alternatives for fast aroma fingerprinting in foods (Ampuero et al. 2004; Lammertyn et al. 2004). Nevertheless, they do not provide actual identification of the individual volatile compounds that constitute the food aroma. Recently, gas chromatography (GC) combined with a hybrid quadrupole time-of-flight (QTOF) mass spectrometry (MS) system was used for the determination of volatile and semi-volatile compounds in honey samples. The method was applied to 19 honey samples where up to 84 compounds could be identified, some of them with the same nominal masses but having different empirical formulae (Moniruzzaman et al. 2014).

More than 600 volatile compounds, originated from various biosynthetic pathways, have been described in honey. Their chemical families include: hydrocarbons, aldehydes, superior alcohols, ketones, fatty and other carboxylic acids, esters, benzene and its derivatives (furan and pyran), norisoprenoids, sesquiterpenes and its derivatives, and sulphur and cyclic compounds (Manyi-Loh et al. 2011a, b). The length of the carboxylic acid carbon chains provides different flavours that can range from spicy to rancid. The short chain carboxylic acids, such as acetic acid, have a spicy aroma and flavour, while butanoic and hexanoic acids provide a rancid aroma (Barra et al. 2010). Benzene derivatives are found at high concentrations in

all honeys; these include benzaldehyde, benzyl alcohol, 2-phenylethanol and phenylacetaldehyde (Radovic et al. 2001). Compounds such as acetic acid (de la Fuente et al. 2005) and acetone (Pérez et al. 2002) are also found at important levels in multiple types of honeys. There are, however, volatile compounds that could be used to discriminate monofloral honeys from different floral origins, and the diversity of volatile compounds that may occur in honey also gives rise to distinct profiles that might represent fingerprints for particular types of honey (Manyi-Loh et al. 2011a, b). For instance, the presence of important levels of benzaldehyde and furfural has been reported in clover, blueberry, wildflower (Overton and Manura 1994) and thistle honeys (Bianchi et al. 2011). Methyl anthranilate, sinensal isomers, lilac alcohol, lilac aldehyde, limonene diol and 1-*p*-menthen-9-al have been proposed as characteristic of citrus honey (Piasenzotto et al. 2003; Alissandrakis et al. 2005; Castro-Vázquez et al. 2009). In chestnut honey, Bonaga et al. (1986) identified 3-aminoacetophenone as the main component of the volatile fraction and suggested it as a specific marker of this type of honey. Radovic et al. (2001) proposed 4-methyl acetophenone as a candidate marker for lime tree honeys, which was later confirmed by Piasenzotto et al. (2003) and Wardencki et al. (2009). 3,9-Epoxy-1-*p*-mentadieno, t-8-*p*-menthan-oxide-1,2-diol and *cis*-rose have been indicated as markers for lemon honey (Castro-Vázquez et al. 2007). Diketones, sulphur compounds and nonanol, nonanal and nonanoic acid are typical of eucalyptus honey. Hexanal and heptanal are the main compounds in the aroma of lavender honeys (Radovic et al. 2001), although nerolidol oxide was proposed to typify lavender honey from Spain (Castro-Vázquez et al. 2009). Vomifoliol and coumarin were indicated to be characteristic of *Prunus mahaleb* L. honey, while methyl-4-hydroxy-3,5-dimethoxybenzoate could be a potential marker for asphodel honey (Jerković et al. 2011a, b). More than 300 compounds were identified in honeydew honey from Slovakia, four of which (2,3-butanediol, 3-hydroxy-2-butanone, acetic acid and methyl ester of 2-hydroxibenzoic acid) were established as markers of that type of honey (Jànošková et al. 2014). Previously, methyl butanal and lilac aldehyde had been proposed as marker compounds in Polish honeydew honey (Wardencki et al. 2009), and n-decane, nonanal, dimethylphenyl acetate, nonanol and 2-methyl heptanoic acid in Turkeys honeydew honey (Senyuva et al. 2009). High acetic acid concentrations have also been reported to be characteristic of honeydew honeys, although its formation by microbial metabolism cannot be ruled out, so that it would not be a good marker compound (Campos et al. 2000). Other examples of volatile compounds proposed as characteristic of some unifloral honeys are given in Table 3.6.

Despite all those observations, there is disagreement about which compounds could actually serve as floral markers for a given honey type, owing to the differences among plant varieties, geographical origins and beekeeping practices. According to the published data, only a few types of honeys might contain a characteristic compound, while the majority of honey samples would be characterized by several compounds (Kaškonienė and Venskutonis 2010). Clearly, the focus to choose markers for discrimination of honey's floral origins must be put on plant-derived compounds and their metabolites, such as benzene, terpenes, and its derivatives, and norisoprenoids (Manyi-Loh et al. 2011a, b). Possibly, further developments

Table 3.6 Specific volatile compounds that proposed to typify unifloral honeys

Honey source	Geographical origin	Compound	Reference
Acacia	Poland	Hexanal	Wardencki et al. (2009)
	European countries	*cis*-Linalool oxide and heptanal	Radovic et al. (2001)
Eucalyptus	Spain	3-Caren-2-ol, *p*-cymene and its derivate alcohol	Castro-Vázquez et al. (2009)
		Acetoin, diacetyl, 2,3-pentanedione, dimethyldisulfide	de la Fuente et al. (2005)
	Turkey	Nonanal, ethylphenyl acetate, phenethyl alcohol	Senyuva et al. (2009)
	European countries	1-Octene or 2,3-pentanedione	Radovic et al. (2001)
	Italy	Nonanoic acid and acetoin	Piasenzotto et al. (2003)
Thyme	Italy	Ethenyl phenyl acetate	Piasenzotto et al. (2003)
	Turkey	3,4,5-Trimethoxybenzaldehyde	Mannas and Altug (2007)
	Greek	1-Phenyl-2,3-butanedione, 3-hydroxy-4-phenyl-2-butanone, 3-hydroxy-1-phenyl-2-butanone, phenylacetonitrile, and carvacrol	Alissandrakis et al. (2007)
Pine	Turkey	3-Carene and another unidentified compound	Tananaki et al. 2007
		Nonanal, benzene, 4-hexen-3-ol, alpha-pinene and 2-heptanone	Silici (2011)
Sunflower	European countries	α-Pinene or 3-methyl-2-butanol	Radovic et al. (2001)
	Slovakia	*trans*-Furanoid linalool	Pazitna et al. (2012)
Buckwheat	Poland	Pentanal, furfural and 2-ethylhexanol	Wardencki et al. (2009)
	USA and England	3-Methylbutanal, 3-hydroxy-4,5-dimethyl-2(5H)-furanone (sotolon), β-damascenone	Zhou et al. (2002)
Fir honeydew	Hungary	Acetonitrile, methyl-2-buten-1-ol, n-hexanol, 3-hexanol, 1-propyne, 2-furanmethanol, 5-methyl-2(5H)-furanone, 4-methylphenol, hexadecanoic acid, methylheptanoate	Lušić et al. (2007)
Oak honeydew	Spain	*trans*-Oak lactone, aminoacetophenone, propylanisol	Castro-Vázquez et al. (2006)

in the analytical techniques could enable the identification and quantification of very minor volatile constituents in honey that might be specific and provide more accurate information on the floral source. The reliability of the results may also be improved by increasing the amount of samples and comparing with honeys from other floral origins. Nevertheless, it should also be taken into account that volatile compounds present in very low concentrations may be the result of contribution of

co-occurring minor floral nectars and could therefore be misinterpreted (Kaškonienė and Venskutonis 2010).

Volatile compounds have also been proposed for the geographical discrimination of honey samples. Radovic et al. (2001) analysed honey samples from England, Denmark, Spain and Portugal, and suggested the existence of some typical patterns, such as the presence of 1-penten-3-ol in English honeys or the absence of 3-methylbutanal in Danish ones, which could be used as a geographical markers. Nevertheless, the limited number of analysed samples makes those results tentative. The influence of the geographical origin in Spanish chestnut honeys was investigated by Castro-Vázquez et al. (2010) through the comparison of their sensory characteristic and the volatile composition; application of principal component analysis to the whole data allowed successful differentiation among samples from north-east, north-west and south-east areas of Spain.

3.8 Phenolic Compounds

Phenolic compounds are one of the largest groups of plant secondary metabolites, biosynthesized mainly for protection against biotic and abiotic stress and oxidative damage, and transferred via the nectar to honey. The phenolic compounds of honey can be classified into two main families: phenolic acids and flavonoids.

Honey may contain various aromatic and aryl-aliphatic carboxylic acids, mainly hydroxyl and methoxy-derivatives of benzoic and cinnamic acid (Fig. 3.1) that contribute to their sensory properties (Anklam 1998). Among others, the presence of benzoic, hydroxybenzoic, salicylic, gallic, vanillic, syringic, protocatechuic, caf-

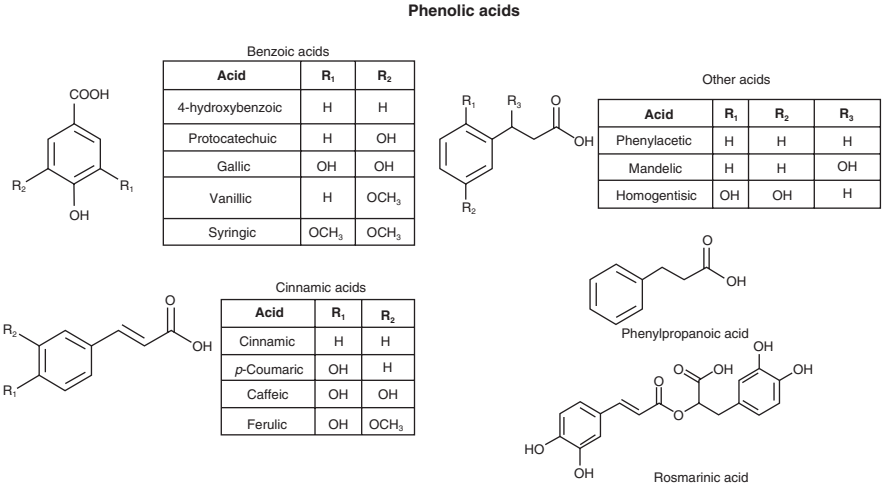


Fig. 3.1 Structures of the principal phenolic acids described in honey

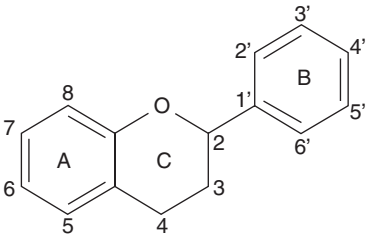


Fig. 3.2 Phenylchromane skeleton

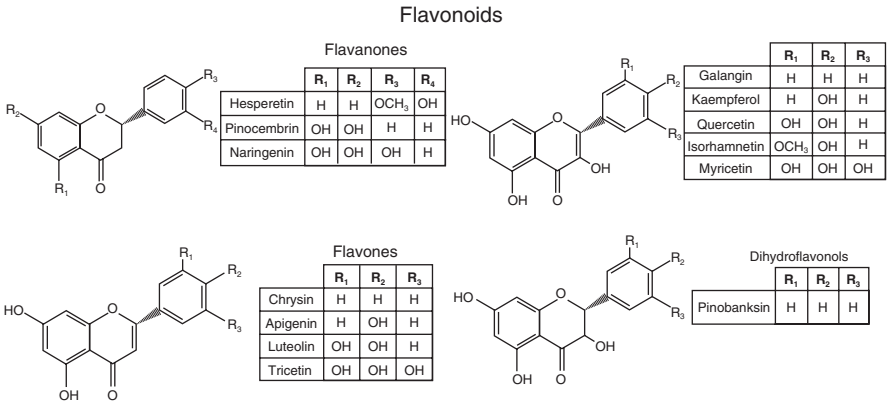


Fig. 3.3 Structures of the principal flavonoids described in honey

feic, coumaric, ferulic, chlorogenic, phenylacetic and rosmarinic acids has been described in honey (Dimitrova et al. 2007; Trautvetter et al. 2009).

The flavonoid family comprises several thousand compounds sharing a common C6-C3-C6 phenylchromane skeleton (Fig. 3.2). Based on the oxidation level of the ring C different flavonoids classes are distinguished: flavones, flavonols, flavanones, flavan-3-ols, anthocyanins, dihydroflavonols and isoflavones, although in honey mainly flavanones, flavones, flavonols and dihydroflavonols have been identified (Fig. 3.3), such as pinocembrin, chrysin, galangin, pinobanksin, apigenin, genistein, quercetin, kaempferol and myricetin (Tomás-Barberán et al. 2001). Although during years it was assumed that only flavonoid aglycones were present in honey, today it is well established that also *C*- and *O*-glycosyl derivatives are present (Truchado et al. 2011).

The total phenolic content in honey, calculated using unspecific methods like Folin-Ciocalteu, has been estimated to range from 20 to 193 mg gallic acid equivalents (GAE)/100 g of honey, and the flavonoid content from 1.1 to 7.5 mg quercetin equivalents (QE)/100 g (Pontis et al. 2014; Ciappini and Stoppani 2014; Alvarez-Suarez et al. 2010a).

Phenolic compounds, as well as other organic compounds, are degraded in honey depending on the environmental conditions to which they are subjected. For instance, some of the flavonoid glycosides are not stable under slightly alkaline

conditions and are sensible to oxidation in the presence of oxidizing agents like hydrogen peroxide present in honey (Truchado et al. 2008). However, in general terms, the impact of industrial processing, including thermal treatments and storage, on the polyphenolic profile is less important than the logical variability due to the year of harvesting (as a consequence of environmental factors) and botanical origin (Escriche et al. 2014).

In general, analytical determination of phenolic compounds involves several steps such as isolation from the sample matrix using solvent or solid-phase extraction (SPE), analytical separation, commonly achieved by HPLC or capillary electrophoresis, identification, usually by UV-Vis and/or mass spectrometry, and quantification. Particular attention must be paid to the extraction and clean-up procedures in order to guarantee an increased concentration of phenolic acids and flavonoids and the removal of any potential interfering compounds, especially sugars and other polar substances (Ciulu et al. 2016). When the interest is focused primarily on aglycones, liquid-liquid extraction using ethyl acetate or ethanol is frequent. In the case of ethyl acetate, repeated extractions with the solvent are performed on a solution obtained dissolving honey in pure water (Tuberoso et al. 2009) or in a 2% NaCl aqueous solution (Kečkeš et al. 2013). More recently, different authors have used dispersive liquid-liquid microextraction to extract phenolic compounds from honeys, obtaining recoveries higher than 70% (Campone et al. 2014; Campillo et al. 2015). There are also examples in the literature describing the use of the so-called "ultrasonic extraction" referring to the extraction based on the solubilisation of honey in water or HPLC mobile phase and the sonication of this solution for a few tens of minutes (Biesaga and Pyrzyńska 2013; Zhang et al. 2013).

Regarding solid-phase extraction, Amberlite XAD-2 is one of the most popular adsorbents for the extraction of phenolic compounds from honeys. The use of this resin was initially proposed by Ferreres et al. (1994a) and afterwards it has been applied by numerous research groups. Briefly, the procedure consists of the solubilisation of honey in acidified water, passing the solution through the column containing the resin, removing sugar and other polar compounds with acidified water and, finally, eluting the phenolic fraction with methanol. The protocol usually includes a subsequent clean-up step based on a liquid-liquid extraction with ethyl ether. Some authors, including the above mentioned group, have further proposed simpler procedures based on the use of SPE cartridges (e.g. C18, Oasis HLB or Strata-X) which allow combining the extraction and clean-up steps (Michalkiewicz et al. 2008; Truchado et al. 2015). Multiwalled carbon nanotubes (Badjah Hadj Ahmed et al. 2014) or carbamate-embedded triacontyl-modified silica (Sil-CBM-C30) (Liu et al. 2016) have also been successfully prepared and used as efficient sorbents for phenolic compounds solid-phase extraction from honeys.

In general, the separation of phenolic acids and flavonoids is carried out by HPLC, almost always in its reverse phase configuration (RP-HPLC) using C18 columns (Alvarez-Suarez et al. 2009; Pyrzynska and Biesaga 2009; Ciulu et al. 2016). In these conditions, the usual elution order of phenolic compounds is benzoic acids, cinnamic acids, flavanone glycosides followed by flavonol and flavone glycosides and then the free aglycones in the same order (Pyrzynska and

Biesaga 2009). A polyethylene glycol reversed phase column (Discovery HS PEG) has also been used for the HPLC separation of flavonoid aglycones in honey (Campillo et al. 2015). Even though HPLC remains the most dominant separation technique for polyphenolic compounds, capillary electrophoresis (CE) has also been employed for the analysis of phenolic compounds in honey (Arráez-Román et al. 2006; Xu et al. 2016). Recently, new chromatographic approaches, such as ultra-high performance liquid chromatography (UHPLC), have been applied to phenolic determination in honey (Wabaidur et al. 2015).

Initially the most used detection system was UV absorption, so that compound identification was made by the comparison of the retention times and/or the peak spiking method, therefore this was only possible when the analyte was available as chromatographic standard. Further introduction as HPLC detectors of diode array spectrophotometers able to provide the UV-visible spectra of the peaks notably improved the chance for compound identification. Nevertheless, more recent methods involve different couplings between HPLC and mass spectrometry, allowing identification of compounds based on their molecular weights. Moreover, the study of the fragmentation patterns allows performing the structural investigations on particular classes of compounds whose discrimination is difficult due to the strong similarities in their structures such as *C*- and *O*- glycosides (Truchado et al. 2009, 2011).

The qualitative and quantitative dissimilarities in the phenolic profile of honeys are a direct consequence of the natural variability of these compounds in the plant from which they originate. The botanical origin may affect the profile of flavonoids and phenolic compounds sufficiently to permit discrimination based on the predominance of some individual components or a group of compounds in honey. One of the most significant examples of specificity of a single phenolic compound as chemical marker of the floral origin is the case of homogentisic acid for the strawberry tree (*Arbutus unedo* L.) honey, proposed by Cabras et al. (1999) and that has never been found in any other unifloral honey. More recently, the same research group has also proposed the presence of *cis, trans*- and *trans, trans*-abscisic acids, together with the volatile compound unedone, as complementary markers for strawberry tree honey (Tuberoso et al. 2010). The occurrence of those isomers of abscisic acid was described in acacia honeys (*Robinia pseudoacacia*) (Tomás-Barberán et al. 2001), heather honey (*Erica* spp.) from Portugal (Ferreres et al. 1996), and sage honeys (*Salvia officinalis* L) (Kenjeric et al. 2008), but not in such high amounts and with a constant ratio of about 1:1 as in arbutus honey.

Although many other compounds have been proposed as chemical markers, most of them have been later found also in honeys of other botanical origin, thus losing any aspect of strict specificity towards a single unifloral honey (Ciulu et al. 2016). This is for example the case of hesperetin, described as a typical marker for citrus honey by Ferreres et al. (1993) and that was later detected in other families of honey such as *Asteraceae*, *Fabaceae*, *Lamiaceae* and *Rosaceae* (Kurtagić et al. 2015).

Nowadays, almost all the studies are focused on the quantification of one or more compounds, rather than the presence/absence of a specific chemical marker, and the subsequent analysis of the results using chemometrics tools. For example, a minimum level of 122.6 mg/kg for methyl syringate (MSYR) can be considered as a

chemical marker in asphodel honeys (*Asphodelus microcarpus* Salzm. et Viv.) (Tuberoso et al. 2009); although this compound is present in several honeys like robinia, rape, chestnut, clover, linden blossom, dandelion, sunflower, thyme and fir, in all of them the described levels were <5 mg/kg (Tuberoso et al. 2009). In Manuka honeys (*Leptospermum scoparium*) methyl syringate is also the major compound, representing approximately 44% of the total phenolic content, however, its content is around 20 mg/kg (Alvarez-Suarez et al. 2016) far enough from the level proposed as marker in asphodel honeys.

Kaempferol and 8-methoxykaempferol were proposed as markers for Rosemary honeys (Tomás-Barberán et al. 2001), although in a recent paper chrysin, pinocembrin, caffeic acid and naringenin have been indicated as complementary markers (Escriche et al. 2014). Quercetin was suggested as a floral marker for sunflower (*Helianthus annuus*) honey by Tomás-Barberán et al. (2001) and later supported by other authors (Hogenboom et al. 2009). More recently, eriodictyol together with quercetin have been suggested as floral markers for Serbian sunflower (Kečkeš et al. 2013). Relatively larger amounts of gallic acid (maximum value 1.45 mg/kg) have also been reported in sunflower honeys compared to acacia, linden (*Tilia cordata*), basil (*Ocimum basilicum*), buckwheat (*Fagopyrum esculentum*), oilseed rape (*Brassica napus*), and goldenrod (*Solidago virgaurea*) honeys (Kečkeš et al. 2013). The same authors described the presence of a relevant amount of apigenin (0.97 mg/kg) as characteristic of buckwheat honey. The significant occurrence of *p*-coumaric acid and the flavonoids myricetin, quercetin and galangin in honeydew honey has been pointed out by Escriche et al. (2014), whereas the abundance of hydroxycinnamates (caffeic, *p*-coumaric and ferulic acids) was indicated as typical for chestnut honey (Cherchi et al. 1994).

In Acacia honeys there is not consensus among the scientific community and different markers have been suggested to typify acacia honeys from different geographic locations, such as *cis, trans*- and *trans, trans*-abscisic acids together with ellagic acid (Tomás-Barberán et al. 2001), cinnamic acid derivatives (Dimitrova et al. 2007), or kaempferol rhamnosides (Truchado et al. 2008). Likewise with heather honeys, for which different markers have been proposed, e.g., tricetin and different myricetin derivatives (Ferreres et al. 1994b); ellagic, *p*-hydroxybenzoic, syringic and *o*-coumaric acids (Andrade et al. 1997), or high amounts of phenyllactic (800 mg/kg) and phenylacetic (170 mg/kg) acids (Dimitrova et al. 2007).

There are also examples in the literature regarding the use of the phenolic profile for discrimination of honeys that are indistinguishable by means of a melissopalynological analysis. Stephens et al. (2010) observed that 2-methoxybenzoic and trimethoxybenzoic acids were characteristic of Manuka honey, while 4-methoxyphenyllactic acid was distinctive of the phenolic composition of the close relative Kanuka honey (*Kunzea ericoides*). The analysis of flavonoids (naringenin, hesperetin, chrysin, galangin, kaempferol, luteolin, pinocembrin, and quercetin) and phenolic acids (caffeic acid and *p*-coumaric acid) and the subsequence application of principal component analysis was shown to be useful for botanical authentication of lemon blossom honey (*Citrus limon*) and orange blossom honey (*Citrus* spp.) (Escriche et al. 2011).

Approaches based on the use of two and three-dimensional fluorescence spectroscopy coupled to chemometric data treatment methods have also been applied to the differentiation and classification of unifloral honeys according to their botanical origin based on the analysis of characteristic fluorescence spectra as determined by the presence of different phenolic phluorophores (Sergiel et al. 2014; Lenhardt et al. 2015).

Attempts of geographical discrimination of honey according to differences in the phenolic profile have also been described in the literature. Thirty-five samples of thyme honey from four different locations in Greece were correctly classified based on the phenolic composition and conventional physicochemical parameters by means of multivariate analysis of the variance (Karabagias et al. 2014). The concentrations of gallic, chlorogenic, caffeic, *p*-coumaric and ferulic acids were related as a function of the production site of Italian sulla (*Hedysarum* spp.) honeys (Gambacorta et al. 2014). Furthermore, because the sunlight exposure promotes the production of phenolic compounds in plants, a significant difference in the content of phenolic compounds of honeys produced in non-arid and arid regions has been found (Habib et al. 2014).

In conclusion, the revised literature suggests different markers based on the comparison of the phenolic profiles of a limited number of samples and honey types. Consequently, although the definition of the qualitative and quantitative profile of phenolic compounds in unifloral honeys could contribute to the characterization of their botanical and/or geographical origin, more data sets should be compared in order to establish adequate phenolic markers able to unequivocally distinguish a unifloral honey type from the rest.

References

Abu-Tarboush HM, Al-Kahtani HA, El-Sarrage M (1993) Floral-type identification and quality evaluation of some honey types. Food Chem 46:13–17

Agila A, Barringer S (2012) Application of selected ion flow tube mass spectrometry coupled with chemometrics to study the effect of location and botanical origin on volatile profile of unifloral American honeys. J Food Sci 77:C1103–C1108

de Alda-Garcilope C, Gallego-Picó A, Bravo-Yagüe J, Garcinuño-Martínez R, Fernández-Hernando P (2012) Characterization of Spanish honeys with protected designation of origin "Miel de Granada" according to their mineral content. Food Chem 135:1785–1788

Alonso-Torre SR, Cavia MM, Fernández-Muiño MA, Moreno G, Huidobro JF, Sancho MT (2006) Evolution of acid phosphatase activity of honeys from different climates. Food Chem 97:750–755

Alissandrakis E, Tarantilis PA, Harizanis PC, Polissiou M (2005) Evaluation of four isolation techniques for honey aroma compounds. J Sci Food Agric 85:91–97

Alissandrakis E, Tarantilis PA, Harizanis PC, Polissiou M (2007) Comparison of the volatile composition in thyme honeys from several origins in Greece. J Agric Food Chem 55:8152–8157

Alqarni AS, Owayss AA, Mahmoud AA, Hannan MH (2014) Mineral content and physical properties of local and imported honeys in Saudi Arabia. J Saudi Chem Soc 18:618–625

Alvarez-Suarez JM, Tulipani S, Romandini S, Vidal A, Battino M (2009) Methodological aspects about determination of phenolic compounds and in vitro evaluation of antioxidant capacity in the honey: a review. Curr Anal Chem 5:293–302

Alvarez-Suarez JM, González-Paramás AM, Santos-Buelga C, Battino M (2010a) Antioxidant characterization of native monofloral Cuban honeys. J Agric Food Chem 58: 9817–9824

Alvarez-Suarez JM, Tulipani S, Romandini S, Bertoli E, Battino M (2010b) Contribution of honey in nutrition and human health: a review. Mediterr J Nutr Metab 3:15–23

Alvarez-Suarez JM, Giampieri F, Damiani E, Astolfi P, Fattorini D, Regoli F, Quiles JL, Battino M (2012) Radical-scavenging activity, protective effect against lipid peroxidation and mineral contents of monofloral Cuban honeys. Plant Foods Hum Nutr 67:31–38

Alvarez-Suarez JM, Giampieri F, Cordero M, Gasparrini M, Forbes-Hernández TY, Mazzoni L, Afrin S, Beltrán-Ayala P, González-Paramás AM, Santos-Buelga C, Varela-Lopez A, Quiles JL, Battino M (2016) Activation of AMPK/Nrf2 signalling by Manuka honey protects human dermal fibroblasts against oxidative damage by improving antioxidant response and mitocondrial function promoting wound healing. J Funct Foods 25:38–49

Alves-Moreira RF, Bastos-de Maria CA (2001) Glicídios no mel. Quim Nova 24:516–525

Ampuero S, Bodganov S, Bosset JO (2004) Classification of unifloral honeys with an MS-based electronic nose using different sampling modes SHS SPME and INDEX. Eur Food Res Technol 218:194–207

Andrade P, Ferreres F, Amaral MT (1997) Analysis of honey phenolic acids by HPLC, its application to honey botanical characterization. J Liq Chromatogr Relat Technol 20:2281–2288

Anjos O, Campos MG, Contreras-Ruiz P, Antunes P (2015) Application of FTIR-ATR spectroscopy to the quantification of sugar in honey. Food Chem 169:218–223

Anklam E (1998) A review of the analytical methods to determine the geographical and botanical origin of honey. Food Chem 63:549–562

Arráez-Román D, Gómez-Caravaca AM, Gómez-Romero M, Segura-Carretero A, Fernández-Gutiérrez A (2006) Identification of phenolic compounds in rosemary honey using solid-phase extraction by capillary electrophoresis-electrospray ionization-mass spectrometry. J Pharm Biomed Anal 41:1648–1656

Atanassova J, Yurukova L, Lazarova M (2012) Pollen and inorganic characteristics of Bulgarian unifloral honeys. Czech J Food Sci 30:520–526

Azeredo LC, Azeredo MAA, de Souza SR, Dutra VML (2003) Protein contents and physicochemical properties in honey samples of *Apis mellifera* of different floral origins. Food Chem 80:249–254

Babacan S, Rand AG (2005) Purification of amylase from honey. J Food Sci 70:C413–C418

Badjah Hadj Ahmed AY, Obbed MS, Wabaidur SM, Al Othman ZA, Al-Shaalan NH (2014) High-performance liquid chromatography analysis of phenolic acid, flavonoid, and phenol contents in various natural Yemeni honeys using multi-walled carbon nanotubes as a solid-phase extraction adsorbent. J Agric Food Chem 62:5443–5450

Baker HG, Baker I (1986) The occurrence and significance of amino acids in floral nectar. Plant Syst Evol 151:175–186

Ball DW (2007) The chemical composition of honey. J Chem Educ 84:1643–1646

Baroni MV, Chiabrando GA, Costa C, Wunderlin DA (2002) Assessment of the floral origin of honey by SDS-page immunoblot techniques. J Agric Food Chem 50:1362–1367

Baroni MV, Arrua C, Nores ML, Faye P, Diaz MP, Chiabrando GA, Wunderlin DA (2009) Composition of honey from Cordoba (Argentina). Assessment of North/South provenance by chemometrics. Food Chem 114:727–733

Barra MPG, Ponce-Díaz MC, Venegas-Gallegos C (2010) Volatile compounds in honey produced in the central valley of Ñuble province, Chile. Chilean J Agric Res 70:75–84

Batista B, Da Silva L, Rocha B, Rodrigues J, Berretta-Silva A, Bonates T, Gomes V, Barbosa R, Barbosa F (2012) Multi-element determination in Brazilian honey samples by inductively coupled plasma mass spectrometry and estimation of geographic origin with data mining techniques. Food Res Int 49:209–215

Battaglini MB, Bossi G (1972) Determination of glucides by GLC and its possibilities for honey qualification. Apiacta 1:12–13

Belitz HD, Grosch W, Schieberle P (2009) Sugars, sugar alcohols and honey In: Food chemistry, vol 2009, 4th edn.; Springer: Berlin, pp 883−890.

Bentabol-Manzanares A, Hernández-García Z, Rodríguez-Galdón B, Rodríguez-Rodríguez E, Díaz-Romero C (2011) Differentiation of blossom and honeydew honeys using multivariate analysis on the physicochemical parameters and sugar composition. Food Chem 126: 664–672

Bernal JL, Nozal MJ, Toribio L, Diego JC, Ruiz A (2005) A comparative study of several HPLC methods for determining free amino acid profiles in honey. J Sep Sci 28:1039–1047

Bianchi F, Mangia A, Mattarozzi M, Musci M (2011) Characterization of the volatile profile of thistle honey using headspace solid-phase microextraction and gas chromatography–mass spectrometry. J Food Chem 129:1030–1036

Biesaga M, Pyrzyńska K (2013) Stability of bioactive polyphenols from honey during different extraction methods. Food Chem 136:46–54

Bilikova EK, Simuth J (2010) New criterion for evaluation of honey: quantification of royal jelly protein Apalbumin 1 in honey. J Agric Food Chem 58:8776–8781

Boffo EF, Tavares LA, Tobias ACT, Ferreira MMC, Ferreira AG (2012) Identification of components of Brazilian honey by ^{1}H NMR and classification of its botanical origin by chemometric methods. LWT Food Sci Technol 49:55e63

Bogdanov S, Baumann E (1988) Determination of sugar composition of honeys by HPLC. Mitt Geb Lebensm Hyg 79:198–206

Bogdanov S, Lüllmann C, Martin P, Von der Ohe W, Russmann H, Vorwohl G et al (1999) Honey quality and international regulatory standards: review by the International Honey Commission. Bee World 80:61e69

Bogdanov S, Haldimann M, Luginbuhl W, Gallmann P (2007) Minerals in honey: environmental, geographical and botanical aspects. J Apic Res 46:269–275

Bogdanov S, Jurendic T, Sieber R, Gallmann P (2008) Honey for nutrition and health: a review. J Am Coll Nutr 27:677–689

Bonaga G, Giumanini AG, Gliozzi G (1986) Chemical composition of chestnut honey: analysis of the hydrocarbon fraction. J Agric Food Chem 34:319–326

Bouseta A, Collin S (1995) Optimized Likens-Nickerson methodology for quantifying honey flavors. J Agric Food Chem 43:1890–1897

Bouseta A, Scheirman V, Collin S (1996) Flavor and free amino acid composition of lavender and *eucalyptus* honeys. J Food Sci 61:683–694

Bradbear N (2009) Definition and uses of honey. In: Bees and their role in forest livelihoods. FAO, Rome, pp 81–88. http://www.fao.org/3/a-i0842e.pdf Accessed Oct 2016

Brokl M, Soria AC, Ruiz-Matute AI, Sanz ML, Ramos L (2010) Separation of disaccharides by comprehensive two-dimensional gas chromatography−time-of-flight mass spectrometry. Application to honey analysis. J Agric Food Chem 58:11561–11567

Brudzynski K, Sjaarda C, Maldonado-Alvarez L (2013) A new look on protein–polyphenol complexation during honey storage: is this a random or organized event with the help of dirigent-like proteins? PLoS One 8:1–9

Cabras P, Angioni A, Tuberoso C, Floris I, Reniero F, Guillou C, Ghelli S (1999) Homogentisic acid: a phenolic acid as a marker of strawberry-tree (*Arbutus unedo*) honey. J Agric Food Chem 47(10):4064–4067

Campillo N, Viñas P, Férez-Melgarejo G, Hernández-Córdoba M (2015) Dispersive liquid-liquid microextraction for the determination of flavonoid aglycone compounds in honey using liquid chromatography with diode array detection and time-of-flight mass spectrometry. Talanta 131:185–191

Campone L, Piccinelli AL, Pagano I, Carabetta S, Di Sanzo R, Russo M, Rastrelli L (2014) Determination of phenolic compounds in honey using dispersive liquid-liquid microextraction. J Chrom A 1334:9–15

Campos G, Nappi U, Raslan D, Augusti R (2000) Substâncias voláteis em mel floral e mel de melato. Cienc Tecnol Aliment 20:18–22

Castro-Várquez L, Pérez-Coello MS, Cabezudo MD (2003) Analysis of volatile compounds of Rosemary honey. Comparison of different extraction techniques. Chromatographia 57:227–233

Castro-Vázquez L, Díaz-Maroto MC, Pérez-Coello MS (2006) Volatile composition and contribution to the aroma of Spanish honeydew honeys. Identification of a new chemical marker. J Agric Food Chem 54:4809–4813

Castro-Vázquez L, Díaz-Maroto MC, Pérez-Coello MS (2007) Aroma composition and new chemical markers of Spanish citrus honeys. Food Chem 103:601–606

Castro-Vázquez L, Díaz-Maroto MC, González-Viñas MA, de la Fuente E, Pérez-Coello MS (2008) Influence of storage conditions on chemical composition and sensory properties of citrus honey. J Agric Food Chem 56:1999–2006

Castro-Vázquez L, Díaz-Maroto MC, González-Viñas MA, Pérez-Coello MS (2009) Differentiation of monofloral citrus, rosemary, eucalyptus, lavender, thyme and heather honeys based on volatile composition and sensory descriptive analysis. Food Chem 112:1022–1030

Castro-Vázquez L, DÍaz-Maroto MC, de Torres C, Pérez-Coello MS (2010) Effect of geographical origin on the chemical and sensory characteristics of chestnut honeys. Food Res Int 43:2335–2340

Cavia MM, Fernández-Muino MA, Alonso-Torre SR, Huidobro JF, Sancho MT (2007) Evolution of acidity of honeys from continental climates: influence of induced granulation. Food Chem 100:1728–1733

Chen H, Fan C, Chang Q, Pang G, Hu X, Lu M (2014) Chemometric determination of the botanical origin for Chinese honeys on the basis of mineral elements determined by ICP-MS. J Agric Food Chem 62:2443–2448

Cherchi A, Spanedda L, Tuberoso C, Cabras P (1994) Solid-phase extraction and high-performance liquid chromatographic determination of organic acids in honey. J Chromatogr A 669: 59–64

Chiba S (1998) α-Glucosidase. In: The Amylase Research Society of Japan (ed) Handbook of amylases and related enzymes. Pergamon Press, Oxford, pp 104–116

Chua LS, Abdul-Rahaman NL, Sarmidi MR, Aziz R (2012) Multi-elemental composition and physical properties of honey samples from Malaysia. Food Chem 135:880–887

Chua LS, Lee JY, Chan GF (2013) Honey protein extraction and determination by mass spectrometry. Anal Bioanal Chem 405:3063–3074

Chua LS, Lee JY, Chan GF (2015) Characterization of the proteins in honey. Anal Lett 48:697–709

Chudzinska M, Baralkiewicz D (2010) Estimation of honey authenticity by multielements characteristics using inductively coupled plasma-mass spectrometry (ICP-MS) combined with chemometrics. Food Chem Toxicol 48:284–290

Chudzinska M, Baralkiewicz D (2011) Application of ICP-MS method of determination of 15 in honey with chemometric approach for the verification of their authenticity. Food Chem Toxicol 49:2741–2749

Ciappini MC, Stoppani FS (2014) Determination of antioxidant capacity, flavonoids, and total phenolic content in eucalyptus and clover honeys. J Apic Sci 58:103–111

Cimpoiu C, Hosu A, Miclaus V, Puscas A (2013) Determination of the floral origin of some Romanian honeys on the basis of physical and biochemical properties. Spectrochim Acta A Mol Biomol Spectrosc 100:149–154

Ciulu M, Solinas S, Floris I, Panzanellia A, Pilo MI, Piu PC, Spanoa N, Sanna G (2011) RPHPLC determination of water-soluble vitamins in honey. Talanta 83:924–929

Ciulu M, Spano N, Pilo MI, Sanna G (2016) Recent advances in the analysis of phenolic compounds in unifloral honeys. Molecules 21:451–482

Codex Alimentarius (2001) Revised codex standard for honey, Codex Stan 12-1981 Rev.2

Cometto PM, Faye PF, di Paola RD, Rubio MA, Aldao MAJ (2003) Comparison of free amino acids profile in honey from three Argentinian regions. J Agric Food Chem 51:5079–5087

Consonni R, Cagliani LR (2015) Recent developments in honey characterization. RSC Adv 5(5):59696–59714

Consonni R, Cagliani LR, Cogliati C (2012) NMR characterization of saccharides in Italian honeys of different floral sources. J Agric Food Chem 60:4526–4534

Consonni R, Cagliani LR, Cogliati C (2013) Geographical discrimination of honeys by saccharides analysis. Food Control 32:543e548
Conte LS, Miorini M, Giomo A, Bertacco G, Zironi R (1998) Evaluation of some fixed components for unifloral honey characterization. J Agric Food Chem 46:1844–1849
Cotte JF, Casabianca H, Chardon S, Lheritier J, Grenier-Loustalot MF (2003) Application of carbohydrate analysis to verify honey authenticity. J Chromatogr A 1021:145–155
Cotte JF, Casabianca H, Chardon S, Lheritier J, Grenier-Loustalot MF (2004a) Chromatographic analysis of sugars applied to the characterisation of monofloral honey. Anal Bioanal Chem 380: 698–705
Cotte JF, Casabianca H, Giroud B, Albert M, Lheritier J, Grenier-Loustalot MF (2004b) Characterization of honey amino acid profiles using high-pressure liquid chromatography to control authenticity. Anal Bioanal Chem 378:1342–1350
Crane E (1990) The traditional hive products: honey and beeswax. In: Crane E (ed) Bees and beekeeping: science, practice and world resources. Heinemann Newnes, Oxford, pp 388–413
Cuevas-Glory LF, Pino JA, Santiago LS, Sauri-Duch LF (2007) A review of volatile analytical methods for determining the botanical origin of honey. Food Chem 103:1032–1043
Daniele G, Maitre D, Casabianca H (2012) Identification, quantification and carbon stable isotopes determinations of organics acids in monofloral honeys. A powerful tool for botanical and authenticity control. Rapid Commun Mass Spectrom 26:1993–1998
Davies AMC (1975) Amino acid analysis of honeys from eleven countries. J Apic Res 14:29–39
Davies AMC (1976) The application of amino acid analysis to the determination of the geographical origin of honey. J Food Technol 11:515–523
Di Girolamo F, D'Amato A, Righetti PG (2012) Assessment of the floral origin of honey via proteomic tools. J Proteomics 75:3688–3693
Dimitrova B, Gevrenova R, Anklam E (2007) Analysis of phenolic acids in honeys of different floral origin by solid-phase extraction and high-performance liquid chromatography. Phytochem Anal 18:24–32
Dobre I, Georgescu LA, Alexe P, Escuredo O, Seijo MC (2012) Rheological behavior of different honey types from Romania. Food Res Int 49:126–132
Doner LW (1977) The sugars of honey—a review. J Sci Food Agric 28:443–456
Dustman JH (1971) Über die katalaseaktivität in bienenhonig ausder tracht der heidekrautgewächse (Ericaceae). Z Lebensm Unters Forsch 145:292–295
Escriche I, Kadar M, Juan-Borrás M, Domenech E (2011) Using flavonoids, phenolic compounds and headspace volatile profile for botanical authentication of lemon and orange honeys. Food Res Int 44:1504–1513
Escriche I, Kadar M, Juan-Borrás M, Domenech E (2014) Suitability of antioxidant capacity, flavonoids and phenolic acids for floral authentication of honey. Impact of industrial thermal treatment. Food Chem 142:135–143
Escuredo O, Míguez M, Fernández-González M, Seijo MC (2013) Nutritional value and antioxidant activity of honeys produced in a European Atlantic area. Food Chem 138:851–856
Escuredo O, Dobre I, Fernández-González M, Seijo MC (2014) Contribution of botanical origin and sugar composition of honeys on the crystallization phenomenon. Food Chem 149: 84–90
EU Council (2002) Council Directive 2001/110/EC of 20 December 2001 relating to honey. Official Journal of European Community L10:47–52
Faostat (2016). http://fenix.fao.org/faostat/beta/es/?#data. Accessed Sept 2016.
Feller-Demalsy MJ, Vincent B, Beaulieu F (1989) Teneur en minéraux et origine géographique des miels du Canada. Apidology 20:77–91
Fernandez-Torres R, Perez-Bernal JL, Bello-Lopez MA, Callejon-Mochon M, Jimenez-Sanchez JC, Guiraum-Perez A (2005) Mineral content and botanical origin of Spanish honeys. Talanta 65:686–691
Ferreres F, García-Viguera C, Tomás-Lorente F, Tomás-Barberán FA (1993) Hesperetin: a marker of the floral origin of citrus honey. J Sci Food Agric 61:121–123

Ferreres F, Tomás-Barberán FA, Soler C, García-Viguera C, Ortiz A, Tomás-Lorente F (1994a) A simple extractive technique for honey flavonoid HPLC analysis. Apidologie 25:21–30

Ferreres F, Andrade P, Tomás-Barberán FA (1994b) Flavonoids from Portuguese heather honey. Z Lebensm Unters Forsch 199:32–37

Ferreres F, Andrade P, Tomás-Barberán FA (1996) Natural occurrence of abscisic acid in heather honey and floral nectar. J Agric Food Chem 44:2053–2056

Flanjak I, Strelec I, Kenjerić D, Primorac L (2016) Croatian produced unifloral honeys characterised according to the protein and proline content and enzyme activities. J Apic Sci 60:39–48

Földházi G (1994) Analysis and quantitation of sugars in honey of different botanical origin using high performance liquid chromatography. Acta Alim 23:229–311

Frazzoli C, D'Ilio B, Bocca B (2007) Determination of Cd and Pb in honey by SF–ICP-MS: validation figures and uncertainty of results. Anal Lett 40:1992–2004

de la Fuente E, Martínez-Castro I, Sanz J (2005) Characterization of Spanish unifloral honeys by solid phase microextraction and gas chromatography-mass spectrometry. J Sep Sci 28:1093–1100

de la Fuente E, Ruiz-Matute AI, Valencia-Barrera RM, Sanz J, Martínez-Castro I (2011) Carbohydrate composition of Spanish unifloral honeys. Food Chem 129:1483–1489

Gambacorta E, Simonetti A, Garrisi N, Intaglietta I, Perna A (2014) Antioxidant properties and phenolic content of sulla (*Hedysarum* spp.) honeys from Southern Italy. Int J Food Sci Technol 49:2260–2268

Gilbert J, Shephard MJ, Wallwork MA, Harris RG (1981) Determination of the geographical origin of honeys by multivariate analysis of gas chromatographic data on their free amino acid content. J Apic Res 20:125–135

Giri KV (1938) The chemical composition and enzyme content of indian honey. Madras Agric J 26:68–72

Godefroot M, Sandra P, Verzele M (1981) New method for quantitative essential oil analysis. J Chromatogr 203:325–335

Goldschmidt S, Burkert H (1955) Observation of some heretofore unknown sugars in honey. Z Physiol Chem 300:188–200

Gómez Bárez JA, Garcia-Villanova RJ, Elvira Garcia S, Rivas Palá T, González Paramás AM, Sánchez Sánchez J (2000) Geographical discrimination of honeys through the employment of sugar patterns and common chemical quality parameters. Eur Food Res Technol 210:437–444

Gómez-Bárez JA, García-Villanova RJ, Elvira-García S, González-Paramás AM (1999) Optimization of the capillary gas chromatographic analysis of mono- and oligosaccharides in honeys. Chromatographia 50:461–469

González-Miret ML, Terrab A, Hernanz D, Fernández-Recamales MA, Heredia FJ (2005) Multivariate correlation between color and mineral composition of honeys and by their botanical origin. J Agric Food Chem 53:2574–2580

González-Paramás AM, Gómez-Bárez JA, García-Villanova RJ, Rivas Palá T, Ardanuy Albajar R, Sánchez J (2000) Geographical discrimination of honeys by using mineral composition and common chemical quality parameters. J Sci Food Agric 80:157–165

González-Paramás AM, Gómez-Bárez JA, Cordón-Marcos C, García-Villanova RJ, Sánchez-Sánchez J (2006) HPLC-fluorimetric method for analysis of amino acids in products of the hive (honey and bee-pollen). Food Chem 95:148–156

Habib HM, Al Meqbali FT, Kamal H, Souka UD, Ibrahim WH (2014) Bioactive components, antioxidant and DNA damage inhibitory activities of honeys from arid regions. Food Chem 153:28–34

Hermosín I, Chicón RM, Cabezudo MD (2003) Free amino acid composition and botanical origin of honey. Food Chem 83:263–268

Hernández OM, Fraga JMG, Jiménez AI, Jiménez F, Arias JJ (2005) Characterization of honey from the Canary Islands: determination of the mineral content by atomic absorption spectrophotometry. Food Chem 93:449–458

Hogenboom AC, van Leerdam JA, de Voogt P (2009) Accurate mass screening and identification of emerging contaminants in environmental samples by liquid chromatography–hybrid linear ion trap Orbitrap mass spectrometry. J Chromatogr A 1216:510–519

Iglesias MT, de Lorenzo C, Polo MC, Martín-Álvarez PJ, Pueyo E (2004) Usefulness of amino acid composition to discrimínate between honeydew and floral honeys. Application to honeys from a small geographic area. J Agric Food Chem 52:84–89

Iglesias MT, Martín-álvarez PJ, Polo MC, de Lorenzo C, González M, Pueyo E (2006) Changes in the free amino acid contents of honeys during storage at ambient temperature. J Agric Food Chem 54:9099–9104

Janiszewska K, Aniołowska M, Nowakowski P (2012) Free amino acids content of honeys from Poland. Pol J Food Nutr Sci 62:85–89

Jànoškovà N, Vyviurska O, Špànik I (2014) Identification of volatile organic compounds in honeydew honeys using comprehensive gas chromatography. J Food Nutr Res 53:353–362

Jerković I, Marijanović Z, Staver MM (2011a) Screening of natural organic volatiles from *Prunus mahaleb* L. honey: coumarin and vomifoliol as nonspecific biomarkers. Molecules 16:2507–2518

Jerković I, Tuberoso CIG, Kasum A, Marijanović Z (2011b) Volatile compounds of *Asphodelus microcarpus* Salzm. et Viv. honey obtained by HS-SPME and USE analyzed by GC/MS. Chem Biodivers 8:587–598

Kadri SM, Zaluski R, de Oliveira Orsi R (2017) Nutritional and mineral contents of honey extracted by centrifugation and pressed processes. Food Chem 218:237–241

Karabagias IK, Vavoura MV, Badeka A, Kontakos S, Kontominas MG (2014) Differentiation of greek thyme honeys according to geographical origin based on the combination of phenolic compounds and conventional quality parameters using chemometrics. Food Anal Methods 7:2113–2121

Kaškonienė V, Venskutonis PR (2010) Floral markers in honey of various botanical and geographic origins: a review. Compr Rev Food Sci Food Saf 9:620–634

Kaškonienė V, Venskutonis PR, Čeksteytė V (2010) Carbohydrate composition and electrical conductivity of different origin honeys from Lithuania. LWT Food Sci Technol 43:801–807

Kečkeš S, Gašić U, Veličković TĆ, Milojković-Opsenica D, Natić M, Tešic Z (2013) The determination of phenolic profiles of Serbian unifloral honeys using ultra-high-performance liquid chromatography/high resolution accurate mass spectrometry. Food Chem 138:32–40

Kenjerić D, Primorac LJ, Mandić ML, Bubalo D, Perl Pirički A, Flanjak I (2006) Dalmatian sage (*Salvia officinalis* L.) honey characterisation. Dtsch Lebensmitt Rundsch 102:479–484

Kenjeric D, Mandic ML, Primorac L, Cacic F (2008) Flavonoid pattern of sage (*Salvia officinalis* L.) unifloral honey. Food Chem 110:187–192

Kešckeš J, Trifković J, Andrić F, Jovetić M, Tešić Ž, Milojković-Opsenica D (2013) Amino acids profile of Serbian unifloral honeys. J Sci Food Agric 93:3368–3376

Krell R (1996) Value-added products from beekeeping. In: FAO agricultural services bulletin no. 124. http://www.fao.org/3/a-w0076e/index.html. Accessed Sept 2016

Kropf U, Jamnik M, Bertoncelj J, Golob T (2008) Linear regression model of the ash mass fraction and electrical conductivity for Slovenian honey. Food Technol Biotechnol 46:335–340

Kurtagić H, Barudanović S, Durmić V (2015) Determination of rutin, quercetin, naringenin and hesperetin in the honey from Bosnia and Herzegovina (B & H) in relation to the composition of pollen. J Environ Sci Eng A 4:615–622

Lammertyn J, Veraverbeke EA, Irudayaraj J (2004) zNose technology for the classification of honey based on rapid aroma profiling. Sens Actuators 98:54–62

Lee DC, Lee SY, Cha SH, Choi YS, Rhee HI (1998) Discrimination of native bee-honey and foreign bee-honey by SDS–PAGE. Korean J Food Sci 30:1–5

Lenhardt L, Bro R, Zeković I, Dramićanin T, Dramićanin MD (2015) Fluorescence spectroscopy coupled with PARAFAC and PLS DA for characterization and classification of honey. Food Chem 75:284–291

León-Ruiz V, Vera S, González-Porto AV, San Andrés MP (2011) Vitamin C and sugar levels as simple markers for discriminating Spanish honey sources. J Food Sci 76:C356–C361

León-Ruiz V, Vera S, González-Porto AV, Andrés MPS (2013) Analysis of water-soluble vitamins in honey by isocratic RP-HPLC. Food Anal Methods 6:488–496

Likens ST, Nickerson GB (1964) Detection of certain hop oil constituents in brewing products. American Society of Brewing Chemists Proceedings, pp 5–13

Liu H, Zhang M, Guo Y, Qiu H (2016) Solid-phase extraction of flavonoids in honey samples using carbamate-embedded triacontyl-modified silica sorbent. Food Chem 204:56–61

Low NH, Sporns P (1988) Analysis and quantitation of minor di- and trisaccharides in honey, using capillary gas chromatography. J Food Sci 53:558–561

Lušić D, Koprivnjak O, Curić D, Sabatini AG, Conte LS (2007) Volatile profile of Croatian lime tree (*Tilia sp.*), fir honeydew (*Abies alba*) and sage (*Salvia officinalis*) honey. Food Technol Biotechnol 45:156–165

Maignial L, Pibrot P, Bonetti G, Chaintrau A, Marion JP (1992) Simultaneous distillation-extraction under static vacuum: isolation of volatile compounds at room temperature. J Chromatogr 606:87–94

Mannas D, Altug T (2007) SPME/GC/MS and sensory flavour profile analysis for estimation of authenticity of thyme honey. Int J Food Sci Technol 42:133–138

Mannina L, Sobolev AP, di Lorenzo A, Vista S, Tenore GC, Daglia M (2015) Chemical composition of different botanical origin honeys produced by Sicilian black honeybees (*Apis mellifera* ssp. *sicula*). J Agric Food Chem 63:5864–5874

Manyi-Loh CE, Clarke AM, Munzhelele T, Green E, Mkwetshana NF, Ndip RN (2010) Selected South African honeys and their extracts possess *in vitro anti-Helicobacter pylori* activity. Arch Med Res 41:324–331

Manyi-Loh CE, Clarke AM, Ndip RN (2011a) Identification of volatile compounds in solvent extracts of honeys produced in South Africa. Afr J Agric Res 6:4327–4334

Manyi-Loh C, Ndip RN, Clarke AM (2011b) Volatile compounds in honey: a review on their involvement in aroma, botanical origin determination and potential biomedical activities. Int J Mol Sci 12:9514–9532

Marshall T, Williams KM (1987) Electrophoresis of honey: characterization of trace proteins from a complex biological matrix by silver staining. Anal Biochem 167:301–303

Mason BS, Slaver HT (1971) A gas chromatographic method for the determination of sugars in food. J Agric Food Chem 19:551–554

Mato I, Huidobro JF, Sánchez MP, Muniategui S, Fernández-Muino MA, Sancho MT (1998) Enzymatic determination of L-malic acid in honey. Food Chem 62:503–508

Mato I, Huidobro JF, Simal-Lozano J, Sancho MT (2007) Analytical methods for the determination of organic acids in honey. Crit Rev Anal Chem 36:3–11

Mazzoni V, Bradesi P, Tomi F, Casanova J (1997) Direct qualitative and quantitative analysis of carbohydrate mixtures using ^{13}C NMR spectroscopy: application to honey. Magn Reson Chem 35:S81–S90

Michalkiewicz A, Biesaga M, Pyrzynska K (2008) Solid-phase extraction procedure for determination of phenolic acids and some flavonols in honey. J Chromatogr A 1187:18–24

Mondragón-Cortez P, Ulloa J, Rosas-Ulloa P, Rodríguez-Rodríguez R, Resendiz Vázquez J (2013) Physicochemical characterization of honey from the West region of México. CyTA J Food 11:7–13

Moniruzzaman M, Rodríguez I, Ramil M, Cela R, Sulaiman SA, Gan SH (2014) Assessment of gas chromatography time-of-flight accurate mass spectrometry for identification of volatile and semi-volatile compounds in honey. Talanta 129:505–515

Moreira RFA, Maria CAB, Pietroluongo M, Trugo LC (2010) Chemical changes in the volatile fractions of Brazilian honeys during storage under tropical conditions. Food Chem 121: 697–704

Morse RA, Lisk DJ (1980) Elemental analysis of honeys from several nations. Am Bee J 120:522–523

Navarrete M, Casado S, Minelli M, Segura A, Bonetti A, Dinelli G, Fernández A (2015) Direct determination of aliphatic acids in honey by coelectroosmotic capillary zone electrophoresis. J Apic Res 44:65–70

Nozal-Nalda MJ, Bernal-Yagüe JL, Diego-Calva JCD, Martín-Gómez MT (2005a) Classifying honeys from the Soria Province of Spain via multivariate analysis. Anal Bioanal Chem 382:311–319

Nozal-Nalda MJ, Bernal JL, Toribio L, Alamo M, Diego JC, Tapia J (2005b) The use of carbohydrate profiles and chemometrics in the characterization of natural honeys of identical geographical origin. J Agric Food Chem 53:3095–3100

Ohashi K, Natori S, Kubo T (1999) Expression of amylase and glucose oxidase in the hypopharyngeal gland with an age-dependent role change of the worker honeybee (*Apis mellifera* L.) Eur J Biochem 265:127–133

Ouchemoukh S, Schweitzer P, Bey MB, Djoudad-Kadji H, Louaileche H (2010) HPLC sugar profiles of Algerian honeys. Food Chem 121:561–568

Overton SV, Manura JJ (1994) Flavor and aroma commercial bee honey: a burge-and-trap thermal desorption technique for the identification and quantification of volatiles and semivolatiles in honey. Am Lab 26:45–53

Özbalci B, Boyaci IH, Topcu A, Kadilar C, Tamer U (2013) Rapid analysis of sugars in honey by processing Raman spectrum using chemometric methods and artificial neural networks. Food Chem 136:1444–1452

Pawlowska M, Armstrong DW (1994) Evaluation of enantiomeric purity of selected amino acids in honey. Chirality 6:270–276

Pažitná A, Janáčová A, Špánik I (2012) The enantiomer distribution of major chiral volatile organic compounds in Slovakian monofloral honeys. J Food Nutr Res 51:236–241

Peña RM, Barciela J, Herrero C, García-Martín S (2004) Solid-phase microextraction gas chromatography-mass spectrometry determination of monoterpenes in honeys. J Sep Sci 27:1540–1544

Pérez RA, Sánchez-Brunete C, Calvo RM, Tadeo JL (2002) Analysis of volatiles from Spanish honeys by solid-phase microextraction and gas chromatography-mass spectrometry. J Agric Food Chem 50:2633–2637

Perna A, Simonetti A, Intaglietta I, Sofo A, Gambacorta E (2012) Metal content of southern Italy honey of different botanical origins and its correlation with polyphenol content and antioxidant activity. Int J Food Sci Technol 47:1909–1917

Persano-Oddo L, Piro R (2004) Main European unifloral honeys: descriptive sheets. Apidologie 35:S38–S81

Persano-Oddo L, Baldi E, Accorti M (1990) Diastatic activity in some unifloral honeys. Apidologie 21:17–24

Persano-Oddo L, Piazza MG, Pulcini P (1999) Invertase activity in honey. Apidologie 30:57–65

Petrov V (1974) Quantitative determination of amino acids in some Australian honeys. J Apic Res 13:61–66

Piasenzotto L, Gracco L, Conte L (2003) Solid phase micro extraction (SPME) applied to honey quality control. J Sci Food Agric 83:1037–1044

Pilz-Güther D, Speer K (2004) Development of a GC method for the simultaneous determination of organic acids in honey. Dtsch Lebensmitt Rundsch 100:84–87

Pirini A, Conte LS, Francioso O, Lercker G (1992) Capillary gas chromatographic determination of free amino acids in honey as a means of discrimination between different botanical sources. J High Resolut Chromatogr 15:165–170

Pisani A, Protano G, Riccobono F (2008) Minor and trace elements in different honey types produced in Siena County (Italy). Food Chem 107:1553–1560

Plutowska B, Chmiel T, Dymerski T, Wardencki WA (2011) Headspace solid-phase microextraction method development and its application in the determination of volatiles in honeys by gas chromatography. Food Chem 126:1288–1298

Pohl P (2009) Determination of metal content in honey by atomic absorption and emission spectrometries. Trends Anal Chem 28:117–128

Pohl P, Stecka H, Sergiel I, Jamroz P (2012) Different aspects of the elemental analysis of honey by flame atomic absorption and emission spectrometry: a review. Food Anal Methods 5:737–751

Pontes M, Marques JC, Cámara JS (2007) Screening of volatile composition from Portuguese multifloral honeys using headspace solid-phase micro-extraction-gas chromatography-quadrupole mass spectrometry. Talanta 74:91–103

Pontis JA, Alves Da Costa LAM, Reis Da Silva SJ, Flach A (2014) Color, phenolic and flavonoid content, and antioxidant activity of honey from Roraima, Brazil. Food Sci Technol Campinas 34:69–73

Primorac L, Angelkov B, Mandić M, Kenjerić D, Nedeljko M, Flanjak I, Perl Pirički A, Arapceska M (2009) Comparison of the Croatian and Macedonian honeydew honey. J Central Eur Agric 10:263–270

Primorac L, Flanjak I, Kenjerić D, Bubalo D, Topolnjak Z (2011) Specific rotation and carbohydrate profile of Croatian unifloral honeys. Czech J Food Sci 29:515–519

Pyrzynska K, Biesaga M (2009) Analysis of phenolic acids and flavonoids in honey. Trends Anal Chem 28:893–902

Qiu PY, Ding HB, Tang YK, Xu RJ (1999) Determination of chemical composition of commercial honey by near-infrared spectroscopy. J Agric Food Chem 47:2760–2765

Radovic BS, Careri M, Mangia A, Musci M, Gerboles M, Anklam E (2001) Contribution of dynamic headspace GC-MS analysis of aroma compounds to authenticity testing of honey. Food Chem 72:511–520

Rebane R, Herodes K (2010) A sensitive method for free amino acids analysis by liquid chromatography with ultraviolet and mass spectrometric detection using precolumn derivatization with diethyl ethoxymethylenemalonate: application to the honey analysis. Anal Chim Acta 672:79–84

Ribeiro ROR, Máralém ET, de Jesús EFO, Carneiro CS, Junior CAC, de Almeida E, Filho VFN (2014) Determination of trace elements in honey from different regions in Rio de Janeiro state (Brazil) by total reflection X-ray fluorescence. J Food Sci 79:T738–T742

Rizelio VM, Gonzaga LV, da Silva GCB, Maltez HF, Oliveira-Costa AC, Fett R (2012) Fast determination of cations in honey by capillary electrophoresis: a possible method for geographic origin discrimination. Talanta 99:450–456

Rodriguez-Otero JL, Paseiro P, Simal J (1990) Intento de caracterización de las mieles naturales de Galicia mediante las fracciones proteicas separadas por electroforesis. Anal Bromatol 42:83–98

Rodriguez-Otero J, Paseiro P, Simal J, Cepeda A (1994) Mineral content of the honeys produced in Galicia (north-west Spain). Food Chem 49:169–171

Rossano R, Larocca M, Polito T, Perna AM, Padula MC, Martelli G, Riccio P (2012) What are the proteolytic enzymes of honey and what they do tell us? A fingerprint analysis by 2-d zymography of unifloral honeys. PLoS One 7:e49164. doi:10.1371/journal.pone.0049164

Ruiz-Matute AI, Brokl M, Soria AC, Sanz ML, Martínez-Castro I (2010) Gas chromatographic–mass spectrometric characterization of tri- and tetrasaccharides in honey. Food Chem 120:637–642

Sánchez MP, Huidobro JF, Mato I, Muniategui S, Sancho MT (2001) Evolution of invertase activity in honey over two years. J Agric Food Chem 49:416–422

Sancho MT, Muniategui S, Huidobro JF, Simal J (1991) Provincial classification of Basque Country (nothern Spain) honeys by their chemical composition. J Apic Res 30:168–172

Sanz S, Pérez C, Herrera A, Sanz M, Juan T (1995) Application of a statistical approach to the classification of honey by geographic origin. J Sci Food Agric 69:135–140

Sanz ML, del Castillo MD, Corzo N, Olano A (2003) 2-Furoylmethyl amino acids and hydroxymethylfurfural as indicators of honey quality. J Agric Food Chem 51:4278–4283

Sanz ML, Sanz J, Martinez-Castro I (2004a) Gas chromatographic−mass spectrometric method for the qualitative and quantitative determination of disaccharides and trisaccharides in honey. J Chromatogr A 1059:143–148

Sanz ML, Gonzalez M, de Lorenzo C, Sanz J, Martinez-Castro I (2004b) Carbohydrate composition and physico chemical properties of artisanal honeys from Madrid (Spain): occurrence of *Echium* sp honey. J Sci Food Agric 84:1577–1584

Sanz ML, Gonzalez M, de Lorenzo C, Sanz J, Martinez-Castro I (2005) A contribution to the differentiation between nectar honey and honeydew honey. Food Chem 91:313–317

Schepartz AI, Subers MH (1964) The glucose oxidase of honey. Biochim Biophys Acta 85: 228–237

Senyuva HZ, Gilbert J, Silici S, Charlton A, Dal C, Gurel N, Cimen D (2009) Profiling Turkish honeys to determine authenticity using physical and chemical characteristics. J Agric Food Chem 57:3911–3919

Sergiel I, Pohl P, Biesaga M, Mironczyk A (2014) Suitability of three-dimensional synchronous fluorescence spectroscopy for fingerprint analysis of honey samples with reference to their phenolic profiles. Food Chem 145:319–326

Serra J, Ventura E (1995) Characterization of citrus honey (*Citrus Spp*.) produced in Spain. J Agric Food Chem 43:2053–2057

Serrano S, Espejo R, Villarejo M, Jodral ML (2007) Diastase and invertase activities in Andalusian honeys. Int J Food Sci Technol 42:76–79

Shaw PE (1988) Handbook of sugar separations in foods by HPLC. CRC Press, Boca Raton

Silici S (2011) Determination of volatile compounds of pine honeys. Turk J Biol 35:641–645

Silici S, Karaman K (2014) Chemometric approaches for the characterization of Turkish rhododendron and honeydew honeys depending on amino acid composition. J Liq Chromatogr Relat Technol 37:864–877

Silici S, Uluozlu OD, Tuzen M, Soylak M (2016) Honeybee and honey as monitors for heavy metal contamination near the thermal power plants in Mugla, Turkey. Toxicol Ind Health 32:507–516

Silva LR, Videira R, Monteiro AP, Valentaõ P, Andrade PB (2009) Honey from Luso region (Portugal): physicochemical characteristics and mineral contents. Microchem J 93:73–77

da Silva PM, Gauche C, Gonzaga LV, Costa ACO, Fett R (2016) Honey: chemical composition, stability and authenticity. Food Chem 196:309–323

Simuth J, Bilikova K, Kovacova E, Kuzmova Z, Schroder W (2004) Immunochemical approach to detection of adulteration in honey: physiologically active royal jelly protein stimulating TNF-alpha release is a regular component of honey. J Agric Food Chem 52:2154–2158

Sing RP, Sing PN (1996) Amino acid and lipid spectra of larvae of honey bee (*Apis cerana* Fabr) feeding on mustard pollen. Apidologie 27:21–28

Smanalieva J, Senge B (2009) Analytical and rheological investigations into selected unifloral German honey. Eur Food Res Technol 229:107–113

Solayman M, Islam MA, Paul S, Ali Y, Khalil MI, Alam N, Gan SH (2016) Physicochemical properties, minerals, trace elements, and heavy metals in honey of different origins: a comprehensive review. Compr Rev Food Sci Food Saf 15:219–233

Stadelmeier M, Bergner KG (1986) Proteine des bienenhonigs. VII. Eigenschaften und herkunft der honigamylase. Z Lebensm Unters Forsch 182:196–199

Stephens JM, Schlothauer RC, Morris BD, Yang D, Fearnley L, Greenwood DR, Loomes KM (2010) Phenolic compounds and methylglyoxal in some New Zealand manuka and kanuka honeys. Food Chem 120:78–86

Suárez-Luque S, Mato I, Huidobro JF, Simal-Lozano J, Sancho MT (2002) Rapid determination of minoritary organic acids in honey by high performance liquid chromatography. J Chromatogr A 955:207–214

Sweeley CC, Bentley R, Makita M, Wells WW (1963) Gas-liquid chromatography of trimethylsilyl derivatives of sugars and related substances. J Am Chem Soc 85:2497–2507

Tananaki C, Thrasyvoulou A, Giraudel JL, Montury M (2007) Determination of volatile characteristics of Greek and Turkish pine honey samples and their classification by using Kohonen self organising maps. Food Chem 101:1687–1693

Terrab A, Vega-Pérez JM, Díez MJ, Heredia FJ (2001) Characterisation of northwest Moroccan honyes by gas chromatographic-mass spectrometric analysis of their sugar components. J Sci Food Agric 82:179–185

Terrab A, Hernanz D, Heredia FJ (2004) Inductively coupled plasma optical emission spectrometric determination of minerals in thyme honeys and their contribution to geographical discrimination. J Agric Food Chem 52:3441–3445

Tomás-Barberán FA, Martos I, Ferreres F, Radovic BS, Anklam E (2001) HPLC flavonoid profiles as markers for the botanical origin of European unifloral honeys. J Sci Food Agric 81:485–496

Tourn ML, Lombard A, Belliardo F, Buffa M (1980) Quantitative analysis of carbohydrates and organic acids in honeydew, honey and royal jelly by enzymatic methods. J Apic Res 19:144–146

Trautvetter S, Koelling-Speer I, Speer K (2009) Confirmation of phenolic acids and flavonoids in honeys by UPLC–MS. Apidologie 40:140–150

Truchado P, Ferreres F, Bortolotti L, Sabatini AG, Tomás-Barberán FA (2008) Nectar flavonol rhamnosides are floral markers of acacia (*Robinia pseudoacacia*) honey. J Agric Food Chem 56:8815–8824

Truchado P, Ferreres F, Tomas-Barberan FA (2009) Liquid chromatography-tandem mass spectrometry reveals the widespread occurrence of flavonoid glycosides in honey, and their potential as floral origin markers. J Chrom A 1216:7241–7248

Truchado P, Vit P, Ferreres F, Tomas-Barberan F (2011) Liquid chromatography–tandem mass spectrometry analysis allows the simultaneous characterization of C-glycosyl and O-glycosyl flavonoids in stingless bee honeys. J Chromatogr A 1218:7601–7607

Truchado P, Vit P, Heard TA, Tomàs-Barberàn FA, Ferreres F (2015) Determination of interglycosidic linkages in O-glycosyl flavones by high-performance liquid chromatography/photodiode-array detection coupled to electrospray ionization ion trap mass spectrometry. Its application to *Tetragonula carbonaria* honey from Australia. Rapid Commun Mass Spectrom 29:948–954

Truzzi C, Annibaldi A, Illuminati S, Finale C, Scarponi G (2014) Determination of proline in honey: comparison between official methods, optimization and validation of the analytical methodology. Food Chem 150:477–481

Tuberoso CIG, Bifulco E, Jerkovic I, Caboni P, Cabras P, Floris I (2009) Methyl syringate: a chemical marker of asphodel (*Asphodelus microcarpus* salzm. et viv.) monofloral honey. J Agric Food Chem 57:3895–3900

Tuberoso CIG, Bifulco E, Caboni P, Cottiglia F, Cabras P, Floris I (2010) Floral markers of strawberry tree (Arbutus unedo L.) honey. J Agric Food Chem 58(1):384–389

Val A, Huidobro JF, Sánchez MP, Muniategui S, Fernández-Muiño MA, Sancho MT (1998) Enzymatic determination of galactose and lactose in honey. J Agric Food Chem 46:1381–1385

Valachová I, Bučeková M, Majtán J (2016) Quantification of bee-derived peptide defensin-1 in honey by competitive enzyme-linked immunosorbent assay, a new approach in honey quality control. Czech J Food Sci 34:233–243

Vanhanen LP, Emmertz A, Savage GP (2011) Mineral analysis of mono-floral New Zealand honey. Food Chem 128:236–240

Viñas P, Balsalobre N, López-Erroz C, Hernández-Córdoba M (2004) Liquid chromatographic analysis of riboflavin vitamers in foods using fluorescence detection. J Agric Food Chem 52:1789–1794

Wabaidur SM, Ahmed YBH, Alothman ZA, Obbed MS, Al-Harbi NM, Al-Turki TM (2015) Ultra high performance liquid-chromatography with mass spectrometry method for the simultaneous determination of phenolic constituents in honey from various floral sources using multiwalled carbon nanotubes as extraction sorbents. J Separ Sci 38:2597–2606

Wang J, Kliks MM, Qu W, Jun S, Shi G, Li QX (2009) Rapid determination of the geographical origin of honey based on protein fingerprinting and barcoding using MALDI TOF MS. J Agric Food Chem 57:10081–10088

Wardencki W, Chmiel T, Dymerski T, Biernacka P, Plutowska B (2009) Application of gas chromatography, mass spectrometry and olfactometry for quality assessment of selected food products. Ecol Chem Eng 16:287–300

Waś E, Rybak-Chmielewska H, Szczęsna T, Kachaniuk K, Teper D (2011) Characteristics of Polish unifloral honeys. III. Heather honey (*Calluna vulgaris* L.) J Apic Sci 55:129–137

Wehling M, von der Ohe K, von der Ohe W (2006) Problemhonig robinie: Zu wenig invertase-aktivitä. Deutsch Bienen J 13:18–19
Weston RJ (2000) The contribution of catalase and other natural products to the antibacterial activity of honey: a review. Food Chem 71:235–239
White JW (1975) Physical characteristics of honey. In: Crane E (ed) From honey: a comprehensive survey. Heinemmann, London, pp 207–239
White JW Jr (1978) Honey. In: Advances in food research. Academic, New York, pp 287–364
White JW, Kushnir I (1967) The enzymes of honey: examination by ion-exchange chromatography, gel filtration, and starch-gel electrophoresis. J Apicult Res 6:69–89
White JW, Schepartz AI, Subers MH (1963) Identification of inhibine, antibacterial factor in honey, as hydrogen peroxide and its origin in a honey glucose-oxidase system. Biochim Biophys Acta 73:57–70
Wilkins AL, Lu Y, Tan S-T (1995) Extractives from New Zealand honeys. 5. Aliphatic dicarboxylic acids in New Zealand rewerewa (*Knightea excelsa*) honey. J Agric Food Chem 43:3021–3025
Won SA, Li C, Kim J, Rhee H (2009) Immunological characterization of honey major protein and its application. Food Chem 113:1334–1338
Wongchawalit J, Yamamoto T, Nakai H, Kim Y, Sato N, Nishimoto M, Okuyama M, Mori H, Saji O, Chanchao C, Wongsiri S, Surarit R, Svasti J, Seiya C, Kimura A (2006) Purification and characterization of α-glucosidase I from Japanese honeybee (*Apis cerana japonica*) and molecular cloning of its cDNA. Biosci Biotechnol Biochem 70:2889–2898
Wootton M, Edwards RA, Faraji-Haremi R (1978) Effect of accelerated storage conditions on the chemical composition and properties of Australian honeys. 3. Changes in volatile components. J Apic Res 17:167–172
Wunnachit W, Jenner F, Sedgley M (1992) Floral and extrafloral nectar production in *Anacardium occidentale* l. (anacardiaceae): an andromonoecious species. Int J Plant Sci 153:413–420
Xu J-J, An M, Yang R, Cao J, Ye L-H, Peng L-Q (2016) Trace amounts of poly-cyclodextrin wrapped carbon nanotubes for the microextraction of flavonoids in honey samples by capillary electrophoresis with light-emitting diode induced fluorescence detection. Electrophoresis 37:1891–1901
Zamora MC, Chirife J (2006) Determination of water activity change due to crystallization in honeys from Argentina. Food Control 17:59–64
Zhang XH, Wu HL, Wang JY, Tu DZ, Kang C, Zhao J, Chen Y, Miu XX, Yu RQ (2013) Fast HPLC-DAD quantification of nine polyphenols in honey by using second-order calibration method based on trilinear decomposition algorithm. Food Chem 138:62–69
Zhou Q, Wintersteen CL, Cadwallader KR (2002) Identification and quantification of aroma-active components that contribute to the distinct malty flavor of buckwheat honey. J Agric Food Chem 50:2016–2021
Zieliński L, Deja S, Jasicka-Misiak I, Kafarski P (2014) Chemometrics as a tool of origin determination of Polish monofloral and multifloral honeys. J Agric Food Chem 62:2973–2981

Chapter 4
Honey Health Benefits and Uses in Medicine

Hana Scepankova, Jorge A. Saraiva, and Letícia M. Estevinho

Contents

4.1 Antioxidant Capacity

The generation of reactive oxygen species (ROS) and other free radicals during metabolism is an essential and normal process that ideally is compensated through the antioxidant system. However, due to many environmental, lifestyle, and pathological situations, free radicals and oxidants can be produced in excess, resulting in oxidative damage of biomolecules (e.g., lipids, proteins, and DNA). This plays a major role in the development of chronic and degenerative illness such as cancer, autoimmune disorders, aging, cataract, rheumatoid arthritis, cardiovascular, and neurodegenerative diseases (Pham-Huy et al. 2008; Willcox et al. 2004). The human body has several mechanisms to counteract oxidative stress by producing

H. Scepankova • J.A. Saraiva
Department of Chemistry, QOPNA, University of Aveiro, Campus Universitário de Santiago, 3810-193 Aveiro, Portugal

L.M. Estevinho (✉)
Agricultural College of Bragança, Polytechnic Institute of Bragança, Campus Santa Apolónia, 5301-855 Bragança, Portugal

Centre of Molecular and Environmental Biology, University of Minho, Braga, Portugal
e-mail: leticia@lpb.pt

J.M. Alvarez-Suarez (ed.), *Bee Products - Chemical and Biological Properties*,
DOI 10.1007/978-3-319-59689-1_4

antioxidants, which are either naturally synthetized *in situ*, or externally supplied through foods, and/or supplements (Pham-Huy et al. 2008).

Research indicates that foods rich in antioxidants such as honey can protect from the damaging effects of free radicals and ROS and thus exhibit beneficial effects on human health; such as cardiovascular protection by preventing ROS-induced low density lipoprotein (LDL) oxidation (Schramm et al. 2003); cell death in some cancer cell lines (Jaganathan et al. 2015); enhance the human antioxidant defense system (Schramm et al. 2003) among others (Ajibola 2015). For instance in animal models, honey showed a protective effect against damage and oxidative stress induced by cigarette smoke in rat testis (Mohamed et al. 2011); honey supplementation exhibited a hepatoprotective and nephroprotective effect in rats with experimental aflatoxicosis due to its antioxidant activity (Yaman et al. 2016).

The antioxidant capacity (or antioxidant activity) of honey is commonly attributed to its phenolic compounds. These compounds exhibit several preventive effects against different diseases like cancer, cardiovascular diseases, inflammatory disorders, neurological degeneration, wound healing, infectious diseases and aging (Khalil et al. 2010).

The main antioxidant phenolic compounds in honey are: (a) phenolic acids: gallic acid, caffeic, ellagic, ferulic and p-coumaric acids, syringic acid, benzoic acid, cinnamic acid; chlorogenic acid, and (b) flavonoids: apigenin, chrysin, galangin, hesperetin, kaempferol, pinocembrin and quercetin (Fig. 4.1) (Rao et al. 2016; Erejuwa et al. 2014). While some of these bioactive compounds are found in most honey samples, others such as hesperetin and naringenin are found in few honey varieties (Erejuwa et al. 2012).

The amount and type of the phenolic antioxidants depend largely upon the honey's floral source and/or variety of the honey (Gheldof et al. 2002). Generally, darker honeys have been shown to have a higher total phenolic content (TPC) and consequently a higher antioxidant capacity than lighter honeys (Eteraf-oskouei and Najafi 2013). Beside this, Ferreira et al. (2007) found that the dark honey contained the highest concentration of other antioxidants such flavonoids, ascorbic acid, and β-carotene compared to the light and amber honeys.

In addition, some *in vivo* studies have shown that the antioxidant compounds of honey are bioavailable to the human body. Schramm et al. (2003) observed that honey fed at 1.5 g/kg body weight increased both phenolic antioxidants and plasma antioxidant capacity inhealthy human subjects. These results supported the concept that phenolic antioxidants from honey are bioavailable and that these compounds may augment oxidative defense in the human body. Similar evidence has was observed by (Gheldof et al. 2003).

The antioxidant activity of phenolic compounds is related to a number of different mechanisms, such as free radical-scavenging, hydrogen-donation, singlet oxygen quenching and/or metal ion chelation (Eteraf-oskouei and Najafi 2013). Therefore, in order to obtain more accurate and representative results, the antioxidant capacity of honey is generally measured by use of various *in vitro* assays such as: in the form of antiradical activity using 1,1-diphenyl-2-picrylhydrazyl (DPPH) scavenging assay; 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) assay; oxygen radical absorbance capacity (ORAC) assay; and commonly used ferric reducing antioxi-

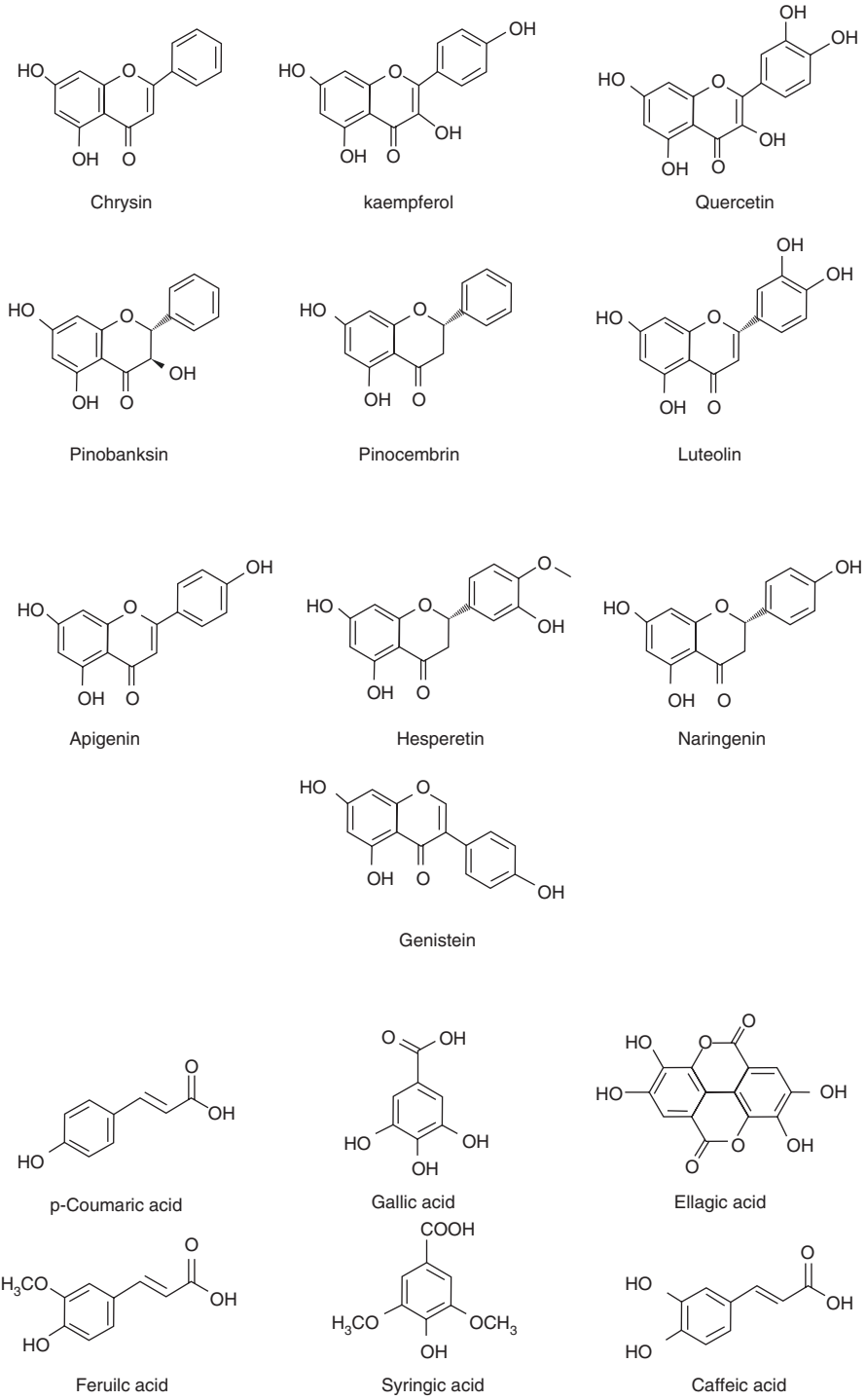


Fig. 4.1 Chemical structures of some flavonoids and phenolic acids in honey (Erejuwa et al. 2014)

dant power (FRAP) assay, that measures the conversion by antioxidants of the oxidized form of iron (Fe3+) to the reduced form (Fe2+) (Erejuwa et al. 2012). Several *in vitro* studies showed that the antioxidant capacity is strongly correlated with the content of the total phenolics in honey (Chua et al. 2013; Sagdic et al. 2013). For instance, a positive correlation was found between antioxidant capacity (ORAC assay) and TPC of various commercial honeys contributed to their antioxidant properties.

However, Gheldof et al. (2002) stated that the levels of single phenolic or other compounds in honey are too low to have a major individual antioxidant significance. Hence, the total antioxidant capacity of honey has been associated to the result of the combined activity and interactions of a wide range of compounds, including both enzymatic: catalase, glucose oxidase, peroxidase and non-enzymatic substances: ascorbic acid, α-tocopherol, carotenoids, amino acids, proteins, organic acids, Maillard reaction products, and other minor components (Nayik et al. 2016; Eteraf-oskouei and Najafi 2013; Ferreira et al. 2007).

Erejuwa et al. (2012) described the synergistic antioxidant effect of honey and thus considered the advantage of honey over other antioxidants, such as vitamins C and E. In fact, these vitamins in their antioxidant action do not end with scavenging or elimination of free radicals. Instead, they can become themselves pro-oxidants which can require other antioxidants for their regeneration into the active or antioxidant form. The advantage of honey is that it comprises several antioxidant constituents and if any of them exhibit pro-oxidant properties, there would be sufficient other antioxidants, which can protect the one against oxidative destruction, and thus lead to the regeneration into the antioxidant form. In fact, honey contains both aqueous and lipophilic antioxidants and thus can act at different cellular levels as an ideal natural antioxidant (Oryan et al. 2016).

Moreover, the quantity of honey consumed in the diet is low compared with the quantity of many of the food sources of antioxidants. According to (Erejuwa et al. 2012), if honey would be used instead of refined sugars as a sweetener for food and drinks it could make a substantial difference to the quantity of antioxidants consumed in the diet.

4.2 Antibacterial Activity

The treatment of bacterial infections is being increasingly complicated by the ability of bacteria to develop resistance to current available antimicrobial agents. This evidence leads to the need of less and better use of antibacterials and antifungals, improved infection control and research on new therapeutic compounds (Feás et al. 2013).

Antibacterial activity of honey is one of the most important findings that was first recognized in 1892 by the Dutch scientist Van Ketel (Eteraf-oskouei and Najafi 2013). The recent research indicates that the effectiveness of honey in many of its medical uses is due to its antibacterial activity that is capable of inhibiting Gram-positive and Gram-negative bacteria, including multidrug resistant strains (Kwakman et al. 2008), and some species of fungi and viruses (Irish et al. 2006; Naama 2009).

For instance, Junie et al. (2016) compared *in vitro* antibacterial activity of several types of honey of different origins against the bacterial resistant strains isolated from patients, including *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Salmonella enterica* serovar *Typhimurium*, *Bacillus cereus*, *Bacillus subtilis*, and *Listeria monocytogenes*. The results showed that all the honey samples presented antibacterial activity against the studied strains and that all the honey samples inhibited bacterial growth. This evidence was similar to other studies conducted elsewhere (Huttunen et al. 2013).

The quantitative determination of the reduction of microbial colonization against a representative panel of bacteria is generally analyzed by *in vitro* tests including (a) determination of minimum inhibitory concentration (MIC) using broth tube dilution methods through visual inspection and (b) by determination of minimum bactericidal concentration (MBC) by sub-culturing tubes showing no visible sign of growth/turbidity (Wasihun and Kasa 2016); these determinations allow a distinction between whether a honey is just stopping the bacteria from growing (bacteriostatic action) or is killing the bacteria (bactericidal action), respectively (Molan 1992). It has been stated that honey possessed a significant antibacterial activity against some bacteria which are resistant to antibiotics (Junie et al. 2016; Mohapatra et al. 2011).

In a study by Wasihun and Kasa (2016) the antibacterial activity of honey was evaluated against multidrug resistant human pathogenic bacterial isolates (*Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, coagulase-negative *Staphylococcus*, *Streptococcus pyogenes* and *Klebsiella pneumoniae*). The MIC and MBC values indicated that the tested honeys had potential bacteriostatic and bactericidal activities against the tested bacteria. Unlike most conventional antibiotics, honey dose may not lead to development of antibiotic-resistant bacteria and it may be used continuously (Eteraf-oskouei and Najafi 2013). According to Alandejani et al. (2009), antibiotics tested (cefazolin, oxacillin, vancomycin, azithromycin, fusidic acid, gentamicin, and linezolid) were not bactericidal to methicillin-sensitive *Staphylococcus aureus*, methicillin-resistant *Staphylococcus aureus* (MRSA), or *Pseudomonas aeruginosa* (PA) biofilms. But the bactericidal rates for the Sidr and Manuka honeys were significantly higher than those seen with the single antibiotics. Thus, the use of honey in a medical setting is considered to be helpful in combating bacterial resistance (Kwakman et al. 2008).

The bacterial strains differ in their sensitivity to honeys. Due to the different floral source, locations, bee species, storage (time and temperature), and processing, the antibacterial potency of different honeys can vary (Grego et al. 2016; Sousa et al. 2016), for some by more than 100-fold (Lusby et al. 2005). Thus it is difficult to standardize honeys and assess their usefulness in a medical application (Sousa et al. 2016). In spite of this, there are medical grade honeys like Revamil source (RS) honey and Manuka (i.e., Medihoney). Having reproducible antibacterial activity, these honeys are produced under controlled conditions in greenhouses and each batch is analyzed individually to assess the Unique Manuka Factor (UMF) that gives a number based on its bactericidal activity (Knight 2013).

However, the mechanism by which honey exerts the activities against a broad spectrum of organisms is still under debate. There are some factors that are closely related to the antibacterial capacity of honey, including the level of hydrogen perox-

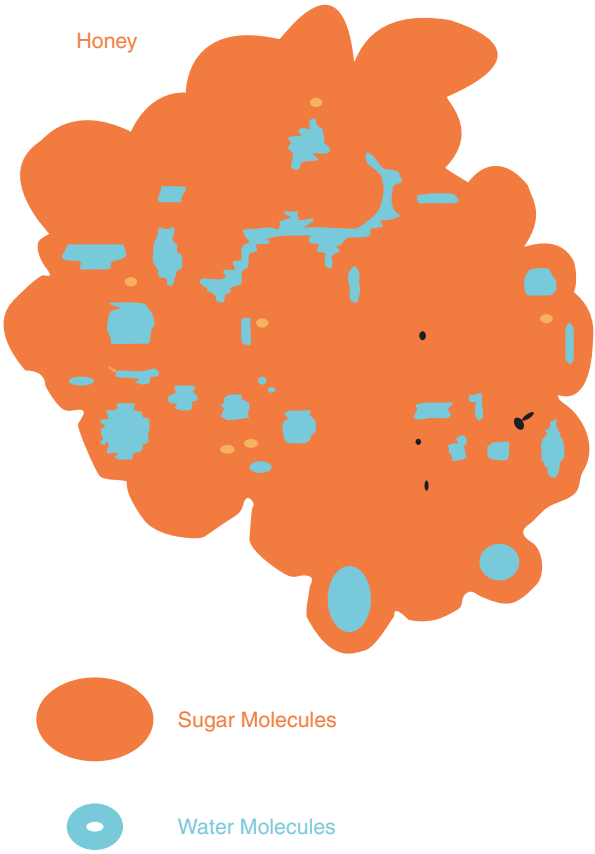


Fig. 4.2 Physical structure of honey (Hadagali and Chua 2014)

ide (H_2O_2), which is formed when honey is diluted (Knight 2013). According to Hadagali and Chua (2014), enzymes convert sucrose into a simple and soluble mixture of monosaccharides. The sugar molecules in the honey solution bind to free water molecules (Fig. 4.2), which means that there is no water available for microbes to use, preventing their survival. The enzyme glucose oxidase (produced by bees) converts glucose into gluconic acid, making the honey too acidic for microbes to grow and survive. The H_2O_2 produced as a by-product of this reaction acts as a sporicidal antiseptic that sterilizes the honey (Fig. 4.3).

Further, the osmolarity of honey, due to about 80% of its composition being sugars, is another important factor to prevent growth of bacteria (Kwakman and Zaat 2012). Different concentrations (or dilutions) of honey used in the *in vitro* tests have been associated with a different antibacterial response (Steinberg et al. 1996).

Beside the sugar content, the low pH (between 3.2 and 4.5 for undiluted honey) is inhibitory to many pathogenic bacteria. However, when consumed orally, the honey would be so diluted by body fluids that any effect of low pH is likely to be lost (Molan 1995). In spite, honeys have substantial antibacterial activity due to non-peroxide components including methylglyoxal and the antimicrobial peptide bee defensing-1. For instance, these compounds have been identified in Manuka and RS honey as antibacterial compounds (Kwakman and Zaat 2012). In addition, fac-

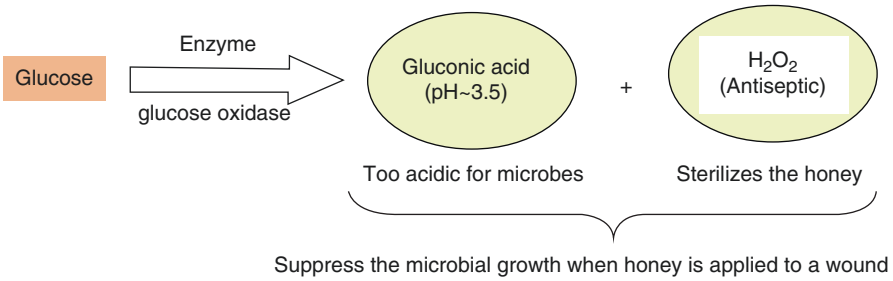


Fig. 4.3 Formation of gluconic acid and hydrogen peroxide (H_2O_2) (Hadagali and Chua 2014)

tors such as phenolic compounds (i.e. flavonoids and phenolic acids) (Kwakman and Zaat 2012; Sousa et al. 2016) and some unknown floral or bee components are being considered as contributing to the antibacterial activity of honey as well (Nishio et al. 2016).

4.3 Anti-inflammatory Capacity

Honey possesses quite a large number of therapeutic properties, including antioxidant and antimicrobial properties, as well as anti-inflammatory activity (Vallianou et al. 2014).

The anti-inflammatory property of honey is mainly related to its antiseptic nature that works by removing infectious bacteria stimulating the inflammatory response, and reduction of the amount of bacteria present in the wound (Hadagali and Chua 2014). In fact, that honey can remove bacteria that cause inflammation, a decrease in wound inflammation after applying honey gauze has been associated to its direct anti-inflammatory properties, such as antioxidant capacity (Yaghoobi et al. 2013). In particular, some of the antioxidant phenolic compounds (i.e. flavonoids) are deeply related to anti-inflammatory effects as previously reported in the literature (González et al. 2011). However, beside the wound inflammation (Tomblin et al. 2014), the correlation of antioxidant capacity of honey with its anti-inflammatory action has been observed in other inflammation models as well (Owoyele et al. 2011).

For instance, the potential protective effect of a honey flavonoid extract (HFE) has been studied on the production of pro-inflammatory mediators by lipopolysaccharide-stimulated N13 microglia. It has been shown that the HFE (containing luteolin, quercetin, apigenin, kaempferol, isorhamnetin, acacetin, tamarixetin, chrysin, and galangin) can inhibit microglial activation and thus be considered as a potential preventive–therapeutic agent for neurodegenerative diseases involving neuroinflammation (Candiracci et al. 2012). The main evidence that considers the antioxidant activity as the anti-inflammatory factor is the ability of antioxidants to inhibit ROS production during the inflammatory process. A number of drugs are available for the treatment of ulcerative colitis. Manuka honey has been shown to specifically decrease the inflammatory response associated with ulcerative colitis, an inflammatory bowel disease characterized by an over-expression of inflammatory cells, possibly by

increasing antioxidant activity (Prakash et al. 2008). In a study by Borsato et al. (2014), honey extract decreased edema, reduced leucocyte infiltration, and inhibited the production of ROS during the inflammatory process induced chemically in mice ear. The anti-inflammatory activity has been associated with a synergetic effect of the honey phenolic compounds, including kaempferol and caffeic acid.

In general, the transcription factor nuclear factor-kappa beta (NF-KB) plays a key role in pathogenesis of inflammation, being known as marker of inflammation (Vallianou et al. 2014). It enhances pro-inflammatory activity, thereby contributing to an amplified inflammatory response, and activates genes encoding pro-inflammatory cytokines – interleukin (IL)-6, IL-8, and tumor necrosis factor-α (TNF-α). These pro-inflammatory cytokines stimulate nitric oxide production (NO), an important mediator of inflammation (Tomblin et al. 2014).

The anti-inflammatory effect of honey has been observed in numerous reports, stating that honey can inhibit the release of pro-inflammatory cytokines, expression of nitric oxide synthase (iNOS), production of ROS (Candiracci et al. 2012), and can decreases prostaglandin levels, one of the major players in the process of inflammation (Al-Waili and Boni 2003). According to an *in vivo* study by Owoyele et al. (2011), honey caused inhibition of NO release in acute and chronic inflammation. Further, Gelam honey has been investigated in an acute inflammation model system showing the reduction of edema in inflamed rat paws. The mechanism was associated with the inhibition of cyclo-oxygenase (COX-2) and iNOS, which resulted in suppressed levels of pro-inflammatory mediators such as NO, PGE2, TNF-α, and IL-6 (Hussein et al. 2012).

In general, acute inflammation is the body's primary response to injurious stimuli, and some of the body's responses are characterized by pains (Hadagali and Chua 2014). Side effects of the available drugs for the treatment of inflammatory pain can sometimes limit the use of these drugs (e.g., NSAID, Indomethacin) (Owoyele et al. 2014). It has been shown that honey significantly decreased production of pro-inflammatory cytokines, which was similar to the effect of the anti-inflammatory drug Indomethacin (NSAID) (Hussein et al. 2012), and also could modulate muscarinic receptors to produce its analgesic effect (Owoyele et al. 2014), thus being potentially useful for treatment of inflammation.

4.4 Wound Healing Activity

Several animal studies and clinical trials have examined the application of honey for acute and chronic wounds (Moore et al. 2001) including burn injuries (Bangroo et al. 2005), and have demonstrated that it limits the amount of edema, improves granulation and epithelization in the proliferative phase while decreasing total wound healing time, reduces scarring and contractures in patients with burn wounds (Mohamed et al. 2015), without adverse effect (allergy or toxicity) at all (Yaghoobi et al. 2013). Due to its low adherence in wound surface, honey causes minimal pain

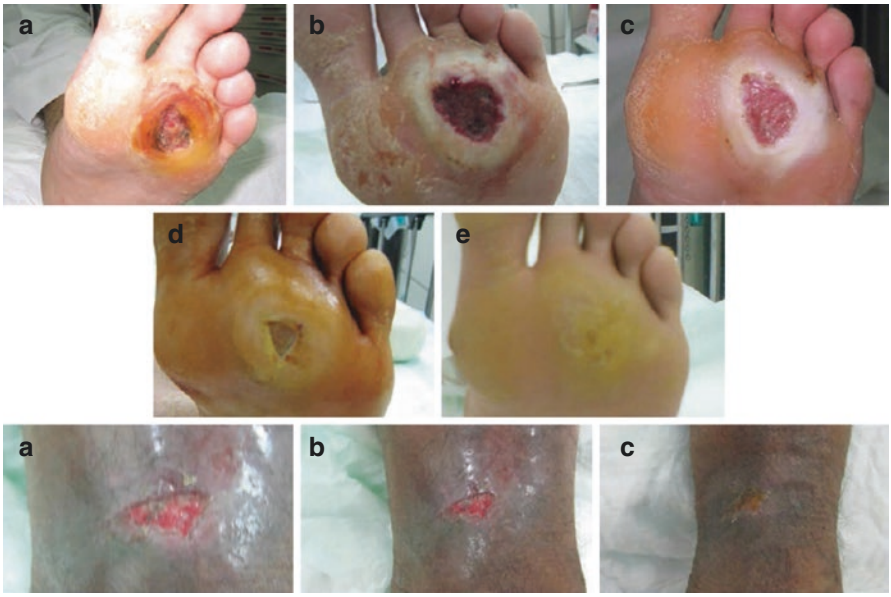


Fig. 4.4 Wound healing activity of honey. Case 1 (**a**–**e**): Diabetic neuropathic ulcer with 3 weeks time to healing. Case 2: (**a**–**c**): Varicose ulcer with 6 weeks time to healing (Mohamed et al. 2015)

during application and upon removal preserving the newly forming granulation tissue (Mohamed et al. 2015).

There is evidence that honey can heal partial thickness burns more quickly (around 4–5 days) than conventional dressings; and post-operative infected wounds can be treated by honey more effectively than by use of antiseptic or gauze (Jull et al. 2008). In a study by (Mohamed et al. 2015), a total of 12 patients with chronic foot ulcers utilized natural honey as an effective alternative to more expensive, advanced wound products. After the wound rinsing with normal saline, natural honey was applied and the wound was covered by glycerin-impregnated gauze. Patients were followed on a daily basis for an average of 4 weeks (Fig. 4.4). The results showed that all ulcers healed with no contractures or scars with a mean healing time of 3 weeks. Moreover, there was a 75% reduction in the dressing budget of the health center and a high level of satisfaction among both health professionals and patients. Also, patients' pain levels were reduced significantly after using natural honey. Similar evidence has been observed when Manuka honey gel was used for treatment of partial-thickness facial burns. The healing time was congruent with or better than what would been expected with standard treatment. No abnormal bacterial growth was reported and the patients reported overall satisfaction with the treatment and cost of the treatment. It has been suggested that Manuka honey is a clinically and economically valuable treatment for partial-thickness facial burns

(Duncan et al. 2016). In addition, a recent study by Aziz et al. (2017) showed that honey dressings can promote better results for burn wounds than the silver-based dressings (i.e., silver sulfadiazine), the currently extensively used method used to treat a variety of acute and chronic wounds.

The presence of antibiotic resistant *S. aureus* in wounds is a cause for concern due to its capacity to acquire resistance to multiple antibiotics that make the treatment of wounds difficult. Jenkins et al. (Jenkins et al. 2012) showed that Manuka honey effectively inhibited the strains of vancomycin-intermediate *S. aureus* (hVISA, VISA) and the clinical strains of vancomycin-sensitive *S. aureus* (VSSA) in the clinical setting. It has been indicated that Manuka honey at low concentration (≤6% (w/v)) can inhibit the growth of clinical isolates of *S. aureus* and thus can be used as a treatment option to help decontaminate wounds infected with antibiotic-resistant organisms like *S. aureus*. Besides that, clinical and laboratory data indicate that natural honey is effective against a variety of common pathogens (see Sect. 4.2).

Honey facilitates wound healing by its ability to create an effective viscous barrier on the wound surface, thus preventing the invasion of microorganisms (Aziz et al. 2017) present in the wounds and can remove any dead tissue that may provide a favourable environment for the growth of microorganisms (Zbuchea 2014). The acidic pH of honey (3.2 to 4.5) inhibits growth of most pathogenic bacteria within wounds, and increases production of hydrogen peroxide from the enzyme glucose oxidase at 1:1000 concentration. This is less than the conventional rinse solutions but enough to inhibit bacterial growth without compromising the new granulation tissue (Mohamed et al. 2015). Thus, when applied topically, honey is capable of cleaning infection from a wound and improving healing (Al-waili et al. 2011). Nevertheless, the wound healing capacity of honey is not only through its antiseptic nature, but also through its immunomodulatory effects, which boost the immune system to fight infection (Fig.4.4). The components in honey related to its immunomodulatory properties have not been yet fully identified, but are being attributed to lipopolysaccharide (LPS), a 5.8 kDa component, major royal jelly protein 1, arabinogalactants, polyphenols, and antioxidants (McLoone et al. 2016). Different types of honey have been shown to act with different mechanisms, and moreover that some of these mechanisms are more efficient than others (Ranzato et al. 2013). For instance, buckwheat honey is used in wound healing products because of its high-polyphenolic content, which make this honey effective in reducing ROS levels causing cell damage and inhibition of wound healing; Manuka honey has notable antibacterial and healing activities, which directly originate from the methylglyoxal it contains, and make this honey useful for treating problematic wounds (Ranzato et al. 2013). The Manuka honey has been claimed to have therapeutic advantages over other honeys and is thus the type of honey most often studied in controlled wound healing studies (Majtan 2011) (Fig. 4.5).

The honey for wound healing is being commonly used as a base for ointments, gels, and in surgical dressings (Shenoy et al. 2012) and some studies successfully demonstrated its healing effect when applied directly in a raw form (Mohamed et al. 2015).

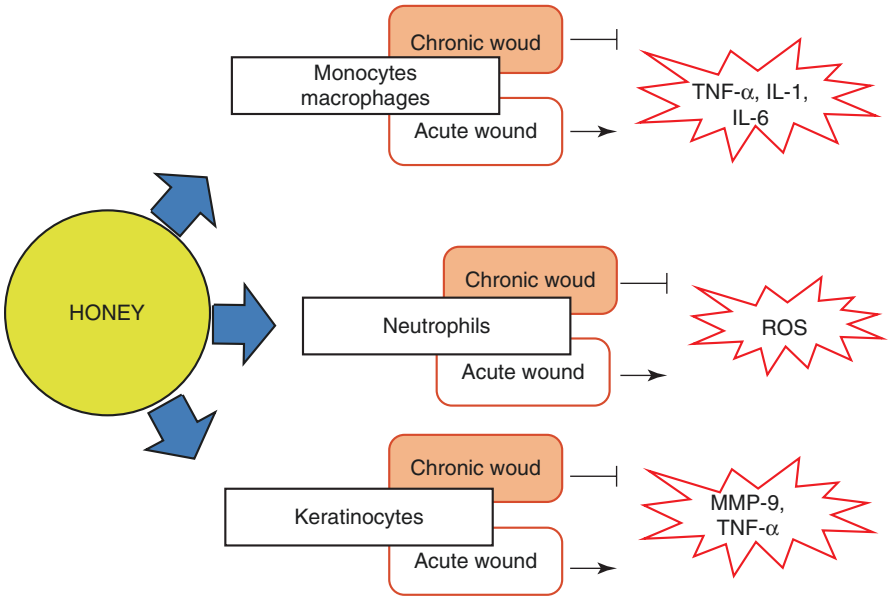


Fig. 4.5 The immunomodulatory action of honey on immune and cutaneous cells involved in wound healing. According to Majtan (2014), honey is able to either stimulate or inhibit the release of certain factors (i.e., cytokines; the MMP-9 protein that plays an important role in normal wound healing through its involvement in re-epithelialisation and extra-cellular matrix remodeling; and ROS) from immune and cutaneous cells depending on wound condition. Honey induces secretion of pro-inflammatory cytokines and MMP-9 during the inflammatory and proliferative wound healing phase, respectively. On the other hand, when the wound inflammation is uncontrolled, honey abrogates prolonged wound inflammation and reduces the elevated levels of pro-inflammatory cytokines, ROS, and MMP-9 (Majtan 2014)

However, natural honey from the comb is not medical grade and should not be used in wound care. Medical grade honey is filtered; gamma irradiated to kill *Clostridium* spores, and produced under exacting standards of hygiene. There are some commercially available sterile honey products like Revamil source (RS) and Manuka honey, the two major medical-grade honeys.

References

Ajibola A (2015) Novel insights into the health importance of natural honey. Malays J Med Sci 22:7–22

Alandejani T, Marsan J, Ferris W, Slinger R, Chan F (2009) Effectiveness of honey on Staphylococcus aureus and Pseudomonas aeruginosa biofilms. Otolaryngol Head Neck Surg 141:114–118

Al-Waili NS, Boni NS (2003) Natural honey lovwers plasma prostaglandin concentrations in normal individuals. J Med Food 6:129–133

Al-waili NS, Salom K, Al-ghamdi AA (2011) Honey for wound healing, ulcers, and burns; data supporting its use in clinical practice. Scientific World J 11:766–787

Aziz Z, Abdul B, Hassan R (2017) The effects of honey compared to silver sulfadiazine for the treatment of burns: a systematic review of randomized controlled trials. Burns 43:50–57

Bangroo AK, Khatri R, Chauhan S (2005) Honey dressing in pediatric burns. J Indian Assoc Pediatr Surg 10:172

Borsato DM, Prudente AS, Döll-Boscardin PM, Borsato AV, Luz CF, Maia BH, Cabrini DA, Otuki MF, Miguel MD, Farago PV, Miguel OG (2014) Topical anti-inflammatory activity of a monofloral honey of Mimosa scabrella provided by Melipona marginata during winterin southern Brazil. J Med Food 17:1–9

Candiracci M, Piatti E, Dominguez-Barragán M, García-Antrás D, Morgado B, Ruano D, Gutiérrez JF, Parrado J, Castaño A (2012) Anti-inflammatory activity of a honey flavonoid extract on lipopolysaccharide-activated N13 microglial cells. J Agric Food Chem 60:12304–12311

Chua LS, Rahaman NL, Adnan NA, Eddie Tan TT (2013) Antioxidant activity of three honey samples in relation with their biochemical components. J Anal Methods Chem 2013:1–8

Duncan CL, Enlow PT, Szabo MM, Tolchin E, Kelly RW, Castanon L, Aballay AM (2016) A pilot study of the efficacy of active Leptospermum honey for the treatment of partial-thickness facial burns. Adv Skin Wound Care 29:349–355

Erejuwa OO, Sulaiman SA, Ab Wahab MS (2012) Honey: a novel antioxidant. Molecules 17:4400–4423

Erejuwa OO, Sulaiman SA, Wahab MSA (2014) Effect of honey and its mechanisms of action on the development and progression of cancer. Molecules 19:2497–2522

Eteraf-oskouei T, Najafi M (2013) Traditional and modern uses of natural honey in human diseases: a review. Iran J Basic Med Sci 16:731–742

Feás X, Iglesias A, Rodrigues S, Estevinho LM (2013) Effect of Erica sp. honey against microorganisms of clinical importance: study of the factors underlying this biological activity. Molecules 18:4233–4246

Ferreira ICFR, Aires E, Barreira JCM, Estevinho LM (2007) Antioxidant activity of Portuguese honey samples: different contributions of the entire honey and phenolic extract. Food Chem 114:1438–1443

Gheldof N, Wang X-H, Engeseth NJ (2002) Identification and quantification of antioxidant components of honeys from various floral sources. J Agric Food Chem 50:5870–7. 5870–5877

Gheldof N, Wang XH, Engeseth NJ (2003) Buckwheat honey increases serum antioxidant capacity in humans. J Agric Food Chem 51:1500–1505

González R, Ballester I, López-Posadas R, Suárez MD, Zarzuelo A, Martínez-Augustin O, Sánchez de Medina F (2011) Effects of flavonoids and other polyphenols on inflammation. Crit Rev Food Sci Nutr 51:331–362

Grego E, Robino P, Tramuta C, Giusto G, Boi M, Colombo R, Serra G, Chiadò-Cutin S, Gandini M, Nebbia P (2016) Evaluation of antimicrobial activity of Italian honey for wound healing application in veterinary medicine. Schweiz Arch Tierheilkd 158:521–527

Hadagali MD, Chua LS (2014) The anti-inflammatory and wound healing properties of honey. Eur Food Res Technol 239:1003–1014

Hussein SZ, Mohd Yusoff K, Makpol S, Mohd Yusof YA (2012) Gelam honey inhibits the production of proinflammatory , mediators NO, PGE 2, TNF-α, and IL-6 in carrageenan-induced acute paw edema in rats. Evid Based Complement Alternat Med 2012:1–13

Huttunen S, Riihinen K, Kauhanen J, Tikkanen-Kaukanen C (2013) Antimicrobial activity of different Finnish monofloral honeys against human pathogenic bacteria. APMIS 121:827–834

Irish J, Carter DA, Shokohi T, Blair SE (2006) Honey has an antifungal effect against Candida species. Med Mycol 44:289–291

Jaganathan SK, Balaji A, Vellayappan MV, Asokan MK, Subramanian AP, John AA, Supriyanto E, Razak SI, Marvibaigi M (2015) A review on antiproliferative and apoptotic activities of natural honey. Anticancer Agents Med Chem 15:48–56

Jenkins R, Wootton M, Howe R, Cooper R (2012) Susceptibility to manuka honey of Staphylococcus aureus with varying sensitivities to vancomycin. Int J Antimicrob Agents 40:88–89
Jull Ab, Rodgers A, Walker N (2008) Honey as a topical treatment for wounds. Cochrane Database Syst Rev 8:CD005083
Junie LM, Vică ML, Glevitzky M, Matei HV (2016) Phisico-chemical characterisation and antibacterial activity of different types of honey tested on strains isolated from hospitalised patients. J Apic Sci 60:5–14
Khalil MI, Sulaiman SA, Boukraa L (2010) Antioxidant properties of honey and its role in preventing health disorder. Open Nutraceuticals J 3:6–16
Knight A (2013) The therapeutic effects of honey. Plymouth Student Sci 6:376–385
Kwakman PHS, Zaat SAJ (2012) Antibacterial components of honey. IUBMB Life 64:48–55
Kwakman PH, Van den Akker JP, Güçlü A, Aslami H, Binnekade JM, de Boer L, Boszhard L, Paulus F, Middelhoek P, te Velde AA, Vandenbroucke-Grauls CM, Schultz MJ, Zaat SA (2008) Medical-grade honey kills antibiotic-resistant bacteria in vitro and eradicates skin colonization. Clin Infect Dis 46:1677–1682
Kwakman PH, Te Velde AA, de Boer L, Vandenbroucke-Grauls CM, Zaat SA (2011) Two major medicinal honeys have different mechanisms of bactericidal activity. PLoS ONE 6:e17709
Lusby PE, Coombes AL, Wilkinson JM (2005) Bactericidal activity of different honeys against pathogenic bacteria. Arch Med Res 36:464–467
Majtan J (2011) Methylglyoxal-a potential risk factor of manuka honey in healing of diabetic ulcers. Evid Based Complement Alternat Med 2011:295494
Majtan J (2014) Honey: an immunomodulator in wound healing. Wound Repair Regen 22:187–192
McLoone P, Warnock M, Fyfe L (2016) Honey an immunomodulatory agent for disorders of the skin. Food Agric Immunol (3):338–349
Mohamed M, Sulaiman SA, Jaafar H, Sirajudeen KN (2011) Antioxidant protective effect of honey in cigarette smoke-induced testicular damage in rats. Int J Mol Sci 12:5508–5521
Mohamed H, Salma MA, Al Lenjawi B, Abdi S, Gouda Z, Barakat N, Elmahdi H, Abraham S, Hamza AH, Al Khozaei D, Al Majid S, Al Majid H, Abdini J, Al Jaber M, Al Masseh F, Al Ali AA (2015) The efficacy and safety of natural honey on the healing of foot ulcers: a case series. Wounds 27:103–114
Mohapatra DP, Thakur V, Brar SK (2011) Antibacterial efficacy of raw and processed honey. Biotechnol Res Int 2011:1–6
Molan P (1992) The antibacterial activity of honey: 2. Variation in the potency of the antibacterial activity. Bee World 73:59–76
Molan P (1995) The antibacterial properties of honey. Chem in NZ 59:10–14
Moore OA, Smith LA, Campbell F, Seers K, McQuay HJ, Moore RA (2001) Systematic review of the use of honey as a wound dressing. BMC Complement Altern Med 1:2
Naama RTA (2009) Evaluation of in-vitro inhibitory effect of honey on some microbial isolate. J Bacteriol Res 1:64–67
Nayik GA, Suhag Y, Majid I, Nanda V (2016) Discrimination of high altitude Indian honey by chemometric approach according to their antioxidant properties and macro minerals. J Saudi Soc Agric Sci. doi:10.1016/j.jssas.2016.04.004
Nishio EK, Ribeiro JM, Oliveira AG, Andrade CG, Proni EA, Kobayashi RK, Nakazato G (2016) Antibacterial synergic effect of honey from two stingless bees: Scaptotrigona bipunctata. Sci Rep 12:21641
Oryan A, Alemzadeh E, Moshiri A (2016) Biological properties and therapeutic activities of honey in wound healing: a narrative review and meta-analysis. J Tissue Viability 25:98–118
Owoyele BV, Adenekan OT, Soladoye AO (2011) Effects of honey on inflammation and nitric oxide production in Wistar rats. Chin J Integr Med 9:447–452
Owoyele BV, Oladejo RO, Ajomale K, Ahmed RO, Mustapha A (2014) Analgesic and anti-inflammatory effects of honey: the involvement of autonomic receptors. Metab Brain Dis 29:167–173
Pham-Huy LA, He H, Pham-Huy C (2008) Free radicals, antioxidants in disease and health. Int J Biomed Sci 4:89–96

Prakash A, Medhi B, Avti PK, Saikia UN, Pandhi P, Khanduja KL (2008) Effect of different doses of manuka honey in experimentally induced inflammatory bowel disease in rats. Phytother Res 22:1511–1519

Premanath R, Lakshmidevi N (2010) Studies on anti-oxidant activity of Tinospora cordifolia (Miers.) leaves using in vitro models. J Am Sci 6:736–743

Ranzato E, Martinotti S, Burlando B (2013) Honey exposure stimulates wound repair of human dermal fibroblasts. Burns Trauma 1:32–38

Rao PV, Krishnan KT, Salleh N, Gan SH (2016) Biological and therapeutic effect of honey produced by honey bees and stingless bees: comparative review. Rev Bras Farmacogn 26:657–664

Sagdic O, Silici S, Ekici L (2013) Evaluation of the phenolic content, antiradical, antioxidant, and antimicrobial activity of different floral sources of honey. Int J Food Prop 16:658–666

Schramm DD, Karim M, Schrader HR, Holt RR, Cardetti M, Keen CL (2003) Honey with high levels of antioxidants can provide protection to healthy human subjects. J Agric Food Chem 51:1732–1735

Shenoy VP, Ballal M, Shivananda P, Bairy I (2012) Honey as an antimicrobial agent against pseudomonas aeruginosa isolated from infected wounds. J Glob Infect Dis 4:102–105

Sousa JM, de Souza EL, Marques G, Meireles B, de Magalhães Cordeiro TT, Gullón B, Pintado MM, Magnani M (2016) Polyphenolic profile and antioxidant and antibacterial activities of monofloral honeys produced by Meliponini in the Brazilian semiarid region. Food Res Int 84:61–68

Steinberg D, Kaine G, Gedalia I (1996) Antibacterial effect of propolis and honey on oral bacteria. Am J Dent 9:236–239

Tomblin V, Ferguson LR, Han DY, Murray P, Schlothauer R (2014) Potential pathway of anti-inflammatory effect by New Zealand honeys. Int J Gen Med 7:149–158

Vallianou NG, Gounari P, Skourtis A, Panagos P, Kazazis C (2014) Honey and its anti-inflammatory, anti-bacterial and anti-oxidant properties. General Med 2:132

Wasihun AG, Kasa BG (2016) Evaluation of antibacterial activity of honey against multidrug resistant bacteria in Ayder Referral and Teaching Hospital. Springerplus 5(1):842

Willcox JK, Ash SL, Catignani GL (2004) Antioxidants and prevention of chronic disease. Crit Rev Food Sci Nutr 44:275–295

Yaghoobi R, Kazerouni A, Kazerouni O (2013) Evidence for clinical use of honey in wound healing as an anti-bacterial, anti-inflammatory, anti-oxidant and anti-viral agent: a review. Jundishapur J Nat Pharm Prod 8:100–104

Yaman T, Yener Z, Celik I (2016) Histopathological and biochemical investigations of protective role of honey in rats with experimental aflatoxicosis. BMC Complement Altern Med 16:1–11

Zbuchea A (2014) Up-to-date use of honey for burns treatment. Ann Burns Fire Disasters XXVII(1):22–30

Part II
Propolis

Chapter 5
Phenolic Composition of Propolis

Celestino Santos-Buelga and Ana M. González-Paramás

Contents

5.1 Introduction

Propolis (bee glue) is a dark-coloured resinous substance collected by honeybees from living plants. It is mixed with wax and used to seal cracks, smooth walls and keep moisture and temperature stable in the hive. Raw propolis is typically composed of 50% plant resins, 30% waxes, 10% essential and aromatic oils, 5% pollens and 5% other organic substances (Huang et al. 2014). Its colour varies from green to brown and reddish depending on its botanical source (Kuropatnicki et al. 2013). Although it is generally accepted that in temperate zones the bud exudates of *Populus* species and their hybrids are the main source of propolis, there is a long list of plants also used by bees as a source of propolis in different parts of the world. In addition to poplars, this list also includes resins of conifers, birch, alder, willow, palm, *Dalbergia ecastaphyllum*, etc. (Crane 1990; Bankova et al. 2000).

Propolis cannot be commercialised as a raw material; it must be purified by extraction with solvents. This process should remove the inert material and preserve the

C. Santos-Buelga • A.M. González-Paramás (✉)
Grupo de Investigación en Polifenoles (GIP-USAL), Facultad de Farmacia, Universidad de Salamanca, Campus Miguel de Unamuno, 37007 Salamanca, Spain
e-mail: paramas@usal.es

J.M. Alvarez-Suarez (ed.), *Bee Products - Chemical and Biological Properties*,
DOI 10.1007/978-3-319-59689-1_5

phenolic fraction. Traditionally, the purification of raw propolis is carried out by soaking, shaking, reflux or Soxhlet extraction, using different solvents to obtain dewaxed propolis extracts rich in polyphenolic components (Pietta et al. 2002). Absolute ethanol is the solvent most commonly employed to prepare propolis extracts, although extraction with aqueous ethanol (70–95%) can result in wax-free tinctures containing higher amounts of phenolic substances (Park and Ikegaki 1998). Commercial propolis can also be obtained by turbo-extraction, which reduces the extraction time to less than one hour, without using heat (Cottica et al. 2011). Applying this technique, higher yields of total phenolic compounds can be obtained using lower percentages of ethanol (around 60%) and higher concentrations of crude propolis (near 30%, m/v); nevertheless, the highest concentrations of flavonoids in the extracts are obtained with higher ethanol percentages in the solvent (maximum 96%) (Cottica et al. 2011). Propolis extraction with pure water (Bankova et al. 1992), hexane and acetone (Pereira et al. 2000), methanol (Pietta et al. 2002; Cao et al. 2004) or chloroform (Negri et al. 2003) has also been used.

More than 500 compounds have been identified in propolis including phenolic components, terpenes, lipid-wax substances, beeswax and other substances such as vitamins, proteins, amino acids and sugars (Walker and Crane 1987; Kurek-Górecka et al. 2014). Among them, plant phenolics constitute the most abundant group of chemical components, including flavonoids, phenolic acids and aldehydes, simple phenols and their esters, coumarins, stilbenes and lignans. The total flavonoid content has been proposed as a quality index for raw propolis; Gardana et al. (2007) suggested that, except for some Brazilian propolis with very low flavonoid content, crude propolis with a content of less than 11% (w/w) should be considered low quality, whereas propolis with contents of 11–14%, 14–17% and >17% could be classified as being of acceptable, good and high quality, respectively. Phenolic contents of between 6.4 and 15.2% (w/w) have been reported for ethanolic extracts of green Brazilian propolis (Woiski and Salatino 1998). Brazilian legislation states that minimum flavonoid and phenolic contents of 0.5% (w/w) and 5% (w/w), respectively, are required in crude propolis (Sawaya et al. 2011).

The different plant sources influence the chemical composition of bee glue, particularly the phenolic profile. In the temperate zone (Europe, Asia and North America) propolis (mostly poplar type) are characterized by similar phenolic patterns consisting of high levels of flavanones and flavones and low levels of phenolic acids and their esters (Marcucci 1995). In propolis from tropical regions, more complex profiles of phenolic compounds have been described, including prenylated *p*-coumaric acids, prenylated flavonoids, caffeoylquinic acid derivatives and lignans (Basnet et al. 1996; Boudourova-Krasteva et al. 1997; Banskota et al. 1998).

5.2 Phenolic Compounds

Phenolic compounds are among the largest and widely distributed groups of secondary metabolites in plants. They differ from other plant metabolites that may also possess a phenolic structure (e.g. terpenes) in their biosynthetic origin. Biogenetically, phenolic compounds derive from two metabolic pathways: the shikimic acid pathway, where mainly phenylpropanoids are formed, and the acetic acid pathway, in

which the main products are simple phenols. The combination of both pathways leads to the formation of flavonoids, the most abundant group of phenolic compounds in nature (Reis Giada 2013).

As indicated above, there are differences in the phenolic composition of propolis depending on the plant source. The most characteristic compounds of temperate propolis, i.e. poplar type propolis, are flavonoids without B-ring substituents, such as pinocembrin, pinobanksin, galangin and chrysin (Walker and Crane 1987). Some recent publications have suggested that poplar propolis can be classified into three main types according to their thin layer chromatography (TLC) behaviour on silica gel 60 or NH_2 plates. Such a grouping is based on the colour of the bands observed after derivatization with polyethylene-glycol and 2-aminoethyldiphenylborinate and illumination under UV light at 366 nm (Ristivojević et al. 2015a). All kinds of poplar propolis show the presence of orange, blue and green chromatographic bands after TLC separation. However, in 'orange type' (O-type) propolis strong orange bands typical of flavonoids such as quercetin are predominant, while in 'blue type' (B-type) light blue bands (characteristic of hydroxycinnamoyl derivatives) are dominant (Bertrams et al. 2013; Ristivojević et al. 2015b; Sârbu and Moț 2011). 'Green type' (G-type), identified in German propolis, is rare and exhibits bands similar to those of O-type, but of lower intensity, in addition to a weak green and one or two stronger green bands corresponding to apigenin, galangin and their methyl-ether derivatives (Bertrams et al. 2013).

In tropical propolis flavonoids are less abundant than other phenolic compounds, such as prenylated derivatives of phenylpropanoids (Brazilian propolis) or benzophenones (characteristic of propolis from Venezuela, Cuba and north eastern regions of Brazil) and caffeolylquinic acids (Salatino et al. 2011). Geranyl flavanones have been reported as characteristic of the so called "Pacific type" propolis, derived from fruit exudates of species of *Macaranga* (Bankova 2005); although initially these flavonoids were used to describe propolis samples from the Pacific region (Taiwan, Okinawa), they have also been found in propolis from African regions (Egypt and Kenya) where such *Macaranga* species are also distributed (Petrova et al. 2010). Propolis from some eastern Mediterranean regions may contain relevant levels of anthraquinones (e.g. chrysophanol), the plant origins of which are still unknown (Salatino et al. 2011).

5.2.1 Flavonoids

The presence of flavonoids in propolis was firstly suggested by Jaubert (1926), who identified the flavone chrysin in bee glue and showed that it could contribute to the colour of beeswax. The relation of the flavonoids present in propolis to the chemical composition of plant exudates was evidenced by Popravko et al. (1970), who identified two flavanones and six other flavonoid pigments in propolis. In parallel, Lavie's research group in France reported the isolation and identification of galangin, pinocembrin, tectochrysin and isalpinin from propolis (Villanueva et al. 1964, 1970). Further advances in chromatographic techniques allowed the separation of more components from propolis and by the end of the 1980s almost 40 flavonoids had been described (Walker and Crane 1987). To date, more than 150 flavonoids have been identified in propolis (Huang et al. 2014).

The composition of raw propolis has been more frequently studied than that of the commercial equivalent. The usual preparation of crude propolis for analysis includes extraction with a solvent (the most usual proportion is 1:10, w/v), solvent evaporation under reduced pressure and dissolution of the residue prior to compound determination. Methods described in the literature for the analysis of flavonoids in propolis include both non-separation, e.g. UV-vis spectrophotometry or NMR, and separation techniques, such as GC, HPTLC, HPLC or HPCE (Gómez-Caravaca et al. 2006; Cuesta-Rubio et al. 2007).

Spectrophotometry is frequently applied to evaluate the total flavonoid content due to its simplicity and affordability. Two colorimetric methods have been used for the quantitative analysis of flavonoids in propolis based on reaction with aluminium chloride for the determination of flavones and flavonols, and with 2,4-dinitrophenylhydrazine (DNP) for the determination of flavanones and dihydroflavonols (Gómez-Caravaca et al. 2006). The sum of the flavonoid contents determined by these two methods closely reflects the actual total flavonoid content of the sample (Chang et al. 2002). The Folin-Ciocalteu reagent has also been employed to quantify the total flavonoid content (Ahn et al. 2004; Popova et al. 2004), although it essentially identifies all phenolic families as well as reducing substances that may be present in the extracts.

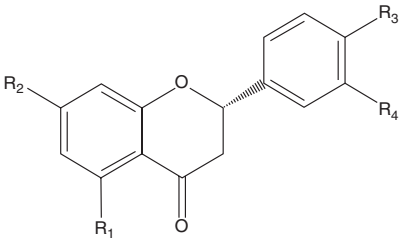
Thin layer chromatography (TLC) is especially useful for the rapid screening of propolis components before detailed instrumental analysis (Luo et al. 2011); the stationary phase has to be carefully selected, taking into account the structure of the phenolic constituents being separated (Park et al. 2002; Popova et al. 2005).

Gas chromatography (GC) can be used for both qualitative and quantitative analysis of phenolic compounds: it provides excellent resolution but an extra derivatization step is required to volatilize the flavonoids. GC coupled with mass spectrometry (MS) is widely used, since it allows the acquisition of molecular mass data and structural information, thus allowing the identification of compounds (Alencar et al. 2007; Campo Fernández et al. 2008; Kalogeropoulos et al. 2009). High-temperature high-resolution gas chromatography (HT-HRGC) has been employed for separating complex mixtures and studying high molecular weight compounds that do not elute on ordinary GC columns (Pereira et al. 1998; Park et al. 2002).

High-performance liquid chromatography (HPLC) is currently the most widely used technique for the analysis of polyphenols and there are multiple examples in the literature of the application of this technique to their study in propolis (Kumazawa et al. 2004a, b; Gardana et al. 2007; Zhou et al. 2008; Lima et al. 2009; Pellati et al. 2011).

Capillary electrophoresis (CE) is considered an alternative to HPLC for flavonoid analysis due to its advantages of rapidity, high separation efficiency and low cost (Jiang et al. 2008). However, it is less versatile and shows inferior repeatability compared to that of HPLC. There have been some reports in the literature on the determination of flavonoids in propolis using CE (Hilhorst et al. 1998; Volpi 2004; Fu et al. 2005; Jiang et al. 2008). Further information on analytical aspects of propolis analysis can be found in reviews by Gómez-Caravaca et al. (2006) and Sawaya et al. (2011).

Flavonoids belonging to flavanone, flavone, flavonol, flavanonol, chalcone, dihydrochalcone, isoflavone, isodihydroflavone, flavan, isoflavan and neoflavonoid groups have been reported in propolis. Most of the identified compounds are



	R_1	R_2	R_3	R_4
Pinocembrin	OH	OH	H	H
Pinostrobin	OCH_3	OH	H	H
Naringenin	OH	OH	OH	H
Sakuranetin	OCH_3	OH	OH	H
Isosakuranetin	OH	OH	OCH_3	OH
Liquiritigenin	H	OH	OH	H

Fig. 5.1 Structures of the principal flavanones described in propolis

aglycones because while collecting propolis, bees secrete β-glucosidase which removes the sugar residues of the glycosylated flavonoids present in plants (Sahinler and Kaftanoglu 2005). Nevertheless, this enzyme could be inefficient at hydrolysing some types of glycosides, such as *β*-diglycosides (Zhang et al. 2011) or *C*-glycosides (Righi et al. 2011). Thus, the presence of rutin (quercetin 3-*O*-rutinoside) and isorhamnetin-3-*O*-rutinoside has been reported in propolis samples from different countries (Bonvehi and Call 1994; Popova et al. 2009), as well as flavone *C*-glycosides (Righi et al. 2011) and luteolin and naringenin glucosides (Zhang et al. 2015) in propolis samples from Brazil and China, respectively. A propolis with an uncommon phenolic profile rich in flavonoid glycosides was described in Portugal, showing more than 10 glycoside derivatives, among which quercetin and kaempferol rutinosides were predominant, together with some glucuronides and glucosides (Falcão et al. 2013).

Flavanones are one of the most significant groups of flavonoids present in propolis, with more than 40 different compounds of this class having been described in this product. These compounds are common in poplar type propolis from the temperate zone, in both the northern and southern hemispheres. Pinocembrin, pinostrobin, naringenin, sakuranetin, isosakuranetin and liquiritigenin (Fig. 5.1) have been reported as the most prevalent flavanones in European, Chinese, non-tropical Asian and North American propolis (Villanueva et al. 1970; Greenaway et al. 1991; Marcucci 1995; Bankova et al. 2000; Shi et al. 2012; de Groot et al. 2014; Ristivojević et al. 2015a). Pinobanksin is frequently found in the form of methylated or esterified derivatives (acetates, propionates, butyrates, pentanoates, methylbutyrates, etc.) (Gardana et al. 2007; Falcão et al. 2010). Other hydroxy- and methoxy-flavanones that have been identified are (*2R*, *3R*)-3,6,7-trihydroxyflavanone in propolis from Nepal (Shrestha et al. 2007a, b), (*2R*, *3R*)-3,7-dihydroxy-6-methoxyflavanone and (*2R*, *3R*)-3,5,7-trihydroxy-6-methoxyflavanone (alnustinol) in Brazilian propolis (Li et al. 2008), and 5-methoxy-3-hydroxyflavanone in propolis from Portugal (Falcão et al. 2010) and Mexico (Li et al. 2010). In addition, the corresponding chalcones of the principal flavanones have been detected in poplar type propolis (Greenaway et al. 1991), Canadian samples from Tacamahaca poplars (Christov et al. 2006) and Brazilian red propolis from *Dalbergia ecastophyllum* (Li et al. 2008). In Portuguese propolis, Falcão et al. (2010) detected the presence of a dimethylated derivative of hesperetin

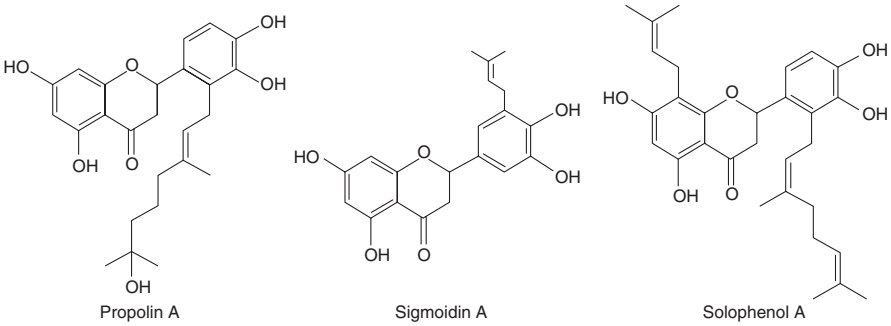
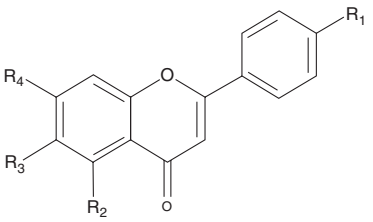


Fig. 5.2 Typical prenylated flavanones described in "Pacific type" propolis

and also identified six peculiar pinocembrin/pinobanksin derivatives containing a phenylpropanoic acid substituent. Two phenylallyl-pinobanksin derivatives with cytotoxic properties were also described by Li et al. (2011) in propolis from Mexico. Prenylated flavanones were shown to be common and abundant constituents in "Pacific type" propolis from Taiwan, Japan or the Solomon Islands. Prenylated flavanones such as propolin A (Fig. 5.2) and propolin B were firstly isolated and characterized by Chen et al. (2003) in Taiwanese propolis, in which propolin E and sigmoidin B (Fig. 5.2) were also present. In propolis from Japan, geranylflavanone derivatives are abundant (Kumazawa et al. 2004a), while solophenol A (Fig. 5.2) and sophoraflavanone A are typical in propolis from the Solomon Islands (Inui et al. 2012). In propolis from tropical zones, particularly those from the south eastern region of Brazil, where flavonoids are not the main phenolic constituents, some flavanones such as isosakuranetin have been described in propolis from *Baccharis dracunculifolia* (Park et al. 2002).

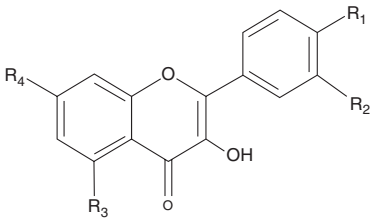
Although less abundant than flavanones, **flavones and flavonols** (Fig. 5.3) are also commonly found in propolis. Typical examples include acacetin, apigenin, chrysin, tectochrysin, galangin, izalpinin, quercetin and kaempferol, and, less commonly, fisetin or pectolinarigenin (Marcucci 1995; Stevens et al. 1995). Kaempferide and luteolin have also been found in Brazilian green propolis (Simões et al. 2004) and Chinese propolis (Cao et al. 2004), respectively. A new flavonol named macarangin (Fig. 5.4) was identified in Kenyan samples from the *Macaranga* genus (Petrova et al. 2010). As mentioned previously, propolis with an unusual flavonoid composition, including quercetin and kaempferol glycosides, was described in Portugal (Falcão et al. 2013).

The presence of **isoflavones** and **isodihydroflavones** (Fig. 5.5) in European propolis is rare, however, compounds of this family (e.g., daidzein, formononetin, xenognosin B, vestitone and calycosin, among others) were found in red Brazilian and red Cuban propolis, likely originating from Leguminous plants, *Dalbergia ecastophyllum* in particular in the case of the Brazilian variety (Piccinelli et al. 2005; Alencar et al. 2007; Campo Fernández et al. 2008; Li et al. 2008). **Isoflavans**, such as vestitol, mucronulatol and neovestitol (Fig. 5.6), and **pterocarpins**



Flavones

	R_1	R_2	R_3	R_4
Chrysin	H	OH	H	OH
Tectochrysin	H	OH	H	OCH_3
Apigenin	OH	OH	H	OH
Acacetin	OCH_3	OH	H	OH
Pectolinarigenin	OCH_3	OH	OCH_3	OH

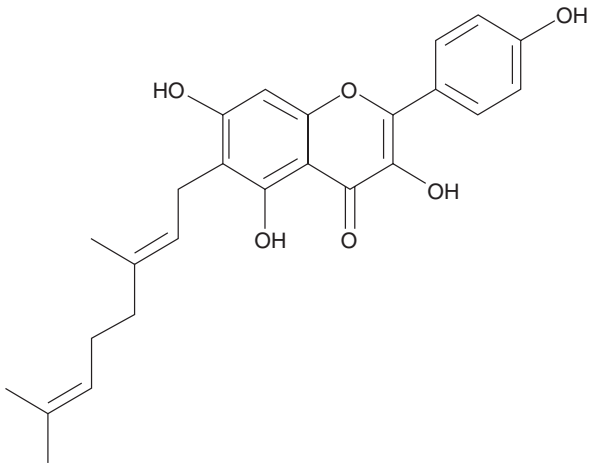


Flavonols

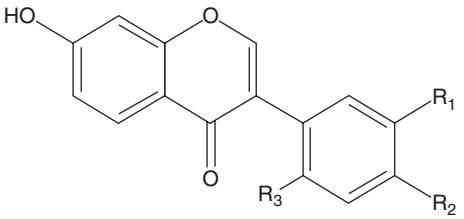
	R_1	R_2	R_3	R_4
Galangin	H	H	OH	OH
Kaempferol	OH	H	OH	OH
Quercetin	OH	OH	OH	OH
Fisetin	OH	OH	H	OH
Izalpinin	H	H	OH	OCH_3

Fig. 5.3 Main flavones and flavonols described in propolis

Fig. 5.4 Structure of macarangin

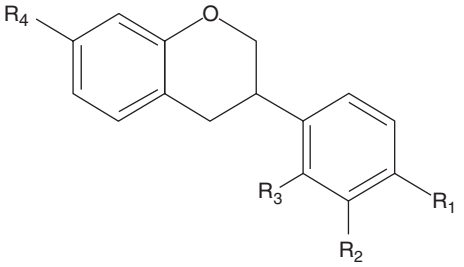


(i.e., neoflavonoids; Fig. 5.7) were also identified in these two types of red propolis (Piccinelli et al. 2005; Campo Fernández et al. 2008; Li et al. 2008). Finally, in samples from Nepal, up to 14 unique open-chain neoflavonoids (Fig. 5.8) were identified and proposed as chemical markers for this type of propolis (Awale et al. 2005; Shrestha et al. 2007b).



	R_1	R_2	R_3
Daidzein	H	OH	H
Formonetin	H	OCH_3	H
Calycosin	OH	OCH_3	H
Xenognosin B	H	OCH_3	OH

Fig. 5.5 Isoflavones described in propolis



	R_1	R_2	R_3	R_4
Vesitol	OCH_3	H	OH	OH
Neovestitol	OH	H	OH	OCH_3
Mucronulatol	OCH_3	OH	OCH_3	OH

Fig. 5.6 Main isoflavans identified in propolis

Fig. 5.7 Basic structure of pterocarpins

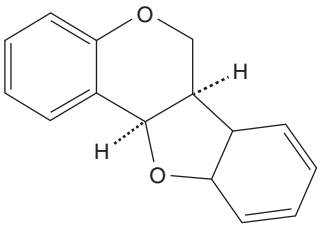
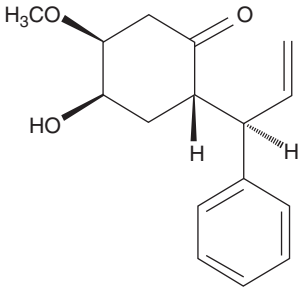


Fig. 5.8 Basic structure of open-chain neoflavonoids



5.2.2 Phenolic Acid Derivatives

Phenolic acids in propolis mainly consist of hydroxybenzoic acids (gallic, gentisic, protocatechuic, salicylic and vanillic acids) and hydroxycinnamic acids (*p*-coumaric, caffeic and ferulic acids). In addition to their free forms, phenolic acids are often present as benzyl-, methylbutenyl-, phenylethyl- and cinnamyl-esters, with caffeic acid

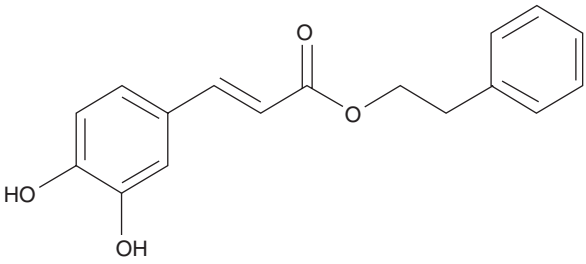


Fig. 5.9 Structure of caffeic acid phenethyl ester (CAPE)

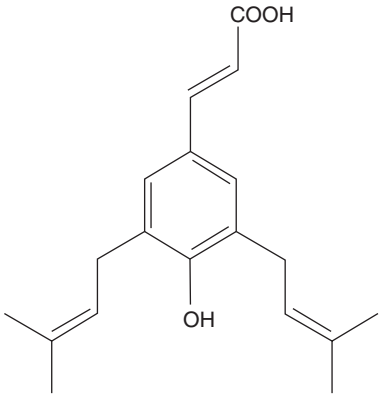


Fig. 5.10 Structure of artepillin C

phenethyl ester (CAPE) (Fig. 5.9) being a major constituent in temperate propolis (Marcucci 1995; Huang et al. 2014; Ristivojević et al. 2015a, b). Hydroxycinnamic acid derivatives were also found in Brazilian (Marcucci et al. 2001; Salatino et al. 2005) and Australian propolis (Abu-Mellal et al. 2012). In Brazilian propolis from *Citrus* spp. chlorogenic acids are particularly abundant (dos Santos Pereira et al. 2003). Furthermore, prenylated phenylpropanoids were shown to be very common in Brazilian green propolis (Salatino et al. 2005). Among them, two different classes of prenylated cinnamic acid-derived compounds have been described: one is characterized by the presence of one or two prenyl groups not involved in ring formation (e.g., artepillin C, Fig. 5.10), and the other by the prenyl group forming a heterocyclic ring giving rise to chromenes (Salatino et al. 2005). These prenylated cinnamic acids may also be present as esters, such as 3-prenyl-cinnamic acid allyl ester (Negri et al. 2003).

5.2.3 Other Phenolic Compounds

Although stilbenes are not very common in plants, there are a few reports identifying this class of compounds in propolis from different geographical origins. Two geranylstilbenes (scheweinfurthin A and B) and 5-farnesyl-3'-hydroxyresveratrol were isolated from propolis produced from *Macaranga* spp. in Kenya and the Solomon Islands, respectively (Petrova et al. 2010; Inui et al. 2012). Different stilbenes, especially in the prenylated form, were identified in propolis from Australian

Kangaroo Island (Abu-Mellal et al. 2012), suggesting that stilbene sources are not limited to only a few plants.

Lignans have been identified in a few propolis samples, especially those from tropical zones, such as Canary Island (Bankova et al. 1998), Kenya (Petrova et al. 2010) and Brazil (Li et al. 2008), as well as in propolis from Chile (Valcic et al. 1998). In Brazilian red propolis in particular, the presence of syringaresinol, pinoresinol and their dimethyl ether derivatives has been reported (Li et al. 2008).

Some coumarins, such as coumarin and daphnetin and the prenylated coumarin suberosin 1, were also identified in some propolis samples (de Castro 2001; Trusheva et al. 2010).

References

Abu-Mellal A, Koolaji N, Duke RK, Tran VH, Duke CC (2012) Prenylated cinnamate and stilbenes from Kangaroo Island propolis and their antioxidant activity. Phytochemistry 77:251–259

Ahn MR, Kumazawa S, Hamasaka T, Bang KS, Nakayama T (2004) Antioxidant activity and constituents of propolis collected in various areas of Korea. J Agric Food Chem 52:7286–7292

Alencar SM, Oldoni TLC, Castro ML, Cabral ISR, Costa-Neto CM, Cury JA, Rosalen PL, Ikegaki M (2007) Chemical composition and biological activity of a new type of Brazilian propolis: red propolis. J Ethnopharmacol 113:278–283

Awale S, Shrestha SP, Tezuka Y, Ueda JY, Matsushige K, Kadota S (2005) Neoflavonoids and related constituents from Nepalese propolis and their nitric oxide production inhibitory activity. J Nat Prod 68:858–864

Bankova V (2005) Chemical diversity of propolis and the problem of standardization. J Ethnopharmacol 100:114–117

Bankova VS, Christov RS, Tejera AD (1998) Lignans and other constituents of propolis from the Canary Islands. Phytochemistry 49:1411–1415

Bankova V, Christov R, Stoev G, Popov S (1992) Determination of phenolics from propolis by capillary gas chromatography. J Chromatogr 607:150–153

Bankova VS, de Castro SL, Marcucci MC (2000) Propolis: recent advances in chemistry and plant origin. Apidologie 31:3–15

Banskota AH, Tezuka Y, Prasain JK, Matsushige K, Saiki I, Kadota S (1998) Chemical constituents of Brazilian propolis and their cytotoxic activities. J Nat Prod 61:896–900

Basnet P, Matsushige K, Hase K, Kadota S (1996) Potent antihepatotoxic activity of dicaffeoyl quinic acids from propolis. Biol Pharm Bull 19:1479–1484

Bertrams J, Muller MB, Kunz N, Krammerer DR, Stintzing FC (2013) Phenolic compounds as marker compounds for botanical origin determination of German propolis samples based on TLC and TLC-MS. J Appl Bot Food Qual 86:143–153

Bonvehi JS, Call FV (1994) Phenolic composition of propolis from China and from South America. Z Naturforsch 49c:712–718

Boudourova-Krasteva G, Bankova V, Sforcin JM, Nikolova N, Popov S (1997) Phenolics from Brazilian propolis. Z Naturforsch 52c:676–679

Campo Fernández M, Cuesta-Rubio O, Rosado Perez A, Montes De Oca Porto R, Márquez Hernández I, Piccinelli AL, Rastrelli L (2008) GC–MS determination of isoflavonoids in seven red Cuban propolis samples. J Agric Food Chem 56:9927–9932

Cao YH, Wang Y, Yuan Q (2004) Analysis of flavonoids and phenolic acid in propolis by capillary electrophoresis. Chromatographia 59:135–140

Chang CC, Yang MH, Wen HM, Chern JC (2002) Estimation of total flavonoid content in propolis by two complementary colorimetric methods. J Food Drug Anal 10:178–182

Chen CN, Wu CL, Shy HS, Lin JK (2003) Cytotoxic prenylflavanones from Taiwanese propolis. J Nat Prod 66:503–506

Christov R, Trusheva B, Popova M, Bankova V, Bertrand M (2006) Chemical composition of propolis from Canada, its antiradical activity and plant origin. Nat Prod Res 20:531–536

Cottica SM, Sawaya ACHF, Eberlin MN, Franco S, Zeoulae LM, Visentainera JV (2011) Antioxidant activity and composition of propolis obtained by different methods of extraction. J Braz Chem Soc 22:927–933

Crane E (1990) The traditional hive products: honey and beeswax. In: Crane E (ed) Bees and beekeeping: science, practice and world resources. Heinemann Newnes, Oxford, pp 388–413

Cuesta-Rubio O, Piccinelli AL, Campo Fernandez M, Márquez Hernández I, Rosado A, Rastrelli L (2007) Chemical characterization of Cuban propolis by HPLC-PDA, HPLC-MS, and NMR: the brown, red, and yellow Cuban varieties of propolis. J Agric Food Chem 55:7502–7509

de Castro SL (2001) Propolis: biological and pharmacological activities. Ann Rev Biomed Sci 3:49–83

de Groot AC, Popova MP, Bankova VS. (2014) An update on the constituents of poplar-type propolis, Wapserveen, The Netherlands: acdegroot publishing, 11. ISBN/EAN: 978-90-813233-0-7.

dos Santos Pereira A, de Miranda Pereira AF, Trugo LC, de Aquino Neto FR (2003) Distribution of quinic acid derivatives and other phenolic compounds in Brazilian propolis. Z Naturforsch C 58:590–593

Falcão SI, Vale N, Gomes P, Domingues MRM, Freire C, Cardoso SM, Vilas-Boas M (2013) Phenolic profiling of Portuguese propolis by LC–MS spectrometry: uncommon propolis rich in flavonoid glycosides. Phytochem Anal 24:309–318

Falcão S, Vilas-Boas M, Estevinho LM, Barros C, Domingues MRM, Cardoso SM (2010) Phenolic characterization of northeast Portuguese propolis: usual and unusual compounds. Anal Bioanal Chem 396:887–897

Fu SH, Yang MH, Wen HM, Chern JC (2005) Analysis of flavonoids in propolis by capillary electrophoresis. J Food Drug Anal 13:43–50

Gómez-Caravaca AM, Gómez-Romero M, Arráez-Román D, Segura-Carretero A, Fernández-Gutiérrez A (2006) Advances in the analysis of phenolic compounds in products derived from bees. J Pharm Biomed Anal 41:1220–1234

Gardana C, Scaglianti M, Pietta P, Simonetti P (2007) Analysis of the polyphenolic fraction of propolis from different sources by liquid chromatography-tandem mass spectrometry. J Pharm Biomed Anal 45:390–399

Greenaway W, May J, Scaysbrook T, Whatley FR (1991) Identification by gas chromatography-mass spectrometry of 150 compounds in propolis. Z Naturforsch 46C:111–121

Hilhorst MJ, Somsen GW, de Jong GJ (1998) Potential of capillary electrophoresis for the profiling of propolis. J High Resolut Chromatogr 21:608–612

Huang S, Zhang C-P, Wang K, Li GQ, Hu F-L (2014) Recent advances in the chemical composition of propolis. Molecules 19:19610–19632

Inui S, Shimamura Y, Masuda S, Shirafuji K, Moli RT, Kumazawa S (2012) A new prenylflavonoid isolated from propolis collected in the Solomon Islands. Biosci Biotechnol Biochem 76:1038–1040

Jaubert GF (1926) Origin of the colour of beeswax and the composition of propolis. C R Hebd Seances Acad Sci 184:1134–1136. (French)

Jiang LQ, Fang GZ, Zhang Y, Cao GJ, Wang S (2008) Analysis of flavonoids in propolis and *Ginkgo biloba* by micellar electrokinetic capillary chromatography. J Agric Food Chem 56:11571–11577

Kalogeropoulos N, Konteles SJ, Troullidou E, Mourtzinos I, Karathanos VT (2009) Chemical composition, antioxidant activity and antimicrobial properties of propolis extracts from Greece and Cyprus. Food Chem 116:452–461

Kumazawa S, Goto H, Hamasaka T, Fukumoto S, Fujimoto T, Nakayama T (2004a) A new prenylated flavonoid from propolis collected in Okinawa, Japan. Biosci Biotechnol Biochem 68:260–262

Kumazawa S, Hamasaka T, Nakayama T (2004b) Antioxidant activity of propolis of various geographic origins. Food Chem 84:329–339
Kurek-Górecka A, Rzepecka-Stojko A, Górecki M, Stojko J, Sosada M, Świerczek-Zięba G (2014) Structure and antioxidant activity of polyphenols derived from propolis. Molecules 19:78–101
Kuropatnicki AK, Szliszka E, Krol W (2013) Historical aspects of propolis research in modern times. J Evidence-Based Complementary Altern Med 2013:Article ID 964149. 11 pages
Li F, Awale S, Tezuka Y, Esumi H, Kadota S (2010) Study on the constituents of Mexican propolis and their cytotoxic activity against PANC-1 human pancreatic cancer cells. J Nat Prod 73:623–627
Li F, Awale S, Tezuka Y, Kadota S (2008) Cytotoxic constituents from Brazilian red propolis and their structure-activity relationship. Bioorg Med Chem 16:5434–5440
Li F, He YM, Awale S, Kadota S, Tezuka Y (2011) Two new cytotoxic phenylallylflavanones from Mexican propolis. Chem Pharm Bull 59:1194–1196
Lima B, Tapia A, Luna L, Fabani MP, Schmeda-Hirschmann G, Podio NS, Wunderlin DA, Feresin GE (2009) Main flavonoids, DPPH activity, and metal content allow determination of the geographical origin of propolis from the province of San Juan (Argentina). J Agric Food Chem 57:2691–2698
Luo C, Zou X, Li Y, Sun C, Jiang Y, Wu Z (2011) Determination of flavonoids in propolis-rich functional foods by reversed phase high performance liquid chromatography with diode array detection. Food Chem 127:314–320
Marcucci MC (1995) Propolis: chemical composition, biological properties and therapeutical activity. Apidologie 26:83–99
Marcucci M, Ferreres F, García-Viguera C, Bankova V, De Castro S, Dantas A, Valente P, Paulino N (2001) Phenolic compounds from Brazilian propolis with pharmacological activities. J Ethnopharmacol 274:105–112
Negri G, Salatino MLF, Salatino A (2003) "Green propolis": unreported constituents and a novel compound from chloroform extracts. J Apic Res 42:53–56
Park YK, Ikegaki M (1998) Preparation of water and ethanolic extracts of propolis and evaluation of the preparations. Biosci Biotechnol Biochem 62:2230–2232
Park YK, Alencar SM, Aguiar CL (2002) Botanical origin and chemical composition of Brazilian propolis. J Agric Food Chem 50:2502–2506
Pellati F, Orlandini G, Pinetti D, Benvenuti S (2011) HPLC-DAD and HPLC-ESI-MS/MS methods for metabolite profiling of propolis extracts. J Pharm Biomed Anal 55:934–948
Pereira AD, Pinto AC, Cardoso JN, Neto FRD, Ramos HFS, Dellamora-Ortiz GM, dos Santos EP (1998) Application of high temperature high resolution gas chromatography to crude extracts of propolis. J High Resolut Chromatogr 21:396–400
Pereira AD, Norsell M, Cardoso JN, Neto FRD (2000) Rapid screening of polar compounds in Brazilian propolis by high-temperature high-resolution gas chromatography−mass spectrometry. J Agric Food Chem 48:5226–5230
Petrova A, Popova M, Kuzmanova C, Tsvetkova I, Naydenski H, Muli E, Bankova V (2010) New biologically active compounds from Kenyan propolis. Fitoterapia 81:509–514
Piccinelli AL, Campo Fernandez M, Cuesta-Rubio O, Márquez Hernández I, de Simone F, Rastrelli L (2005) Isoflavonoids isolated from Cuban propolis. J Agric Food Chem 53:9010–9016
Pietta PG, Gordana C, Pietta AM (2002) Analytical methods for quality control of propolis. Fitoterapia 73:S7–S20
Popova M, Bankova V, Butovska D, Petkov V, Nikolova-Damyanova B, Sabatini AG, Marcazzan GL, Bogdanov S (2004) Validated methods for the quantification of biologically active constituents of poplar-type propolis. Phytochem Anal 15:235–240
Popova MP, Chinou IB, Marekov IN, Bankova VS (2009) Terpenes with antimicrobial activity from Cretan propolis. Phytochemistry 70:1262–1271
Popova M, Silici S, Kaftanoglu O, Bankova V (2005) Antibacterial activity of Turkish propolis and its qualitative and quantitative chemical composition. Phytomedicine 112:221–228
Popravko SA, Gurevich AI, Kolosov MN (1970) Isolation and identification of the main components of propolis. In: Proceedings of the 22nd international beekeeping congress: summaries, pp 163–164

Reis Giada ML (2013) Food phenolic compounds: main classes, sources and their antioxidant power. In: Morales-González JA (ed) Oxidative stress and chronic degenerative diseases - a role for antioxidants, InTech. doi: 10.5772/45722

Righi AA, Alves TR, Negri G, Marques LM, Breyer H, Salatino A (2011) Brazilian red propolis: unreported substances, antioxidant and antimicrobial activities. J Sci Food Agric 91:2363–2370

Ristivojević P, Trifković J, Andrić F, Milojković-Opsenica D (2015a) Poplar-type propolis: chemical composition, botanical origin and biological activity. Nat Prod Commun 10:1869–1876

Ristivojević P, Trifković J, Gašić U, Andrić F, Nedić N, Tešić Ž, Milojković-Opsenica D (2015b) Ultrahigh-performance liquid chromatography and mass spectrometry (UHPLC–LTQ/Orbitrap/MS/MS) study of phenolic profile of Serbian poplar type propolis. Phytochem Anal 26:127–136

Sahinler N, Kaftanoglu O (2005) Natural product propolis: chemical composition. Nat Prod Res 19:183–188

Salatino A, Weinstein Teixeira E, Negri G, Message D (2005) Origin and chemical variation of Brazilian propolis. J Evidence-Based Complementary Altern Med 2:33–38

Salatino A, Fernandes-Silva CC, Righi AA, Salatino MLF (2011) Propolis research and the chemistry of plant products. Nat Prod Rep 28:925–936

Sârbu C, Moț AC (2011) Ecosystem discrimination and fingerprinting of Romanian propolis by hierarchical fuzzy clustering and image analysis of TLC patterns. Talanta 85:1112–1117

Sawaya ACHF, Cunha IBS, Marcucci MC (2011) Analytical methods applied to diverse types of Brazilian propolis. Chem Cent J 5:27

Shrestha SP, Narukawa Y, Takeda T (2007a) Chemical constituents of Nepalese propolis (II). Chem Pharm Bull 55:926–929

Shrestha SP, Narukawa Y, Takeda T (2007b) Chemical constituents of Nepalese propolis: isolation of new dalbergiones and related compounds. J Nat Med 61:73–76

Shi H, Yang H, Zhang X, Yu LL (2012) Identification and quantification of phytochemical composition and anti-inflammatory and radical scavenging properties of methanolic extracts of Chinese propolis. J Agric Food Chem 60:12403–12410

Simões LM, Gregório LE, Da Silva Filho AA, de Souza ML, Azzolini AE, Bastos JK, Lucisano-Valim YM (2004) Effect of Brazilian green propolis on the production of reactive oxygen species by stimulated neutrophils. J Ethnopharmacol 94:59–65

Stevens JF, Hart HT, Wollenweber E (1995) The systematic and evolutionary significance of exudates flavonoids in *Aeonium*. Phytochemistry 39:805–813

Trusheva B, Todorov I, Ninova M, Najdenski H, Daneshmand A, Bankova V (2010) Antibacterial mono-and sesquiterpene esters of benzoic acids from Iranian propolis. Chem Cent J 4:8

Valcic S, Montenegro G, Timmermann BN (1998) Lignans from Chilean propolis. J Nat Prod 61:771–775

Villanueva VR, Barbier M, Gonnet M, Lavie P (1970) Les flavonoides de la propolis. Isolement d'une nouvelle substance bacteriostatique: la pinocembrine. Ann Inst Pasteur 118:84–87

Villanueva VR, Bogdanovsky D, Barbier M, Gonnet M, Lavie P (1964) Sur l'identification de la 3, 5, 7-trihydroxy flavones (galangine) a partir de la propolis. Ann Inst Pasteur 106:292–302

Volpi N (2004) Separation of flavonoids and phenolic acids from propolis by capillary zone electrophoresis. Electrophoresis 25:1872–1878

Walker P, Crane E (1987) Constituents of propolis. Apidologie 18:327–334

Woiski RG, Salatino A (1998) Analysis of propolis: some parameters and procedures for chemical quality control. J Apicult Res 37:99–105

Zhang C-P, Zheng H-Q, Hu F-L (2011) Extraction, partial characterization, and storage stability of β-glucosidase from propolis. J Food Sci 76:C75–C79

Zhang CP, Huang S, Wei WT, Ping S, Shen XG, Li YJ, Hu FL (2014) Development of high-performance liquid chromatographic for quality and authenticity control of Chinese propolis. J Food Sci 79:C1315-C1322

Zhou J, Li Y, Zhao J, Xue X, Wu L, Chen F (2008) Geographical traceability of propolis by high-performance liquid-chromatography fingerprints. Food Chem 108:749–759

Chapter 6
Propolis and Geopropolis Volatiles

Maria Graça Miguel and Ana Cristina Figueiredo

Contents

6.1 Introduction

Propolis, also known as bee glue, is a resinous and sticky bee product that bees use both as a building and sheltering material. Of varying odour, texture and colour (from light to dark), propolis is a complex mixture of plant buds and resins, as well as other plant secretions and volatiles, that honeybees (*Apis* spp.) blend with pollen

M.G. Miguel
Universidade do Algarve, Faculdade de Ciências e Tecnologia, MeditBio, Campus de Gambelas, 8005-139 Faro, Portugal
e-mail: mgmiguel@ualg.pt

A.C. Figueiredo
Centro de Estudos do Ambiente e do Mar (CESAM), Faculdade de Ciências da Universidade de Lisboa, Centro de Biotecnologia Vegetal (CBV), DBV, C2, Campo Grande, 1749-016 Lisboa, Portugal

J.M. Alvarez-Suarez (ed.), *Bee Products - Chemical and Biological Properties*,
DOI 10.1007/978-3-319-59689-1_6

and beeswax (the mixture being called cerum or cerumen). Occasionally, other constituents are also found in propolis, particularly when plant resources are low. Another type of propolis, geopropolis, is collected by stingless bee species (*Melipona* spp.) native to tropical countries. Geopropolis is composed by combining soil elements with plant exudates and beeswax (Bankova et al. 2000, 2014; Salatino et al. 2005, 2011; Toreti et al. 2013; Huang et al. 2014).

Although they account for only a minor portion of propolis constituents, volatiles impair particular aromas to propolis from different origins and have been considered as a possible quality criterion for propolis freshness (Woisky and Salatino 1998).

Increasing interest in the medicinal properties of propolis has inspired research on numerous topics related to this product, and several reviews have addressed the chemical composition, botanical origin and biological properties of propolis (Bankova et al. 2000, 2014; Salatino et al. 2011; Toreti et al. 2013; Huang et al. 2014). However, literature addressing the volatile profile of propolis or geopropolis is scarce compared to that of other chemical components, with just one previous review focusing on this subject (Bankova et al. 2014). The present review examines the volatile constituents of propolis and geopropolis (for simplicity they will both be referred to as propolis), their isolation and analysis.

6.2 Propolis Volatile Fraction

Propolis volatiles have been studied by several researchers, Table 6.1, as recently reviewed by Bankova et al. (2014). Despite the use of different sampling techniques, all allow the characterization of the propolis volatile fraction in a more or less specific way, so they will all be considered here.

As a bee product, the chemical composition of propolis in general, and volatiles in particular, is dependent on a number of factors, such as geographical origin, bee type, local flora, and also the methodology used for isolation and analysis of the volatiles, as discussed below.

6.2.1 Propolis Origin

In total, propolis and geopropolis volatiles from 25 countries were evaluated, Table 6.1, with between one and 106 samples evaluated per country. Propolis from different geographical origins showed different chemical profiles, Table 6.1, which usually correlate with the natural flora available at the hive location and the preferences of the bee type.

Traditionally (Bankova et al. 2014), propolis types have been divided according to their continent of origin. Nevertheless, considering the diversity and specificity of the flora in different countries, for instance those in northern and southern Europe, some additional subgroups may have to be considered.

Table 6.1 Studies on propolis and geopropolis volatiles

Propolis and geopropolis origin[a]	Hives bee type	S[b]	Volatiles isolation	Volatiles analysis	Propolis main volatile compounds (%, ≥3%)[c]	References
Albania (Tirana)		1	Steam distillation for 4 h followed by extraction with diethyl ether/ pentane 1:1	GC-MS	Cadinene (isomer) 11, methoxyacetophenone 9, heptacosane 7, tricosane 5, nonacosane 5, heneicosane 4, pentacosane 4, hentriacontane 4	Bankova et al. (1994)
Algeria (El-malha, Benibelaîd, Kaous)		3	Hydrodistillation for 4 h	GC-MS	2-Hexenal 1-11, myristic acid 2-6, cyclotetradecane nd-6, thymol nd-5, linoleic acid 2-5, isooctane nd-4, α-cedrol nd-3	Segueni et al. (2010)
Argentina (Andes)		1	HS-SPME for 30 min at 42 °C	HS-SPME-GC–MS	Similarity between the volatile profile of *Larrea nitida* and the corresponding studied propolis, with 17 constituents detected in both samples	Agüero et al. (2011)
Brazil (Ceara, Parana, São Paulo)		4	Steam distillation for 4 h followed by extraction with ethyl ether/n-hexane 1:1	GC-MS	2-*cis*,6-*trans*-Farnesol nd-17, diprenyl acetophenone nd-11, prenyl acetophenone nd-8, ledol 0.1-6, ethylphenol nd-5, decanoic acid nd-5, tricosane nd-5, pentacosane nd-4, 3-phenyl-propanol nd-4, heneicosane nd-4, δ-cadinene 1-3, acetophenone nd-3, octadecane nd-3, nonadecane nd-3, heptacosane nd-3	Bankova et al. (1995)
Brazil (Botucatu)	Honeybees: *Apis mellifera* (Africanized honeybees)	1	Distillation-extraction using a Likens-Nickerson apparatus for 4 h	GC and GC-MS	Spathulenol 9-14, (2-*cis*,6-*trans*)-farnesol 8-12, benzoic acid 2-12, dihydrocinnamic acid 0.4-11, diprenyl acetophenone 0.2-9, prenyl acetophenone 7-8, benzyl benzoate 0.3-8, δ-cadinene 2-4, β-caryophyllene 1-3, ledol 2-3, (*trans*,*trans*)-8,10-dodecadienol 1-3, heptacosane nd-3	Bankova et al. (1998a)

(continued)

Table 6.1 (continued)

Propolis and geopropolis origin[a]	Hives bee type	S[b]	Volatiles isolation	Volatiles analysis	Propolis main volatile compounds (%, ≥3%)[c]	References
Brazil (Botucatu)	Honeybees: *Apis mellifera ligustica* (European honeybees)	1	Distillation-extraction using a Likens-Nickerson apparatus for 4 h	GC and GC-MS	Benzyl benzoate 1-18, spathulenol 3-12, (2-*cis*,6-*trans*)-farnesol 2-15, benzoic acid 0.2-5, diprenyl acetophenone 1-8, *p*-methoxy acetophenone 1-7, prenyl acetophenone 0.2-6, dihydrocinnamic acid nd-6, δ-cadinene 0.2-5, ledol 1-3, (*trans*,*trans*)-8,10-dodecadienol 1-3, pentacosane 1-3, β-caryophyllene nd-3	Bankova et al. (1998a)
Brazil (Piaui and Parana)	Stingless bees: *Melipona compressites*, *Tetragona clavipes*, *Melipona quadrifasciata anthidioides*	1	Essential oil	GC-MS	Aliphatic hydrocarbons C8-C25 11-28, spathulenol 1-10, nerolidol nd-12, *p*-cymen-8-ol nd-11, ethylphenol nd-10, verbenone nd-7, cinnamic acid 1-4, alkylbenzenes and alkylnaphtalenes nd-5, T-muurolol nd-4, ledol nd-3, verbenol nd-3, palmitic acid nd-3, dihydrocinnamic acid nd-3	Bankova et al. (1999)
Brazil (Minas Gerais)		1	Steam distillation for 5 h	NMR of the fractions obtained by CC	2,2-Dimethyl-8-prenyl-6- vinylchromene, 2,6-diprenyl-4-vinylphenol, 2,2-dimethyl-6-vinylchromene, acetophenone, 2-prenyl-4-vinylphenol, 3,4-dimethoxy-styrene, 3,4-dimethoxy-allylbenzene, 4-hydroxy-3,5-diprenylbenzaldehyde, (-)-spathulenol	Kusumoto et al. (2001)
Brazil (Minas Gerais)	Honeybees: *Apis mellifera*	1	Chloroform extraction for 24 h at room temperature	GC-MS	Derivatives of benzoic acid, benzaldehyde, prenylated and non-prenylated cinnamic acid, chromane, chromene	Negri et al. (2003a)
Brazil (São Paulo)	Honeybees: *Apis mellifera*	1	Chloroform extraction for 24 h at 50 °C	GC-MS	α-Amorphene, β-cadinene, δ-cadinene, *trans*-caryophyllene, α-humulene, ledene, *trans*-nerolidol, spathulenol	Negri et al. (2003b)

Brazil	Honeybees: *Apis mellifera*	1	Distillation-extraction for 2 h	GC-MS	Propene, acetophenone, β-linalool, 2-methyl-1-hepten-6-one, caryophyllene	Atungulu et al. (2007)
Brazil (Minas Gerais)	Honeybees: *Apis mellifera*	1	Hydrodistillation for 3 h	GC and GC-MS	*trans*-Nerolidol 17, β-caryophyllene 13, selina-3,7(11)-diene 10, aromadendrene 8, germacrene A 8, δ-amorphene 5, spathulenol 5, *cis*-calamenene 4, α-copaene 4, viridiflorene 4, γ-muurolene 4, α-humulene 3	de Albuquerque et al.(2008)
Brazil (southeastern)	Honeybees: *Apis mellifera*	1	Hydrodistillation for 1 h and methylene chloride extraction of decoction water	GC-MS	Nerolidol 7, 1-phenyl-ethanone 5, linalool 5, spathulenol 4, *trans*-caryophyllene 4, aromadendrene 3, δ-cadinene 3	Maróstica Junior et al. (2008)
Brazil (Piaui)		1	Micro-hydrodistillation for 3 h followed by extraction with Cl_2CH_2	GC-MS	α-Pinene 0.3-34, 1,8-cineole 2-24, β-caryophyllene 3-17, α-copaene 1-8, α-gurjunene 1-7, hexadecanoic acid 0.4-8, β-caryophyllene oxide 0.2-8, β-selinene 0.3-7, β-pinene nd-8, terpinen-4-ol nd-8, fenchone nd-6, *p*-menta-1,5-dien-8-ol nd-5, δ-cadinene 1-5, ethyl phenol nd-5, trans-pinocarveol nd-4, α-humulene nd-4, linalool nd-3, sphatulenol 1-3, hexanoic acid nd-3, benzyl alcohol nd-3, nonanal nd-3, phenylethanol nd-3	Torres et al. (2008)
Brazil (São Paulo)	Stingless bees: from South America	1	Hydrodistillation for 4 h	GC and GC-MS	α-Pinene 18, β-pinene 7, δ-cadinene 7, germacrene D 6, α-copaene 4, *trans*-nerolidol 4, *trans*-pinocarveol 3, viridiflorol 3, isopimara-9(11)-15-diene 3	Ioshida et al. (2010)

(continued)

Table 6.1 (continued)

Propolis and geopropolis origin[a]	Hives bee type	S[b]	Volatiles isolation	Volatiles analysis	Propolis main volatile compounds (%, ≥3%)[c]	References
Brazil (Rio de Janeiro)	Honeybees: *Apis mellifera*	1	Steam distillation for 6 h	GC-MS	β-Caryophyllene 13, acetophenone 12, linalool 6, γ-elemene 6, γ-cadinene 6, γ-muurolene 4, α-copaene 3, spathulenol 3	Oliveira et al. (2010)
Brazil		1	Microwave-assisted extraction (MAE) for 15 min at 35 °C	GC-MS	Longipinene 25, α-eudesmol 7, β-eudesmol 6, β-caryophyllene 5, guaiol 5, benzenecarboxylic acid 4, β-Maaliene 4, 3,4-dihydro-2-naphthoic acid 4	Li et al. (2012)
Brazil (Minas Gerais)		8	HS 15 min at 100 °C	HS-GC–MS	Alkenes, alcohols, esters, terpenes, aldehydes and ketones	Nunes and Guerreiro (2012)
Brazil (Rio Grande do Sul)		3	Hydrodistillation for 4 h	GC and GC-MS, and CPGC	α-Pinene 57-63, β-pinene 13-31, limonene nd-11	Simionatto et al. (2012)
Brazil		1	HS (static) 10 min at 50 °C	HS-GC–MS	α-Pinene 53, β-pinene 21, β-methyl crotonaldehyde 10, hexanal 3	Kaškoniene et al. (2014)
Brazil		1	Maceration with ethanol at room temperature followed by extraction with hexane	GC-MS	Sesquiterpenes, fatty acids and other compounds	Bittencourt et al. (2015)
Brazil (Mato Grosso)	Honeybees: *Apis mellifera*	1	Hydrodistillation	GC-MS	β-Caryophyllene 8, δ-cadinene 8, spathulenol 7, viridiflorene 5, α-copaene 4, aromadendrene 4, α-*trans*-bergamotene 4, *trans*-nerolidol 4, germacrene D 3, γ-muurolene 3	Fernandes et al. (2015)

Brazil (Minas Gerais)		1	Steam distillation	GC-MS	3-Prenylcinnamic acid allyl ester 26, spathulenol 23, 7-phenyl-5-oxo-heptanol 13, benzenepropanoic acid methyl ester 9, benzenepropanoic acid ethyl ester 6, δ-cadinene 4, β-caryophyllene 3, β-copaene 3	Fernandes-Silva et al. (2015)
Bulgaria (Russe)		1	Steam distillation for 4 h followed by extraction with diethyl ether/pentane 1:2	GC-MS	β-Eudesmol 9, δ-cadinene 5, γ-muurolene 5, tricosane 5, pentacosane 4, methoxyacetophenone 3, guaiol 3, cadinene (isomer) 3, heptacosane 3	Bankova et al. (1994)
Canary Islands (Gran Canaria)		1	Distillation for 3 h using a Likens-Nickerson apparatus	GC-MS	Nerolidol 3-11, ethyl oleate 3-7, spathulenol 3-8, ledol 2-4, cinnamic acid 1-4, ethyl palmitate 1-4, δ-cadinene 1-3, aromadendrene 0.3-3	Bankova et al. (1998b)
China (23 regions)		23	Trapped on Tenax	DHS and GC-MS, and GC-O	(concentrations in μg/L) 2-Hydroxy-benzoic acid formate nd-96, acetic acid nd-45, 3-methyl-3-buten-1-ol nd-32, nonanal nd-31, benzyl alcohol 0.4-22, 2-methyl-2-buten-1-ol nd-20, phenylethyl alcohol 1-17, limonene 1-14, hexanal nd-14, guaiol nd-11, propanoic acid nd-11, benzaldehyde 2-10, eucalyptol nd-10, α-pinene nd-9, 2-methyl-3-buten-2-ol t-6, ethyl acetate nd-6, pentanal nd-6, toluene nd-3	Yang et al. (2010)

(continued)

Table 6.1 (continued)

Propolis and geopropolis origin[a]	Hives bee type	S[b]	Volatiles isolation	Volatiles analysis	Propolis main volatile compounds (%, ≥3%)[c]	References
China (4 regions)		4	Thermal desorption and cryofocusing of the volatile compounds. Incubation at 50 °C for 10 min	DHS and GC-MS, and GC-MS-O	Acetic acid 9-63, benzyl alcohol 1-14, α-cedrene nd-17, 1-(1,5-dimethyl-4-hexenyl)-4-methyl-benzen 0.1-11, 3-methyl-3-buten-1-ol t-7, 3-methyl-2-buten-1-ol t-7, acetic acid methyl ester nd-7, 3-methyl-2-buten-1-ol acetate nd-5, acetic acid phenylmethyl ester 0.3-4, phenylethyl alcohol 0.3-4, 2-methyl-2-buten-1-ol nd-4, toluene nd-4, benzaldehyde 1-3, 2-methyl-2-butenoic acid nd-3, 3-methyl-3-buten-1-ol acetate nd-3, eucalyptol nd-3	Cheng et al. (2013)
China		1	HS (static) 10 min at 50 °C	HS-GC–MS	3-Methyl-3-buten-1-ol 40, 3-methyl-2-buten-1-ol 12, 4-penten-1-yl acetate 9, α-longipinene 9, 3-methyl-2-buten-1-ol acetate 4	Kaškoniene et al. (2014)
Croatia (Dalmatia and Slavonia)		2	Hydrodistillation for 4 h	GC-MS	Benzoic acid 1-27, benzyl alcohol 3-18, naphthalene nd-18, limonene 6-11, γ-terpinene 1-6, benzyl benzoate 4	Borčić et al. (1996)
Croatia (8 areas)		8	HS-SPME of 40 min from sample heated for 1 h at 60 °C	GC and GC-MS	Thymol nd-76, α-bisabolol nd-23, γ-cadinene nd-21, vanillin nd-20, α-eudesmol nd-20, β-eudesmol nd-13, *cis*-calamenene nd-11, 2-phenylethanol nd-10, bornyl acetate nd-9, α-copaene nd-9, nonanal nd-7, guaiol nd-7, α-cadinol nd-7, γ-eudesmol nd-7, T-cadinol nd-7, *ar*-curcumene nd-6, benzyl alcohol nd-5, α-pinene nd-4, decanal nd-4, α-muurolene nd-4, β-bourbonene nd-3, α-guaiene nd-3, *allo*-aromadendrene nd-3, α-amorphene nd-3	Jercović et al. (2016)

Croatia (8 areas)		8	Distillation-extraction using a Likens-Nickerson apparatus for 3 h	GC and GC-MS	Thymol nd-44, 4-vinyl-2-methoxyphenol nd-18, γ-cadinene nd-17, α-eudesmol nd-16, α-bisabolol nd-15, β-eudesmol nd-12, benzyl cinnamate nd-12, γ-eudesmol nd-11, T-cadinol nd-10, decanal nd-8, docosane nd-7, benzyl alcohol nd-5, nonanal nd-5, guaiol nd-5, tricosane nd-5, benzoic acid nd-4, bornyl acetate nd-4, *cis*-calamenene nd-4, α-muurolene nd-3, heneicosane nd-3	Jercović et al. (2016)
Cuba (3 locations in Pinar del Río)	Honeybees: *Apis mellifera*	3	Distillation-extraction using a Likens-Nickerson apparatus for 1.5 h	GC-MS	(data in ppm) 1,4-naphtoquinone 142-427, anethole 51-85, methyleugenol 30-64, dehydro-α-lapachole 17-33, eicosane 18-36	Bracho (2000)
England (Oxfordshire)		1	Trapped on Tenax GC, desorbed by flash heating	GC-MS	Benzoic acid 22, ester of 2-methylpropanoic acid 17, 3-methyl-3-butenyl acetate 15, 3-methyl-3-buten-1-ol 5, 3-methyl-2-butenyl acetate 5, acetic acid 5, benzyl alcohol 4, 3-methylbutyl acetate 3, 3-methyl-2-buten-1-ol 3, 2-methylbutanoic acid 3	Greenaway et al. (1991)
Estonia		1	HS (static) 10 min at 50 °C	HS-GC–MS	Eucalyptol 26, α-pinene 21, benzaldehyde 11, β-pinene 9, heptane 5, benzyl alcohol 3	Kaškoniene et al. (2014)
Ethiopia (Assela)	Honeybees: *Apis mellifera monticola*	1	Hydrodistillation for 3 h	GC-MS	5,6,7,8-Tetramethylbicyclo[4,1,0]hept-4-en-3-one 15, acoradien 14, epicedrol 7, (6E,6E)-3,7,11,15-tetramethyl-1,6,10,14-hexadecatetraen-3-ol 6, α-cadinol 4, hexadecane 4, calamenene 4, 3-Isopropyl-6-methyl-2-oxo-1-(3-oxobutyl)-cyclohexanecarbaldehyde 5, *cis*-verbenol 3	Haile et al. (2012)

(continued)

Table 6.1 (continued)

Propolis and geopropolis origin[a]	Hives bee type	S[b]	Volatiles isolation	Volatiles analysis	Propolis main volatile compounds (%, ≥3%)[c]	References
Ethiopia (Haramaya)	Honeybees: *Apis mellifera jementica*	1	Hydrodistillation for 3 h	GC-MS	Calamenene 14, 4-terpineol 9, *epi*-bicyclosesquiphellandrene 8, 4-(2-acetyl-5,5-dimethylcyclopent-2-enylidene) butan-2-one 8, 3-Isopropyl-6-methyl-2-oxo-1-(3-oxobutyl)-cyclohexanecarbaldehyde 6, α-himachalene 5, *cis*-verbenol 4, *trans*-pinocarveol 3, camphor 3, α-phellandren-8-ol 3, *p*-menth-1-en-8-ol 3, cedrol 3, 3-thujen-2-one 3, *p*-cymen-8-ol 3	Haile et al. (2012)
France (Berry and Lyonnais)		4	Hydrodistillation	GC and GC-MS	β-Eudesmol, guaiol, β-caryophyllene, β-selinene, α-guaiene, naphtalene, benzyl benzoate, benzyl cinnamate, benzyl acetate, trimethylbenzene, methylethylbenzene	Clair and Peyron (1981)
Greece (Chalkidiki, Agrinio, Arta, Preveza and Andros)		5	Hydrodistillation for 3 h	GC and GC-MS	α-Pinene 8-46, *n*-decanal 3-10, junipene nd-12, α-eudesmol nd-12, δ-cadinene 0.3-8, *trans*-β-terpineol 2-7, manoyl oxide 0.1-7, β-eudesmol 1-5, cedrol nd-6, *n*-nonanal nd-5, α-muurolene nd-5, guaiol nd-5, manool nd-5, hexadecen-1 nd-4, γ--eudesmol nd-4, α-cadinol nd-4, α-longipinene nd-3, γ-terpinene 1-3, *trans*-totarol 0.1-3, acetovanillone nd-3	Melliou et al. (2007)
Hungary (20 samples)		20	Steam distillation	GC and GC-MS	β-Eudesmol + sesquiterpene alcohol 10-60, benzyl benzoate 1-40, cyclohexyl benzoate 1-8, vanilin 1-6, benzyl alcohol 1-4	Petri et al. (1988)

India (Maharashtra)	Honeybees: *Apis mellifera*	1	Hydrodistillation	GC and GC-MS	Tricosane 14, hexacosane 12, heptacosane 8, palmitic acid 8, linalool 7, geraniol 6, methyleugenol 6, palmitoleic acid 6, heneicosane 5, *cis*-ethyl cinnamate 5, catechol 3	Naik et al. (2013)
Italy (Pise and Livourne)		2	Steam distillation for 1 h	GC and GC-MS	Anethole, α-pinene and β-pinene for Pise sample. Vanillin, eugenol and borneol for Livourne sample	Corsi (1981)
Italy (1 region, South)	Honeybees: *Apis mellifera*	1	Hydrodistillation	GC–MS	Similar volatile profile. HS-SPME-GC–MS technique allowed to obtain higher % relative peak area values for δ-cadinene, T-cadinol and α-cadinol, but lower for 3-methyl-3-buten-1-ol and 2-methyl-2-buten-1-ol (quantitative data not provided)	Pellati et al. (2013)
Italy (9 regions from North and South)	Honeybees: *Apis mellifera*	9	HS-SPME of 20 min from sample heated for 30 min at 75 °C	HS-SPME-GC–MS	Benzoic acid 1-30, β-eudesmol 2-13, α-pinene nd-13, benzyl benzoate nd-13, δ-cadinene nd-13, α-cadinol nd-10, T-cadinol nd-10, γ-cadinene nd-9, α-muurolene nd-7, germacrene D-4-ol nd-6, cubenol 1-5, *trans*-verbenol nd-5, α-bisabolol nd-5, α-terpenyl acetate nd-5, vanillin nd-5, calamenene 1-4, benzyl alcohol nd-4, γ-muurolene nd-4, γ-eudesmol nd-4, δ-cadinol 1-3, tricosane 1-3, menthol nd-3, thymol nd-3, *allo*-aromadendrene nd-3, caryophyllene oxide nd-3, cedrol nd-3, benzyl cinnamate nd-3	Pellati et al. (2013)

(continued)

Table 6.1 (continued)

Propolis and geopropolis origin[a]	Hives bee type	S[b]	Volatiles isolation	Volatiles analysis	Propolis main volatile compounds (%, ≥3%)[c]	References
Mexico (Yucatán)	Honeybees: *Apis mellifera*	1	Distillation for 1 h using a Likens-Nickerson apparatus	GC and GC-MS	(values in mg/kg) α-pinene 2, limonene 1, trans-verbenol 1, pinocarvone 1, β-pinene 0.3, spathulenol 0.3, α-campholenal 0.2, *trans*-pinocarveol 0.3, myrtenal 0.3, myrtenol 0.3, verbenone 0.6, caryophyllene oxide 0.6, δ-cadinene 0.5, camphene 0.1, α-copaene 0.1, β-bourbonene 0.1, β-caryophyllene 0.1, α-humulene 0.1, *allo*-aromadendrene 0.1, bicyclogermacrene 0.1, viridiflorol 0.1, α-humulene epoxide II 0.1	Pino et al. (2006)
Mexico (Yucatán)	Stingless bees: *Melipona beecheii*	1	Distillation for 1 h using a Likens-Nickerson apparatus	GC and GC-MS	(values in mg/kg) α-pinene 82, β-caryophyllene 55, spathulenol 45, caryophyllene oxide 44, β-bourbonene 43, β-pinene 32, α-copaene 22, *trans*-verbenol 19, viridiflorol 11, bicyclogermacrene 7, *trans*-pinocarveol 6, myrtenal 6, verbenone 6, α-humulene 6, α-humulene epoxide II 6, δ-cadinene 5, myrtenol 5, β-cubebene 5, pinocarvone 4, α-cubebene 4, camphene 4, limonene 4, α-campholenal 4, *allo*-aromadendrene 3	Pino et al. (2006)
Mexico (Veracruz)	Stingless bees: *Melipona beecheii*	1	HS-SPME of 15 min from sample heated for 30 min at 45 °C	HS-SPME-GC–MS	β-Fenchene 15, styrene 9, benzaldehyde 8, (*cis*)-ocimenone 6, α-pinene 4, *m*-cymene 4, limonene 3, heptanal 3, *trans*-isocarveol 3, (4-*trans*)-decenal 3, verbenone 3	Torres-González et al. (2016)

Mongolia (Ulan Bator)		1	Steam distillation for 4 h followed by extraction with diethyl ether /pentane 1:3	GC-MS	Benzyl benzoate 9, calamenene 3	Bankova et al. (1994)
Mongolia (Inner Mongolia)[d]		1	Hydrodistillation (H), simultaneous distillation extraction (SD) and dynamic headspace sampling (DHS)	GC-MS	H: 3-methyl-2-buten-1-ol 27, phenyl ethyl alcohol 17, 1,2,3,4,4a,5,6,7-octahydro-α, α,4a,8-tetra-methyl-2-naphthalenemetha-nol 15, 2-methoxy-4-vinylphenol 10, α-bisabolol 4. SD: α-bisabolol 20, 2-methyl-3-buten-2-ol 11, 3-methyl-2-buten-1-ol 8, azulene 5. DHS: heptadecane 7, phennathrene 4, ar-tumerone 4, 1-(1, 5-dimethyl-4-hexenyl)-4-methyl-benzene 3, octadecane 3, 1-(1,5-dimethyl-4-hexenyl)-4-methyl-benzene 3, hexadecane 3	Fu et al. (2009)
Poland	Honeybees: *Apis mellifera carnica*	1	Steam distillation of the ethanolic extract	GC-MS	Guaiol, farnesol, dehydroeudesmol, nerolidol	Maciejewicz et al. (1983)
Portugal (Algarve)		70	Hydrodistillation for 3 h	GC and GC-MS	Thymol t-79, viridiflorol 3-38, tricosane 3-22, nonadecane 3-18, heneicosane 2-14, labd-7-en-15-ol t-15, 15-nor-labdan-8-ol 1-11, pentacosane 1-11, ambroxide t-6, heptadecane t-6, guaiol t-5, palmitic acid t-5, ledol t-4, ethyl linoleate t-4, 8-epi-13-*nor*-ambreinolide t-4, pinocarvone t-3, β-eudesmol t-3, 13-*nor*-ambreinolide t-3, eicosane t-3, lab-8(17)-en-15-ol t-3, labd-8-en-15-ol t-3, eicosanol t-3	Miguel et al. (2013)

(continued)

Table 6.1 (continued)

Propolis and geopropolis origin[a]	Hives bee type	S[b]	Volatiles isolation	Volatiles analysis	Propolis main volatile compounds (%, ≥3%)[c]	References
Portugal (mainland), Azores archipelago and Madeira Island (six different geographical locations)		36	Hydrodistillation for 3 h	GC and GC-MS	Thymol nd-64, α-bisabolol nd-45, β-eudesmol 1-26, α-eudesmol nd-25, α-pinene nd-19, γ-eudesmol 3-18, valerianol nd-14, viridiflorol nd-10, cedrol nd-10, α-bisabolol oxide B nd-10, benzyl salicylate nd-10, T-cadinol 1-9, *trans*-nerolidol nd-9, α-cadinol nd-9, nonadecane nd-9, δ-cadinene 0.3-8, hexadecanol nd-8, manool nd-8, tetracosane nd-8, *trans*-totarol nd-7, β-caryophyllene oxide nd-6, tricosane nd-6, heptadecane 0.2-5, 2,6,6-trimethyl cyclohexanone nd-5, ledol nd-5, α-muurolol nd-5, labd-7-en-15-ol nd-5, α-copaene nd-4, γ-cadinene nd-4, β-oplopenone nd-4, 1,2-dehydroviridiflorol nd-4, benzyl benzoate nd-4, phenylethyl octanoate nd-4, α-terpineol nd-3, globulol nd-3, decanoic acid nd-3, β-bourbonene nd-3, *allo*-aromadendrene nd-3, guaiol nd-3, δ-cadinol nd-3, 1,2-dehydroglobulol nd-3, hexadecanoic acid nd-3, pentacosane nd-3	Falcão et al. (2016)
Turkey (Anatolia)		1	HS-SPME of propolis ethanolic extracts for 15 min at 50 °C	HS-SPME-GC–MS	Cedrol 4-16, α-bisabolol nd-14, phenyl ethyl alcohol 1-8, decanal nd-7, ethyl benzoate 0.1-7, benzyl alcohol 0.4-7, δ-cadinene 0.2-6, nonanal nd-5, *trans*-pinocarveol 1-4, α-eudesmol 0.3-4, verbenone nd-4, *trans*-verbenol 1-3, calamenene 1-3, β-eudesmol 1-3, γ-cadinene 0.3-3	Hames-Kocabas et al. (2013)

Uruguay		3	HS (static) 10 min at 50 °C	HS-GC–MS	α-Pinene 23-53, β-pinene 24-27, limonene 2-16, *cis*-β-ocimene nd-7, 1,3-dichloro-benzene nd-6, camphene 4-5, styrene 0.1-5, 3-methyl-3-buten-1-ol 0.1-4, β-myrcene nd-4	Kaškoniene et al. (2014)
Venezuela (Falcón)		1	Hydrodistillation for 4 h	GC and GC-MS	Germacrene D 27, β-caryophyllene 10, β-elemene 8, α-cadinol 8, bicyclogermacrene 7, δ-cadinene 5, germacrene A 4, α-humulene 3, γ-muurolene 3, T-muurolol 3	Rios et al. (2014)
Wales (Cardiff)		1	Trapped on Tenax GC, desorbed and then analysed	GC and GC-MS	6-Methyl-5-hepten-2-one 16, benzyl alcohol 14, isopentyl acetate 10, benzaldehyde 9, 3-methylbut-3-enol 6, 3-methylbut-2-enol 5, 2-methylbut-2-enoic acid 5, 2-methylbutyric acid 5, 2-phenylethyl alcohol 4, hexanal 3	Greenaway et al. (1989)

[a]Up to 4 hive locations mentioned

[b]*S* Number of samples studied, *CC* column chromatography, *CPGC* chiral phase gas chromatography, *DHS* dynamic headspace sampling, *GC* gas chromatography, *GC-MS* gas chromatography-mass spectrometry, *HS* headspace, *MAE* microwave-assisted extraction, *NMR* nuclear magnetic resonance spectroscopy, *SPME* solid-phase microextraction

[c]Whe n compounds were quantified and unless otherwise stated, *nd* not detected

[d]Paper in Chinese (data from abstract only)

6.2.2 Bee Type

Studies on Africanized bees (*Apis mellifera*) and European bees (*Apis mellifera ligustica*) showed more quantitative than qualitative variations in propolis volatiles. This may indicate that both types of bees collected propolis from the same plants, but, depending on when the propolis was collected, some plants may have been more dominant than others (Bankova et al. 1998a). According to Haile et al. (2012), the variability in the volatiles of propolis obtained from two locations in Ethiopia could in part be due to the dominance of *Apis mellifera monticola* in the Assela region compared to the Haramaya region, where *Apis mellifera jementica* was the dominant honeybee.

As with honeybees, stingless bees also collect plant exudates but produce their particular form of propolis, geopropolis, using different plant sources from those used by honeybees (Table 6.1). Foraging workers of three species of *Frieseomelitta* stingless bee have been shown to carry complex mixtures of plant-derived mono-, sesqui-, di- and tri-terpenes in the posterior tibia (Patricio et al. 2002). Although studies on volatiles from stingless bee propolis are limited, they have confirmed a different chemical profile from propolis obtained from honeybees of the same region, as this is dependent on the flora preferences of the bees (Bankova et al. 1999; Pino et al. 2006; Bankova and Popova 2007; Ioshida et al. 2010; Huang et al. 2014; Isidorov et al. 2016; Torres-González et al. 2016).

6.2.3 Natural Flora

Natural local flora determines the chemical composition of propolis, including that of their volatile fraction. In addition to exudates from trees, bees may also use secretions from aromatic plants (lavender, rosemary, sage, thyme) or material from other sources, such as asphalt or tar (Borčić et al. 1996; Alqarni et al. 2015). However, current knowledge on the plant sources of propolis is based more on observation studies of bee behaviour, or comparative studies of the phenolic composition of plants and propolis, than on the corresponding volatile composition.

Indeed, although several of the studies on propolis volatiles report the natural local flora (Corsi 1981; Petri et al. 1988; Greenaway et al. 1989, 1991; Bankova et al. 1994, 1998a, b; Pino et al. 2006; de Albuquerque et al. 2008; Agüero et al. 2011; Haile et al. 2012; Miguel et al. 2013; Torres-González et al. 2016), only some simultaneously compared the volatile profiles of propolis with those of the local flora (Maróstica Junior et al. 2008; Falcão et al. 2016).

Maróstica Junior et al. (2008) showed the chemical similarities between the volatile compounds found in Brazilian green propolis and those from its botanical origin, *Baccharis dracunculifolia* (Asteraceae/Compositae).

According to Falcão et al. (2016), the chemical composition of *Populus x canadensis* (Salicaceae) and *Cistus ladanifer* (Cistaceae) essential oils showed some correlation with that of certain Portuguese propolis samples.

6.2.4 Detection of Acaricides and Adulterants

Acaricides are used to control bee parasites and can be detected in many hive products, such as propolis and beeswax. Similarly, the use of adulterants such as benzoic acid and eugenol has been reported in propolis, with the objective of enhancing the antibacterial activity (Bonvehí et al. 1994; Woisky and Salatino 1998). Some studies on propolis volatiles reported the presence of acaricides such as Vanquard BT 2-4, Coumaran and Apiguard (Bankova et al. 1998b; Bankova et al. 1999; Miguel et al. 2013), which may be responsible, for instance, for the high thymol content seen in some cases (Miguel et al. 2013).

6.2.5 Isolation and Analysis of Volatiles

The propolis volatile fraction comprises a mixture of volatile compounds that can be obtained because they either vaporize naturally or after the use of specific techniques.

In many studies propolis was extracted with ethanol. Although volatile compounds can also be isolated by this organic solvent, this is not an extraction procedure specific to the isolation of volatile compounds and, for this reason, these studies were not included in Table 6.1. According to Woisky and Salatino (1998), propolis extraction with aqueous ethanol (70%) is better than with absolute ethanol as it provides wax-free tinctures containing higher amounts of phenolic substances. Some studies in which less polar solvents were used for propolis extraction were also included for comparison (Table 6.1).

Depending on the technique used for the isolation of propolis volatiles, Table 6.1, some authors also evaluated the composition of propolis essential oil [obtained by hydro, steam or dry distillation from any plant part or by expression of citrus species (Council of Europe 2010)]. Although they do not provide essential oils, other conventional volatile extraction techniques used included solvent extraction combined with distillation, simultaneous distillation-extraction, microwave-assisted extraction, static or dynamic headspace sampling, and solid-phase microextraction.

Needless to say that all of these techniques provide different and complementary information on propolis volatiles, as they are based on diverse principles. Whereas some methodologies are humid-heat based extraction procedures, such as hydrodistillation or distillation-extraction, others are solvent-free extractions which sample the vapour phase of propolis, such as headspace techniques. Bracho et al. (1996), Fu et al. (2009), Pellati et al. (2013), Pellati (2014) and Jercović et al. (2016) compared different methodologies of propolis extraction, obtaining different proportions of volatile compounds (Table 6.1).

After isolation, and independently of the separation procedure, propolis volatiles are then analysed in order to identify and quantify the components, Table 6.1. Gas chromatography (GC) and gas chromatography coupled mass spectrometry (GC-MS), or just GC-MS, were the techniques most frequently used for this purpose. One of the factors that impairs direct comparison between data is the quantification

procedure. In some cases no quantification was performed, in others quantification was performed by GC or GC-MS; and whereas sometimes the data is reported in percentages, in others it is reported in absolute amounts (Table 6.1).

One study combined gas chromatography–olfactometry–mass spectrometry (GC-O-MS) and sniffing tests (Yang et al. 2010). In the sniffing studies, propolis odour was mainly described as fruity, pungent, acidic, grassy, herbaceous, stale and smelly, and it was confirmed that components with high concentrations do not always play a major role in odour contribution. One study (Simionatto et al. 2012) also evaluated the variability in enantiomeric composition of monoterpenes α-pinene, β-pinene and limonene in the volatile oils from three samples of Brazilian propolis. (-)-β-Pinene predominated in all samples, whereas the enantiomeric excess of α-pinene and limonene was dependent on the harvesting location.

6.3 Main Volatile Compounds in Propolis

As stated above, direct comparison of the quantitative composition of propolis volatiles should be avoided, due to the use of different isolation procedures and quantification methods, Table 6.1. For this reason, herein, comparisons are made based on the relative frequency of occurrence of a compound in different propolis volatile extracts.

β-Eudesmol was found in most samples, Fig. 6.1 and Table 6.1. Guaiol, tricosane and thymol were also frequently reported in studies on propolis volatiles (Fig. 6.1, Table 6.1).

It is important to consider that, in some cases, a high frequency of occurrence of a compound in propolis does not mean that it is a natural component resulting from collection by bees. For instance, thymol, as mentioned above, may occur in propolis as a result of treatment against varroa, which uses an acaricide containing thymol as the chief constituent (Miguel et al. 2013).

Though the number of samples studied is still relatively low, in some cases propolis volatiles can be divided according to their continent of origin (Table 6.1); African (Algeria, Canary Islands and Ethiopia), American (Argentina, Cuba, Brazil, Mexico, Uruguay, Venezuela), Asian (China, India, Mongolia, Turkey), and European (Albania, Bulgaria, Croatia, England, Estonia, France, Greece, Hungary, Italy, Poland, Portugal, Wales). This is just one possible classification of propolis volatiles and it includes very different geographical areas in each group, as well as corresponding variability in bee type and local flora. Nevertheless, it offers a first approach to the characterization of propolis volatile compounds.

6.3.1 Africa

Though the Canary Islands are a Spanish archipelago, they are located off the coast of north western Africa. For this reason, herein, they are considered together with Algeria and Ethiopia, located on the opposite side of the African continent in the Horn of Africa.

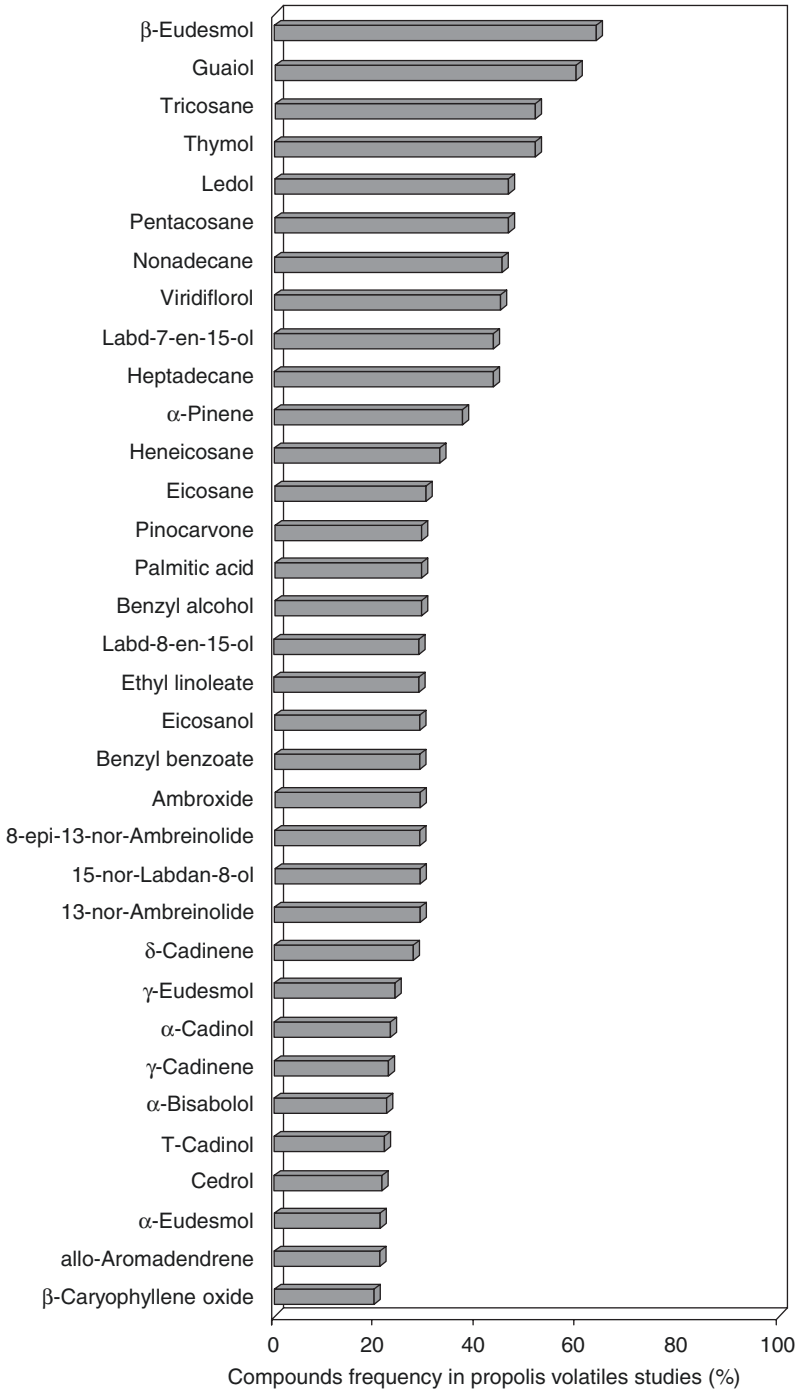


Fig. 6.1 Compound frequency (≥20%) in propolis volatiles studies

Six propolis volatile samples were studied in total, Table 6.1. Calamene and *cis*-verbenol were the only compounds that occurred in amounts ≥3%, found analogously in two studies. Differences in volatiles were also reported for propolis of Ethiopian origin, as they were produced by different *Apis* species, Table 6.1.

6.3.2 America

Ranging from not detected in some samples to variable amounts in others, sesquiterpenes δ-cadinene, β-caryophyllene and spathulenol, and monoterpenes α-pinene, β-pinene, and limonene characterized North American (Cuba and Mexico) and South American (Argentina, Brazil, Uruguay and Venezuela) propolis volatile samples (43 in total), Table 6.1. As there have been more studies performed on green propolis, the most commercialized form of Brazilian propolis, sesquiterpenes are more frequently reported and recognized as propolis components due to the fact that these are main constituents of this form of propolis. However, high levels of other components, such as α-pinene, are also characteristic of some Mexican and Uruguayan propolis volatile samples, Table 6.1.

Although not providing quantitative data, Agüero et al. (2011) showed 17 common constituents between the dichloromethane extracts of *Larrea nitida* (Zygophyllaceae) and the corresponding propolis. o-Cymene and limonene were the most abundant monoterpenes present in *L. nitida*, both of which were detected in the propolis volatiles.

According to Maróstica Junior et al. (2008), α-pinene, 1-phenyl-ethanone, linalool, β-caryophyllene, δ-cadinene, nerolidol, spathulenol and globulol were the components present in concentrations >1% in both *B. dracunculifolia* and propolis volatiles, indicating the similarities in their volatile profiles.

6.3.3 Asia

Overall, 32 Asian propolis volatile samples were evaluated, from China, India, Mongolia and Turkey (Table 6.1). Phenyl ethyl alcohol, benzyl alcohol and 3-methyl-3-buten-1-ol were the compounds most frequently reported in these propolis volatile samples. 1,8-Cineole (eucalyptol), 2-methyl-2-buten-1-ol, acetic acid, benzaldehyde and toluene were also frequently found in these samples, Table 6.1.

6.3.4 Europe

As recently reported by Bankova et al. (2014), the oxygen-containing sesquiterpene β-eudesmol was the most common constituent of European propolis volatiles, Table 6.1. Other common sesquiterpenes included guaiol, viridiflorol, ledol, γ-eudesmol,

γ-cadinene, α-bisabolol, T-cadinol, δ-cadinene and α-cadinol. As for monoterpenes, thymol and α-pinene were the most frequently reported volatile constituents. As previously mentioned, high thymol levels and frequency of occurrence may be a consequence of the long-term use of a thymol-rich acaricide by beekeepers, as reported by Miguel et al. (2013). Labdane diterpenes were abundant in volatiles from some propolis samples collected in Portugal, mainly in *C. ladanifer*-rich regions (Miguel et al. 2013; Falcão et al. 2016). Hydrocarbons such as heptadecane, nonadecane, heneicosane, tricosane and pentacosane were also commonly found in these types of propolis, depending on the extraction procedure. Aromatic compounds, such as benzyl benzoate and benzyl alcohol, which have frequently been identified in several European propolis volatiles, were not detected or were present in trace amounts in *P. canadensis* leaf-buds and *C. ladanifer* branch essential oils (Falcão et al. 2016).

6.4 Conclusion

Much remains to be known and understood about the volatile fraction of propolis. Additional comparative studies on the volatiles of local natural flora and corresponding propolis are required in order to better understand the relation between the two. Further research in this area will reveal to what extent flora volatile components can be used as propolis markers and in the characterization of this unique bee product.

Acknowledgments The invaluable help of Dr M. Lurdes Saramago (Biblioteca da Faculdade de Ciências da Universidade de Lisboa) on providing several references is greatly acknowledged. This study was partially funded by Fundação para a Ciência e a Tecnologia (FCT), under UID/AMB/50017/2013, FEDER PT2020-Compete 2020.

References

Agüero MB, Svetaz L, Sánchez M, Luna L, Lima B, López ML, Zacchino S, Palermo J, Wunderlin D, Feresin GE, Tapia A (2011) Argentinean Andean propolis associated with the medicinal plant Larrea nitida Cav. (Zygophyllaceae). HPLC–MS and GC–MS characterization and antifungal activity. Food Chem Toxicol 49:1970–1978

de Albuquerque IL, Alves LA, Lemos TLG, Dorneles CA, de Morais MO (2008) Constituents of the essential oil of Brazilian green propolis from Brazil. J Essent Oil Res 20:414–415

Alqarni AS, Rushdi AI, Owayss AA, Raweh HS, El-Mubarak AH, Simoneit BRT (2015) Organic tracers from asphalt in propolis produced by urban honey bees, Apis mellifera Linn. PLoS One 10:e0128311

Atungulu G, Miura M, Atungulu E, Satou Y, Suzuki K (2007) Activity of gaseous phase steam distilled propolis extracts on peroxidation and hydrolysis of rice lipids. J Food Eng 80:850–858

Bankova V, Popova M (2007) Propolis of stingless bees: a promising source of biologically active compounds. Pharmacogn Rev 1:88–92

Bankova V, Christov R, Popov S, Pureb O, Bocari G (1994) Volatile constituents of propolis. Z Naturforsch 49c:6–10

Bankova V, Christov R, Kujumgiev A, Marcuccic MC, Popov S (1995) Chemical composition and antibacterial activity of Brazilian propolis. Z Naturforsch 50c:167–172

Bankova V, Boudourova-Krasteva G, Popov S, Sforcin J, Funari SC (1998a) Seasonal variations in essential oil from Brazilian propolis. J Essent Oil Res 10:693–696

Bankova VS, Christov RS, Delgado Tejera A (1998b) Lignans and other constituents of propolis from the Canary islands. Phytochemistry 49:1411–1415

Bankova V, Christov R, Popov S, Marcucci MC, Tsvetkovac I, Kujumgiev A (1999) Antibacterial activity of essential oils from Brazilian propolis. Fitoterapia 70:190–193

Bankova V, de Castro SL, Marcucci MC (2000) Propolis: recent advances in chemistry and plant origin. Apidologie 31:3–15

Bankova V, Popova M, Trusheva B (2014) Propolis volatile compounds: chemical diversity and biological activity: a review. Chem Cent J 8:28

Bittencourt MLF, Ribeiro PR, Franco RLP, Hilhorst HWM, de Castro RD, Fernandez LG (2015) Metabolite profiling, antioxidant and antibacterial activities of Brazilian propolis: use of correlation and multivariate analyses to identify potential bioactive compounds. Food Res Int 76:449–457

Bonvehí JS, Coll FC, Jordà RE (1994) The composition active components of bacteriostatic activity of propolis in dietetics. JAOCS 71:529–532

Borčić I, Radonić A, Grzunov K (1996) Comparison of the volatile constituents of propolis gathered in different regions of Croatia. Flavour Fragr J 11:311–313

Bracho JC (2000) Constituyentes volátiles del propóleo, una realidade acerca de su rica composición química. Bol Soc Quim Peru 66:198–209

Bracho JC, Rosado A, Pino JA (1996) Comparison of isolation methods for propolis volatiles. J Essent Oil Res 8:665–668

Cheng H, Qin ZH, Guo XF, Hu XS, Wu JH (2013) Geographical origin identification of propolis using GC–MS and electronic nose combined with principal component analysis. Food Res Int 51:813–822

Clair G, Peyron L (1981) Contribuition à l'étude de l'huile essentille de propolis. Riv ital EPPOS 63:168–170

Corsi M (1981) Les huilles essentielles de la propolis. In: Meletinov C (ed) XXVIIIth international congress of apiculture. Apimondia, Acapulco, Mexico, pp 407–411

Council of Europe (2010) European Pharmacopoeia, 7th edn. Strasbourg, France, p. 241

Falcão SI, Freire C, Figueiredo AC, Villas-Boas M (2016) The volatile composition of Portuguese propolis towards its origin discrimination. Rec Nat Prod 10:176–188

Fernandes FH, Guterres ZR, Violante IMP, Lopes TFS, Garcez WS, Garcez FR (2015) Evaluation of mutagenic and antimicrobial properties of brown propolis essential oil from the Brazilian Cerrado biome. Toxicol Rep 2:1482–1488

Fernandes-Silva CC, Lima CA, Negri G, Salatini MLF, Salatino A, Mayworm MAS (2015) Composition of the volatile fraction of a sample of Brazilian green propolic and its phytotoxic activity. J Sci Food Agric 95:3091–3095

Fu Y-X, Xu Y-J, Chen B, Li Y, Luo L-P (2009) Analysis of volatile components from inner Mongolia propolis by gas chromatography-mass spectrometry. Chin J Anal Chem 37:745–748

Greenaway W, Scaysbrook T, Whatley FR (1989) Headspace volatiles from propolis. Flavour Fragr J 4:173–175

Greenaway W, May J, Scaysbrook T, Whatley FR (1991) Identification by gas chromatography-mass spectrometry of 150 compounds in propolis. Z Naturforsch 46c:111–121

Haile K, Kebede T, Dekebo A (2012) A comparative study of volatile components of propolis (bee glue) collected from Haramaya University and Assela Beekeeping Centers, Ethiopia. Bull Chem Soc Ethiop 26:353–360

Hames-Kocabas EE, Demirci B, Uzel A, Demirci F (2013) Volatile composition of Anatolian propolis by headspace-solid-phase microextraction (HS-SPME), antimicrobial activity against food contaminants and antioxidant activity. J Med Plant Res 7:2140–2149

Huang S, Zhang C-P, Wang K, Li GQ, Hu F-L (2014) Recent advances in the chemical composition of propolis. Molecules 19:19610–19632

Ioshida MDM, Young MCM, Lago JHG (2010) Chemical composition and antifungal activity of essential oil from Brazilian propolis. J Essent Oil Bear Pl 13:633–637
Isidorov VA, Bakier S, Pirożnikow E, Zambrzycka M, Swiecicka I (2016) Selective behaviour of honeybees in acquiring European propolis plant precursors. J Chem Ecol 42:475–485
Jercović I, Marijanović Z, Kuś PM, Tuberoso CIG (2016) Comprehensive study of Mediterranean (Croatian) propolis peculiarity: headspace, volatiles, anti-Varroa-treatment residue, phenolics, antioxidant properties. Chem Biodivers 13:210–2018
Kaškoniene V, Kaškonas P, Maruška A, Kubiliene L (2014) Chemometric analysis of volatiles of propolis from different regions using static GC-MS. Cent Eur J Chem 12:736–746
Kusumoto T, Miyamoto T, Higuchi R, Doi S, Sugimoto H, Yamada H (2001) Isolation and structures of two new compounds from the essential oil of Brazilian propolis. Chem Pharm Bull 49:1207–1209
Li Y-J, Xuan H-Z, Shou Q-Y, Zhan Z-G, Lu X, Hu F-L (2012) Therapeutic effects of propolis essential oil on anxiety of restraint-stressed mice. Hum Exp Toxicol 31:157–165
Maciejewicz W, Scheller S, Daniewski M (1983) Gas chromatography-mass spectrometry investigation of propolis, analysis of sesquiterpenes. Acta Pol Pharm 40:251–253
Maróstica Junior MR, Daufsch A, Moraes CS, Queiroga CL, Pastore GM, Park YK (2008) Comparison of volatile and polyphenolic compounds in Brazilian green propolis and its botanical origin Baccharis dracunculifolia. Ciênc Tecnol Aliment 28:178–181
Melliou E, Stratis E, Chinou I (2007) Volatile constituents of propolis from various regions of Greece - antimicrobial activity. Food Chem 103:375–380
Miguel MG, Nunes S, Cruz C, Duarte J, Antunes MD, Cavaco AM, Mendes MD, Lima AS, Pedro LG, Barroso JG, Figueiredo AC (2013) Propolis volatiles characterization from acaricide-treated and -untreated beehives maintained at Algarve (Portugal). Nat Prod Res 27:743–749
Naik DG, Vaidya HS, Namjoshi TP (2013) Essential oil of Indian propolis: chemical composition and repellency against the honeybee Apis florea. Chem Biodivers 10:649–657
Negri G, Salatino MLF, Salatino A (2003a) "Green propolis": unreported constituents and a novel compound from chloroform extracts. J Apic Res 42:1–3
Negri G, Salatino MLF, Salatino A (2003b) Unusual chemical composition of a sample of Brazilian propolis as assessed by analysis of a chloroform extract. J Apic Res 42:53–56
Nunes CA, Guerreiro M (2012) Characterization of Brazilian green propolis throughout the seasons by headspace GC/MS and ESI-MS. J Sci Food Agric 93:433–438
Oliveira AP, França HS, Kuster RM, Teixeira LA, Rocha LM (2010) Chemical composition and antibacterial activity of Brazilian propolis essential oil. J Venom Anim Toxins incl Trop Dis 16:121–130
Patricio EFLRA, Cruz-López L, Maile R, Tentschert J, Jones GR, Morgan ED (2002) The propolis of stingless bees: terpenes from the tibia of three Frieseomelitta species. J Insect Physiol 48:249–254
Pellati F (2014) Innovative methods for the extraction and chromatographic analysis of honey bee products. In: Jayprakasha GK, Patil BS, Pellati F (eds) Instrumental methods for the analysis and identification of bioactive molecules, Chapter 2, ACS symposium series. American Chemical Society, Washington, pp 33–49
Pellati F, Prencipe FP, Benvenuti S (2013) Headspace solid-phase microextraction-gas chromatography–mass spectrometry characterization of propolis volatile compounds. J Pharm Biomed Anal 84:103–111
Petri G, Lemberkovics E, Foldvari M (1988) Examination of differences between propolis (bee glue) produced from different floral environments. In: Lawrence BM, Mookherjee BD, Williams BJ (eds) Flavors and fragrances: a world perspective. Elsevier, Amsterdam, pp 439–446
Pino JA, Marbot R, Delgado A, Zumarraga C, Sauri E (2006) Volatile constituents of propolis from honey bees and stingless bees from Yucatán. J Essent Oil Res 18:53–56
Rios N, Yánez C, Rojas L, Mora F, Usubillaga A, Vit P (2014) Chemical composition of essential oil of Apis mellifera propolis from Falcón State, Venezuela. Emir J Food Agric 26:639–642
Salatino A, Teixeira ÉW, Negri G, Message D (2005) Origin and chemical variation of Brazilian propolis. Evid Based Complement Alternat Med 2:33–38

Salatino A, Fernandes-Silva CC, Righi AA, Salatino MLF (2011) Propolis research and the chemistry of plant products. Nat Prod Rep 28:925–936

Segueni N, Khadraoui F, Moussaoui F, Zellagui A, Gherraf N, Lahouelc M, Rhouati S (2010) Volatil constituents of Algerian propolis. Ann Biol Res 1:103–107

Simionatto E, Facco JT, Morel AF, Giacomelli SR, Linares CEB (2012) Chiral analysis of monoterpenes in volatile oils from propolis. J Chil Chem Soc 57:1240–1243

Toreti VC, Sato HH, Pastore GM, Park YK (2013) Recent progress of propolis for its biological and chemical compositions and its botanical origin. Evid Based Complement Alternat ID 697390

Torres RNS, Lopes JAD, Neto JMM, Citó AMGL (2008) Constituintes voláteis de própolis Piauiense. Quim Nova 31:479–485

Torres-González A, López-Rivera P, Duarte-Lisci G, López-Ramírez Á, Correa-Benítez A, Rivero-Cruz JF (2016) Analysis of volatile components from Melipona beecheii geopropolis from Southeast Mexico by headspace solid-phase microextraction. Nat Prod Res 30:237–240

Woisky RG, Salatino A (1998) Analysis of propolis: some parameters and procedures for chemical quality control. J Apic Res 37:99–105

Yang C, Luo L, Zhang H, Yang X, Lva Y, Song H (2010) Common aroma-active components of propolis from 23 regions of China. J Sci Food Agric 90:1268–1282

Chapter 7
The Chemical and Biological Properties of Propolis

Weam Siheri, Sameah Alenezi, Jonans Tusiimire, and David G. Watson

Contents

7.1 General Overview of Propolis

Bees produce several different products which have health benefits. There is no doubt that honey has a highly significant status in medical treatments, while other apian materials, such as wax, royal jelly and propolis, have fewer medical applications, despite the fact that propolis has been used by people since ancient times (Burdock 1998; Ghisalberti 1979). The term propolis comes from two Greek words, pro (which means for or in defence of) and polis (which means the city); thus propolis means in defence of the city or beehive (Ghisalberti 1979). Propolis is a sticky resinous substance, which is gathered from buds and the bark of trees. It is also known as "bee glue" as bees use it to cover surfaces, seal holes and close gaps in their hives, thus providing a sterile environment that protects them from microbes

W. Siheri • S. Alenezi • J. Tusiimire • D.G. Watson (✉)
Strathclyde Institute of Pharmacy and Biomedical Sciences, 161, Cathedral Street, Glasgow G4 0RE, UK
e-mail: d.g.watson@strath.ac.uk

J.M. Alvarez-Suarez (ed.), *Bee Products - Chemical and Biological Properties*,
DOI 10.1007/978-3-319-59689-1_7

and spore-producing organisms, including fungi and molds (Wagh 2013). It is therefore considered to be a potent chemical weapon against bacteria, viruses, and other pathogenic microorganisms that may invade the bee colony. Also, bees use propolis as an embalming substance, to mummify invaders such as other insects, that have been killed and are too heavy to remove from the colony (Bankova et al. 2005a; Wagh 2013). Humans have certainly observed the behaviour of honeybees and the ways in which they use propolis in their hives, which might have inspired interest in the biological properties of bee glue. Therefore, it is not surprising that propolis has become a subject of interest in natural product studies in recent times. The literature on propolis is vast and even a lengthy review cannot cover everything, but can only give an indication of the areas of interest in propolis research. There have been many reviews of the chemical and biological properties of propolis over the years and the most recent ones are listed here: Bankova et al. (2016), Sforcin (2016), Bankova et al. (2014), Silva-Carvalho et al. (2015), Chandna et al. (2014), Toreti et al. (2013), Wagh (2013), Sforcin and Bankova (2011).

7.2 Propolis in History

The ancient Greeks, Romans, and Egyptians were the first to use propolis, with applications in wound healing and as a disinfection substance (Sforcin 2007). The long history of the use of propolis as a medicine is claimed to be as old as the use of other honeybee products, with the former being used from at least 300 BC (Ghisalberti 1979; Burdock 1998; Sforcin 2007).

According to Egyptian history, propolis was one of the main ingredients used in an embalming recipe for mummification, in which it serves as a preservative agent (Mejanelle et al. 1997; Kuropatnicki et al. 2013). Many other ancient civilizations, such as Chinese, Indian, and Arabian, all believed in the power of propolis to treat medical conditions like sores, ulcers, and some skin lesions, so it was used both internally and externally (Kuropatnicki et al. 2013). For a comprehensive review of the history of propolis use see Kuropatnicki et al. (2013). Despite such early use, propolis is often still considered a “folk medicine” and remains an unofficial drug in the field of pharmacy (Valenzuela-Barra et al. 2015; Kuropatnicki et al. 2013; Toreti et al. 2013). However, over the last two decades, its use has begun to gain scientific backing. It is considered to be a promising natural source for the discovery of new pharmaceutical products to treat several types of diseases. Thus, it has been subjected to intensive studies investigating its antioxidant, antimicrobial, anti-inflammatory, immune-stimulating, and anticancer properties (Banskota et al. 2001b). Nevertheless, propolis is still not considered an official conventional medicine in healthcare because of a lack of standardization of its composition due to the variability of its chemical components and thus its biological activity, which varies according to the different geographic locations of its collection (Silva-Carvalho et al. 2015). In addition, there is presently inadequate data regarding therapeutic efficacy from clinical trial studies involving propolis. As a result, there are a few propolis products which have undergone FDA approval (Fitzmaurice et al. 2011).

The bioactivity of propolis mainly depends on its chemical composition. Bankova stated that knowledge of the chemical composition of propolis leads to a prediction of

its biological activities (Bankova et al. 2005a). In general, the term biological activity describes the pharmacological activity of a substance in a living organism. However, when the therapeutic product is a complex mixture, the biological activity could be broadly based due to a multiplicity of active ingredients and thus the product could have many therapeutic indications (Jackson et al. 2007). This is the case with propolis, which contains many active components, leading to numerous pharmacological activities. Propolis has been demonstrated to be safe and non-toxic for human use. However, some cases of allergic reactions such as contact dermatitis have been reported by beekeepers. There is some variability in the toxic and safe dosages of propolis reported by different studies, probably due to a lack of standardized extraction methods (Burdock 1998). The effectiveness of propolis preparations is dependent on the method of preparation, including the solvents used during the extraction process (Silva-Carvalho et al. 2015). The following sections will give an overview of some of the biological activity studies that have been conducted on propolis samples from different parts of the world.

7.3 The Biological Properties of Propolis

7.3.1 The Antioxidant Properties of Propolis

Metabolic processes within the human body consist of multiple complex reactions which generate natural free radicals. The body also has natural enzymatic antioxidants, which include superoxide dismutase, catalase, and glutathione peroxidase, and non-enzymatic antioxidants including lipid soluble, e.g. vitamin E, and water soluble, e.g. vitamin C and glutathione, compounds for defence against the harmful effects of the reactive oxygen species (ROS) (Valko et al. 2007). A free radical in the body is simply an atom or molecule containing one or more unpaired electrons in its outer orbital, such as the oxygen atom shown in Fig. 7.1. The unpaired electron

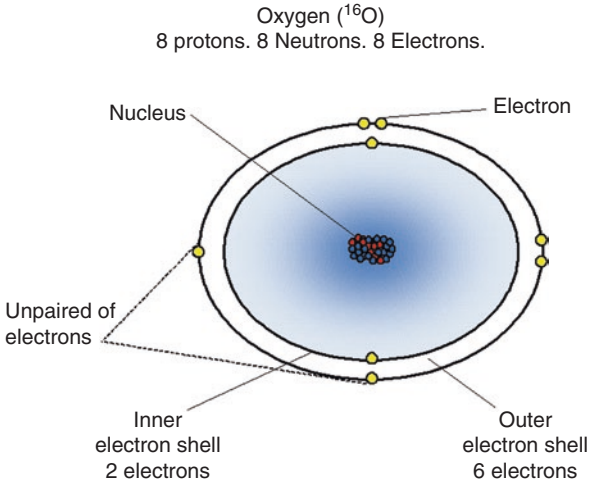


Fig. 7.1 Schematic showing the orbitals of the oxygen atom

allows the ROS to participate in many reactions with other free radicals. In low to moderate concentrations, ROS play vital roles in the biological processes of the human body, including stimulating pathways in response to changes in the extracellular environment (cellular signalling), mitogenic response, and immune response for defence against infections in the intracellular environment (Valko et al. 2007; Halliwell and Gutteridge 2015).

As can be seen in Fig. 7.2, oxygen molecules can accept energy in the form of electrons as an outcome of an inflammation process, leading to the production of oxygen-centred free radicals also known as ROS. The generation of ROS is

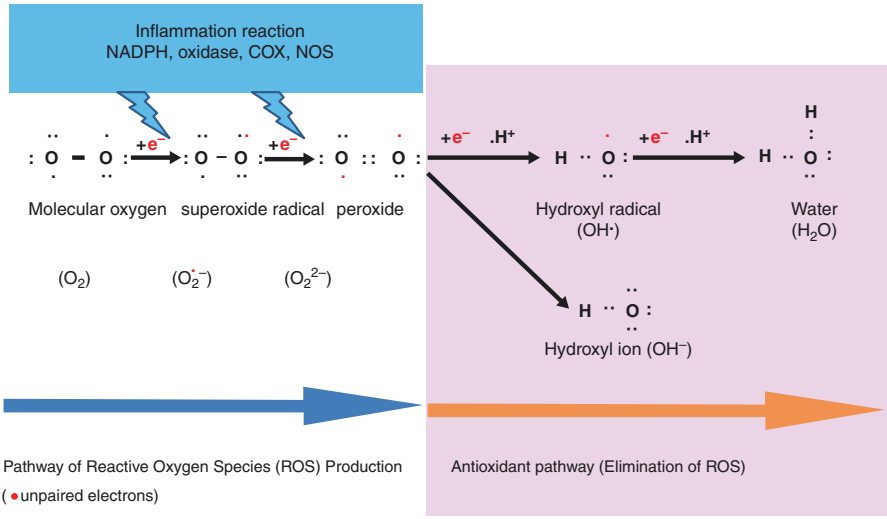


Fig. 7.2 Diagram illustrating the formation of Reactive Oxygen Species (ROS). When the oxygen molecule accepts an electron, it becomes a superoxide radical which, upon further electronation, produces peroxide. The latter can undergo further reactions with electrons and protons to produce potent hydroxyl radicals. Antioxidants act by donating protons (H+) to free radicals, leading to the formation of water, as shown in Fig. 7.3

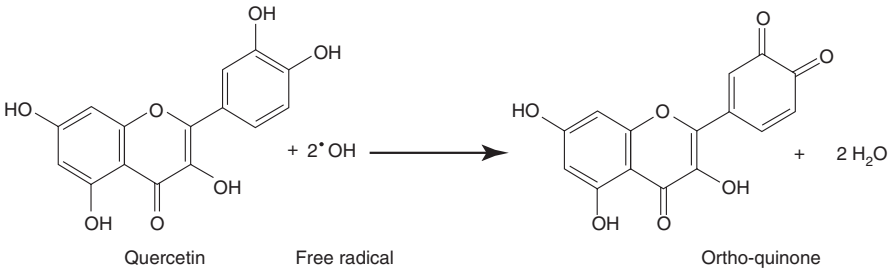


Fig. 7.3 Illustration of the antioxidant effect of quercetin, a flavonoid, on hydroxyl radicals. Quercetin reduces the free radical to water while it is oxidised to an *ortho*-quinone

regulated by the action of the enzyme RO synthase and over production results from both the mitochondrial electron transport chain and excessive production of NADH (Valko et al. 2007). Once formed, these ROS are highly reactive and produce a chain of deleterious reactions resulting in damage to cell structures (lipids, membranes, proteins and DNA) and modulation of many biological processes including inflammation and immune response. Oxidative stress occurs when there is an imbalance between the production of free radicals and physiologically active antioxidant metabolites in the body (Valko et al. 2007). The excessive production of ROS may be responsible for causing a large number of diseases, such as cancer (Kinnula and Crapo 2004), cardiovascular disease and inflammatory disorders such as rheumatoid arthritis. In addition, ROS can induce mutations or cause direct damage in DNA which leads to cell transformation and the possibility of developing a variety of malignant conditions (Valko et al. 2007). Also, active free radicals are the main factor involved in cellular aging and are responsible for the development of many CNS related medical conditions such as Parkinson's and Alzheimer's diseases. Antioxidant agents can serve as a defensive factor against free radicals in neuronal cells (Metodiewa and Kośka 1999; Martin and Grotewiel 2006; Valko et al. 2007).

Many scientific papers have been published on the antioxidant effects of propolis (Bittencourt et al. 2015; Olczyk et al. 2013; Piccinelli et al. 2013). The relationship between antioxidant activity and the chemical composition of propolis from different origins has been investigated by several authors (Isla et al. 2001; Kalogeropoulos et al. 2009; Mello and Hubinger 2012; Piccinelli et al. 2013). These studies confirmed that the significant antioxidant activity of propolis is related to the high content of polyphenolic compounds, such as flavonoids, in the sample. Additionally, it has been reported that the essential oil constituents of *Thymus vulgaris* (thyme) could act as antioxidant agents (Deans et al. 1992). Since one of the main components of propolis has been proven to be essential oils (Bankova et al. 2014; Marcucci 1995), it might be possible that these components contribute to its antioxidant effects. The study conducted by Kumazawa et al. suggested that propolis could act as an antioxidant agent due to the presence of anti-oxidative compounds such as kaempferol and phenethyl caffeate (Kumazawa et al. 2007). Their conclusion came following the investigation of antioxidant activities of various propolis samples from different geographical origins using the DPPH assay.

Another approach to verifying the antioxidant action for potential in human medicine is to analyse the potential alleviating effect of propolis in neurodegeneration by means of cell viability assays on neuronal cells (Imamura et al. 2006). It is well known that the main factor in CNS disorders is oxidative stress. Thus, antioxidant properties play a vital role in the management of CNS disorders induced by oxidative stress. Shimazawa et al. assayed and reported the neuroprotective effect of green Brazilian propolis both *in vitro* and *in vivo*. First, the *in vitro* assay was conducted by exposure of neuronal cell cultures to hydrogen peroxide (H_2O_2), followed by addition of propolis to the neuronal cells. On the other hand, the *in vivo* experiments studied the effect of propolis against lipid peroxidation in

the forebrain of mice and DPPH-induced free radical production (Shimazawa et al. 2005). Furthermore, a recent study found that Turkish propolis contains phenolic components which have the ability to minimize DNA damage by inhibiting the effects of H_2O_2 in cultured fibroblasts (Darendelioglu et al. 2016). An effective natural antioxidant agent such as propolis could provide a safe and novel treatment for oxidative stress-related diseases, especially in elderly people whose conditions tend to be complex in nature and include cases of neurodegeneration corresponding to aging. In addition, propolis could play a key role in the management and prevention of various disease conditions in which ROS have a causative effect, such as some inflammatory disorders, cancer, cardiovascular and immune diseases.

7.3.2 The Antimicrobial Activity of Propolis

Until now, the most widely investigated property of propolis is its antimicrobial activity, with hundreds of publications on this topic having appeared in the last 40 years (Bogdanov 2012). These findings explain why propolis plays such an important role in bee hives since it can be considered as a chemical weapon against pathogenic microorganisms (Fokt et al. 2010; Bankova 2005a). Different propolis types contain many chemical constituents responsible for their antimicrobial properties (Bankova 2005a) and it seems that the sum of the propolis antimicrobial components, rather than individual substances, is responsible for the observed antimicrobial effect (Kujumgiev et al. 1999; Bogdanov 2012). Propolis shows antibacterial (Silici and Kutluca 2005; Kujumgiev et al. 1999; Grange and Davey 1990), antifungal (Kartal et al. 2003; Kujumgiev et al. 1999; Ota et al. 2001), antiviral (Amoros et al. 1992a, b), antiprotozoal (Freitas et al. 2006; Dantas et al. 2006a, b), anti-tumour (Callejo et al. 2001; Komericki and Kränke 2009; Banskota et al. 2000; Su et al. 1994), anti-inflammatory (Khayyal et al. 1992; Dobrowolski et al. 1991; Fokt et al. 2010), local-anaesthetic (Marcucci 1995), antioxidant (Russo et al. 2002; Fokt et al. 2010; Kumazawa et al. 2007), immunostimulating (Dimov et al. 1992; Oršolić et al. 2004), cytostatic (Banskota et al. 1998) and hepatoprotective (Banskota et al. 2001a; Won Seo et al. 2003) activities.

There are many components which are responsible for the biological activity of propolis and these vary with propolis sample type and the solvents used in its extraction (Ugur and Arslan 2004). Flavonoids and esters of phenolic acids are generally regarded as bioactive compounds which are responsible for antimicrobial activity (Fokt et al. 2010). However, there are many other components with such activity; these are summarised in Tables 7.1 and 7.2 for different types of propolis and two of the main types, respectively.

Table 7.1 Biological effects of propolis components adapted from Bogdanov (2012)

Propolis active ingredient and propolis type	Biological activity
Polyphenols and flavonoids Mostly poplar and all propolis types	Antibacterial, antiviral, antifungal
Caffeic acid phenethyl ester (CAPE) and other caffeates Poplar and Bacharis	Antibacterial, antiviral, fungicidal
Caffeic acid (CA) Poplar and Baccharis	Antiviral
Terpenes Greece, Crete, Croatia, Brazil	Antibacterial, antifungal
Essential oils Brazil, Poland	Antibacterial
Furfuran lignans Canary islands	Antibacterial

Table 7.2 Biologically active ingredients in Poplar and Baccharis propolis adapted from Bogdanov (2012)

Biological activity	Propolis type, active ingredient
Antibacterial	*Poplar*: Different flavonones, flavones, phenolic acids and their esters
	Bacharis: Prenylated *p*-coumaric acids, labdane diterpenes
Antifungal	*Poplar*: Pinocembrin, galangin, benzoic acid, salicylic acid, vanillin
	Baccharis: Mono and sesquiterpenes, Artipellin C
Antiviral	*Poplar*: Polyphenols, phenyl carboxylic acids, and esters of substituted cinnamic acids, caffeic acid, quercetin, luteolin, fisetin, and quertecagetin
	Baccharis: Activity detected but no substances identified

7.3.2.1 Antibacterial Activity of Propolis

Antimicrobial activity is recognised as the most important property of propolis, particularly activity against bacteria. Several studies have been performed to evaluate this property against a large group of Gram-positive and Gram-negative bacteria; both aerobic and anaerobic types. The bacteria studied are summarized in Table 7.3. These bacteria were either from laboratory collections or isolated from clinical samples. The studies used propolis of different geographical origins and chemical composition, and employed different experimental approaches such as disc diffusion and disc dilution to investigate the antibacterial activity. In the disc diffusion method, antibacterial activity is determined by measuring the diameter of the bacterial growth inhibition zone in the agar layer surrounding a disc containing propolis extracts (Kujumgiev et al. 1999). The dilution method is used to determine the minimal inhibitory concentration (MIC) and the minimal bactericidal concentration

Table 7.3 Bacteria used in the determination of the antibacterial activity of propolis adapted from Fokt et al. (2010)

Type	Gram-positive	Gram-negative
Aerobic	*Bacillus* spp. • *B. cereus* • *B. subtilis*	*Aeromonas hydrophila*
		Brucella abortus
	Enterococcus spp. • *E. faecalis*	*Corynebacterium* sp. • *C. pseudotuberculosis*
	Micrococcus luteus	*Escherichia coli*
	Nocardia asteroids	*Helicobacter pylori*
	Rhodococcus equi	
	Staphylococcus spp. • *S. aureus* • *S. auricularis* • *S. capitis* • *S. epidermidis* • *S. haemolyticus* • *S. hominis* • *S. mutans* • *S. warnerii*	*Klebsiella pneumoniae*
		Salmonella sp. • *S. enteritidis*, • *S. typhi* • *S. typhimurium*
	Streptococcus spp. • *S. cricetus* • *S. faecalis* • *S pneumioniae* • *S. pyogenes* • *S. β-haemolyticus* • *S. mutans* • *S. sobrinus* • *S. viridians*	*Pseudomonas aeruginosa*
		Proteus spp. • *P. mirabilis* • *P. vulgaris*
		Shigella dysenteriae
Anaerobic	*Actinomyces naeslundii*	*Actinobacillus actinomycetemcomitans*
	Lactobacillus acidophilus	*Capnocytophaga gingivalis*
	Peptostreptococcus micros	*Porphyromonas* spp. • *P. anaerobius* • *P. gingivalis*
		Fusobacterium nucleatum
		Prevotella spp. • *P. intermedia* • *P. melaninogenica* • *P. oralis*
		Veillonella parvula

(MBC) which are, respectively, the lowest concentrations that inhibit visible bacterial growth and the lowest concentration that kills bacteria (Grange and Davey 1990; Stepanović et al. 2003). The vast majority of antibacterial activity studies were carried out using *in vitro* bioassays, as mentioned above. Although the composition of propolis differs considerably depending on its botanical origin, all examined types of propolis have revealed strong antibacterial activity (Kujumgiev et al. 1999; Bankova 2005b; Bankova et al. 2007). Also, the activity of propolis may depend on the type of bee collecting it since it was found that poplar propolis collected by *Apis mellifera*

caucasica had a higher antibacterial activity than that collected by *Apis mellifera anatolica* and *Apis mellifera carnica* (Silici and Kutluca 2005).

Tests for the antibacterial activity of propolis were carried out against a range of different pathogenic bacteria in several studies, as summarised in Table 7.3 (Banskota et al. 2001b; Burdock 1998; Ghisalberti 1979; Grange and Davey 1990). It has been reported that propolis is more active against Gram-positive pathogens, but many Gram-negative bacteria are also inhibited (see Table 7.3) (Fokt et al. 2010; Wagh 2013).

The data collected from a range of studies on the antibacterial properties of propolis support the fact that propolis is active mainly against Gram-positive bacteria and either displays much lower activity against Gram-negative ones or is not active at all (Marcucci 1995; Silici and Kutluca 2005; Kujumgiev et al. 1999; Drago et al. 2007; Grange and Davey 1990; Kartal et al. 2003; Dobrowolski et al. 1991; Fadaly and El-Badrawy 2001).

Such results can be seen in the study by Kujumgiev et al., who evaluated propolis samples from different geographic regions (tropical and temperate zones) against *Staphylococcus aureus* and *Escherichia coli*. All of the extracts exhibited significant antibacterial activity against *S. aureus* but none were active against *E. coli* (Kujumgiev et al. 1999).

However, it was reported that ethanolic extracts from propolis (EEP) completely inhibited the growth of *S. aureus*, *Enterococcus* spp. and *Bacillus cereus*, and moderately inhibited the Gram-negative organisms *Pseudomonas aeruginosa* and *E. coli*. (Grange and Davey 1990). The antibacterial activity of EEP from Brazilian propolis, collected during four seasons, was found to inhibit the growth of Gram-positive bacteria and higher concentrations of EEP were needed to inhibit Gram-negative bacterial growth, but the extracts had no effect on *Klebsiella pneumoniae*.

More recent research has revealed antibacterial activity of propolis against *Micrococcus luteus*, *Salmonella typhimurium* (Uzel et al. 2005) and *K. pneumonae* (Victorino et al. 2007); and although in earlier studies (Grange and Davey 1990) it was stated that *Listeria monocytogenes* is not sensitive to propolis, more recent studies revealed significant activity against this organism (Ozcan et al. 2004; Yang et al. 2006). It was also found that propolis showed strong antibacterial activity against 13 different bacterial plant pathogens (Basim et al. 2006).

The antibacterial effect of propolis is bactericidal (Grange and Davey 1990) and it is proposed to work by inhibiting bacterial mobility. In addition, it has been shown that the antibacterial activity of poplar propolis is based on inhibition of quorum sensing (QSI), with the flavonoid pinocembrin being an important QSI agent (Savka et al. 2015).

The flavonoids galangin, pinocembrin and pinostrobin have been most associated with the antibacterial properties of propolis, as shown in Table 7.1 (Dimov et al. 1992), but it has also been reported that propolis samples containing only traces of flavonoids demonstrate antibacterial action (Tomás-Barberán et al. 1993). In addition, ferulic and caffeic acids, prenylated coumaric acid, benzophenone derivatives and diterpenic acids have also been reported as antibacterial compounds (Ghisalberti 1979; Burdock 1998; Castaldo and Capasso 2002; Kujumgiev et al. 1999; Popova et al. 2007; Mirzoeva et al. 1997).

In recent years, there has been considerable interest in using propolis in hospitals as an antibacterial agent due to the increase in antibiotic resistance (Bogdanov 2012). It has been shown that the components in propolis act synergistically against bacteria (Onlen et al. 2007; Orsi et al. 2006; Scazzocchio et al. 2006; Speciale et al. 2006; Stepanović et al. 2003). Several authors point out that the antimicrobial activity of propolis is related to its highly complex and variable constituents and their synergistic action (Bonvehí and Coll 1994; Burdock 1998; Freitas et al. 2006; Scazzocchio et al. 2006; Mirzoeva et al. 1997; Takaisi-Kikuni and Schilcher 1994).

Compounds which were active against *Mycobacterium marinum*, the closest genetic relative to *Mycobacterium tuberculosis*, were isolated from Saudi Arabian propolis. The strongest activity was found for the flavonoid psiadiarabin which showed an activity only 5 times less than that of the gentamycin control (Almutairi et al. 2014a). Twelve ethanolic extracts of propolis from different areas within Libya were tested against *M. marinum* in order to determine whether or not the observed activity was associated with specific components in the samples. The extracts showed moderate to strong activity against *M. marinum* (Siheri et al. 2016).

Commonly, the biological activity of a natural medicinal product decreases with increasing storage time, but Meresta (1997) stated that ethanolic solutions of propolis stored for 10–15 years had increased antibacterial activity (Meresta 1997).

7.3.2.2 Antiviral Activity of Propolis

Many recent reviews have reported on the various antiviral activities of propolis samples from different geographical origins against different strains of viruses, such as Adenovirus, HSV, Influenza A and B viruses, Newcastle disease virus, Polio virus, Vaccinia, Rotavirus, vesicular stomatitis virus (VSV), and Corona virus (Starzyk et al. 1977; Fokt et al. 2010; Bogdanov and Bankova 2012), as summarised in Table 7.4. Studies have reported that propolis has significant antiviral activity and interferes with the replication of some different viruses that cause human diseases, including *Herpes simplex*, *genitalis* and *zoster*, influenza and smallpox (De Castro 2001; Bogdanov 2012; Silva-Carvalho et al. 2015).

Studies over the past two decades have provided further important information on the antiviral properties of propolis. The effect of propolis on several DNA and RNA viruses, including herpes simplex type 1 (HSV-1), an acyclovir resistant mutant, herpes simplex type 2 (HSV-2), adenovirus type 2, VSV, and poliovirus type 2, was studied. The inhibition of poliovirus propagation was observed through a plaque reduction test and a multistep virus replication assay. At a concentration of 30 μg/ml, propolis reduced the titer of HSV by 1000, whereas VSV and adenovirus were less susceptible. The antiviral effect of propolis along with the major flavonoids found therein, such as galangin, kaempferol, chrysin, apigenin, luteolin and quercetin, against HSV was also studied. Flavonols were found to be more active than flavones, with the order of importance from least to most active being galangin, kaempferol and quercetin, demonstrating that activity increases with the number of hydroxyl groups in the molecule. The efficacy of binary flavone-flavonol combinations against HSV-1 was also investigated. It was concluded that synergism might occur between two or more compounds, leading to enhanced antiviral activity of propolis (Amoros et al. 1992a).

Table 7.4 Antiviral activity of the different propolis constituents from different geographical origins (1992–2016) adapted from Silva-Carvalho et al. (2015)

Propolis type/plant source/origin Type extract/isolated compounds	Species/cells/viruses	Antiviral effect
European type propolis Caffeic acid, p-coumaric acid, benzoic acid, galangin, pinocembrin, and chrysin	RC-37 cells, HSV-1 strain KOS	High anti-HSV-1 activity for both extracts when cells were treated prior to viral infection
European propolis PEE and PWE	RC-37 cells, HSV-2	High antiherpetic activity for both extracts when viruses were pre-treated prior to infection
Brazilian Brown propolis/HPE	HSV-2 strain propagated in Vero cells, female BALB/c mice	Effective against HSV-2 infection
Characteristic of Brazilian propolis Isopentyl ferulate isolatefrom and PEE	Influenza viruses	Suppression of influenza virus A/Hong Kong reproduction *in vitro*
Green Brazilian propolis/*B. dracunculifolia B. eriocladа*, *Myrceugenia euosma*/Brazil, PEE	Influenza A virus	Reduction of body weight loss of infected mice and virus yields in the bronchoalveolar lavage fluids of lungs
European propolis/Populus Nigeria/ France PEE	RC-37 cells, HSV-1 strain H29S, acyclovir resistant mutant HSV1-R strain H29R, HSV-2, adenovirus type 2, poliovirus type 2, and VSV	Reduction of titre of HSV, VSV and adenovirus, which was less susceptible; virucidal action on the enveloped viruses HSV and VSV
Geopropolis from the stingless bee *Scaptotrigona postica*/Brazil/ Hydromethanolic extract	African green monkey kidney cells (ATCC CCL-81); herpes simplex virus strain	Inhibition of HSV replication and entry into cells
Characteristic of Brazilian red and green propolis/synthesised/ Homoisoflavonoids, especially 3-benzyl-4-chromones	BGM (Buffalo GreenMonkey) cells, coxsackie viruses B3, B4, and A9 and echovirus 30	Good antiviral activity against the coxsackie viruse s B3, B4, and A9 andechovirus 30
European propolis/*P. trichocarpa* and *P. tremuloides*/Canada/PEE	HSV-1 and HSV-2 virus replicated in MDBK cells	Impairing the ability of the virus to adsorb or to penetrate the host cells
Green propolis/*Baccharis dracunculifolia* Characteristic of Brazilian green propolis. /Brazil/Water extracts, 3,4-dicaffeoylquinic acid (Isolated from Brazilian propolis)	Female BALB/c mice, Influenza A virus strain A/WSN/33 (H1N1)	Extension of the lifetime of mice. 3,4-dicaffeoylquinic acid which increases mRNA levels of tumor necrosis factor-related apoptosis-inducing and decreases H1N1 hemagglutinin mRNA

(continued)

Table 7.4 (continued)

Propolis type/plant source/origin Type extract/isolated compounds	Species/cells/viruses	Antiviral effect
Characteristic of Brazilian green pro polis Brazil/Melliferone, moronic acid, anwuweizonic acid and betulonic acid (isolated from Brazilian propolis)	H9 lymphocytes, HIV-1	Moronic acid inhibiting anti-HIV replication
Mediterranean propolis/*Populus* spp., *Eucalyptus* spp., and *Castanea sativa*/ Israel/PWE Characteristic of European propolis	Jurkat, uninfected human T-cell lines, and MT2 (HTLV-1 infected human T cells) cells	Inhibition of the activation of NF-κB-dependent promoter by Tax and prevention of NF-κB Tax binding to IκBα and its degradation
Nanometer propolis Flavone/ Nanometer propolis Flavone provided by Binzhou Animal Science and Veterinary.Medicine Academy of Shandong Province	Kidney cells (PK-15) Porcine parvovirus (PPV) Britain White guinea pigs	Inhibition of PPV infecting porcine kidney- (PK-) 15 cells. Restraining of PPV copy in lung, gonad, and blood, decrease of the impact of PPV on weight of guinea pigs and increase of hemagglutination inhibition of PPV in serum as well as improving the contents of IL-2, IL-6, and γ-IFN
European propolis/*Populus nigra* Brazil Green propolis/*Baccharis* USA and China Brazil/PEE	Peripheral blood mononuclear cells obtained from blood of healthy donors, microglial cells isolated from human fetal brain tissue, HIV-1AT, HIV-1SF162	Inhibition of HIV-1 variants expression
Propolis samples Turkey (Hatay region) Hatay, Turkey/PEE	HSV-1 and HSV-2 in Hep-G2 cell cultures	Quite effective against the replication of HSV-1 and HSV-2

A number of other studies have suggested an association between the antiviral activity of propolis and certain compounds which are found therein. Some flavonoids have an inhibitory effect on human immunodeficiency virus (HIV) infection and replication. It was found that luteolin was more active than quercetin, but less active than caffeic acid and some esters of substituted cinnamic acids found in propolis. Isopentyl ferulate significantly inhibited the infectious activity of influenza virus A. It has previously been observed that the antiviral activity is due to both the major constituents in propolis and the minor components such as 3-methylbut-2-enyl caffeate and 3-methylbutyl ferulate (Amoros et al. 1992a; Maksimova-Todorova et al. 1985; Vanden Berghe et al. 1986; Marcucci 1995). 3-Methylbut-2-enyl caffeate showed strong inhibition of HSV-1 growth (Amoros et al. 1992a, b).

In addition, it has been reported that some propolis constituents and their analogues (esters of substituted cinnamic acids) significantly inhibited infection by influenza virus A/Hong Kong (H3N2) (Serkedjieva et al. 1992). A study of the antiviral effect of caffeic acid, a constitutent of propolis, found that *Vaccinia* and adenovirus were more sensitive to caffeic acid than polio and parainfluenza viruses, but it exhibited only minor activity against influenza virus (Fokt et al. 2010).

The antiviral activity of aqueous and ethanol extracts of propolis and constituents, such as flavonoids caffeic acid, p-coumaric acid, benzoic acid, galangin, pinocembrin and chrysin, was tested against herpes simplex virus type 1 (HSV-1). Both propolis extracts demonstrated high levels of antiviral activity against HSV-1 in viral suspension tests; plaque formation was significantly reduced by >98%. Galangin and chrysin were proposed to greatly contribute to the observed activity (Schnitzler et al. 2010).

Besides its inhibitory effect on viral growth, propolis also shows virucidal action on enveloped viruses HSV and VSV (Marcucci 1995). The activity of Brazilian propolis against HSV-1 infection was studied after its oral administration to infected mice three times daily and on days 0–6 after treatment. The results revealed a significant effect on the development of herpetic skin lesions (Shimizu et al. 2011).

The antiviral activities of four propolis samples from Austria, Egypt, France and Germany were investigated against avian reovirus (ARV) and infectious bursal disease virus (IBDV). The results indicated that all propolis samples reduced the viral infectivity to a different degree and that the Egyptian propolis showed the highest antiviral activity against ARV and IBDV (Hegazi et al. 2000; El Hady and Hegazi 2002). The activity of 13 ethanol extracts of Brazilian green propolis against viruses was investigated. The extracts displayed antiviral activity against influenza virus *in vitro* and *in vivo*. The effect was attributed to the flavonoid and phenolic acid constituents (Shimizu et al. 2008).

The aqueous and ethanolic extracts of propolis were evaluated against HSV-1 and HSV-2. The anti-herpetic effect was analysed in cell culture and both propolis extracts exhibited high levels of antiviral activity against HSV-2. Infectivity was significantly reduced by 49% and direct concentration- and time-dependent antiherpetic activity was demonstrated (Nolkemper et al. 2010).

A hydromethanolic extract of geopropolis (HMG) was evaluated using viral DNA quantification experiments and electron microscopy. The study showed a reduction of viral DNA from herpes virus by about 98% under all conditions and concentrations of HMG tested (Coelho et al. 2015).

The antiviral activity of EEP of Turkish propolis on the replication of both HSV-1 and HSV-2 was investigated. HSV-1 and HSV-2 were suppressed in the presence of 25, 50, and 100 μg/mL of propolis extract when infection of a Hep-G2 cell line was examined. Synergistic effects of propolis with acyclovir were identified against these viruses. The results showed a significant decrease in the number of viral copies (Yildrim et al. 2016).

It was found that propolis suppresses the replication of human immunodeficiency virus type 1 (HIV-1), in addition to modulating immune responses. The antiviral activity of propolis samples against HIV-1 from several geographic regions was

investigated in CD4+ lymphocyte and microglial cell cultures. The results showed inhibited viral expression in a concentration-dependent manner (maximal suppression of 85 and 98% was observed at 66.6 μg/ml propolis in CD4+ lymphocyte and microglial cell cultures, respectively) (Gekker et al. 2005).

Propolis flavonoids act by preventing the virus from entering the host cell and by reducing intracellular replication activities. This process contributes to suppression of the growth and development of the virus. Other possible mechanisms of antiviral activity include inhibition of reverse transcriptase and stimulation of the immune system to fight back against the infection (Schnitzler et al. 2010; Boukraâ et al. 2013).

Propolis extracts were screened in a plaque reduction assay and exhibited anti-influenza activity. Mice were infected intranasally with the influenza virus, and the four extracts were orally administered at 10 mg/kg three times daily for seven successive days after infection; the EEP was found to possess anti-influenza activity and to ameliorate influenza symptoms in mice (Shimizu et al. 2008).

7.3.2.3 Antiprotozoal and Antihelminthic Activity of Propolis

Recently, attention has been focused on the antiparasitic activity of propolis since improvements on existing drugs against several tropical diseases caused by different protozoa are required. Numerous assessments have been performed using different *in vivo* and *in vitro* experiments to investigate the activity of raw propolis and active compounds isolated from propolis. Accordingly, significant effects against different parasitic species including *Cholomonas paramecium*, *Eimeria magna*, *Media perforans*, *Giardia lambia*, *Giardia duodenalis*, *Trichomonas vaginalis*, *Trypanosoma cruzi* and *Trypanosoma evansi* have been reported in the literature (Freitas et al. 2006; Falcão et al. 2013; Bogdanov 2012; Parreira et al. 2010). Several studies have been performed that show the activity of propolis and its components against a range of protozoan parasites which cause various human diseases, including *Trypanosoma brucei* which causes sleeping sickness and *Trypanosoma cruzi* which causes Chagas disease (Higashi and De Castro 1994; De Castro and Higashi 1995; Marcucci et al. 2001; Dantas et al. 2006a, b; Salomão et al. 2010; Falcão et al. 2013; Almutairi et al. 2014b; Siheri et al. 2014, 2016; Omar et al. 2016). Antiprotozoal effects of different propolis samples were reported against *Leishmania donovani*, which causes visceral leishmaniasis, and for other strains of leishmania (Duran et al. 2008; Pontin et al. 2008; Ozbilge et al. 2010; Monzote et al. 2011; Amarante et al. 2012; Da Silva et al. 2013; Siheri et al. 2016). Recent studies have reported antiprotozoal effects of propolis extracts against *Plasmodium falciparum*, *Plasmodium malariae*, *Plasmodium vivax* and *Plasmodium ovale*, *all of* which cause malaria (Olayemi 2014; Siheri et al. 2016). Propolis is also effective against *Entamoeba histolytica* and *Giardia lamblia*, which cause intestinal infections (dysentery and diarrhoea), as well as multicellular organisms such as intestinal worms, including helminths such as Schistosoma spp., cestodes such as tapeworms, nematodes such as roundworms, and trematodes such as flukes (Freitas et al. 2006; Issa 2007; Hegazi et al. 2007; Abdel-Fattah and Nada 2007; Noweer and Dawood 2008;

Alday-Provencio et al. 2015; Hassan et al. 2016). Some of the studies are described in more detail below.

Extracts of Portuguese propolis and its potential sources such as poplar buds were screened against different protozoa, including *Plasmodium falciparum*, *Leishmania infantum*, *Trypanosoma brucei* and *Trypanosoma cruzi* (Falcão et al. 2013). The toxicity of the extracts against MRC-5 fibroblast cells was also evaluated to assess toxic selectivity. The propolis extracts showed moderate activity against these parasites, with the highest inhibitory effect being observed against *Trypanosoma brucei* (Falcão et al. 2013).

Recently, extracts from 12 samples of propolis collected from different regions of Libya were tested for their activity against *Trypanosoma brucei*, *Leishmania donovani*, *Plasmodium falciparum* and *Crithidia fasciculate*, while the cytotoxicity of the extracts was also tested against mammalian cells. All of the extracts were active to some degree against all of the protozoa, exhibiting a range of EC_{50} values between 1.65 and 53.6 μg/ml (Siheri et al. 2016), while only exhibiting moderate to negligible cytotoxicity.

The activity of propolis against Chagas disease (caused by *Trypanosoma cruzi*) was assessed in comparison with crystal violet, a standard drug recommended to prevent the transmission of Chagas disease via blood (De Castro and Higashi 1995). The relationship between trypanocidal activity and the chemical composition of propolis has been widely investigated by several authors and these studies confirmed that Brazilian green propolis is highly active against *T. cruzi* transmission (Dantas et al. 2006b; De Castro and Higashi 1995; Higashi and De Castro 1994).

The activity of ethanol extracts from Brazilian (Et-Bra) and Bulgarian (Et-Blg) propolis against *T. cruzi* were tested and it was found that, although there were differences in the chemical composition between both extracts, they were both active against *T. cruzi*. The study also confirmed that in European samples biological activity was associated with the presence of flavonoids and aromatic acids and their esters. In Brazilian propolis, amyrins occur as components that might contribute to the anti-trypanosomal activity (Salomão et al. 2004; Higashi and De Castro 1994).

The activity of acetone and ethanol extracts of two Bulgarian propolis samples (Bur and Lov) against *T. cruzi* was evaluated. Both extracts showed similar chemical compositions with a high content of flavonoids and strong inhibitory activity against *T. cruzi* proliferative epimastigotes, which were more susceptible than trypomastigotes. While in the presence of blood, the activity of Et-Bur or Et-Lov against trypomastigotes was similar to that of the standard drug, crystal violet (Prytzyk et al. 2003). It was also found that two different samples from Bulgarian propolis showed significant activity against *T. cruzi in vitro* (Salomão et al. 2004, 2009; Dantas et al. 2006a, b).

Current therapy for *T. evansi* infections is not effective for the vast majority of animals with relapsing parasitemia and clinical signs. The susceptibility of *T. evansi* to a propolis extract *in vitro* and *in vivo* was evaluated. A dose-dependent trypanocidal activity of the propolis extract was observed *in vitro*. All trypomastigotes were killed within 1 h after incubation with 10 μg/ml of the extract. However, *in vivo* assessment of concentrations of 100, 200, 300 and 400 mg/kg administered orally

for 10 consecutive days presented no curative effect, and the rats died from the disease. However, rats treated with the two highest concentrations of propolis extract showed higher longevity than the other groups. Based on these data the study concluded that, despite the lack of curative efficacy observed *in vivo* at the concentrations tested, propolis extract can prolong life in rats infected with the protozoan (Gressler et al. 2012).

A comprehensive chemical profiling study was carried out on 22 African propolis samples collected from the sub-Saharan region. Results revealed that triterpenoids were the major chemical components in more than half of the propolis samples analysed in this study and some others were classified as temperate and Eastern Mediterranean types of propolis. Based on comparative chemical profiling, one propolis sample from southern Nigeria stood out from the others by containing prenylated isoflavonoids, which indicated that it was more like Brazilian red propolis (Zhang et al. 2014). This propolis was further investigated and ten phenolic compounds were isolated, including a new dihydrobenzofuran. All the isolated compounds were tested against *T. brucei* and displayed moderate to high activity. Some of the compounds tested showed similar activity against wild type *T. brucei* and two strains displaying pentamidine resistance. The Nigerian propolis from Rivers State showed some similarities to Brazilian red propolis and exhibited antitrypanosomal activity at a potentially useful level (Omar et al. 2016).

The chemical profile and antitypanosomal activity of Ghanian propolis against *T. brucei* was also investigated. Two compounds were isolated; a prenylated tetrahydroxy stilbene and a geranylated tetrahydroxy stilbene. These compounds exhibited moderate activity against *T. brucei*. In the same paper, isolation of a new phloroglucinone analogue from Cameroon propolis was reported. The compound was found to possess high potency, comparable to that of suramin (Almutairi et al. 2014b).

The EEP of Libyan propolis was tested for its activity against *T. brucei*. One of the samples was fractionated and yielded a number of active fractions. Three of the active fractions contained single compounds, found to be 13-epitorulosal, acetyl-13-epi-cupressic acid and 13-epi-cupressic acid, which had been identified previously in Mediterranean propolis. Two of the compounds had a MIC value of 1.56 μg/mL against *T. brucei* (Siheri et al. 2014).

The chemical composition and biological activity of a propolis sample collected from Saudi Arabia were investigated. A new diterpene, propsiadin, was isolated along with two flavonoids and a known diterpene, psiadin. The compounds had MICs in the range 30.9–78.1 μM against *T. brucei*. The propolis was thought to originate from *Psiadia arabica* and *Psiadia punctulata*, representing a new type of propolis (Almutairi et al. 2014a).

Leishmaniasis has been reported as an endemic disease in 88 countries in tropical and sub-tropical regions across the world, affecting more than 12 million people. There are no vaccines available for any form of the disease and the chemotherapy of this disease is still inadequate and expensive (Kayser et al. 2003; Croft et al. 2005). An intense search for potential natural products isolated from plants or propolis for the treatment of Leishmaniasis has been carried out during the last decades. The previous literature contains several reports on the activity of a variety of crude natu-

ral extracts against Leishmania, especially from plants collected in tropical zones (Croft et al. 2006).

Previous studies have reported that propolis samples from various origins possess activity as anti-leishmanial agents due to the presence of flavonoids and amyrins (Machado et al. 2007).

A study of propolis from Turkey investigated the effects of propolis against *Leishmania tropica* and it was observed with microscopic examination that propolis inhibited parasite growth at ≥32 μg/ml concentration. It was also found that the antileishmanial effects of propolis increased with increasing concentrations and incubation periods (Ozbilge et al. 2010).

The activity of *Baccharis dracunculifolia*, which is the most important plant source of Brazilian green propolis, against promastigote forms of *L. donovani* was investigated and IC_{50} values of 42 μg/ml were obtained. The extract also displayed high activity in a schistosomicidal assay (Parreira et al. 2010).

The activity of eighteen Cuban propolis extracts collected in different geographic areas were screened against *Leishmania amazonensis* and *Trichomonas vaginalis*. The study observed that all propolis extracts produced an inhibitory effect on intracellular amastigotes of *L. amazonensis*. Only five samples decreased the viability of *T. vaginalis* trophozoites at concentrations lower than 10 μg/ml (Monzote et al. 2011).

Brazilian green propolis was tested against *L. braziliensis* by experimental infection of mice. The results showed an IC_{50} value of 18.1 μg/ml against promastigote forms of *L. brasiliensis*. IC_{50} values were in the range 78–148 μg/ml against the M2904 strain of *L. brasiliensis* and the extract also had antiproliferative activity on *L. brazilensis* promastigotes at 100 μg/ml (Da Silva et al. 2013).

The EEP of Libyan propolis collected from North East Libya was found to be active against *L. donovani*, and four compounds, three diterpenes and a lignan, were isolated. These compounds exhibited moderate to strong activity against *L .donovani*, with IC_{50} values in the range 5.1–21.9 μg/ml (Siheri et al. 2014). These results were replicated in subsequent assays on *L. donovani* involving twelve extracts of Libyan propolis where IC_{50} values ranged from 2.67 to 16.2 μg/ml (Siheri et al. 2016).

The activity of methanolic extracts of ten Bolivian propolis samples was studied against *L. amazonensis* and *L. braziliensis*. The most active samples towards *Leishmania* species had IC_{50} values in the range 78–121 μg/ml against *L. amazonensis* and *L brasiliensis* (Nina et al. 2016).

It was reported that an ethanolic extract of European propolis showed activity against *Toxoplasma gonodi* (De Castro 2001).

The activity of Nigerian propolis was tested against *Plasmodium berghei* using mice experimentally infected with *P berghei*, with chloroquine as a positive control. The propolis significantly reduced the level of parasitemia in treated mice, and there was no significant difference from mice treated with chloroquine (Olayemi 2014).

Propolis extract inhibited the growth of the intestinal parasites *Giardia lamblia*, *Giardia intestinalis* and *Giardia duodenalis*. The extract decreased the growth of trophozoites and the level of inhibition varied according to the extract concentration and incubation times. Significant decreases in parasite growth were detected in

cultures exposed to 125, 250 and 500 µg/ml of propolis, respectively, for all incubation periods (24, 48, 72 and 96 h). Growth reduction of 50% was observed in cultures treated with 125 µg/ml of the extract, and concentrations of 250 and 500 µg/ml were able to inhibit growth by more than 60% (Freitas et al. 2006).

Mice were orally infected with axenically cultivated *Giardia lamblia* trophozoites. The trophozoite count in the intestines, measurements of interferon-gamma serum levels, and histopathological examination of duodenal and jejunal sections were carried out. The results showed that propolis as a prophylaxis resulted in a significant decrease in the intensity of infection. As a treatment, propolis caused a more significant decrease in trophozoite count than that obtained by metronidazole. However, mice treated with propolis alone showed a reversed $CD4^+$: $CD8^+$ T-lymphocyte ratio resulting in a strong immune enhancing effect, which resulted in an adverse increase in inflammatory response at the intestinal level. Combined therapy of metronidazole and propolis was more effective in reducing the parasite count than by each drug alone (Abdel-Fattah and Nada 2007).

Propolis was used as a foliar application or soil drench on fava bean plants. Propolis treatment increased total chlorophyll and carotenoid content and the magnitude of increase was more noticeable after applying a higher concentration (1000 mg/l). It was found that fava bean plants treated with propolis extract, either as a foliar application or soil drench, were able to overcome the inhibitory influence of nematode infection on chlorophyll formation (Noweer and Dawood 2008).

A study was carried out in BALB/c mice to investigate the synergistic effect of the EEP of Egyptian propolis and immunization with *Taenia saginata* crude antigen for the prevention of bovine cysticercosis. After 24 weeks of challenge the mice in G2 (given both EEP and immunisation) showed the highest level of protection (100%) with no cyst being detected as for mice in G1 (which received only immunisation). The latter showed just 33.3% protection. Additionally, the ELISA results in this study showed higher antibody titres in G2, with reduction in the alteration of liver and kidney functions, compared to mice in G1 (Kandil et al. 2015).

There are several papers on the antihelmintic effects of propolis extracts. Propolis inhibited the growth of the helminth parasite *Fasciola gigantica* (Hegazi et al. 2007). In tests against schistosomiasis in mice, a significant reduction in the number of schistosomules of 59.2% was obtained in the group treated with propolis compared to a reduction of 98.9% in the praziquantel treated group (Issa 2007). A study was carried out to evaluate the effect of Egyptian propolis against *Toxocara vitulorum*. Adult worms were incubated for 24 h in several concentrations of EEP (100, 50, 25, 12 and 6 µg/ml) and assessed by light and scanning electron microscopy following 24 h incubation. It was observed that the extract possessed anthelmintic efficacy and the mortality rate was concentration dependent: LC_{25} was 6.9 µg/ml, LC_{50} was 12.5 µg/ml, and LC_{90} was 53.4 µg/ml. The authors thus confirmed the nematodicidal effect of Egyptian propolis (Hassan et al. 2016).

7.3.2.4 Antifungal Properties of Propolis

The activity of an ethanolic extract of Italian propolis was tested against a range of zoophilic fungi and Candida species. The extracts were effective at 5% w/v in inhibiting fungal growth (Cafarchia et al. 1999).

The activity of Brazilian propolis against 80 strains of Candida yeast was studied: 20 strains of *Candida albicans*, 20 strains of *Candida tropicalis*, 20 strains of *Candida krusei*, and 15 strains of *Candida guilliermondii*. The propolis showed clear antifungal activity with the following order of sensitivity: *C. albicans* > *C. tropicalis* > *C. krusei* > *C. guilliermondii*. MICs were in the range 8–12 mg/ml. Patients with full dentures who used a hydroalcoholic propolis extract showed a decrease in the number of Candida in their saliva (Ota et al. 2001). In a further study patients treated with a commercial ethanol extract of propolis showed lesion regression similar to that observed in patients treated with nystatin (Santos et al. 2005).

An alcoholic extract of Brazilian propolis was tested against the fungal isolates *Candida parapsilosis*, C. *tropicalis*, *C. albicans* and other yeast species obtained from onychomycosis lesions. The concentration capable of inhibiting all of the yeasts contained 50 μg/ml flavonoids while 20 μg/ml flavonoids promoted yeast cell-death. Trichosporon sp. were the most sensitive species (Oliveira et al. 2006).

The antifungal activity of propolis ethanol extract (PE) and propolis microparticles (PM) obtained from a sample of Brazilian propolis was tested against vulvovaginal candidiasis (VVC). Yeast isolates obtained from vaginal exudates of patients with VVC were exposed to PE and PM, as well as to conventional drugs used in the treatment of VVC (Fluconazole, Voriconazole, Itraconazole, Ketoconazole, Miconazole and Amphotericin B). Some *Candida albicans* isolates showed resistance or dose-dependent susceptibility for the azole drugs and Amphotericin B. All yeasts were inhibited by PE and PM, with small variations, independent of the species of yeast. While the activity of the azole drugs was much higher than both PE and PM, the extracts inhibited resistant lines in the range 33–1100 to 174–5574 μg/ml, respectively (Dota et al. 2011).

The antifungal activity of propolis extracts from Argentinian propolis was tested against a range of fungi and yeasts. The most susceptible species were *Microsporum gypseum*, *Trichophyton mentagrophytes*, and *Trichophyton rubrum*. All the dermatophytes and yeasts tested were strongly inhibited by different propolis extracts (MICs between 16 and 125 μg/ml). The main bioactive compounds in the extracts were found to be 2′,4′-dihydroxy-3-methoxychalcone and 2′,4′-dihydroxychalcone. Both were highly active against clinical isolates of *T. rubrum* and *T. mentagrophytes* (MICs and MFCs between 1.9 and 2.9 μg/ml) (Agüero et al. 2009).

7.4 Studies of the Protective Effect of Propolis on Bees

The role of propolis in protecting beehives from infection has become of increasing interest. Poplar propolis has been found to be active against various bee pathogens and pests, including *Varroa* mite (Simone-Finstrom and Spivak 2010). It has been observed that bee colonies exposed to *Ascophaera apis* (chalkbrood fungus) increased their foraging for poplar propolis and that increased propolis levels in the hive reduced the intensity of infection (Simone-Finstrom and Spivak 2012).

The sources of propolis in the vicinity of beehives in Minnesota were studied. Despite the availability of a wide range of poplar species, the bees foraged discriminately for resin from *Poplar deltoides* (Eastern Cottonwood) and *Poplar balsamifera* (Balsam Poplar). The compositions of the resins from these trees did not exhibit much seasonal variation in composition. The resins were active against *Paenibacillus larvae* in the range of 50–175 μg/ml (Wilson et al. 2013).

The differences between French bee colonies tolerant to *Varroa destructor* compared with colonies from the same apiary which were non-tolerant to the mites were also studied. The results indicated that non-tolerant colonies collected more poplar propolis than the tolerant ones but the percentage of four compounds, caffeic acid and three pentenyl caffeates, was higher in propolis from tolerant colonies (Popova et al. 2014).

The effect of a propolis envelope on beehive viability was studied by installing propolis traps in the 'treatment' hives. Brood areas were similar between treated and untreated groups but colonies with propolis traps had significantly more worker brood than controls. However, it was not possible to replicate this in a second experiment. Colony survivorship in the treatment group was significantly higher in the first year of the experiment but not replicated in the second year. There were no differences in Varroa or virus levels between treatment and control and only marginal differences in Nosema. The transcription of six key genes involved in the immune response were lower in the treatment group. The protein vitellogenin was higher in treatment bees, indicating improved nutritional status. The presence of the propolis envelope reduced the expression of immunogenic genes and thus reduced the costly investment of energy in the immune system of individual bees (Borba et al. 2015).

7.5 Wound Healing Properties of Propolis

Propolis, along with other honeybee products, has been widely used as an external treatment for wounds and burns. Propolis is believed to possess antimicrobial, antiseptic, anti-inflammatory, antioxidant and immunomodulatory effects (Castaldo and Capasso 2002). These properties probably enhance cell proliferation in the skin and activate remodelling of the skin tissue. The wound healing process is a natural reaction of the body involving a sequence of several biochemical factors and multiple cellular reactions which can be divided into four stages, namely, hemostasis (blood clotting), inflammation, proliferation of new skin tissue, and remodelling of mature tissue (Gurtner et al. 2008). Hemostasis is an initial natural reaction that occurs

immediately in the first few minutes of injury. During this stage, platelets in the blood accumulate at the site of injury where they release chemical signals. These signals cause fibrin, a blood protein, to form a mesh that works as a glue to bind the platelets, leading to the formation of thrombi (blood clots), which seal the injury and control bleeding. Immediately after formation of the blood clot the inflammation process starts and immune cells such as macrophages and inflammatory cells such as neutrophils secrete large amounts of pro-inflammatory cytokines and reactive oxygen species (ROS), respectively. The generated ROS arrive in the wound lesion and act as a defence system against pathogens. However, any excess amount of superoxides may damage the surrounding normal tissue, such as occurs in diabetes. The proliferative phase then occurs two or three days after the injury and involves the movement of epithelial cells until they meet each other from either migration side. This occurs via stimulation of fibroblasts in the dermis layer along with keratinocytes in the epidermis. New blood vessels start to form to provide nutrition and oxygen to repair the injured tissue. Then, proliferative fibroblasts begin to secrete matrix proteins and collagen to build up the extracellular matrix that acts as a connective tissue to form initial dermal granulation tissue. After that, the fibroblasts differentiate into myo-fibroblasts which cause contraction of the wound size due to increases in collagen synthesis relative to the proliferative process. Lastly, the remodelling and maturation phase occurs, which is characterised by scar formation as a result of replacement of type III collagen by type I collagen (Guo and DiPietro 2010; Gurtner et al. 2008). The wound healing process is illustrated in Fig. 7.4.

Some recent studies have confirmed the therapeutic efficacy of propolis in different types of wounds, such as gastric ulcers, surgical wounds, infected wounds and burns (de Barros et al. 2007; Barroso et al. 2012; Martin et al. 2013). In addition, propolis accelerates the healing process in some medical conditions where there is a delay in wound healing, for example in patients of advanced age, or diabetic and immune-suppressed patients (McLennan et al. 2008).

According to several papers, propolis may aid wound healing by activation of all stages involved. Among these, there was a study which investigated the anti-inflammatory activity and wound healing potential of an alcohol extract of green Brazilian propolis. It was reported that the propolis sample could potentially control the processes involved in the early phases of wound healing, including hemostasis and inflammation (Moura et al. 2011). Several studies carried out *in vivo* have reported that propolis activity in the management of burns apparently occurs via regulation of the immune response during the inflammatory phase (Dimov et al. 1992; Mirzoeva and Calder 1996). Another study in rats showed that the skin penetration of a topical propolis ointment formulation on cutaneous wounds depends on the healing stage and mainly stimulates the proliferation phase of wound healing by stimulating the production of keratinocytes (Sehn et al. 2009). Propolis was also reported to be useful during the remodelling and maturation phase in which it stimulated the repair of granulation cells in burnt skin tissue (Olczyk et al. 2013).

A previous study of propolis in an induced diabetic rat model found that propolis showed significant effects in the acceleration of wound healing in diabetes by increasing the re-epithelialization process. The same study demonstrated a decrease in the inflammation phase, mainly by normalizing the physiological count of neutrophil and

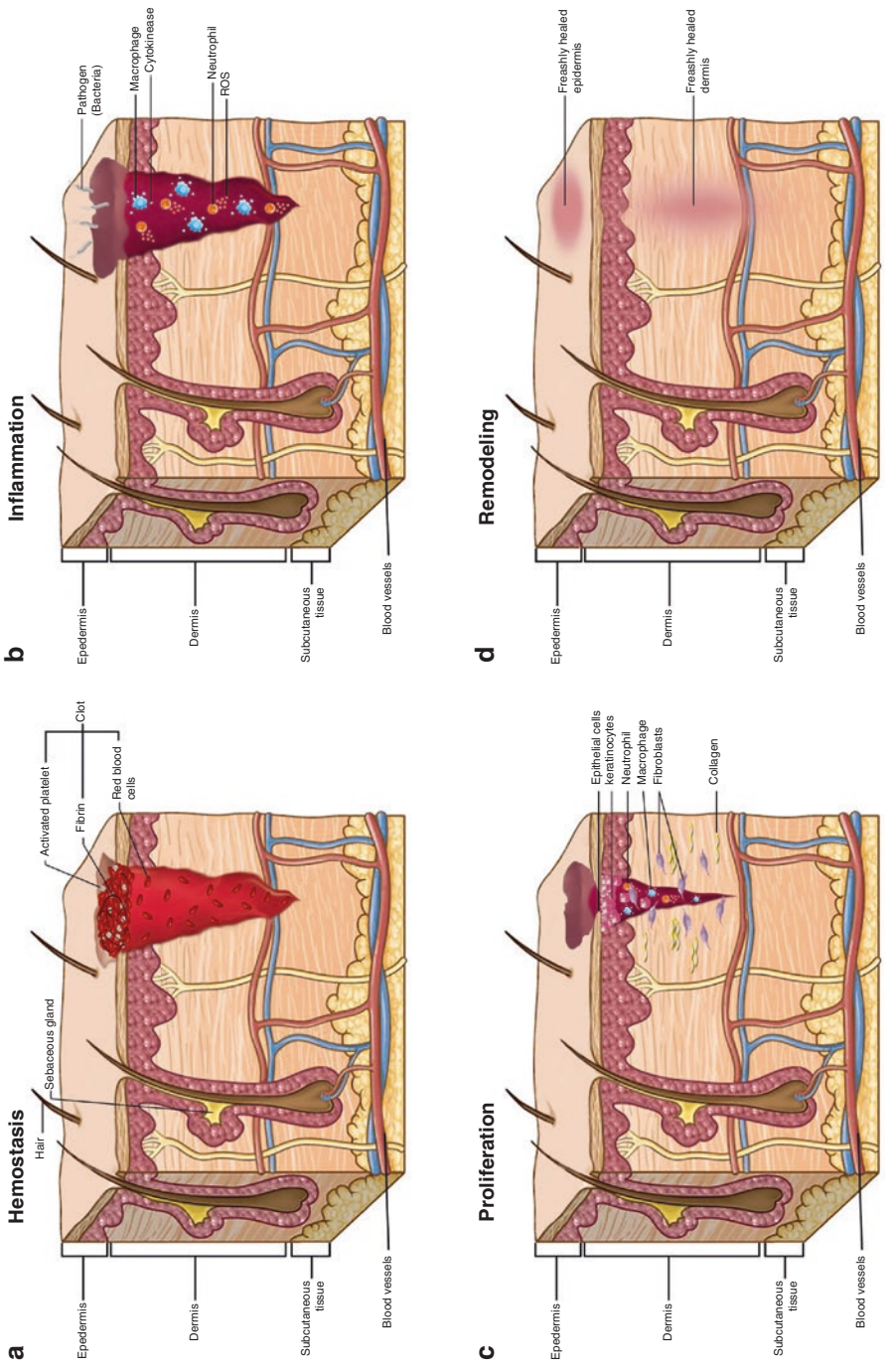


Fig. 7.4 Illustration of the process of wound healing

macrophage influx. As a result, persistent inflammation, which is frequently seen in diabetes, was prevented (McLennan et al. 2008). A recent study on wounds in a diabetic rodent model treated with a topical cream consisting of a mixture of natural products, namely: propolis, honey, royal jelly and olive oil, showed a significant beneficial effect on increasing the rate of wound healing due to antimicrobial, anti-inflammatory, and antioxidant activities (Rashidi et al. 2016).

Many scientific papers have investigated the chemical composition of propolis extracts, which could be responsible for wound healing. The study conducted by Olczyk et al. found that flavonoids have the ability to reduce lipid peroxidation and inhibit cellular necrosis (Olczyk et al. 2013). Moreover, flavonoids present in propolis reported to have antioxidant, anti-inflammatory, immunomodulatory and antimicrobial properties consequently aid in skin tissue repair and accelerate healing during wound treatment (Castaldo and Capasso 2002; Barroso et al. 2012). Another study pointed out that there is a close link between the presence of caffeic acid in propolis samples and the anti-inflammatory process *in vivo* (Rossi et al. 2002). This observation was supported by findings of a study by Song et al. which confirmed potent antioxidant and anti-inflammatory activities of caffeic acid, leading to wound healing in mice (Song et al. 2008).

In clinical trials on patients with similar minor burns, it was found that a propolis cream improved skin tissue healing and decreased wound inflammation more effectively compared to a topical silver sulfadiazine treatment (Gregory et al. 2002). Another clinical study on mouth wounds and dental sockets after tooth extraction found that the topical application of propolis stimulated epithelial cellular repair, but had no significant effect on wound healing in dental sockets (Filho and Carvahlo 1990). Thus, propolis is quite potent in accelerating wound healing because of its broad-spectrum activity encompassing all wound healing phases. Along with other bee products, propolis has multiple modes of action which give it a higher possibility of therapeutic success than drugs with a single mechanism of action.

7.6 Propolis in the Treatment of Diabetes and Cardiovascular Disease

Nitric oxide (NO) is believed to be an effector molecule that induces destruction of pancreatic β-cells resulting in type I diabetes. It has been reported that streptozotocin (SZO) induces destruction of pancreatic β-cells via a free radical mechanism which includes the production of NO. Rats were treated with water or methanol extracts of Brazilian green propolis. Untreated and propolis treated rats were treated with SZO. The rats treated with SZO alone developed elevated levels of glucose, suffered weight loss and had elevated triglyceride levels; propolis treatment inhibited these changes. It was believed that propolis acted via inhibition of nitric oxide synthase and free radical activity (Matsushige et al. 1996).

It was observed that an extract of Brazilian propolis was able to inhibit the postprandial rise in glucose levels in Sprague-Dawley rats. It was observed that the mech-

anism of action of the extract was via inhibition of maltase activity by the propolis extract, thus delaying the release of glucose from starch. The most active constituent in the propolis extract was 3,4,5-tri-caffeoylquinic acid (Matsui et al. 2004).

Type 2 diabetes has reached epidemic proportions in the developed and developing world. The anti-diabetic properties of propolis have been studied by several groups. Most commonly, rat or mouse models have been used. Type 2 diabetes was induced in rats by injection with alloxan and the rats were then treated by intragastric injection of water and ethanol extracts of Chinese propolis. Propolis treatment prevented elevation of glucose levels with time in treated compared to control diabetic animals, although levels remained well above those of normal controls. Also, propolis reduced the levels of oxidative stress and nitric oxide levels in treated compared with control diabetic animals (Fuliang et al. 2005).

SZO induced type I diabetes in rats was treated with a commercial ethanol extract of Brazilian green propolis by oral gavage. Treatment significantly increased body weight and reduced urinary albumin excretion in diabetic animals in comparison to untreated diabetic controls. In addition, treatment also increased levels of glutathione (GSH), superoxide dismutase (SOD) and catalase (CTA) and decreased malonyldialdehyde (MDA) in the renal tissue of treated diabetic animals. This suggested the potential of propolis for treating diabetic nephropathy (Abo-Salem et al. 2009).

Type I diabetes was induced in rats by injection of SZO. In the treated animals there were reductions in animal body weight and significant increases in serum glucose, triglycerides, total cholesterol and low density lipoprotein-cholesterol (LDL-C), and a decrease in serum high density lipoprotein-cholesterol (HDL-C) (51%) as compared to the control normal group. In addition, there was a significant elevation in malondialdehyde (MDA) and a marked reduction in glutathione (GSH), catalase (CAT), and pancreatic superoxide dismutase (SOD) in SZO-treated rats. Oral treatment of animals with a commercial ethanol extract of Brazilian green propolis showed reduced weight loss and alterations in glucose, lipids, lipoproteins, NO, GSH, CAT, pancreatic MDA, and SOD levels (El-Sayed et al. 2009).

The hypolipidemic effect of propolis in a mouse obesity model induced by a high fat-diet was observed in C57BL/6N mice fed a high-fat diet and given Brazilian propolis extract intragastrically. Propolis inhibited weight gain and the formation of visceral adipose tissue. In addition, propolis reduced the levels of free fatty acids and glucose in serum, and triglycerides in liver and serum. Realtime PCR results suggested that the anti-obesity effects of the propolis extract could be attributed to reduced expression of fatty acid synthesis genes in the liver (Koya-Miyata et al. 2009).

An extract of Brazilian red propolis was found to promote the differentiation of pre-adipocytes into adipocytes *in vitro* via its activity on peroxisome proliferator-activated receptor γ (PPAR γ). Several genes associated with adipocyte formation were upregulated, thus providing a potential mechanism for the observed effects of propolis in promoting lipid metabolism (Iio et al. 2010).

Diabetes was induced in rats by a single dose of SZO (35 mg/kg). Rats with a high level of glycaemia were treated with an extract of Brazilian green propolis. Treatment over seven days had no effect in reducing blood glucose or free fatty acid

levels, or in reducing weight loss (Sartori et al. 2009). This contrasts with other findings. However, in other studies longer term administration of SZO was carried out, thus inducing more severe type I diabetes. In another study, type I diabetes was induced in rats treated with a single dose of SZO (60 mg/kg) and propolis was found to reduce blood glucose and increase insulin levels in treated diabetic rats. Animals treated with propolis had a reduced level of thioabarbituric acid reactive substances in their serum. There was no effect of propolis treatment on calcitonin or parathyroid hormone levels (Al-Hariri et al. 2011).

The effect of propolis on SZO induced type I diabetes in rats was studied. Chinese and Brazilian propolis extracts (10 mg/ml) were administered orally. Propolis was found to inhibit weight loss in diabetic rats and to reduce blood glucose levels. There was some reduction in glycated haemoglobin levels and oxidative stress was reduced in propolis treated rats in comparison to untreated controls (Zhu et al. 2011).

Type 2 diabetes was induced in a rat model by a combination of a high fat diet and injection of low dose SZO. Animals were treated with Chinese propolis encapsulated in cyclodextrin by spray drying and the propolis was administered via gavage. The propolis was able to decrease blood glucose levels, improve lipid profiles, and improve insulin sensitivity in the treated animals in comparison to untreated diabetic controls (Li et al. 2011).

Type I diabetes was induced in mice by treatment with alloxan. The mice were treated by intra-peritoneal administration of extracts from Croatian propolis. The lifespans of the propolis treated mice were significantly increased in comparison to untreated controls and they also gained weight in comparison with controls. The propolis treatment reduced the degree of cellular vacuolisation in the livers of treated mice due to protection against reactive oxygen species and improved fatty acid metabolism. The treated mice had less infiltration of lymphocytes and eosinophils into their kidney tissues (Oršolić et al. 2012).

The ob/ob mice are genetically obese and serve as a model of type 2 diabetes. An extract of Brazilian green propolis was administered via intra-peritoneal injection. Treatment had no effect on body weight or food intake but blood glucose and plasma cholesterol levels were lowered in comparison to untreated mice, and glucose tolerance and insulin sensitivity were improved. The propolis-treated mice showed lower weight gain in mesenteric adipose tissue. It was proposed that Brazilian propolis improved diabetes in *ob*/*ob* mice through modulation of immune cells in mesenteric adipose tissues (Kitamura et al. 2013).

The effect of oral administration of propolis on Otsuka Long-Evans Tokushima Fatty rats, which have the symptoms of type 2 diabetes, was studied. Glucose and insulin levels and systolic blood pressure were all lowered in the treated rats compared to controls after eight weeks of treatment. Interstitial fluid pH was higher in ascites, liver, and skeletal muscle after propolis treatment, suggesting that the effects of propolis might be mediated via suppression of metabolic acidosis (Aoi et al. 2013).

BALB/c mice were treated with S961 peptide, an antagonist of the insulin receptor, in order to induce type 2 diabetes. The mice were treated orally with an ethanolic extract of Indonesian propolis. Propolis treatment was found to decrease

blood glucose levels and levels of interferon-γ via a reduction in the level of activated T-cells, thus potentially reducing T-cell mediated damage (Rifa'i and Widodo 2014).

The effect of Brazilian green propolis, and four flavonoids isolated from it, on systolic blood pressure in spontaneously hypertensive rats was observed. The four flavonoids all lowered systolic blood pressure after 28 days with isosakuranetin being the most potent. In addition, the four flavonoids and various fractions of Brazilian green propolis were found to relax aorta from spontaneously hypertensive rats in a concentration dependent manner (Maruyama et al. 2009).

The effect of Brazilian propolis on the development of atherosclerosis in rabbits fed high levels of cholesterol was also studied. The cholesterol-enriched diet promoted increases in serum total cholesterol (TC), triglycerides, low density lipoprotein cholesterol (LDLC), concentrations of thiobarbituric acid-reactive substances (TBARS), and a decrease in high density lipoprotein-cholesterol (HDLC) and glutathione (GSH) levels compared to the control group. Administration of propolis reduced levels of TC, LDLC, triglycerides and TBARS, while increasing HDLC and GSH. Propolis lowered the levels of endothelial damage and thickened foam cells in aorta and reversed the damage to the kidneys induced by the high cholesterol diet as observed by histopathology (Nader et al. 2010).

7.7 Anti-cancer Effects of Propolis

The anti-cancer effects of propolis have been widely studied in *in vitro* and animal models. There is an overlap between anti-cancer effects and the immunomodulatory effects of propolis, which largely seems to exert its anti-cancer activity via immunomodulation. The anticancer properties of propolis have been recently reviewed (Watanabe et al. 2011).

A methanol extract of Netherlands propolis had antiproliferative activity towards murine colon 26-L5 carcinoma with an EC_{50} value of 3.5 mg/ml. Fractionation of the extract resulted in the isolation of four flavonoids, seven cinnamic acid derivatives and glycerol derivatives. The isolated compounds were tested for their antiproliferative activity. The three most active had EC_{50} values of 0.288, 1.76 and 0.114 μM towards colon 26-L5 carcinoma (Banskota et al. 2002).

Polyphenolic compounds isolated from propolis and a water soluble extract of Croatian propolis were investigated for their effects on the growth and metastatic potential of mammary carcinoma in mice. Metastases in the lung were generated by intravenous injection of tumour cells. Oral dosing of the compounds and extract (50 mg/kg) significantly decreased the number of tumour nodules in the lung (Oršolić et al. 2004).

The effect of propolis in combination with paclitaxel against induced experimental breast cancer was investigated in female Sprague Dawley rats. Administration of paclitaxel and propolis effectively suppressed breast cancer, which was revealed by a decrease in the extent of lipid peroxidation with an increase in the activities of

superoxide dismutase, catalase and glutathione peroxidase, and the levels of glutathione, Vitamin C and Vitamin E when compared to animals treated with either paclitaxel or propolis alone. Thus, it was proposed that co-administration could reduce the side-effects of paclitaxel (Padmavathi et al. 2006).

The effects of ethanolic extracts of two samples of Brazilian propolis and the bud resin of *B. dracunculifolia* on the proliferation of human prostate cancer cells was studied. The Brazilian propolis extracts showed significant inhibitory effects on the proliferation of human prostate cancer cells. Inhibition was achieved through regulation of protein expression of cyclins D1 and B1, and cyclin dependent kinase, as well as expression of the p21 protein (Paredes-Guzman et al. 2007).

An ethanolic extract of green Brazilian propolis (EEBP) significantly reduced the number of newly formed vessels in an *in vivo* angiogenesis assay. The ethanolic extract of propolis (EP) also showed antiangiogenic effects in an *in vitro* tube formation assay. The major constituent of EP, artepillin C, was found to significantly reduce the number of newly formed vessels in an *in vivo* angiogenesis assay. Thus, artepillin C, at least in part, is responsible for the antiangiogenic activity of EEBP *in vivo* and might prove effective in counteracting tumour angiogenesis (Ahn et al. 2007).

The prenylated flavanoid propolin G, isolated from Taiwanese propolis, was able to efficiently induce apoptosis in brain cancer cell lines, glioma and glioblastoma, with IC_{50} values of 5 and 7.5 μg/ml, respectively, against the two cell lines. The results suggested that apoptosis might have occurred through a mitochondrial- and caspase-dependent pathway. Propolin G and Taiwanese and Brazilian propolis extracts were also found to protect against oxidative stress in rat primary cortical neurons (Huang et al. 2007).

The activity of extracts of three Mexican propolis samples against human colonic, human lung, and cervical cancer cells was examined. The extracts exhibited strong anti-proliferative activities and analysis of DNA isolated from treated cells showed the presence of intra-nucleosomal DNA cleavage (Hernandez et al. 2007).

An extract of propolis was found to inhibit the proliferation of U937 cells in a dose-dependent manner by inducing apoptosis and blocking cell cycle progression in the G2/M phase. Western blot analysis showed that propolis increased the expression of p21 and p27 proteins, and decreased the levels of cyclin B1, cyclin A, Cdk2 and Cdc2, causing cell cycle arrest. The results suggested that propolis-induced apoptosis was related to the selective activation of caspase-3 and induction of Bcl-2/Bax regulation (Motomura et al. 2008).

A methanol extract of red Brazilian propolis was found to kill 100% of nutrient starved human pancreatic cancer cells at a concentration of 10 μg/mL. Forty three compounds were isolated from the extract with the most potent compound producing 100% kill at a concentration of 12.5 μM. The mechanism of cell death was believed to be via necrosis. The targeting of nutrient deprived cells presents a method of targeting cells which are resistant to nutrient starvation (Li et al. 2008).

A methanolic extract of propolis from Myanmar inhibited the growth of human pancreatic cancer cells preferentially under nutrient-deprived conditions (NDM), with a PC_{50} value of 9.3 μg/mL. Two new cycloartane-type triterpenes, 13 cycloartanes, and four known prenylated flavanones were isolated from the extract. One of

the newly identified triterpene acids exhibited the most potent preferential cytotoxicity (PC_{50} 4.3 μM) in a concentration- and time-dependent manner and induced apoptosis-like morphological changes of PANC-1 cells within 24 h of treatment (Li et al. 2010).

The anticancer effect of ethanol extracts of Chinese and Brazilian propolis on four human colon carcinoma cell-lines was studied. The extracts of both Chinese and Brazilian propolis caused a marked dose-dependent growth inhibition of the cells, with IC_{50} values in the range 4–41 μg/ml (Ishihara et al. 2009).

The effect of propolis on the production of IL-2, IF-γ, IL-4 and IL-10 cytokines by T-helper cells in melanoma-bearing mice submitted to immobilization stress was studied. Spleen cells were assessed for cytokine expression. Stress induced a higher rate of tumour growth, while propolis-treated mice, stressed or not, showed melanoma development similar to the control. Cytokine production was inhibited in melanoma-bearing mice and propolis administration to melanoma-bearing mice submitted to stress promoted IL-2 and IFN-γ production, indicating the activation of antitumor cell-mediated immunity. Propolis also stimulated IL-10 expression and production (Missima et al. 2010).

Extracts from two Portuguese propolis samples were tested for their anticancer properties on human renal cell carcinoma cells, with IC_{50} values of 56.5 and 56.1 μg/ml. The extracts were much less toxic against normal human renal cells (Valente et al. 2011).

Tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) is an important endogenous anti-cancer cytokine that induces apoptosis selectively in tumour cells. However, some cancer cells are resistant to TRAIL-mediated apoptosis. Phenolic and polyphenolic compounds sensitize TRAIL-resistant cancer cells and augment the apoptotic activity of TRAIL. The cytotoxic and apoptotic effects of an ethanolic extract of Brazilian green propolis (EEP) was examined against LNCaP prostate cancer cells. The extract sensitised TRAIL-resistant prostate cancer cells to TRAIL-mediated apoptosis, and when prostate cancer cells were co-treated with 100 ng/ml TRAIL and 50 μg/ml EEP, the percentage of apoptotic cells was increased to 65.8%—several fold greater than the level induced by TRAIL alone (Szliszka et al. 2011a, b).

An ethanolic extract of stingless bee propolis from Thailand was tested for antiproliferative activity against five cancer cell lines and two normal cell lines. The cell viability (%) and IC_{50} values were calculated. The hexane extract provided the highest *in vitro* activity against the five cancer cell lines and the lowest cytotoxicity against the two normal cell lines. Fractionation of the extract produced a fraction with high antiproliferative activity with IC_{50} values in the range 4.09–14.7 μg/ml against cancer cells but with limited activity against normal cells (Umthong et al. 2011).

PC-3 prostate cancer cells were incubated with dimethyl sulfoxide and water extracts of Turkish propolis and the treatments were found to significantly reduce cell viability to 24.5% and 17.7%, respectively. Statistically significant discriminatory peaks in the proteomic profiles were observed between control PC-3 cells and those treated with the dimethyl sulfoxide extract of propolis. Surface enhanced laser desorption ionization time of flight mass spectrometry was used to examine changes in the proteome as a result of the propolis treatments and it was found that

the treatment promoted changes in the proteome, suggesting that this might be a mechanism for the cytotoxic action (Barlak et al. 2011).

Extracts of *Apis mellifera* propolis collected in Thailand were assayed for cytotoxic activity against five human cancer cell lines and a control cell line. Crude hexane and dichloromethane extracts of propolis displayed anti-proliferative/cytotoxic activities with IC_{50} values across the five cancer cell lines ranging from 41.3 to 52.4 μg/ml and from 43.8 to 53.5 μg/ml, respectively. Two main bioactive components, a cardanol and a cardol, were isolated and IC_{50} values across the five cancer cell lines ranging from 10.8 to 29.3 μg/ml were obtained for the cardanol and <3.13 to 5.97 μg/ml for the cardol. Both compounds induced cytotoxicity and cell death without DNA fragmentation in the cancer cells, but only an anti-proliferation response in the control Hs27 cells (Teerasripreecha et al. 2012).

An ethanolic extract of Brazilian red propolis was found to significantly reduce the viability of MCF-7 breast cancer cells through the induction of mitochondrial dysfunction, caspase-3 activity, and DNA fragmentation, but did not affect these factors in a control cell line. In addition the extract was found to promote apoptosis via endoplasmic reticulum stress (Kamiya et al. 2012).

7.8 Immunomodulatory Effects of Propolis

The immunological effects of propolis have been recently reviewed (Sforcin 2007).

A water soluble extract of propolis (WSP) was used to modulate the alternative complement activation pathway (AP) in mice. The extract was administered via the oral, intravenous, and intraperitoneal routes. The most significant effect was obtained from intraperitoneal administration which inhibited the AP and also caused a moderate fall in zymosan-induced paw odema (Ivanovska et al. 1995). In a related study, the WSP was found to inhibit the complement pathway (CP) to a greater extent than AP in human serum, whereas in mice the reverse was the case (Ivanovska et al. 1995).

Paracoccidoides brasiliensis affects individuals living in endemic regions, such as certain areas in Brazil, through inhalation of airborne conidia or mycelial fragments. Peritoneal macrophages isolated from BALB/c mice were stimulated with Brazilian or Bulgarian propolis and subsequently challenged with *P. brasiliensis*. There was an increase in the fungicidal activity of macrophages treated with either propolis extract (Murad et al. 2002).

Macrophages were pre-stimulated with extracts of Brazilian or Bulgarian propolis and subsequently challenged with *Salmonella typhimurium* at different macrophage/bacteria ratios.

To assess the bactericidal activity, the number of colony-forming units of *S. typhimurium* after 60 min was counted. Propolis from Brazil and Bulgaria enhanced the bactericidal activity of macrophages, depending on its concentration, with Brazilian propolis being more effective than that from Bulgaria (Orsi et al. 2005).

The effect of intraperitoneal or dietary administration of propolis on innate immune response of gilthead seabream was evaluated. Fish were intraperitoneally injected with 5 mg water (WEP), ethanol (EEP) or both WEP and EEP extracts of propolis. Humoral (alternative complement activity and peroxidase content) and cellular (leucocyte peroxidase, phagocytosis, cytotoxicity and respiratory burst activity) immune responses were evaluated in both cases. The results suggested that propolis had limited immunostimulatory effects although intraperitoneal administration was more effective than dietary intake (Cuesta et al. 2005).

The adjuvant capacity of an ethanol extract of green propolis associated with inactivated Suid herpesvirus type 1 (SuHV-1) vaccine preparations was tested. Mice inoculated with the SuHV-1 vaccine plus aluminium hydroxide plus 5 mg propolis extract produced higher levels of antibodies when compared to animals that received the vaccine plus aluminium hydroxide without propolis. The SuHV-1 vaccine with propolis extract alone did not induce a significant increase in antibodies, however, in this case the cellular immune response increased based on an increase in the expression of mRNA for IFN-γ. Using propolis as an adjuvant increased the percentage of animals surviving challenge with a lethal dose of SuHV-1 (Fischer et al. 2007).

The interaction between stress and immunity has been widely investigated and has been found to involve the neuroendocrine system and several organs. The effect of propolis on activated macrophages in BALB/c mice submitted to immobilization stress was investigated and histopathological analysis of the thymus, bone marrow, spleen and adrenal glands was also carried out. Stressed mice showed higher hydrogen peroxide generation by peritoneal macrophages, and propolis treatment potentiated hydrogen peroxide generation and inhibited nitric oxide (NO) production by these cells. Histopathological analysis showed no alterations in the thymus, bone marrow and adrenal glands, but increased germinal centers in the spleen. Propolis treatment counteracted the alterations found in the spleen of stressed mice (Missima and Sforcin 2008).

The effect of Turkish propolis samples on peripheral blood mononuclear cells (PBMC) was studied. All propolis samples decreased mitogen-induced neopterin release and tryptophan degradation in both stimulated and non-stimulated PBMC. In addition, TNF-α and IFN-γ release in stimulated PBMC was inhibited by propolis extracts (Girgin et al. 2009).

The effect of oral propolis administration in mice on IL-2, IFN-γ, IL-4 and IL-10 production by T-helper 1 (Th-1) and T-helper 2 (Th-2) cells was studied. Propolis administration to mice did not affect IL-2, IL-4 and IL-10 expression and production, but IFN-γ production was inhibited in the splenocyte cultures, when both unstimulated or stimulated by conclavin A. These findings support the proposed anti-inflammatory effects of propolis (Orsatti et al. 2010a, b).

The antimicrobial activity of Brazilian stingless bee (*Melipona fasciculata*) geopropolis against oral pathogens and its effects on *Streptoccus mutans* biofilms was studied. Activity was observed against *S. mutans* and *Candida albicans* but not against *Lactobacillus acidophilus*. The propolis extract increased the levels of anti-inflammatory cytokines IL-4 and IL-10 in the blood of mice which were treated in their oral cavity with a gel containing propolis (Liberio et al. 2011).

The effect of propolis on Toll-like receptor (TLR-2 and TLR-4) expression and on the production of pro-inflammatory cytokines (IL-1β and IL-6) was evaluated. Male BALB/c mice were treated with propolis (200 mg/kg) by oral gavage for three consecutive days. TLR-2 and TLR-4 expression was increased in the peritoneal macrophages of the propolis-treated mice. TLR-2 and TLR-4 expression and IL-1β and IL-6 production were also upregulated in the spleen cells of propolis-treated mice. This suggests that propolis activates the initial steps of the immune response by upregulating TLR expression and by the production of pro-inflammatory cytokines in mice (Orsatti et al. 2010a, b).

The effect of propolis on chronically stressed mice was assessed by evaluating TLR-2 and TLR-4 expression by spleen cells, and corticosterone levels. Propolis administration to the stressed mice prevented inhibition of TLR-2 and TLR-4 expression. No effect was seen on the corticosterone levels among the groups. It was concluded that propolis exerted an immunomodulatory action in chronically stressed mice by upregulating TLR-2 and TLR-4 mRNA expression, thus supporting the immune system (Orsatti and Sforcin 2012).

An extract of Brazilian green propolis, given orally at a dose of 5 mg/kg, was shown to inhibit the production of pro-inflammatory cytokines and promote the production of anti-inflammatory cytokines in mice challenged either with a cotton pellet granuloma implanted in subcutaneous tissue or by intranasal administration of lipopolysaccharide (Machado et al. 2012).

Cinnamic acid (CA) is a major component of propolis. The effects of CA were evaluated on human monocytes by assessing the expression of Toll-like receptors HLA-DR and CD80. Cytokine production (TNF-α and IL-10) and the fungicidal activity of monocytes were also evaluated. CA was found to downregulate TLR-2, HLA-DR, and CD80 and upregulate TLR-4 expression by human monocytes. High concentrations of CA inhibited both TNF-α and IL-10 production, and induced a higher fungicidal activity against *Candida albicans*. TNF-α and IL-10 production was decreased by blocking TLR-4, while the fungicidal activity of monocytes was not affected by blocking TLRs (Conti et al. 2013).

The antileishmanial and immunomodulatory effects of Brazilian propolis were evaluated in an experimental *Leishmania braziliensis* infection. In the immunomodulatory assay, macrophages were pre-treated with propolis and then infected with *L. braziliensis in vivo*. Supernatants from liver cells and peritoneal exudates of BALB/c mice were pretreated with propolis and infected with *Leishmania* promastigotes, and TNF-α and IL-12 were measured. Macrophages incubated with propolis showed a significant increase in interiorization and greater killing of parasites. Increased TNF-α production was seen in mice pretreated with propolis, whereas IL-12 was downregulated during the infection. Brazilian propolis showed direct action on the parasite and displayed immunomodulatory effects on macrophages (Da Silva et al. 2013).

The expression of TLR-2, TLR-4, human leukocyte antigen-DR and cluster of differentiation (CD) 80 by human monocytes was assessed following incubation with Brazilian propolis. In addition, production of TNF-α and IL-10 was measured. Propolis was found to upregulate TLR-4 and CD80 expression, and inhibited TNF-α and IL-10 production at 100 μg/ml, but stimulated their production at lower concentrations. The propolis also increased the fungicidal activity of monocytes. Propolis did not affect cell viability (Bufalo et al. 2014).

References

Abdel-Fattah N, Nada O (2007) Effect of propolis versus metronidazole and their combined use in treatment of acute experimental giardiasis. J Egypt Soc Parasitol 37(2 Suppl):691–710

Abo-Salem OM, El-Edel RH, Harisa G, El-Halawany N, Ghonaim MM (2009) Experimental diabetic nephropathy can be prevented by propolis: effect on metabolic disturbances and renal oxidative parameters. Pak J Pharm Sci 22(2):205–210

Agüero MB, Gonzalez M, Lima B, Svetaz L, Sánchez M, Zacchino S, Feresin GE, Schmeda-Hirschmann G, Palermo J, Wunderlin D (2009) Argentinean propolis from Zuccagnia punctata Cav.(Caesalpinieae) exudates: phytochemical characterization and antifungal activity. J Agric Food Chem 58:194–201

Ahn M-R, Kunimasa K, Ohta T, Kumazawa S, Kamihira M, Kaji K, Uto Y, Hori H, Nagasawa H, Nakayama T (2007) Suppression of tumor-induced angiogenesis by Brazilian propolis: major component artepillin C inhibits in vitro tube formation and endothelial cell proliferation. Cancer Lett 252:235–243

Alday-Provencio S, Diaz G, Rascon L, Quintero J, Alday E, Robles-Zepeda R, Garibay-Escobar A, Astiazaran H, Hernandez J, Velazquez C (2015) Sonoran propolis and some of its chemical constituents inhibit in vitro growth of Giardia lamblia trophozoites. Planta Med 81:742–747

Al-Hariri M, Eldin TG, Abu-Hozaifa B, Elnour A (2011) Glycemic control and anti-osteopathic effect of propolis in diabetic rats. Diabetes Metab Syndr Obes 4:377–384

Almutairi S, Edrada-Ebel R, Fearnley J, Igoli JO, Alotaibi W, Clements CJ, Gray AI, Watson DG (2014a) Isolation of diterpenes and flavonoids from a new type of propolis from Saudi Arabia. Phytochem Lett 10:160–163

Almutairi S, Eapen B, Chundi SM, Akhalil A, Siheri W, Clements C, Fearnley J, Watson DG, Edrada-Ebel R (2014b) New anti-trypanosomal active prenylated compounds from African propolis. Phytochem Lett 10:35–39

Amarante MK, Watanabe MAE, Conchon-Costa I, Fiori LL, Oda JMM, Búfalo MC, Sforcin JM (2012) The effect of propolis on CCL5 and IFN-γ expression by peripheral blood mononuclear cells from leishmaniasis patients. J Pharm Pharmacol 64:154–160

Amoros M, Sauvager F, Girre L, Cormier M (1992a) In vitro antiviral activity of propolis. Apidologie 23:231–240

Amoros M, Simõs C, Girre L, Sauvager F, Cormier M (1992b) Synergistic effect of flavones and flavonols against herpes simplex virus type 1 in cell culture. Comparison with the antiviral activity of propolis. J Nat Prod 55:1732–1740

Aoi W, Hosogi S, Niisato N, Yokoyama N, Hayata H, Miyazaki H, Kusuzaki K, Fukuda T, Fukui M, Nakamura N (2013) Improvement of insulin resistance, blood pressure and interstitial pH in early developmental stage of insulin resistance in OLETF rats by intake of propolis extracts. Biochem Biophys Res Commun 432:650–653

Bankova V (2005a) Chemical diversity of propolis and the problem of standardization. J Ethnopharmacol 100(1):114–117

Bankova V (2005b) Recent trends and important developments in propolis research. Evid Based Complement Alternat Med 2:29–32

Bankova V, Popova M, Trusheva B (2007) Plant origin of propolis: latest developments and importance for research and medicinal use. In: Marghitas LA, Dezmirean D (eds) Apicultura-De la stiinta la agribusiness si apiterapie. Editura Academic Press, Cluj Napoca, pp 40–46

Bankova V, Popova M, Trusheva B (2014) Propolis volatile compounds: chemical diversity and biological activity: a review. Chem Cent J 8:1

Bankova V, Popova M, Trusheva B (2016) New emerging fields of application of propolis. Maced J Chem Chem Eng 35:1–11

Banskota AH, Tezuka Y, Prasain JK, Matsushige K, Saiki I, Kadota S (1998) Chemical constituents of Brazilian propolis and their cytotoxic activities. J Nat Prod 61:896–900

Banskota AH, Tezuka Y, Adnyana IK, Midorikawa K, Matsushige K, Message D, Huertas AA, Kadota S (2000) Cytotoxic, hepatoprotective and free radical scavenging effects of propolis from Brazil, Peru, the Netherlands and China. J Ethnopharmacol 72:239–246

Banskota A, Tezuka Y, Adnyana I, Ishii E, Midorikawa K, Matsushige K, Kadota S (2001a) Hepatoprotective and anti-Helicobacter pylori activities of constituents from Brazilian propolis. Phytomedicine 8:16–23

Banskota AH, Tezuka Y, Kadota S (2001b) Recent progress in pharmacological research of propolis. Phytother Res 15:561–571

Banskota AH, Nagaoka T, Sumioka LY, Tezuka Y, Awale S, Midorikawa K, Matsushige K, Kadota S (2002) Antiproliferative activity of the Netherlands propolis and its active principles in cancer cell lines. J Ethnopharmacol 80:67–73

Barlak Y, Değer O, Çolak M, Karataylı SC, Bozdayı AM, Yücesan F (2011) Effect of Turkish propolis extracts on proteome of prostate cancer cell line. Proteome Sci 9:1

de Barros MP, Sousa JPB, Bastos JK, de Andrade SF (2007) Effect of Brazilian green propolis on experimental gastric ulcers in rats. J Ethnopharmacol 110:567–571

Barroso PR, Lopes-Rocha R, Pereira EMF, Marinho SA, de Miranda JL, Lima NL, Verli FD (2012) Effect of propolis on mast cells in wound healing. Inflammopharmacology 20:289–294

Basim E, Basim H, Özcan M (2006) Antibacterial activities of Turkish pollen and propolis extracts against plant bacterial pathogens. J Food Eng 77:992–996

Bittencourt ML, Ribeiro PR, Franco RL, Hilhorst HW, de Castro RD, Fernandez LG (2015) Metabolite profiling, antioxidant and antibacterial activities of Brazilian propolis: use of correlation and multivariate analyses to identify potential bioactive compounds. Food Res Int 76:449–457

Bogdanov S (2012) Propolis: composition, health, medicine: a review. Bee Product Science, www bee-hexagon net

Bogdanov S, Bankova V (2012) The Propolis Book. Bee hexagon

Bonvehí JS, Coll FV (1994) The composition, active components and bacteriostatic activity of propolis in dietetics. J Am Oil Chem Soc 71:529–532

Borba RS, Klyczek KK, Mogen KL, Spivak M (2015) Seasonal benefits of a natural propolis envelope to honey bee immunity and colony health. J Exp Biol 218:3689–3699

Boukraâ L, Abdellah F, Ait-Abderrahim L (2013) Antimicrobial properties of bee products and medicinal plants

Bufalo MC, Bordon-Graciani AP, Conti BJ, Assis Golim M, Sforcin JM (2014) The immunomodulatory effect of propolis on receptors expression, cytokine production and fungicidal activity of human monocytes. J Pharm Pharmacol 66:1497–1504

Burdock GA (1998) Review of the biological properties and toxicity of bee propolis (propolis). Food Chem Toxicol 36:347–363

Cafarchia C, De Laurentis N, Milillo M, Losacco V, Puccini V (1999) Antifungal activity of Apulia region propolis. Parassitologia 41:587–590

Callejo A, Armentia A, Lombardero M, Asensio T (2001) Propolis, a new bee-related allergen. Allergy 56:579–579

Castaldo S, Capasso F (2002) Propolis, an old remedy used in modern medicine. Fitoterapia 73:S1–S6

Chandna P, Adlakha VK, Das S, Singh S (2014) Complementary and Alternative Medicine (CAM): a review of propolis in dentistry. Technology 4:6

Coelho GR, Mendonça RZ, Vilar KS, Figueiredo CA, Badari JC, Taniwaki N, Namiyama Oliveira MI, Curti SP, Evelyn Silva P (2015) Antiviral action of hydromethanolic extract of geopropolis from Scaptotrigona postica against antiherpes simplex virus (HSV-1). Evid Based Complement Alternat Med 2015:296086

Conti BJ, Búfalo MC, Golim MA, Bankova V, Sforcin JM (2013) Cinnamic acid is partially involved in propolis immunomodulatory action on human monocytes. Evid Based Complement Alternat Med 2013:109864

Croft SL, Barrett MP, Urbina JA (2005) Chemotherapy of trypanosomiases and leishmaniasis. Trends Parasitol 21:508–512

Croft SL, Seifert K, Yardley V (2006) Current scenario of drug development for leishmaniasis. Indian J Med Res 123:399

Cuesta A, Rodríguez A, Esteban MA, Meseguer J (2005) In vivo effects of propolis, a honeybee product, on gilthead seabream innate immune responses. Fish Shellfish Immunol 18:71–80

Da Silva SS, Thomé GS, Cataneo AHD, Miranda MM, Felipe I, CGTdJ A, MAE W, Piana GM, Sforcin JM, Pavanelli WR (2013) Brazilian propolis antileishmanial and immunomodulatory effects. Evid Based Complement Alternat Med 2013:673058

Dantas AP, Olivieri BP, Gomes FHM, De Castro SL (2006a) Treatment of Trypanosoma cruzi-infected mice with propolis promotes changes in the immune response. J Ethnopharmacol 103:187–193

Dantas AP, Salomão K, Barbosa HS, De Castro SL (2006b) The effect of Bulgarian propolis against Trypanosoma cruzi and during its interaction with host cells. Mem Inst Oswaldo Cruz 101(2):207–211

Darendelioglu E, Aykutoglu G, Tartik M, Baydas G (2016) Turkish propolis protects human endothelial cells in vitro from homocysteine-induced apoptosis. Acta Histochem 118:369–376

De Castro S (2001) Propolis: biological and pharmacological activities. Therapeutic uses of this bee-product. Annu Rev Biomed Sci 3:49–83

De Castro S, Higashi K (1995) Effect of different formulations of propolis on mice infected with Trypanosoma cruzi. J Ethnopharmacol 46:55–58

Deans S, Simpson E, Noble R, MacPherson A, Penzes L (1992) Natural antioxidants from Thymus vulgaris (thyme) volatile oil: the beneficial effects upon mammalian lipid metabolism. Acta Hortic 332:177–182

Dimov V, Ivanovska N, Bankova V, Popov S (1992) Immunomodulatory action of propolis: IV. Prophylactic activity against gram-negative infections and adjuvant effect of the water-soluble derivative. Vaccine 10:817–823

Dobrowolski JW, Vohora S, Sharma K, Shah SA, Naqvi S, Dandiya P (1991) Antibacterial, antifungal, antiamoebic, antiinflammatory and antipyretic studies on propolis bee products. J Ethnopharmacol 35:77–82

Dota KFD, Consolaro MEL, Svidzinski TIE, Bruschi ML (2011) Antifungal activity of Brazilian propolis microparticles against yeasts isolated from Vulvovaginal candidiasis. Evid Based Complement Alternat Med 2011:201953

Drago L, De Vecchi E, Nicola L, Gismondo M (2007) In vitro antimicrobial activity of a novel propolis formulation (Actichelated propolis). J Appl Microbiol 103:1914–1921

Duran G, Duran N, Culha G, Ozcan B, Oztas H, Ozer B (2008) In vitro antileishmanial activity of Adana propolis samples on Leishmania tropica: a preliminary study. Parasitol Res 102:1217–1225

El Hady FKA, Hegazi AG (2002) Egyptian propolis: 2. Chemical composition, antiviral and antimicrobial activities of East Nile Delta propolis. Z Naturf C 57(3–4):386–394

El-Sayed e-S, Abo-Salem OM, Aly HA, Mansour AM (2009) Potential antidiabetic and hypolipidemic effects of propolis extract in streptozotocin-induced diabetic rats. Pak J Pharm Sci 22:168–174

Fadaly H, El-Badrawy EEY (2001) Flavonoids of propolis and their antibacterial activities. Pakistan J Biol Sci 21:204–207

Falcão SI, Vale N, Cos P, Gomes P, Freire C, Maes L, Vilas-Boas M (2013) In vitro evaluation of portuguese propolis and floral sources for antiprotozoal, antibacterial and antifungal activity. Phytother Res 28:437–443

Filho OM, Carvahlo ACPD (1990) Application of propolis to dental sockets and skin wounds. J Nihon Univ Sch Dent 32(1):4–13

Fischer G, Conceição FR, Leite FPL, Dummer LA, Vargas GDA, de Oliveira Hübner S, Dellagostin OA, Paulino N, Paulino AS, Vidor T (2007) Immunomodulation produced by a green propolis extract on humoral and cellular responses of mice immunized with SuHV-1. Vaccine 25:1250–1256

Fitzmaurice S, Sivamani RK, Isseroff RR (2011) Antioxidant therapies for wound healing: a clinical guide to currently commercially available products. Skin Pharmacol Physiol 24:113–126

Fokt H, Pereira A, Ferreira A, Cunha A, Aguiar C (2010) How do bees prevent hive infections? The antimicrobial properties of propolis. In: Current research, technology and education. Topics in applied microbiology and microbial biotechnology, vol 1. World Scientific, Singapore, pp 481–493

Freitas SF, Shinohara L, Sforcin JM, Guimarães S (2006) In vitro effects of propolis on Giardia duodenalis trophozoites. Phytomedicine 13:170–175

Fuliang H, Hepburn H, Xuan H, Chen M, Daya S, Radloff S (2005) Effects of propolis on blood glucose, blood lipid and free radicals in rats with diabetes mellitus. Pharmacol Res 51:147–152

Gekker G, Hu S, Spivak M, Lokensgard JR, Peterson PK (2005) Anti-HIV-1 activity of propolis in CD4+ lymphocyte and microglial cell cultures. J Ethnopharmacol 102:158–163

Ghisalberti E (1979) Propolis: a review. Bee World 60:59–84

Girgin G, Baydar T, Ledochowski M, Schennach H, Bolukbasi DN, Sorkun K, Salih B, Sahin G, Fuchs D (2009) Immunomodulatory effects of Turkish propolis: changes in neopterin release and tryptophan degradation. Immunobiology 214:129–134

Grange J, Davey R (1990) Antibacterial properties of propolis (bee glue). J R Soc Med 83:159–160

Gregory SR, Piccolo N, Piccolo MT, Piccolo MS, Heggers JP (2002) Comparison of propolis skin cream to silver sulfadiazine: a naturopathic alternative to antibiotics in treatment of minor burns. J Altern Complement Med 8:77–83

Gressler LT, Da Silva AS, Machado G, Rosa LD, Dorneles F, Gressler LT, Oliveira MS, Zanette RA, de Vargas ACP, Monteiro SG (2012) Susceptibility of Trypanosoma evansi to propolis extract in vitro and in experimentally infected rats. Res Vet Sci 93(3):1314–1317

Guo S, DiPietro LA (2010) Factors affecting wound healing. J Dent Res 89:219–229

Gurtner GC, Werner S, Barrandon Y, Longaker MT (2008) Wound repair and regeneration. Nature 453(7193):314–321

Halliwell B, Gutteridge JM (2015) Free radicals in biology and medicine. Oxford University Press, New York

Hassan SE, Abou-El-Dobal SK, Hegazi AG (2016) Bioassay of Egyptian Propolis on Toxocara vitulorum adult worms. World Appl Sci J 34:283–289

Hegazi A, Abd El Hady F, Shalaby H (2000) Egyptian propolis: 4—Chemical composition of Siwa Oasis propolis with reference to its effect on Fasciola gigantica eggs. In: Proceedings of the 1st international forum on apitherapy, Athens, Greece, 2006

Hegazi AG, El Hady FKA, Shalaby HA (2007) An in vitro effect of propolis on adult worms of Fasciola gigantica. Vet Parasitol 144:279–286

Hernandez J, Goycoolea FM, Quintero J, Acosta A, Castañeda M, Dominguez Z, Robles R, Vazquez-Moreno L, Velazquez EF, Astiazaran H (2007) Sonoran propolis: chemical composition and antiproliferative activity on cancer cell lines. Planta Med 73:1469–1474

Higashi KO, De Castro SL (1994) Propolis extracts are effective against Trypanosoma cruzi and have an impact on its interaction with host cells. J Ethnopharmacol 43:149–155

Huang W-J, Huang C-H, Wu C-L, Lin J-K, Chen Y-W, Lin C-L, Chuang S-E, Huang C-Y, Chen C-N (2007) Propolin G, a prenylflavanone, isolated from Taiwanese propolis, induces caspase-dependent apoptosis in brain cancer cells. J Agric Food Chem 55:7366–7376

Iio A, Ohguchi K, Inoue H, Maruyama H, Araki Y, Nozawa Y, Ito M (2010) Ethanolic extracts of Brazilian red propolis promote adipocyte differentiation through PPARγ activation. Phytomedicine 17:974–979

Imamura K, Takeshima T, Kashiwaya Y, Nakaso K, Nakashima K (2006) D-β-hydroxybutyrate protects dopaminergic SH-SY5Y cells in a rotenone model of Parkinson's disease. J Neurosci Res 84:1376–1384

Ishihara M, Naoi K, Hashita M, Itoh Y, Suzui M (2009) Growth inhibitory activity of ethanol extracts of Chinese and Brazilian propolis in four human colon carcinoma cell lines. Oncol Rep 22:349

Isla M, Moreno MN, Sampietro A, Vattuone M (2001) Antioxidant activity of Argentine propolis extracts. J Ethnopharmacol 76:165–170

Issa RM (2007) Schistosoma mansoni: the prophylactic and curative effects of propolis in experimentally infected mice. Rawal Med J 32:94–98

Ivanovska N, Dimov V, Bankova V, Popov S (1995) Immunomodulatory action of propolis. VI. Influence of a water soluble derivative on complement activity in vivo. J Ethnopharmacol 47:145–147

Jackson CM, Esnouf MP, Winzor DJ, Duewer DL (2007) Defining and measuring biological activity: applying the principles of metrology. Accred Qual Assur 12:283–294

Kalogeropoulos N, Konteles SJ, Troullidou E, Mourtzinos I, Karathanos VT (2009) Chemical composition, antioxidant activity and antimicrobial properties of propolis extracts from Greece and Cyprus. Food Chem 116:452–461

Kamiya T, Nishihara H, Hara H, Adachi T (2012) Ethanol extract of Brazilian red propolis induces apoptosis in human breast cancer MCF-7 cells through endoplasmic reticulum stress. J Agric Food Chem 60:11065–11070

Kandil OM, Nassar SA, Nasr SM, Shalaby HAM, Hendawy S, El Moghazy FM (2015) Synergetic effect of Egyptian propolis in immunization of BALB/c mice against bovine cysticercosis. Asian Pac J Trop Biomed 5:324–330

Kartal M, Yıldız S, Kaya S, Kurucu S, Topçu G (2003) Antimicrobial activity of propolis samples from two different regions of Anatolia. J Ethnopharmacol 86:69–73

Kayser O, Olbrich C, Yardley V, Kiderlen A, Croft S (2003) Formulation of amphotericin B as nanosuspension for oral administration. Int J Pharm 254:73–75

Khayyal M, El-Ghazaly M, El-Khatib A (1992) Mechanisms involved in the antiinflammatory effect of propolis extract. Drugs Exp Clin Res 19:197–203

Kinnula VL, Crapo JD (2004) Superoxide dismutases in malignant cells and human tumors. Free Radic Biol Med 36:718–744

Kitamura H, Naoe Y, Kimura S, Miyamoto T, Okamoto S, Toda C, Shimamoto Y, Iwanaga T, Miyoshi I (2013) Beneficial effects of Brazilian propolis on type 2 diabetes in ob/ob mice: possible involvement of immune cells in mesenteric adipose tissue. Adipocytes 2:227–236

Komericki P, Kränke B (2009) Maculopapular exanthem from propolis: case report and review of systemic cutaneous and non-cutaneous reactions. Contact Derm 61:353–355

Koya-Miyata S, Arai N, Mizote A, Taniguchi Y, Ushio S, Iwaki K, Fukuda S (2009) Propolis prevents diet-induced hyperlipidemia and mitigates weight gain in diet-induced obesity in mice. Biol Pharm Bull 32:2022–2028

Kujumgiev A, Tsvetkova I, Serkedjieva Y, Bankova V, Christov R, Popov S (1999) Antibacterial, antifungal and antiviral activity of propolis of different geographic origin. J Ethnopharmacol 64:235–240

Kumazawa S, Ueda R, Hamasaka T, Fukumoto S, Fujimoto T, Nakayama T (2007) Antioxidant prenylated flavonoids from propolis collected in Okinawa, Japan. J Agric Food Chem 55:7722–7725

Kuropatnicki AK, Szliszka E, Krol W (2013) Historical aspects of propolis research in modern times. Evid Based Complement Alternat Med 2013:964149

Li F, Awale S, Tezuka, Y, Kadota S (2008) Cytotoxic constituents from Brazilian red propolis and their structure–activity relationship. Bioorg Med Chem. 16:5434–5440

Li F, Awale S, Tezuka Y, Esumi H, Kadota S (2010) Study on the constituents of Mexican propolis and their cytotoxic activity against PANC-1 human pancreatic cancer cells. J Nat Prod 73:623–627

Li Y, Chen M, Xuan H, Hu F (2011) Effects of encapsulated propolis on blood glycemic control, lipid metabolism, and insulin resistance in type 2 diabetes mellitus rats. Evid Based Complement Alternat Med 2012:981896

Liberio SA, Pereira ALA, Dutra RP, Reis AS, Araújo MJA, Mattar NS, Silva LA, Ribeiro MNS, Nascimento FRF, Guerra RN (2011) Antimicrobial activity against oral pathogens and immu-

nomodulatory effects and toxicity of geopropolis produced by the stingless bee Melipona fasciculata Smith. BMC Complement Altern Med 11:108
Machado GMC, Leon LL, Castro SL (2007) Activity of Brazilian and Bulgarian propolis against different species of Leishmania. Mem Inst Oswaldo Cruz 102(1):73–77
Machado JL, Assunçao AKM, da Silva MCP, Reis AS, Costa GC, Arruda DS, Rocha BA, MMdOLL V, AMdA P, RNM G (2012) Brazilian green propolis: anti-inflammatory property by an immunomodulatory activity. Evid Based Complement Alternat Med 2012:157652
Maksimova-Todorova V, Manolova N, Gegova G, Serkedzhieva I, Uzunov S (1985) Antiviral action of fractions isolated from propolis. Acta Microbiol Bulg 17:79
Marcucci MC (1995) Propolis: chemical composition, biological properties and therapeutic activity. Apidologie 26(2):83–99
Marcucci MC, Ferreres F, García-Viguera C, Bankova VS, De Castro SL, Dantas AP, PHM V, Paulino N (2001) Phenolic compounds from Brazilian propolis with pharmacological activities. J Ethnopharmacol 74:105–112
Martin I, Grotewiel MS (2006) Oxidative damage and age-related functional declines. Mech Ageing Dev 127:411–423
Martin LFT, Rocha EM, Garcia SB, Paula JS (2013) Topical Brazilian propolis improves corneal wound healing and inflammation in rats following alkali burns. BMC Complement Altern Med 13:1
Maruyama H, Sumitou Y, Sakamoto T, Araki Y, Hara H (2009) Antihypertensive effects of flavonoids isolated from brazilian green propolis in spontaneously hypertensive rats. Biol Pharm Bull 32:1244–1250
Matsui T, Ebuchi S, Fujise T, Abesundara KJ, Doi S, Yamada H, Matsumoto K (2004) Strong antihyperglycemic effects of water-soluble fraction of Brazilian propolis and its bioactive constituent, 3, 4, 5-tri-O-caffeoylquinic acid. Biol Pharm Bull 27:1797–1803
Matsushige K, Basnet P, Hase K, Kadota S, Tanaka K, Namba T (1996) Propolis protects pancreatic β-cells against the toxicity of streptozotocin (STZ). Phytomedicine 3:203–209
McLennan SV, Bonner J, Milne S, Lo L, Charlton A, Kurup S, Jia J, Yue DK, Twigg SM (2008) The anti-inflammatory agent Propolis improves wound healing in a rodent model of experimental diabetes. Wound Repair Regen 16:706–713
Mejanelle P, Bleton J, Goursaud S, Tchapla A (1997) Identification of phenolic acids and inositols in balms and tissues from an Egyptian mummy. J Chromatogr A 767:177–186
Mello BC, Hubinger MD (2012) Antioxidant activity and polyphenol contents in Brazilian green propolis extracts prepared with the use of ethanol and water as solvents in different pH values. Int J Food Sci Nutr 47:2510–2518
Meresta T (1997) Changes in antibacterial activity of propolis extracts during long storage. Medycyna Wet
Metodiewa D, Kośka C (1999) Reactive oxygen species and reactive nitrogen species: relevance to cyto (neuro) toxic events and neurologic disorders. An overview. Neurotox Res 1:197–233
Miguel MG, Antunes MD (2011) Is propolis safe as an alternative medicine? J Pharm Bioallied Sci 3:479
Mirzoeva O, Calder P (1996) The effect of propolis and its components on eicosanoid production during the inflammatory response. Prostaglandins Leukot Essent Fatty Acids 55:441–449
Mirzoeva O, Grishanin R, Calder P (1997) Antimicrobial action of propolis and some of its components: the effects on growth, membrane potential and motility of bacteria. Microbiol Res 152:239–246
Missima F, Sforcin JM (2008) Green Brazilian propolis action on macrophages and lymphoid organs of chronically stressed mice. Evid Based Complement Alternat Med 5:71–75
Missima F, Pagliarone A, Orsatti C, Araújo J, Sforcin J (2010) The Effect of propolis on Th1/Th2 cytokine expression and production by melanoma-bearing mice submitted to stress. Phytother Res 24:1501–1507

Monzote FL, Sariego RI, García PM, Cuesta-Rubio O, Márquez HI, Campo FM, Piccinelli AL, Rastrelli L (2011) Activity of Cuban propolis extracts on Leishmania amazonensis and Trichomonas vaginalis. Nat Prod Commun 6:973–976

Motomura M, Kwon KM, Suh S-J, Lee Y-C, Kim Y-K, Lee I-S, Kim M-S, Kwon DY, Suzuki I, Kim C-H (2008) Propolis induces cell cycle arrest and apoptosis in human leukemic U937 cells through Bcl-2/Bax regulation. Environ Toxicol Pharmacol 26:61–67

Moura SAL, Negri G, Salatino A, Lima LDC, LPA D, Mendes JB, Andrade SP, Ferreira MAND, Cara DC (2011) Aqueous extract of Brazilian green propolis: primary components, evaluation of inflammation and wound healing by using subcutaneous implanted sponges. Evid Based Complement Alternat Med 2011:748283

Murad J, Calvi S, Soares A, Bankova V, Sforcin J (2002) Effects of propolis from Brazil and Bulgaria on fungicidal activity of macrophages against Paracoccidioides brasiliensis. J Ethnopharmacol 79:331–334

Nader MA, El-Agamy DS, Suddek GM (2010) Protective effects of propolis and thymoquinone on development of atherosclerosis in cholesterol-fed rabbits. Arch Pharm Res 33:637–643

Nina N, Lima B, Feresin GE, Giménez A, Salamanca Capusiri E, Schmeda-Hirschmann G (2016) Antibacterial and leishmanicidal activity of Bolivian propolis. Lett Appl Microbiol 62:290–296

Nolkemper S, Reichling J, Sensch KH, Schnitzler P (2010) Mechanism of herpes simplex virus type 2 suppression by propolis extracts. Phytomedicine 17(2):132–138

Noweer E, Dawood M (2008) Efficiency of propolis extract on faba bean plants and its role against nematode infection. Commun Agric Appl Biol Sci 74:593–603

Olayemi KI (2014) Therapeutic potentials of Nigerian insect—propolis against the malarial parasite, Plasmodium berghei (Haemosporida: Plasmodidae). Am J Drug Discov Develop 4(4):241–247

Olczyk P, Komosinska-Vassev K, Winsz-Szczotka K, Stojko J, Klimek K, Kozma EM (2013) Propolis induces chondroitin/dermatan sulphate and hyaluronic acid accumulation in the skin of burned wound. Evid Based Complement Alternat Med 2013:290675

Oliveira ACP, Shinobu CS, Longhini R, Franco SL, Svidzinski TIE (2006) Antifungal activity of propolis extract against yeasts isolated from onychomycosis lesions. Mem Inst Oswaldo Cruz 101(5):493–497

Omar RM, Igoli J, Gray AI, Ebiloma GU, Clements C, Fearnley J, Ebel E, Angeli R, Zhang T, De Koning HP (2016) Chemical characterisation of Nigerian red propolis and its biological activity against Trypanosoma brucei. Phytochem Anal 27(2):107–115

Onlen Y, Duran N, Atik E, Savas L, Altug E, Yakan S, Aslantas O (2007) Antibacterial activity of propolis against MRSA and synergism with topical mupirocin. J Altern Complement Med 13(7):713–718

Orsatti CL, Sforcin JM (2012) Propolis immunomodulatory activity on TLR-2 and TLR-4 expression by chronically stressed mice. Nat Prod Res 26:446–453

Orsatti C, Missima F, Pagliarone A, Bachiega TF, Búfalo M, Araújo J, Sforcin J (2010a) Propolis immunomodulatory action in vivo on Toll-like receptors 2 and 4 expression and on pro-inflammatory cytokines production in mice. Phytother Res 24:1141–1146

Orsatti CL, Missima F, Pagliarone AC, Sforcin JM (2010b) Th1/Th2 cytokines' expression and production by propolis-treated mice. J Ethnopharmacol 129:314–318

Orsi RO, Sforcin JM, Funari SR, Bankova V (2005) Effects of Brazilian and Bulgarian propolis on bactericidal activity of macrophages against Salmonella typhimurium. Int Immunopharmacol 5:359–368

Orsi RO, Sforcin JM, Funari SRC, Fernandes Junior A, Bankova V (2006) Synergistic effect of propolis and antibiotics on the Salmonella typhi. Braz J Microbiol 37:108–112

Oršolić N, Knežević AH, Šver L, Terzić S, Bašić I (2004) Immunomodulatory and antimetastatic action of propolis and related polyphenolic compounds. J Ethnopharmacol 94:307–315

Oršolić N, Sirovina D, Končić MZ, Lacković G, Gregorović G (2012) Effect of Croatian propolis on diabetic nephropathy and liver toxicity in mice. BMC Complement Altern Med 12(1):1

Ota C, Unterkircher C, Fantinato V, Shimizu M (2001) Antifungal activity of propolis on different species of Candida. Mycoses 44(9–10):375–378

Ozbilge H, Kaya EG, Albayrak S, Silici S (2010) Anti-leishmanial activities of ethanolic extract of Kayseri propolis. Afr J Microbiol Res 4:556–560

Ozcan M, Sagdic O, Özkan G (2004) Antibacterial effects of Turkish pollen and propolis extracts at different concentrations. Arch Leb 55:39–40

Padmavathi R, Senthilnathan P, Sakthisekaran D (2006) Therapeutic effect of propolis and paclitaxel on hepatic phase I and II enzymes and marker enzymes in dimethylbenz (a) anthracene-induced breast cancer in female rats. Comp Biochem Physiol C Toxicol Pharmacol 143:349–354

Paredes-Guzman JF, Daugsch A, Park YK, Rhim JS (2007) Antiproliferation of human prostate cancer cells by ethanolic extracts of Brazilian propolis and its botanical origin. Int J Oncol 31:601–606

Parreira NA, Magalhães LG, Morais DR, Caixeta SC, de Sousa JP, Bastos JK, Cunha WR, Silva ML, Nanayakkara N, Rodrigues V (2010) Antiprotozoal, schistosomicidal, and antimicrobial activities of the essential oil from the leaves of Baccharis dracunculifolia. Chem Biodivers 7:993–1001

Piccinelli AL, Mencherini T, Celano R, Mouhoubi Z, Tamendjari A, Aquino RP, Rastrelli L (2013) Chemical composition and antioxidant activity of Algerian propolis. J Agric Food Chem 61:5080–5088

Pontin K, Da Silva Filho AA, Santos FF, e Silva MLA, Cunha WR, Nanayakkara ND, Bastos JK, de Albuquerque S (2008) In vitro and in vivo antileishmanial activities of a Brazilian green propolis extract. J Parasitol Res 103:487–492

Popova MP, Bankova VS, Bogdanov S, Tsvetkova I, Naydenski C, Marcazzan GL, Sabatini A-G (2007) Chemical characteristics of poplar type propolis of different geographic origin. Apidologie 38(3):306–311

Popova M, Reyes M, Le Conte Y, Bankova V (2014) Propolis chemical composition and honeybee resistance against Varroa destructor. Nat Prod Res 28:788–794

Prytzyk E, Dantas AP, Salomão K, Pereira AS, Bankova VS, De Castro SL, Neto FRA (2003) Flavonoids and trypanocidal activity of Bulgarian propolis. J Ethnopharmacol 88:189–193

Rashidi MK, Mirazi N, Hosseini A (2016) Effect of topical mixture of honey, royal jelly and olive oil-propolis extract on skin wound healing in diabetic rats. Wound Medicine 12:6–9

Rifa'i M, Widodo N (2014) Significance of propolis administration for homeostasis of CD4+ CD25+ immunoregulatory T cells controlling hyperglycemia. Spring 3(1):1

Rossi A, Longo R, Russo A, Borrelli F, Sautebin L (2002) The role of the phenethyl ester of caffeic acid (CAPE) in the inhibition of rat lung cyclooxygenase activity by propolis. Fitoterapia 73:S30–S37

Russo A, Longo R, Vanella A (2002) Antioxidant activity of propolis: role of caffeic acid phenethyl ester and galangin. Fitoterapia 73:S21–S29

Salomão K, Dantas AP, Borba CM, Campos LC, Machado DG, Aquino Neto FR, Castro SL (2004) Chemical composition and microbicidal activity of extracts from Brazilian and Bulgarian propolis. Lett Appl Microbiol 38:87–92

Salomão K, de Souza EM, Henriques-Pons A, Barbosa HS, de Castro SL (2009) Brazilian green propolis: effects in vitro and in vivo on Trypanosoma cruzi. Evid Based Complement Alternat Med 185918:11

Salomão K, De Souza EM, Barbosa HS, de Castro SL (2010) Propolis and derivatives of megazol: in vitro and in vivo activity on Trypanosoma cruzi, mechanism of action and selectivity. Int J Infect Dis 14:e441–e442

Santos V, Pimenta F, Aguiar M, Do Carmo M, Naves M, Mesquita R (2005) Oral candidiasis treatment with Brazilian ethanol propolis extract. Phytother Res 19:652–654

Sartori D, Kawakami C, Orsatti C, Sforcin J (2009) Propolis effect on streptozotocin-induced diabetic rats. J Venom Anim Toxins Incl Trop Dis 15:93–102

Savka MA, Dailey L, Popova M, Mihaylova R, Merritt B, Masek M, Le P, Nor SRM, Ahmad M, Hudson AO (2015) Chemical composition and disruption of quorum sensing signaling in geographically diverse United States propolis. Evid Based Complement Alternat Med 472593:10

Scazzocchio F, D'auria F, Alessandrini D, Pantanella F (2006) Multifactorial aspects of antimicrobial activity of propolis. Microbiol Res 161:327–333

Schnitzler P, Neuner A, Nolkemper S, Zundel C, Nowack H, Sensch KH, Reichling J (2010) Antiviral activity and mode of action of propolis extracts and selected compounds. Phytother Res 24:S20–S28

Sehn E, Hernandes L, Franco S, Gonçalves C, Baesso M (2009) Dynamics of reepithelialisation and penetration rate of a bee propolis formulation during cutaneous wounds healing. Anal Chim Acta 635:115–120

Serkedjieva J, Manolova N, Bankova V (1992) Anti-influenza virus effect of some propolis constituents and their analogues (esters of substituted cinnamic acids). J Nat Prod 55:294–297

Sforcin J (2007) Propolis and the immune system: a review. J Ethnopharmacol 113:1–14

Sforcin JM (2016) Biological properties and therapeutic applications of propolis. Phytother Res 30:894–905

Sforcin JM, Bankova V (2011) Propolis: is there a potential for the development of new drugs? J Ethnopharmacol 133:253–260

Sforcin J, Kaneno R, Funari S (2002) Absence of seasonal effect on the immunomodulatory action of Brazilian propolis on natural killer activity. J Venom Anim Toxins 8:19–29

Shimazawa M, Chikamatsu S, Morimoto N, Mishima S, Nagai H, Hara H (2005) Neuroprotection by Brazilian green propolis against in vitro and in vivo ischemic neuronal damage. Evid Based Complement Alternat Med 2:201–207

Shimizu T, Hino A, Tsutsumi A, Park YK, Watanabe W, Kurokawa M (2008) Anti-influenza virus activity of propolis in vitro and its efficacy against influenza infection in mice. Antivir Chem Chemother 19:7–13

Shimizu T, Takeshita Y, Takamori Y, Kai H, Sawamura R, Yoshida H, Watanabe W, Tsutsumi A, Park YK, Yasukawa K (2011) Efficacy of Brazilian propolis against herpes simplex virus type 1 infection in mice and their modes of antiherpetic efficacies. Evid Based Complement Alternat Med 2011:976196

Siheri W, Igoli JO, Gray AI, Nasciemento TG, Zhang T, Fearnley J, Clements CJ, Carter KC, Carruthers J, Edrada-Ebel R (2014) The isolation of antiprotozoal compounds from Libyan propolis. Phytother Res 28:1756–1760

Siheri W, Zhang T, Ebiloma GU, Biddau M, Woods N, Hussain MY, Clements CJ, Fearnley J, Ebel RE, Paget T (2016) Chemical and antimicrobial profiling of propolis from different regions within Libya. PLoS One 11(5):e0155355

Silici S, Kutluca S (2005) Chemical composition and antibacterial activity of propolis collected by three different races of honeybees in the same region. J Ethnopharmacol 99:69–73

Silva-Carvalho R, Baltazar F, Almeida-Aguiar C (2015) Propolis: a complex natural product with a plethora of biological activities that can be explored for drug development. Evid Based Complement Alternat Med 2015:206439

Simone-Finstrom M, Spivak M (2010) Propolis and bee health: the natural history and significance of resin use by honey bees. Apidologie 41:295–311

Simone-Finstrom MD, Spivak M (2012) Increased resin collection after parasite challenge: a case of self-medication in honey bees? PLoS One 7:e34601

Song HS, Park TW, Sohn UD, Shin YK, Choi BC, Kim CJ, Sim SS (2008) The effect of caffeic acid on wound healing in skin-incised mice. Korean J Physiol Pharmacol 12:343–347

Speciale A, Costanzo R, Puglisi S, Musumeci R, Catania M, Caccamo F, Iauk L (2006) Antibacterial activity of propolis and its active principles alone and in combination with macrolides, beta-lactams and fluoroquinolones against microorganisms responsible for respiratory infections. J Chemother 18:164–171

Starzyk J, Scheller S, Szaflarski J, Moskwa M, Stojko A (1977) Biological properties and clinical application of propolis. II. Studies on the antiprotozoan activity of ethanol extract of propolis. Arzneimittelforschung 27:1198

Stepanović S, Antić N, Dakić I, Švabić-Vlahović M (2003) In vitro antimicrobial activity of propolis and synergism between propolis and antimicrobial drugs. Microbiol Res 158:353–357

Su Z, Lin J, Prewett M, Goldstein N, Fisher P (1994) Apoptosis mediates the selective toxicity of caffeic acid phenethyl ester (CAPE) toward oncogene-transformed rat embryo fibroblast cells. Anticancer Res 15:1841–1848

Szliszka E, Czuba ZP, Bronikowska J, Mertas A, Paradysz A, Krol W (2011a) Ethanolic extract of propolis augments TRAIL-induced apoptotic death in prostate cancer cells. Evid Based Complement Alternat Med 2011:1–11

Szliszka E, Zydowicz G, Janoszka B, Dobosz C, Kowalczyk-Ziomek G, Krol W (2011b) Ethanolic extract of Brazilian green propolis sensitizes prostate cancer cells to TRAIL-induced apoptosis. Int J Oncol 38:941–953

Takaisi-Kikuni NB, Schilcher H (1994) Electron microscopic and microcalorimetric investigations of the possible mechanism of the antibacterial action of a defined propolis provenance. Planta Med 60:222–227

Teerasripreecha D, Phuwapraisirisan P, Puthong S, Kimura K, Okuyama M, Mori H, Kimura A, Chanchao C (2012) In vitro antiproliferative/cytotoxic activity on cancer cell lines of a cardanol and a cardol enriched from Thai Apis mellifera propolis. BMC Complement Altern Med 12(1):1

Tomás-Barberán FA, García-Viguera C, Vit-Olivier P, Ferreres F, Tomás-Lorente F (1993) Phytochemical evidence for the botanical origin of tropical propolis from Venezuela. Phytochemistry 34:191–196

Toreti VC, Sato HH, Pastore GM, Park YK (2013) Recent progress of propolis for its biological and chemical compositions and its botanical origin. Evid Based Complement Alternat Med 2013:697390

Torres D, Hollands I, Palacios E (1990) Effect of an alcoholic extract of propolis on the in vitro growth of Giardia lamblia. Revista Cubana de Ciencias Veterinarias 21:15–19

Ugur A, Arslan T (2004) An in vitro study on antimicrobial activity of propolis from Mugla province of Turkey. J Med Food 7:90–94

Umthong S, Phuwapraisirisan P, Puthong S, Chanchao C (2011) In vitro antiproliferative activity of partially purified Trigona laeviceps propolis from Thailand on human cancer cell lines. BMC Complement Altern Med 11:1

Uzel A, Önçağ Ö, Çoğulu D, Gençay Ö (2005) Chemical compositions and antimicrobial activities of four different Anatolian propolis samples. Microbiol Res 160:189–195

Valente MJ, Baltazar AF, Henrique R, Estevinho L, Carvalho M (2011) Biological activities of Portuguese propolis: protection against free radical-induced erythrocyte damage and inhibition of human renal cancer cell growth in vitro. Food Chem Toxicol 49:86–92

Valenzuela-Barra G, Castro C, Figueroa C, Barriga A, Silva X, de las Heras B, Hortelano S, Delporte C (2015) Anti-inflammatory activity and phenolic profile of propolis from two locations in Región Metropolitana de Santiago, Chile. J Ethnopharmacol 168:37–44

Valko M, Leibfritz D, Moncol J, Cronin MT, Mazur M, Telser J (2007) Free radicals and antioxidants in normal physiological functions and human disease. Int J Biochem Cell Biol 39:44–84

Vanden Berghe D, Vhetinck A, Van Hoof L (1986) Plant products as potential antiviral agents. Bulletin de l'Institut Pasteur, France

Victorino FR, Franco SL, Estivalet Svidzinski TI, Avila-Campos MJ, Nakamura Cuman RK, Hidalgo MM, Bersani-Amado CA (2007) Pharmacological evaluation of propolis solutions for endodontic use. Pharm Biol 45:721–727

Wagh VD (2013) Propolis: a wonder bees product and its pharmacological potentials. Adv Pharmacol Sci 308249:11

Watanabe MAE, Amarante MK, Conti BJ, Sforcin JM (2011) Cytotoxic constituents of propolis inducing anticancer effects: a review. J Pharm Pharmacol 63:1378–1386

Wilson MB, Spivak M, Hegeman AD, Rendahl A, Cohen JD (2013) Metabolomics reveals the origins of antimicrobial plant resins collected by honey bees. PLoS One 8(10):e77512

Won Seo K, Park M, Jung Song Y, Kim SJ, Ro Yoon K (2003) The protective effects of propolis on hepatic injury and its mechanism. Phytother Res 17:250–253

Yang HY, Chang CM, Chen YW, Chou CC (2006) Inhibitory effect of propolis extract on the growth of Listeria monocytogenes and the mutagenicity of 4-nitroquinoline-N-oxide. J Sci Food Agr 86:937–943

Yildirim A, Duran GG, Duran N, Jenedi K, Bolgul BS, Miraloglu M, Muz M (2016) Antiviral activity of hatay propolis against replication of herpes simplex virus type 1 and type 2. Med Sci Monitor: Int Med J Exp Clin Res 22:422–430

Zhang T, Omar R, Siheri W, Al Mutairi S, Clements C, Fearnley J, Edrada-Ebel R, Watson D (2014) Chromatographic analysis with different detectors in the chemical characterisation and dereplication of African propolis. Talanta 120:181–190

Zhu W, Chen M, Shou Q, Li Y, Hu F (2011) Biological activities of Chinese propolis and Brazilian propolis on streptozotocin-induced type 1 diabetes mellitus in rats. J Evid Based Complement Altern Med 2011:468529

Part III
Royal Jelly

Chapter 8
Chemical Composition of Royal Jelly

Xiaofeng Xue, Liming Wu, and Kang Wang

Contents

8.1 Sugar Composition

Sugars consist mostly of fructose and glucose, with a chemical composition similar to that of honey. The oldest reports reveal that the average global composition of the glycid fraction in Royal Jelly (RJ) ranged from 7.5 to 15% (Rembold 1965; Rembols and Lackner 1978). Earlier gas chromatography has been used to identify simple sugars such as glucose, fructose and saccharose in Royal Jelly, along with other neutral constituents (Lercker 1986). Generally, the total content of fructose and glucose accounts for 90% of the total sugars contained in royal jelly. The sucrose content varies considerably from one sample to another. Other sugars seen in much smaller quantities are maltose, trehalose, melibiose, ribose and erlose (Lercker et al. 1992). Based on HPLC analysis of RJ, fructose and glucose content varies between 4.06 and 6.4%, and 5.07 and 8.2%, respectively, with a relatively high content of sucrose (0.8 and 3.6%). Maltose and trehalose were present in smaller amounts (0.3% for both) (Popescu et al. 2009).

In authentic French royal jelly samples, the minimal, maximal, and average values for the three main sugars were reported to be 2.3–7.6% for fructose, average 4.9%;

X. Xue (✉) • L. Wu • K. Wang
Institute of Apicultural Research, Chinese Academy of Agricultural Science, Beijing 100093, China
e-mail: xue_xiaofeng@126.com

J.M. Alvarez-Suarez (ed.), *Bee Products - Chemical and Biological Properties*,
DOI 10.1007/978-3-319-59689-1_8

2.9–8.1% for glucose, average 5.5%; and not detected to 1.8% for sucrose, average 0.5%. So, the total sugar content was between 7.9 and 17.9% with an average of 13.1% (Wytrychowski et al. 2013). The other most abundant sugars found in French royal jelly are maltose (not detected to 1.0%, average 0.3%), galactose (not detected to 0.3%, average 0.2%) and trehalose (not detected to 0.5%, average 0.1%) (Wytrychowski et al. 2013). Another earlier study on French royal jelly reported similar findings for fructose (2.3–7.3%, mean 5.0%), glucose (2.9–8.0%, mean 5.5%), sucrose (not detected to 1.7%, mean 0.4%) and total sugar content (8.1–16.8%, mean 12.4%) (Daniele and Casabianca 2012). It has been indicated that the content of sucrose and erlose in French RJ is less than 1.7 and 0.3%, respectively, but they reach 3.9 and 2.0% in some commercial samples (Daniele and Casabianca 2012). Another study reported similar findings for French royal jelly, with sucrose and erlose contents of less than 1.8 and 0.4%, respectively, and the values rise up to 3.9 and 2.0% in some commercial samples and 7.7 and 1.7% in RJs produced by sugarcane feeding (Marine Wytrychowski et al. 2013). Italian royal jelly showed minor variations compared to French RJ, i.e. fructose (2.3–6.9%, mean 4.6%), glucose (3.7–8.2%, mean 5.8%), sucrose (<0.1–2.1%, mean 1.0%) and total sugar content (6.9–16.0%, mean 11.4%) (Sesta 2006).

8.2 Protein Content

Proteins account for >50% of RJ dry weight and almost 80% of soluble RJ proteins are major royal jelly proteins (MRJPs), as they are considered to play specific physiological roles in queen honeybee development (Schmitzova et al. 1998; Nozaki et al. 2012). Studies have shown that the MRJP family, of which there are nine members (MRJP1, MRJP2, MRJP3, MRJP4, MRJP5, MRJP6, MRJP7, MRJP8 and MRJP9), accounts for most of the soluble proteins in RJ (31%) (Albert and Klaudiny 2004; Drapeau et al. 2006; Schonleben et al. 2007; Tamura et al. 2009; Ramadan and Al-Ghamdi 2012; Buttstedt et al. 2013). MRJP1 to 8 have been characterized by the cloning and sequencing of their respective cDNAs (Albert and Klaudiny 2004). French RJ had a minimal and maximal protein content of 11.4 and 16.9%, respectively, with a mean protein content of 13.9% (Wytrychowski et al. 2013).

MRJP1 is a weak acidic glycoprotein (pI 4.9–6.3, 55–57 kDa as monomer) which forms a 350 kDa hexamer after gel-filtration chromatography (Kimura et al. 1995). Another study reported MRJPs as 280 kDa proteins which can be separated as 55 kDa bands by both reducing and non-reducing SDS-PAGE, and also separated into several spots by 2-DE (pH 4.2–6.5) (Tamura et al. 2009). The two different oligomerisation processes tend to be pH dependent with the pentamer formed at pH 6 and 7 and hexamer at pH 8 (Cruz et al. 2011). These studies also indicated that the MRJP1 oligomer is comprised of 55 kDa protein subunits bound by non-covalent bonds (Santos et al. 2005; Tamura et al. 2009). The binding pattern of the MRJP1 oligomer based on Superose 12 HPLC and SDS–PAGE results indicated that the oligomer is a pentamer of MRJP1 monomers (Tamura et al. 2009; Xu and Gao 2013). MRJP1 was thought to be made up of an oligomer complex linked by non-covalent bonds under natural conditions (Tamura et al. 2009). MRJP1 contains a relatively high content of essential amino acids (48%), as it is considered a potential

ingredient of functional foods (Judova et al. 2004). The secondary structure of MRJP1, based on circular dichroism (CD) measurements, consists of 9.6% α-helices, 38.3% β-sheets and 20% β-turns (Cruz et al. 2011).

Soluble RJ proteins were completely separated into five peaks at approximately 640, 280, 100, 72 and 4.5 kDa by size-exclusion HPLC on a Superose 12 column (Tamura et al. 2009). Among these, the 280 and 72 kDa corresponded to the major components of RJ proteins, but the intensity of the 280 kDa peak tends to differ across the original RJ samples (Tamura et al. 2009). Another study showed separation of water soluble proteins in RJ from queen larva analyzed by SDS-PAGE into six bands at 85, 79, 68, 60, 56 and 49 kDa, representing glucose oxidase, MRJP5, MRJP3, MRJP4, MRJP1 and MRJP2, respectively (Xu and Gao 2013). The study also demonstrated that the main components of queen larvae proteins were mainly MRJP1 and MRJP2, but they were quite distinct from the MRJPs of RJ in composition and structure (Xu and Gao 2013).

The open reading frame (ORF) of the AmMRJP1 gene from the western honeybee is a fragment of 1299 bp, which encodes a protein of 422 amino acid residues with a predicted molecular weight of 48 kDa for a deglycosylated protein (Tamura et al. 2009). Mature MRJP1 cDNA of the Chinese honeybee (*Apis cerana cerana* MRJP1, or AccMRJP1) expressed in *Pichia pastoris* showed that recombinant AccMRJP1 was identical in molecular weight to the glycosylated AmMRJP1 from the Western honeybee (*Apis mellifera*) (Shen et al. 2010). The molecular weight of recombinant AccMRJP1 decreased from 57 to 48 kDa after deglycosylation, indicating that AccMRJP1 is in the glycosylated form. MRJP2, MRJP3, MRJP4 and MRJP5 are glycoproteins of 49, 60–70, 60 and 80 kDa, respectively (Li et al. 2007; Schmitzova et al. 1998). MRJP2 to 5 exist mainly in the basic pI range (pI 6.3–8.3) (Li et al. 2007; Sano et al. 2004; Santos et al. 2005; Schonleben et al. 2007). Tamura et al. (2009) reported that the RJ protein of the 72 kDa peak could be separated into several spots that were focused at 51 kDa and ranged from pH 6.2 to 7.9; they were identified as MRJP2. The wide pI range of the MRJP proteins is generally due to post-translational modification by glycosylation and/or phosphorylation (Tamura et al. 2009). Studies indicate that methylation is the most important post-translational modification of MRJPs, which triggers polymorphism of MRJP1 to 5 in the RJ proteome (Zhang et al. 2012). Only one study on RJ of Africanized honeybees was able to identify MRJP8 (Santos et al. 2005). Both MRJP8 and 9 are rare in RJ but were detected in honeybee venom (Peiren et al. 2005, 2008).

The physiological functions of RJ proteins have been widely reported. They are known to have proliferation activity in cell lines derived from humans, rats, and insects. Studies have shown that RJ proteins could stimulate the proliferation of mouse osteoblast-like MC3T3-E1 cells (Narita et al. 2006), U-937 human myeloid cell lines (Watanabe et al. 1998), and human monocytes (Kimura et al. 2003). Recombinant AccMRJP1 from the Chinese honeybee could significantly stimulate Tn-5B-4 cell growth, similar to Western honeybee (*Apis mellifera*) RJ and fetal bovine serum, and affected cell shape and adhesion to the substrate (Shen et al. 2010). Natural MRJP1 from the Western honeybee (*Apis mellifera*) can stimulate the proliferation of rat hepatocytes (Kamakura et al. 2001), promote hepatocyte DNA synthesis, and increase albumin production in the absence of fetal bovine serum (FBS) (Kamakura et al. 2001; Kamakura 2002). It has been reported that crude RJ

protein could inhibit the bisphenol A-induced proliferation of human breast cancer cell lines (Nakaya et al. 2007). Okamoto et al. (2003) identified that MRJP3 could suppress IL-4 production, showing potent antiallergic properties in a mouse model of immediate hypersensitivity. In their *in vitro* studies, MRJP3 not only suppressed the production of IL-4, but also that of IL-2 and IFN-g by T cells, concomitant with inhibition of proliferation. MRJP3-mediated suppression of IL-4 production was also observed when lymph node cells from OVA/alum-immunized mice were stimulated with OVA plus antigen presenting cells (Okamoto et al. 2003). In spite of its antigenicity, as MRJP3 itself is an extraneous foreign protein, intraperitoneal administration of MRJP3 inhibited serum anti-OVA IgE and IgG1 production in immunized mice. Designing MRJP3-derived antiallergic peptides by identifying the associated polypeptide regions would be of great clinical significance (Okamoto et al. 2003). A recent study showed that MRJP1 could hinder vascular smooth muscle cell proliferation by reducing the supply of energy and genetic material, revealing that the function and mechanism correlated with the anti-hypertensive activity (Fan et al. 2016). MRJP1 and MRJP2 can stimulate mouse macrophages to release TNF-α (Simuth et al. 2004). It was found that honey can stimulate TNF-α secretion from murine macrophages, whereas deproteinized honey had no effect on the release of TNF-α, indicating that the immunostimulatory effect of honey is based on its RJ protein content (Majtan et al. 2006). After 1 h ultra-centrifugation, SRJPs are present in the supernatant of the upper, middle and lower layers. The upper and middle layers contained MRJP2 (52 kDa) and MRJP3 (60–70 kDa), while the lower layer contained MRJP1 (290 kDa). SRJPs separated by ultra-centrifugation produced 20-fold higher levels of SRJPs compared to conventional methods involving dialysis and centrifugation, where dialysis is a time-consuming process (Nozaki et al. 2012).

8.3 Content of Free Amino Acids, Lipids, Vitamins, and Bioactive Substances

RJ is rich in amino acids, containing at least 17, including 8 essential ones plus 5 non-identified related compounds. They are valine (1.6%), glycine (3.0%), isoleucine (1.6%), leucine (3.0%), proline (3.9%), threonine (2.0%), serine (2.9%), methionine (3.7%), phenylalanine (0.5%), aspartic acid (2.8%), glutamic acid (8.3%), tyrosine (4.9%), lysine (2.9%), arginine (3.3%), and tryptophan (3.4%) (Bărnuțiu et al. 2011). Rapid ultra-performance liquid chromatography (UPLC) allowed the separation and quantification of 26 amino acids in royal jelly. The results showed that the average amounts of FAA and TAA in fresh royal jelly were 9.21 and 111.27 mg/g, respectively, in which the major FAAs were Pro, Gln, Lys, Glu, and the most abundant TAAs were Asp, Glu, Lys and Leu. However, during storage, the total content of Met and free Gln decreased significantly and continuously, indicating that they can be used as a parameter to predict the quality of royal jelly (Wu et al. 2009).

Table 8.1 Principal vitamins identified in Royal jelly

Vitamins in royal jelly	mg/100 g
Vitamin A	1.10
Vitamin E	5.00
Vitamin B1	2.06
Vitamin D	0.2
Vitamin B2	2.77
Vitamin B6	11.90
Vitamin B12	0.15
Vitamin B5 (Panthotenic acid)	52.80
Niacin (PP)	42.42
Vitamin C (Ascorbic acid)	2.00
Vitamin B9 (Folic acid)	0.40

Other compounds identified in RJ include several nucleotides as free bases (adenosine, uridine, guanosine, iridin, and cytidine) and phosphates such as adenosine 5′-monophosphate (AMP), adenosine 5′-diphosphate (ADP), and adenosine 5′-triphosphate (ATP) (Xue et al. 2009a, b; Zhou et al. 2012). Adenosine is a naturally occurring purine nucleoside, a component of ribonucleic acid (RNA), consisting of adenine attached to a ribofuranose via a b-N9-glycosidic bond. *In vivo*, adenosine is produced by the metabolic degradation of intracellular adenosine triphosphate (ATP), which is the primary cellular energy source for transport systems and enzymatic activities of proteins. ATP is converted to adenosine diphosphate (ADP) and then adenosine monophosphate (AMP) by successive dephosphorylation (Kim and Lee 2011). A UPLC method showed that ATP in RJ sequentially degrades to adenosine 5′-diphosphate (ADP), adenosine 5′-monophosphate (AMP), inosine monophosphate (IMP), inosine (HxR), and hypoxanthine (Hx) (Zhou et al. 2009). A study has shown that significantly higher levels of ATP, ADP and AMP were seen in fresh RJ samples, and IMP, uridine, guanosine, and thymidine were identified in commercial RJ samples (Wu et al. 2015).

RJ also contains vitamins B1, B2, B6, B5, B8, B9, C (in trace amounts), and PP (Table 8.1) (Bărnuțiu et al. 2011).

The volatile organic compound (VOC) profiles of RJ were rich in acid, ester and aldehyde compounds, but their contents were different, as exemplified by the data for acetic acid, benzoic acid methyl ester, hexanoic acid and octanoic acid. Esters and aldehydes were the most abundant VOCs among the 40 detected, accounting for 25 and 17.5%, respectively, and contribute the most to the RJ flavor. Other classes of compounds, such as ketones (15%), acids (10%) and alcohols (10%), were also detected (Zhao et al. 2016).

Several studies have indicated that peptides have antioxidative activity (Shahidi and Zhong 2011). Antioxidative peptides were isolated from RJ, such as Ala-Leu, Phe-Lys, Phe- Arg, Ile-Arg, Lys-Phe, Lys-Leu, Lys-Tyr, Arg-Tyr, Tyr-Asp, Tyr-Tyr, Leu-Asp-Arg, and Lys-Asn-Tyr-Pro, with strong hydroxyl radical scavenging activity. Three dipeptides (Lys-Tyr, Arg-Tyr and Tyr-Tyr) containing Tyr residues at the C-terminal showed strong hydroxyl radical and hydrogen peroxide scavenging activity (Guo et al. 2009).

8.4 Lipid Content

A unique and interesting feature of RJ is its lipid and fatty acid content (Li et al. 2013). The lipid composition of RJ reported to date is 80–85% fatty acids, 4–10% phenols, 5–6% waxes, 3–4% steroids, and 0.4–0.8% phospholipids (Terada et al. 2011). RJ fatty acids are free short-chain fatty acids containing 8–12 carbons and are usually either hydroxyl or dicarboxylic fatty acids, different from fatty acids in animals and plants which are mainly in the tri, di, and monoglyceride forms with minor amounts of free fatty acids (Melliou and Chinou 2005). Lercker et al. (1981) originally discovered that fatty acids in RJ mainly consist of 10-hydroxy-2-decenoic (10-HDA) and 10-hydroxydecanoic acids (10-HDDA), constituting between them at least 60–80% of the total amount of organic acids found in RJ. The fatty acid fraction consists of 32% *trans*-10-hydroxy-2-decenoic acid, 24% gluconic acid, 22% 10-hydroxydecanoic acid, 5% dicarboxylic acids, and several other acids (Terada et al. 2011, Echigo et al. 1986). The major fatty acid in royal jelly is *trans*-10-hydroxy-2-decenoic acid, and to date no other natural product containing this has been reported, even in other bee products (Melliou and Chinou 2005). In Greek royal jelly, the most abundant were 10-hydroxy-2-decenoic acid, 10-hydroxydecanoic acid, and sebacic acid (Melliou and Chinou 2005). Seven different fatty acid derivatives were reported in Greek royal jelly, i.e., 10-acetoxydecanoic acid, trans-10-acetoxydec-2-enoic acid, 11-oxododecanoic acid, (11*S*)-hydroxydodecanoic acid, (10*R*, 11*R*)-dihydroxydodecanoic acid, 3,11-dihydroxydodecanoic acid, and (11*S*), 12-dihydroxydodecanoic acid (Melliou and Chinou 2005). In the past, 10-HDA content was adopted as a marker for RJ and used as a means to evaluate bee products containing RJ (Bloodworth et al. 1995). Beekeepers and manufacturers also utilised 10-HDA content as a freshness indicator for royal jellies, i.e., royal jellies with a 10-HDA content greater than 1.8% were considered as fresh and authentic samples (Antinelli et al. 2003). During a storage period of 12 months, 10-HDA loss rates were 0.1 and 0.2% at −18 and 4 °C, respectively, for samples from France and Thailand. At room temperature storage, corresponding losses were 0.4 and 0.6% for French and Thai samples, respectively (Antinelli et al. 2003).

Fatty acids in RJ are known to poses many medicinal properties, adding commercial and medicinal value to the product. Earlier studies have shown that fatty acids in RJ show anticancer properties (Townsend et al. 1961). Fatty acids in RJ also exhibited several immunomodulatory activities. Earlier studies have shown that 10-HDA stimulated transforming growth factor-β (a potent immunosuppressive cytokine) production in fibroblast cell lines (Koya-Miyata et al. 2004; Wahl et al. 2004). Dendritic cells (DCs) play an important role in the initiation and regulation of the immune response against pathogens, tumors and other antigens. At high concentrations, 10-HDA and 3,10-dihydroxy-decanoic acid (3,10-DDA) could inhibit the proliferation of allogeneic T-cells in rat DC–T-cell cocultures, indicating their immunomodulatory activity (Vucevic et al. 2007). 3,10-DDA could stimulate the maturation of human monocyte-derived dendritic cells *in vitro* by up-regulating the expression of CD40, CD54, CD86 and CD1a, and enhance the allostimulatory and

Th1 polarising capability of these cells, which could be beneficial for anti-tumour and anti-viral immune responses (Tanja Dzopalic et al. 2011). Fatty acids such as 3-hydroxydodecanedioic acid, 11-oxododecanoic acid, and (11*S*)-hydroxydodecanoic acid from RJ exhibited strong broad spectrum antimicrobial activity against pathogenic bacteria and fungi (Melliou and Chinou 2005). 3-Hydroxydodecanedioic acid, *trans*-10-acetoxydec-2-enoic acid, and (11*S*)-hydroxydodecanoic acid exhibited activity against *S. aureus*, *S. epidermidis*, *S. mutans* and *S. viridans* with MIC values ranging from 0.17 to 0.36 mg/mL. Sebacic acid (MIC values of 0.15 to 0.20 mg/mL) and 11-oxododecanoic acid (MIC values of 0.26–0.67 mg/mL) showed higher antifungal activity against different fungi (Melliou and Chinou 2005). Transient receptor potential ankyrin 1 (TRPA1) and vanilloid 1 (TRPV1), belonging to the TRP family, are Ca^{2+} permeable nonselective cation channels (Dhaka et al. 2006, Caterina et al. 1997). TRPA1 and TRPV1 are expressed in dosal root ganglion and nodose ganglion neurons innervating the stomach and in nerve fibers in the gastric wall (Patterson et al. 2003; Ward et al. 2003). Ingestion of TRPA1- and TRPV1-active food components can be effective against obesity via energy expenditure enhancement (Yoshioka et al. 1998). Terada et al. (2011) searched for novel agonists of TRPA1 and TRPV1 in RJ and found that hexane extract activates TRPA1. They found that most of the fatty acids, such as 10-HDA and 10-HDDA which make up approximately 10% of RJ, had a higher affinity for TRPA1 than TRPV1 with EC_{50} values for the latter being 2–3 times higher and maximal responses less than half compared to those for TRPA1 (Terada et al. 2011). Other fatty acids such as 1,8-octanedicarboxylic acid and 1,6-hexanedicarboxylic acid showed relatively strong effects on both receptors, but they only constitute a small percentage of RJ. The study indicated that 10-HDA and 10-HDDA can activate TRPA1 because they contain a 10-carbon chain that renders them moderately hydrophobic (Terada et al. 2011).

References

Albert S, Klaudiny J (2004) The MRJP/YELLOW protein family of *Apis mellifera*: identification of new members in the EST library. J Insect Physiol 50(1):51–59

Antinelli FJ, Zegganea S, Davicoa R, Rognonea C, Faucona JP, Lizzani L (2003) Evaluation of (E)-10-hydroxydec-2-enoic acid as a freshness parameter for royal jelly. Food Chem 80:85–89

Bărnuțiu LI, Al L, Mărghitaş DDS, Mihai CM, Bobiş O (2011) Chemical composition and antimicrobial activity of royal jelly–review. J Anim Sci Biotechnol 44(2):67–72

Bloodworth BC, Harn CS, Hock CT (1995) Liquid chromatographic determination of trans-10-hydroxy-2-decenoic acid content of commercial products containing royal jelly. J AOAC Int 78:1019–1023

Buttstedt A, RF M, Erler S (2013) Origin and function of the major royal jelly proteins of the honeybee (*Apis mellifera*) as members of the *yellow* gene family. Biol Rev Camb Philos Soc 89:255–269. doi:10.1111/brv.12052

Caterina MJ, Schumacher MA, Tominaga M, Rosen TA, Levine JD, Julius D (1997) The capsaicin receptor: a heat-activated ion channel in the pain pathway. Nature 389:816–824

Cruz GCN, Garcia L, Silva AJ, Barbosa JARG, Ricart CAO, Freitas SM, Sousa MV (2011) Calcium effect and pH-dependence on self-association and structural stability of the *Apis mellifera* major royal jelly protein. Apidologie 42:252–269

Daniele G, Casabianca H (2012) Sugar composition of French royal jelly for comparison with commercial and artificial sugar samples. Food Chem 134:1025–1029
Dhaka A, Viswanath V, Patapoutian A (2006) TRP ion channels and temperature sensation. Annu Rev Neurosci 29:135–161
Drapeau MD, Albert S, Kucharski R, Prusko C, Maleszka R (2006) Evolution of the yellow/major royal jelly protein family and the emergence of social behavior in honey bees. Genome Res 16:1385–1394
Dzopalic T, Vucevic D, Tomic S, Djokic J, Chinou I, Colic M (2011) 3,10-Dihydroxy- decanoic acid, isolated from royal jelly, stimulates Th1 polarising capability of human monocyte-derived dendritic cells. Food Chem 126:1211–1217
Echigo T, Takenaka T, Yatsunami K (1986) Comparative studies on chemical composition of honey, royal jelly and pollen loads. Bull Fac Agr Tamagawa Univ 26:1–12
Fan P, Han B, Feng M, Fang Y, Zhang L, Hu H, Hao Y, Qi Y, Zhang X, Li J (2016) Functional and proteomic investigations reveal major royal jelly protein 1 associated with antihypertension activity in mouse vascular smooth muscle cells. Sci Rep 6:30230. doi:10.1038/srep30230
Guo H, Kozuma Y, Yonekura M (2009) Structures and properties of antioxidative peptides derived from royal jelly protein. Food Chem 113:238–245
Judova J, Sutka R, Klaudiny J, Liskova D, OW DW, Simuth J (2004) Transformation of tobacco plants with cDNA encoding honeybee royal jelly MRJP1. Biol Plant 48:185–191
Kamakura M (2002) Signal transduction mechanism leading to enhanced proliferation of primary cultured adult rat hepatocytes treated with royal jelly 57-kDa protein. J Biochem 132: 911–912
Kamakura M, Suenobu N, Fukushima M (2001) Fifty-seven kDa protein in royal jelly enhances proliferation of primary cultured rat hepatocytes and increases albumin production in the absence of serum. Biochem Biophys Res Commun 282:865–874
Kim J, Lee J (2011) Observation and quantification of self-associated adenosine extracted from royal jelly products purchased in USA by HPLC. Food Chem 126:347–352
Kimura Y, Washino N, Yonekura M (1995) N-linked sugar chains of 350-kDa royal jelly glycoprotein. Biosci Biotechnol Biochem 59:507–509
Kimura M, Kimura Y, Tsumura K, Okihara K, Sugimoto H, Yamad H, Yonekura M (2003) 350-kDa royal jelly glycoprotein (apisin), which stimulates proliferation of human monocytes, bears the β1-3 galactosylated N-glycan: analysis of the N-glycosylation site. Biosci Biotechnol Biochem 67:2055–2058
Koya-Miyata S, Okamoto I, Ushio S, Iwaki K, Ikeda M, Kurimoto M (2004) Identification of a collagen production-promoting factor from an extract of royal jelly and its possible mechanism. Biosci Biotechnol Biochem 68:767–773
Lercker G, Capella P, Conte LS, Ruini F, Giordani G (1981) Components of royal jelly: I Identification of the organic acid. Lipids 16(12):912–919
Lercker G (1986) Carbohydrate determination of royal jelly by high resolution gas chromatography (HRGC). Food Chem 19:255–264
Lercker G, Caboni MF, Vecchi MA, Sabatini AG, Nanetti A (1992) Characterizaton of the main constituents of royal jelly. APIC 410:27–37
Li J, Wang T, Zhang Z, Pan Y (2007) Proteomic analysis of royal jelly from three strains of western honeybees (Apis mellifera). J Agric Food Chem 55:8411–8422
Li X, Huang C, Xue Y (2013) Contribution of lipids in honeybee (*Apis mellifera*) royal jelly to health. J Med Food 16:96–102
Majtan J, Kovacova E, Blikova K, Simuth J (2006) The immunostimulatory effect of the recombinant apalbumin 1-major honeybee royal jelly protein-on TNFα release. Int Immunopharmacol 6(2):269–278
Melliou E, Chinou I (2005) Chemistry and bioactivity of royal jelly from Greece. J Agric Food Chem 53(23):8987–8992
Nakaya M, Onda H, Sasaki K, Yukiyoshi A, Tachibana H, Yamada K (2007) Effect of royal jelly on bisphenol A-induced proliferation of human breast cancer cells. Biosci Biotechnol Biochem 71:253–255

Narita Y, Nomura J, Ohta S, Inoh Y, Suzuki KM, Araki Y, Okada S, Matsumoto I, Isohama Y, Abe K, Miyata T, Mishima S (2006) Royal jelly stimulates bone formation: physiologic and nutrigenomic studies with mice and cell lines. Biosci Biotechnol Biochem 70:2508–2514

Nozaki R, Tamura S, Ito A, Moriyama T, Yamaguchi K, Kono T (2012) A rapid method to isolate soluble royal jelly proteins. Food Chem 134:2332–2337

Okamoto I, Taniguchi Y, Kunikata T, Kohno K, Iwaki K, Ikeda M, Kurimoto M (2003) Major royal jelly protein 3 modulates immune responses *in vitro* and *in vivo*. Life Sci 73:2029–2045

Patterson LM, Zheng H, Ward SM, Berthoud HR (2003) Vanilloid receptor (VR1) expression in vagal afferent neurons innervating the gastrointestinal tract. Cell Tissue Res 311:277–287

Peiren N, Vanrobaeys F, De Graaf DC, Devreese B, Van Beeumen J, Jacobs FJ (2005) The protein composition of honeybee venom reconsidered by a proteomic approach. Biochim Biophys Acta 1752:1–5

Peiren N, De Graaf DC, Vanrobaeys F, Danneels EL, Devreese B, Van Beeumen J, Jacobs FJ (2008) Proteomic analysis of the honey bee worker venom gland focusing on the mechanisms of protection against tissue damage. Toxicon 52:72–83

Popescu O, Mărghitas LA, Bobis O, Stanciu O, Bonta V, Moise A, Dezmirean D (2009) Sugar profile and total proteins content of fresh royal jelly. Bull UASVM Anim Sci Biotechnol 66:265–269

Ramadan MF, Al-Ghamdi A (2012) Bioactive compounds and health-promoting properties of royal jelly: a review. J Func Foods 4:39–52

Rembold H (1965) Biologically active substances in royal jelly. Vitam Horm 23:359–382

Rembold H, Lackner B (1978) Vergleichende Analyse von Weiselfuttersäften. Mitteilungen der Deutschen Gesellschaft für allgemeine und angewandte Entomologie 1(2/3/4):299–301

Sano O, Kunikata T, Kohno K, Iwaki K, Ikeda M, Kurimoto M (2004) Characterization of royal jelly proteins in both Africanized and European honeybees (*Apis mellifera*) by two-dimensional gel electrophoresis. J Agric Food Chem 52(1):15–20

Santos KS, Dos Santos LD, Mendes MA, De Souza BM, Malaspina O, Palma MS (2005) Profiling the proteome complement of the secretion from hypopharyngeal gland of Africanized nurse-honeybees (*Apis mellifera* L.) Insect Biochem Mol Biol 35:85–91

Schmitzova J, Klaudiny J, Albert S, Schroder W, Schreckengost W, Hanes J, Judova J, Simuth J (1998) A family of major royal jelly proteins of the honeybee *Apis mellifera* L. Cell Mol Life Sci 54(9):1020–1030

Schonleben S, Sickmann A, Mueller MJ, Reinders J (2007) Proteome analysis of *Apis mellifera* royal jelly. Anal Bioanal Chem 389(4):1087–1093

Sesta G (2006) Determination of sugars in royal jelly by HPLC. Apidologie 37:84–90

Shahidi F, Zhong Y (2011) Revisiting the polar paradox theory: a critical overview. J Agric Food Chem 59:3499–3504

Shen L, Zhang W, Jin F, Zhang L, Chen Z, Liu L, Parnell LD, Lai C, Li D (2010) Expression of recombinant AccMRJP1 protein from royal jelly of Chinese honeybee in *Pichia pastoris* and its proliferation activity in an insect cell line. J Agric Food Chem 58:9190–9197

Simuth J, Bilikova K, Kovacova E, Kuzmova Z, Schroder W (2004) Immunochemical approach to detection of adulteration in honey: physiologically active royal jelly protein stimulating TNF-α is a regular component of honey. J Agric Food Chem 52:2154–2158

Tamura S, Amano S, Kono T, Kondoh J, Yamaguchi K, Kobayashi S, Ayabe A, Moriyama T (2009) Molecular characteristics and physiological functions of major royal jelly protein 1 oligomer. Proteomics 9:5534–5543

Terada Y, Narukawa M, Watanabe T (2011) Specific hydroxy fatty acids in royal jelly activate TRPA1. J Agric Food Chem 59:2627–2635

Townsend GF, Brown WH, Felauer EE, Hazlett B (1961) Studies on the *in vitro* antitumor activity of fatty acids. IV. The esters of acids closely related to 10-hydroxy-2-decenoic acids from royal jelly against transplantable mouse leukemia. Can J Biochem Physiol 39:1765–1770

Vucevic D, Melliou E, Vasilijic S, Gasic S, Ivanovski P, Chinou I, Colic M (2007) Fatty acids isolated from royal jelly modulate dendritic cell-mediated immune response *in vitro*. Int Immunopharmacol 7:1211–1220

Wahl SM, Swisher J, McCartney-Francis N, Chen W (2004) TGF-{beta}: the perpetrator of immune suppression by regulatory T cells and suicidal T cells. J Leukoc Biol 76:15–24

Ward SM, Bayguinov J, Won KJ, Grundy D, Berthoud HR (2003) Distribution of the vanilloid receptor (VR1) in the gastrointestinal tract. J Comp Neurol 465:121–135

Watanabe K, Shinmoto H, Kobori M, Tsushida T, Shinohara K, Kanaeda J, Yonekura M (1998) Stimulation of cell growth in the U-937 human myeloid cell line by honey royal jelly protein. Cytotechnology 26:23–27

Wu L, Zhou J, Xue X, Li Y, Zhao J (2009) Fast determination of 26 amino acids and their content changes in royal jelly during storage using ultra-performance liquid chromatography. J Food Comp Anal 22:242–249

Wu L, Chen L, Selvaraj JN, Wei Y, Wang Y, Li Y, Zhao J, Xue X (2015) Identification of the distribution of adenosine phosphates, nucleosides and nucleobases in royal jelly. Food Chem 173:1111–1118

Wytrychowski M, Chenavas S, Daniele G, Casabianca H, Batteau M, Guibert S, Brion B (2013) Physicochemical characterisation of French royal jelly: comparison with commercial royal jellies and royal jellies produced through artificial bee-feeding. J Food Comp Anal 29:126–133

Xu X, Gao Y (2013) Isolation and characterization of proteins and lipids from honeybee (*Apis mellifera* L.) queen larvae and royal jelly. Food Res Int 54:330–337

Xue XF, Wang F, Zhou JH, Chen F, Li Y, Zhao J (2009a) Online cleanup of accelerated solvent extractions for determination of adenosine 5′-triphosphate (ATP), adenosine 5′-diphosphate (ADP), and adenosine 5′-monophosphate (AMP) in royal jelly using high performance liquid chromatography. J Agric Food Chem 57:4500–4505

Xue X, Zhou J, Wu L, Fu L, Zhao J (2009b) HPLC determination of adenosine in royal jelly. Food Chem 115:715–719

Yoshioka M, St-Pierre S, Suzuki M, Tremblay A (1998) Effects of red pepper added to high-fat and high-carbohydrate meals on energy metabolism and substrate utilization in Japanese women. Br J Nutr 80:503–510

Zhang L, Fang Y, Li R, Feng M, Han B, Zhou T, Li J (2012) Towards posttranslational modification proteome of royal jelly. J Proteome 75:5327–5341

Zhao Y, Li Z, Tian W, Fang X, Su S, Peng W (2016) Differential volatile organic compounds in royal jelly associated with different nectar plants. J Integr Agric 15:1157–1165

Zhou J, Xue X, Chen F, Zhang J, Li Y, Wu L, Chen L, Zhao J (2009) Simultaneous determination of seven fluoroquinolones in royal jelly by ultrasonic-assisted extraction and liquid chromatography with fluorescence detection. J Sep Sci 32(7):955–964

Zhou L, Xue X, Zhou J, Li Y, Zhao J, Wu L (2012) Fast determination of adenosine 5′-triphosphate (ATP) and its catabolites in royal jelly using ultra performance liquid chromatography. J Agric Food Chem 60:8994–8999

Chapter 9
Volatile Compounds of Royal Jelly

Maria G. Miguel and Soukaïna El-Guendouz

Contents

9.1 Introduction

Royal jelly (RJ) is a secretion rich in protein and lipids produced by the hypopharyngeal and mandibular glands, respectively, of *Appis mellifera* L. young worker honeybees (called nurses) (Fujita et al. 2013). RJ is used by nurses to feed young larvae of workers and drones for the first 3 days, and is the exclusive food of the queen bee throughout its life. After this 3 day period, worker larvae are fed a mixture of RJ, pollen and honey (Boselli et al. 2003; Ferioli et al. 2014; Wang et al. 2016). Workers and queens are females developed from fertilized eggs, whereas unfertilized eggs become males, called drones (Wang et al. 2016).

RJ is a viscous and creamy product. It is relatively acidic (pH 3.1–3.9) with a high buffering capacity ranging between 4 and 7, has a density of 1.1 g/mL, is partially soluble in water, ranges in colour from white to yellow, and has a slightly pungent odour and taste (Ramadan and Al-Ghamdi 2012; Nabas et al. 2014; Mureşan et al. 2016).

M.G. Miguel (✉)
Faculdade de Ciências e Tecnologia, Universidade do Algarve, MeditBio, Campus de Gambelas, 8005-139 Faro, Portugal
e-mail: mgmiguel@ualg.pt

S. El-Guendouz
Laboratory of Physiology-Pharmacology-Environmental Health, Faculty of Sciences Dhar El Mehraz, University Sidi Mohamed Ben Abdallah, BP 1796, Atlas, Fez 30 000, Morocco

J.M. Alvarez-Suarez (ed.), *Bee Products - Chemical and Biological Properties*,
DOI 10.1007/978-3-319-59689-1_9

RJ is mainly composed of water (60–70%), proteins (9–18%), carbohydrates (7–18%), lipids (3–8%), minerals (0.8–3%), vitamins, phenols and amino acids (Balkanska et al. 2014).

Major RJ proteins (MRJPs) are water soluble, constituting more than 80% of RJ proteins. The MRJP family has nine members, MRJP1-MRJP9, with molecular masses of 49–87 kDa (Bărnuţiu et al. 2011; Ramadan and Al-Ghamdi 2012 and references therein). In some MRJPs there are repetitive pentapeptide regions concentrated with nitrogen-rich amino acids, and others with repetitive tripeptide regions concentrated with methionine (MRJP5) (Drapeau et al. 2006). Peptides are also constituents of RJ which possess antioxidant activity, mainly due to their capacity to scavenge hydroxyl free radicals, particularly those containing tyrosyl residues at their C-termini (Guo et al. 2009). Free amino acids are also found in RJ, with proline, glycine, lysine and glutamic acid being the most abundant (Liming et al. 2009); although Boselli et al. (2003) also reported alanine, aspartic acid, homoserine, isoleucine, hydroxyproline, ornitine, phenylalanine, serine, threonine, tyrosine and valine as being present in considerable amounts.

Fructose and glucose are the main carbohydrates of RJ (over 90% of the total sugars), although fructose is the most predominant. Sucrose (in high variable concentrations) and oligosccharides trehalose, maltose, gentiobiose, isomaltose, raffinose, erlose, and melezitose (in very small concentrations) are also carbohydrates present in RJ.

The lipid fraction is predominantly composed of organic acids (80–90%), almost all in the free form (mono and dihydroxy acids and dicarboxylic acids with 8 and 10 carbon atoms) with characteristic arrangements. Phenols (4–10%), waxes (5–6%), steroids (3–4%) and phospholipids (0.4–0.8%) are also constituents of the lipid fraction. The fatty acid fraction predominantly consists of monocarboxylic acids *trans*-10-hydroxy-2-decenoic acid (HDEA or 10-HDA) (32%), gluconic acid (24%) and 10-hydroxydecanoic acid (HDAA) (22%). Other monocarboxylic acids have also been reported in RJ (7- and 8-hydroxyoctanoic, 3-hydroxydecanoic, 9-hydroxydecanoic, 9-hydroxy-2-decenoic, 10-hydroxydecanoic, and 3,10-dihydroxydecanoic acids). Dicarboxylic acids are also present in RJ, but in lower amounts than hydroxycarboxylic acids (e.g., 2-octene-1,8-dioic and 2-decene-1,10-dioic acids). Mono- and di-esters of 10-hydroxy-2-*trans*-decenoic acid in which the hydroxyl group is esterified by another fatty acid were also reported in RJ, along with one hydroxyl-2-*trans*-decenoic acid 10-phosphate (Noda et al. 2005; Sabatini et al. 2009; Terada et al. 2011; Ramadan and Al-Ghamdi 2012; Ferioli et al. 2014).

In the lipid fraction of RJ, Lercker et al. (1982) also detected sterols in the unsaponifiable extracts, such as 24-methylenecholesterol, the most abundant, along with sitosterol and Δ5-avenasterol in much lower amounts. More recently, other authors (Ferioli et al. 2014; Isidorov et al. 2012) also reported the presence of such phytosterols in Italian and Polish RJ samples. Isidorov et al. (2012) reported the presence of 25-hydroxy-24-methylcholesterol in Polish RJ samples. Campesterol was also detected in Polish RJ by Isidorov et al. (2016).

Only water soluble vitamins have been reported in RJ. Pantothenic acid (B5) is the predominant vitamin in RJ, although others may also be present but in lower amounts: e.g., thiamine (B1), riboflavin (B2), niacin (B3), pyridoxal (B6), biotin (B7), folic acid and ascorbic acid (vitamin C) (only in trace amounts) (Ciulu et al. 2013; Bogdanov et al. 2016).

The most abundant elements in RJ include, in descending order: K, Ca, Na, Mg, Zn, Fe, Cu, Mn. Trace elements may also be found, such as Al, Ba, Sr, Bi, Cd, Hg, Pb, Sn, Te, Tl, W, Sb, Cr, Ni, Ti, V, Co, Mo (Sabatini et al. 2009; Nabas et al. 2014).

Several authors (Nagai and Inoue 2004; Liu et al. 2008; Ramadan and Al-Ghamdi 2012; Nabas et al. 2014) refer to the presence of phenols in RJ but do not provide a phenolic profile. In a review by Gómez-Caravaca et al. (2006) focusing on the analysis of phenolic compounds in bee-derived products, the authors stated that no studies analysing the phenolic profile of royal jelly had been found. Generally, the antioxidant activity of samples is attributed to phenols. Some authors (Guo et al. 2009) reported that the antioxidant activity of royal jelly is attributed to the tyrosyl residues of amino acids, which contain a phenolic group. The quantification of total phenols in these studies could have therefore resulted from the presence of this amino acid in RJ. However, Isidorov et al. (2012) identified several phenolic compounds either in the volatile fraction, obtained by head space-solid phase microextraction (HS-SPME), or in the ether extracts. The phenolic compounds are presented in Fig. 9.1.

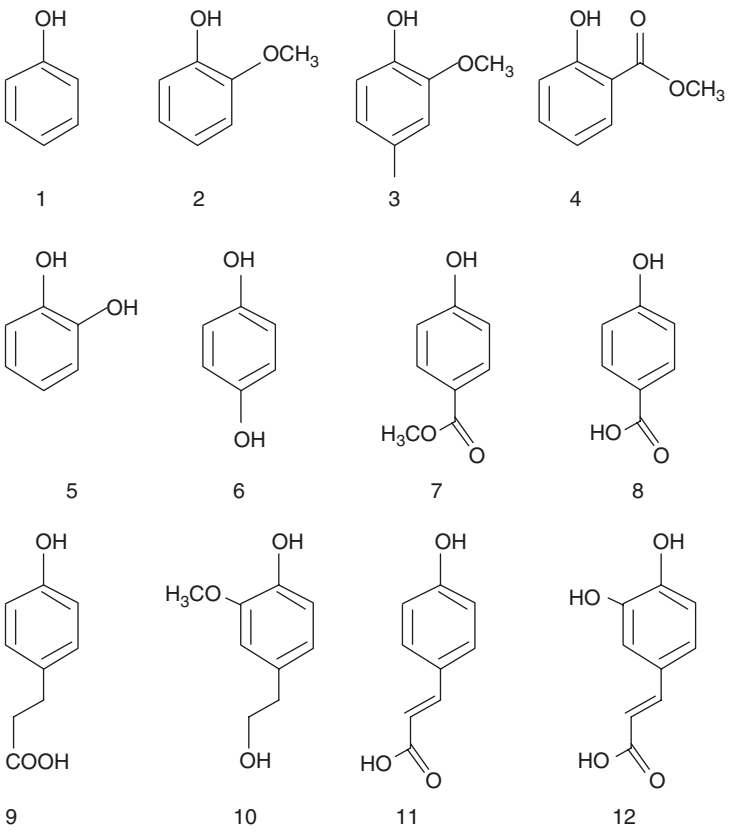


Fig. 9.1 Phenols detected in Polish RJ. In *volatile fraction*: **1**: phenol; **2**: 2-methoxyphenol (*o*-guaiacol); **3**: 2-methoxy-*p*-cresol; **4**: methyl salicylate. In *ether extract*: **5**: pyrocatechol; **6**: hydroquinone; **7**: 4-hydroxybenzoic acid, methylester; **8**: 4-hydroxybenzoic acid; **9**: 4-hydroxyhydrocinnamic acid; **10**: 4-hydroxy-3-methoxyphenylethanol; **11**: *p*-coumaric acid; **12**: caffeic acid

In general, the composition of RJ depends on seasonal and regional conditions (Ramadan and Al-Ghamdi 2012).

9.2 Volatiles in Royal Jelly

The volatiles in RJ have not been studied in detail; for this reason, very few references which included the identification of such compounds in RJ were found (Boch et al. 1979; Lercker et al. 1981; Drijfhout et al. 2005; Nazzi et al. 2009; Isidorov et al. 2012; Zhao et al. 2016).

Royal jelly from colonies consisting of *Apis melliefera ligustica* and *A. mellifera carnica*, located in north eastern Italy, was extracted with diethyl ether and derivatized by silylation (Nazzi et al. 2009). After this procedure, the authors identified 26 compounds by chromatography coupled to mass spectrometry (GC-MS). The compounds were predominantly carboxylic acids (23 aliphatic carboxylic acids and 1 aromatic carboxylic acid). The remaining two compounds identified were hydroquinone and methyl-4-hydroxybenzoate. Two other compounds were not identified. Seventeen compounds found by Nazzi et al. (2009) had already been reported by other authors (Boch et al. 1979; Lercker et al. 1981; Drijfhout et al. 2005). The new compounds found in RJ by Nazzi et al. (2009) were 2-methylbutanoic acid, 3-methylbutanoic acid, pentanoic acid, 2-hexenoic acid, 2-hydroxy-4-methylpentanoic acid, 2-hydroxyhexanoic acid, phenylacetic acid, hydroquinone and methyl-4-hydroxybenzoate.

None of the compounds reported above were detected 3 years later by Isidorov et al. (2012) in volatile fractions of RJ from two colonies of *A. mellifera carnica* situated in two distinct regions of Poland (north eastern and south western), obtained after extraction by HS-SPME and analysed by GC-MS. According to the authors, the volatile fraction predominantly consisted of carbonyl compounds (37.2–43.3%), particularly ketones, with 2-heptanone alone accounting for 20%. The other carbonyl compounds identified were, in descending order of quantity: acetone > 2-nonanone > benzaldehyde > 2-butanone > 2-pentanone, hexanal (all of them in significantly lower percentages than 2-heptanone). The second major component was the alkene 1-pentadecene (17–21%). Phenols were the third major group of compounds (7.2–12%), in descending order of quantity: phenol > methyl salicylate > 2-methoxyphenol > 2-methoxy-*p*-cresol.

In the same work, the authors (Isidorov et al. 2012) evaluated the effect of storage temperature (−18 °C, 4 °C and room temperature) on the volatile profile of RJ. They verified that samples kept for 10 months at room temperature showed the greatest quantitative modifications, particularly the phenol content which decreased twofold (phenol from 12 to 7% and methyl salicylate from 7 to 3%), as well as the volatile C2 and C4 acid content (acetic acid from traces to 7% and butyric acid from not detected to 5%) and C5 aldehyde content (2-methylbutanal, 3-methylbutanal and 2-methyl-2-butenal were absent or in trace amounts in the fresh material while after 10 months their percentages were about 1%). The authors suggested that the

increase in C2 and C4 carboxylic acids in RJ kept at room temperature may have been due to rancidity (Isidorov et al. 2012).

More recently, Zhao et al. (2016) also used GC-MS to analyse the volatile fraction of 30 RJ samples collected from 30 apiaries during flowering seasons of different nectar plants (ten types of RJ: *Schisandra chinensis*, *Medicago sativa*, *Vitex negundo*, *Citrullus lanatus*, *Sophora alopecuroides*, *Brassica campestris*, *Robinia pseudoacacia*, *Helianthus annus*, *Litchi chinensis*, *Tilia tuan*) in China after extraction by HS-SPME. A total of 89 compounds were identified by the authors, distributed amongst the following compound classes: esters and aldehydes (25 and 17.5%, respectively), ketones (15%), acids (10%), and alcohols (10%). Although 89 compounds were identified, the authors prudently decided that only 40 of these could be constitutive volatile components of RJ samples because they were present in relatively high amounts and in at least 2 types of RJ.

Zhao et al. (2016) found that 2-nonanone and acetic acid were common to all RJ samples, whereas octanoic acid, benzaldehyde and phenol were present in 9 RJ types, toluene and octanoic acid were present in 8 RJ types, and 2-pentanone and (benzoic acid, methyl ester) were present in 7 RJ types. On the other hand, there were 14 compounds that were only present in 2–4 RJ types, thus making them unsuitable for classification purposes: (1-propen-2-ol, formate), ethanol, (butanoic acid, ethyl ester), (acetic acid, butyl ester), hexanal, ethylbenzene, octanal, (benzene, 1,2,3,5-tetramethyl-), (2-furancarboxaldehyde, 5-methyl-), benzoxazole, 2-furanmethanol, methyl salicylate, (4H–pyran-4-one,2,3-dihydro-3,5-dihydroxy-6-methyl-), and 2-furan-carboxaldehyde, 5-(hydroxymethyl)-). Ethanol and furfural, which are not constitutive compounds of RJ, were present in some samples possibly due to the fermentation of carbohydrates and heating during the extraction process, respectively (Zhao et al. 2016).

The results obtained by Zhao et al. (2016) allowed the classification of all samples into three main groups; nevertheless, there was one group which only contained RJ from the flowering season of *Tilia tuan*, which was considerably different from the samples belonging to the other two groups. This sample mainly consisted of alcohols, whereas acids, esters, aldehydes and ketones predominated in diverse proportions in the other samples, allowing them to be grouped in the remaining classes.

In short, there are differences in the chemical composition of the volatile fractions of RJ samples studied by several authors (Nazzi et al. 2009; Isidorov et al. 2012; Zhao et al. 2016). Several factors were pointed out by Zhao et al. (2016) as being responsible for these differences: honeybee species, harvesting time, region, storage method and processing technology. In fact, when comparing the results obtained by these three groups it is possible to verify differences, even within the same group when working with different RJ of varying origin. Nevertheless, when only comparing the results of Zhao et al. (2016) and Isidorov et al. (2012), who extracted the volatile fraction of RJ samples by the same procedure (HS-SPME) although using different fibers, a common compound is found in all samples: 2-nonanone. This seems to be a general characteristic of the volatile fraction of RJ, independent of the nectar plant, fiber, region, honeybee species, storage method and processing technology. Other components were also common to both works:

ethanol, acetic acid, ethylacetate, 2-pentanone, 2-methylbutanal, toluene, hexanal, benzaldehyde, phenol, octanoic acid, and methylsalicilate; however, Zhao et al. (2016) found that these compounds could also be absent in at least one sample in the study. Such absences in RJ of different nectar origin reveal that the presence of these compounds may be dependent on the nectar plant. This is reinforced by the fact that octanoic acid was also identified in the study of Nazzi et al. (2009), despite the use of a different extraction method to that of Zhao et al. (2016). However, the group also verified that octanoic acid was absent in the flowering season of *Sophora alopecuroides*.

The presence of carbonyl and volatile phenolic compounds along with octanoic acid in RJ may give rise to repellent effects against the mite *Varroa destructor* and antibacterial activity (Isidorov et al. 2012).

References

Balkanska R, Karadjova I, Ignatova M (2014) Comparative analyses of chemical composition of royal jelly and drone brood. Bulg Chem Commun 46:412–416

Bărnuţiu LI, Mărghitas LA, Dezmirean DS, Mihai CM, Bobiş O (2011) Chemical composition and antimicrobial activity of royal jelly – review. J Anim Sci Biotechnol 44:67–72

Boch R, Shearer DA, Shuel RW (1979) Octanoic and other volatile acids in the mandibular glands of the honeybee and in royal jelly. J Apicult Res 18:250–252

Bogdanov S (2016) Royal jelly, bee brood: composition, health, medicine: A review. Bee Product Science, from www.bee-hexagon.net. April 2016

Boselli E, Caboni MF, Sabatini AG, Marcazzan GL, Lercker G (2003) Determination and changes of free amino acids in royal jelly during storage. Apidologie 34:129–137

Ciulu M, Floris I, Nurchi VM, Panzanelli A, Pilo MI, Spano N, Sanna G (2013) HPLC determination of pantothenic acid in royal jelly. Anal Methods 5:6682–6685

Drijfhout FP, Kochansky J, Lin S, Calderone NW (2005) Components of honeybee royal jelly as deterrents of the parasitic varroa mite, Varroa destructor. J Chem Ecol 31:1747–1764

Drapeau MD, Albert S, Kucharski R, Prusko C, Maleszka R (2006) Evolution of the yellow/major royal jelly protein family and the emergence of social behaviour in honey bees. Genome Res 16:1385–1394

Ferioli F, Armaforte E, Caboni MF (2014) Comparison of the lipid content, fatty acid profile and sterol composition in local Italian and commercial royal jelly samples. J Am Oil Chem Soc 91:875–884

Fujita T, Kozuka-Hata H, Ao-Kondo H, Kunieda T, Oyama M, Kubo T (2013) Proteomic analysis of the royal jelly and characterization of the functions of its derivation glands in the honeybee. J Proteome Res 12:404–411

Gómez-Caravaca AM, Gómez-Romero M, Arráez-Román D, Segura-Carretero A, Fernández-Gutíerrez A (2006) Advances in the analysis of phenolic compounds in products derived from bees. J Pharm Biomed Anal 41:1220–1234

Guo H, Kouzuma Y, Yonekura M (2009) Structure and properties of antioxidative peptides derived from royal jelly proteína. Food Chem 113:238–245

Isidorov VA, Bakier S, Grzech I (2012) Gas-chromatographic-mass spectrometric investigation of volatile and extractable compounds of crude royal jelly. J Chromatogr B Analyt Technol Biomed Life Sci 885–886:109–116

Isidorov VA, Bakier S, Stocki M (2016) GC-MS investigation of the chemical composition of honeybee drone and queen larva homogenate. J Apic Sci 60. doi:10.1515/JAS-2016-0011.

Lercker G, Capella P, Conte LS, Ruini F (1981) Components of royal jelly: I. Identification of the organic acids. Lipids 16:912–919

Lercker G, Capella P, Conte LS, Ruini F, Giordani G (1982) Components of royal jelly II: the lipid fraction, hydrocarbons and sterols. J Apic Res 21:178–184

Liming W, Jinhui Z, Xiaofeng X, Yi L, Jing Z (2009) Fast determination of 26 amino acids and their content changes in royal jelly during storage using ultra-performance liquid chromatography. J Food Compos Anal 22:242–249

Liu J-R, Yang Y-C, Shi L-S, Peng C-C (2008) Antioxidant properties of royal jelly associated with larval age and time of harvest. J Agric Food Chem 56:11,447–11,452

Mureşan CI, Mărghitaş LA, Dezmirean DS, Bobiş O, Bonta V, Zacharias I, Mărgãoan R, Paşca C (2016) Quality parameters for commercialized royal jelly. Bull UASVM Anim Sci Biotechnol 73:1. doi:10.15835/buasvmcn-asb:11630

Nabas Z, Haddadin MSY, Haddadin J, Nazer IK (2014) Chemical composition of royal jelly and effects on symbiotic with two different locally isolated probiotic strains on antioxidant activities. Pol J Food Nutr Sci 64:171–180

Nagai T, Inoue R (2004) Preparation and the functional properties of water extract and alkaline extract of royal jelly. Food Chem 84:181–186

Nazzi F, Bortolomeazzi R, della Vedova G, del Piccolo F, Annoscia D, Milani N (2009) Octanoic acid confers to royal jelly varroa - repellent properties. Naturwissenschaften 96:309–314

Noda N, Umebayashi K, Nakatani T, Miyahara K, Ishiyama K (2005) Isolation and characterization of some hydroxy fatty and phosphoric acid and esters of 10-hydroxy-2-decenoic acid from the royal jelly of honey bees (*Apis mellifera*). Lipids 40:833–838

Ramadan MF, Al-Ghamdi A (2012) Bioactive compounds and health-promoting properties of royal jelly: a review. J Funct Foods 4:39–52

Sabatini AG, Marcazzan GL, Caboni MF, Bogdanov S, de Almeida-Muradian LB (2009) Quality and standardisation of royal jelly. JAAS 1:1–6

Terada Y, Narukava M, Watanabe M (2011) Specific hydroxyl fatty acids in royal jelly activate TRPA1. J Agric Food Chem 59:2627–2635

Wang Y, Ma L, Zhang W, Cui X, Wang H, Xu B (2016) Comparison of the nutrient composition of royal jelly and worker jelly of honey bees (*Apis mellifera*). Apidologie 47:48–56

Zhao Y-z, Li Z-g, Tian W-l, Fang X-m, Su S-k, Peng W-j (2016) Differential volatile organic compounds in royal jelly associated with different nectar plants. J Integr Agric 15:1157–1165. doi:10.1016/S2095–3119(15)61274-6

Chapter 10
Royal Jelly: Health Benefits and Uses in Medicine

Manuel Viuda-Martos, José A. Pérez-Alvarez, and Juana Fernández-López

Contents

10.1 Introduction

The hypopharyngeal glands of the honeybee (*Apis mellifera* L.) produce royal jelly (RJ), which is essential for feeding and raising broods and queens. RJ is the sole food source for all bee larvae between hatching and the third day of life; those larvae which are selected to develop into queens are fed with RJ until the fifth day of larval life (the time at which the cell is operculated), and it then remains a main source of food for the queen bee for the duration of her life (Fratini et al. 2016). It has a gelatinous consistency, often not homogenous due to the presence of undissolved granules of varying size composed mainly of pollen grains derived from the foraging

M. Viuda-Martos • J.A. Pérez-Alvarez • J. Fernández-López (✉)
IPOA Research Group (UMH-1 and REVIV-Generalitat Valenciana), AgroFood Technology Department, Escuela Politécnica Superior de Orihuela, Miguel Hernández University, Crta. Beniel km. 3,2, 03312 Orihuela, Alicante, Spain
e-mail: j.fernandez@umh.es

J.M. Alvarez-Suarez (ed.), *Bee Products - Chemical and Biological Properties*,
DOI 10.1007/978-3-319-59689-1_10

activity of honeybees. It has a distinctively sharp odor and taste, and is partially soluble in water and highly acidic (pH 3.4–4.5) (Sabatini et al. 2009). Chemically, RJ is comprised of water (50–60%), proteins (18%), carbohydrates (15%), lipids (3–6%), mineral salts (1.5%) and vitamins (Nagai and Inoue 2004), together with a huge number of bioactive compounds: principally peptides, such as royalisin, jelleines, aspimin and royalactina (Fratini et al. 2016); polyphenols, mainly phenolic acids and flavonoids (López-Gutiérrez et al. 2014); and lipids, such as 10-Hydroxy-2-Decenoic Acid (10-HDA) (Caparica-Santos and Marcucci 2007). Nevertheless, RJ composition can vary with climate; environmental, seasonal and regional conditions; metabolites and changes in the physiology of nurse bees and/or with larval age; as well as with bee genetics and race (Antinelli et al. 2003; Attalla et al. 2007; Sabatini et al. 2009).

Due to its high content of bioactive compounds, RJ exhibits lots of health-promoting properties, such as antibacterial, antioxidant, anti-inflammatory, vasodilative, hypotensive, hipocholesterolemiant, hepatoprotective, and antitumor activity (Matsui et al. 2002; Koya-Miyata et al. 2004; Jamnik et al. 2007; Rihar et al. 2013; Bilikova et al. 2015). Although the cause and effect relationship has not yet been established between the consumption of RJ and its physiological functions (Agostoni et al. 2011), RJ has been used extensively for improving human health in the food and nutraceutical industry.

This chapter will examine the current literature on the bioactive compound content and health benefits of Royal Jelly, with particular focus on its use in medicine.

10.2 Antibacterial Activity

For many years, a range of different chemical and synthetic compounds have been used as antibacterial and antifungal agents to inhibit microbial spoilage. However, owing to economic impact and consumers' growing concerns over the safety of products containing synthetic chemicals, much attention has been focused on natural or naturally-derived compounds (Alzoreky and Nakahara 2003). Additionally, the scientific community is interested in biologically active compounds obtained and isolated from natural sources for eliminating pathogenic microorganisms due to concerns over antibiotic resistance. As such, the antibacterial properties of RJ against Gram-positive and Gram-negative bacteria have been widely demonstrated by several authors. Ratanavalachai and Wongchai (2002) evaluated the antibacterial activity and potency of intact RJ from northern Thailand against several bacterial strains. The authors found that the RJ showed minimum inhibitory concentration values of 0.3, 15.5 and 15.5 mg/mL against *Micrococcus luteus*, *Proteus vulgaris* and *Pseudomonas aeruginosa*, respectively. In another study, Eshraghi (2005) found that RJ concentrations of 200, 330 and 1000 mg/mL inhibited the growth of *Streptomyces griseus* (ATCC 11746), *Staphylococcus aureus* (ATCC 14776) and *Escherichia coli* (ATCC 29532), respectively. Similarly, Melliou and Chinou (2005) studied the antibacterial activity of RJ from Greece against two Gram-positive

bacteria, *S. aureus* and *Staphylococcus epidermidis*, and four Gram-negative bacteria, *E. coli*, *Enterobacter cloacae*, *Klebsiella pneumoniae* and *Ps. aeruginosa*. The authors reported minimum inhibitory concentration values of 0.67, 0.74, 0.95, 1.20, 0.98 and 0.94 mg/mL, respectively. They attributed the antibacterial activity mainly to the fatty acids present in RJ, such as *trans*-10-hydroxydec-2-enoic acid, 3-hydroxydodecanoic acid, 11-oxododecanoic acid, and 11-*S*-hydroxydodecanoic acid. Likewise, Moselhy et al. (2013) investigated the antimicrobial effects of royal jelly produced by honeybees and collected from Egypt and China against four different types of bacteria (*E. coli*, *Ps. aeruginosa*, *S. aureus*, and *Bacillus subtilis*). The authors found that both Egyptian and Chinese royal jelly had minimum inhibitory concentration values of 500 μg/mL for *S. aureus* and *B. subtilis*, while they were inactive against *E. coli* and *Ps. aeruginosa.* In another study, Garcia et al. (2013) analyzed the antibacterial activity of RJ from Argentina and they reported that the growth of all the tested bacterial strains was inhibited by the RJ samples studied. These authors found minimum inhibitory concentration values of 53.3 mg/g for *Staphylococcus* sp., 69.5 mg/g for *Enterococcus* sp., and 74.5 mg/g for *Streptococcus* sp. Dinkov et al. (2016) investigated the antibacterial effect of royal jelly obtained from beekeepers from the region of Stara Zagora, Bulgaria, against Methicillin-resistant *S. aureus* (MRSA). The authors reported that at least 3 decimal reductions in MRSA count were observed in Tryptone Soy with concentrations of both 20 and 30% royal jelly.

According to Eshraghi (2005), the antibacterial action of RJ could be attributed to the ether-soluble fraction, particularly the 10-carbon fatty acid molecule 10-hydroxydecenoic acid (10-DHA). However, it should be born in mind that other substances, such as peptides, could also contribute to the antibacterial activity. Protein and peptides in RJ can participate in honeybee defense against microbial pathogens by means of direct inactivation of microorganisms occurring in honeybee products, as well as via induction of cytokines participating in the regulation of transcription of defensive proteins and peptides (Bărnuțiu et al. 2011). As mentioned above, the main peptides found in RJ are royalisin, jelleines, aspimin and royalactin. Bilikova et al. (2002) reported that royalisin, an antibiotic polypeptide previously isolated from the RJ of *Apis mellifera*, showed clearly weaker inhibition of *B. subtilis* at a concentration of 180 μg/mL as compared to tetracyclin at a concentration 50 μg/mL. In a similar study, Shen et al. (2012) analyzed the antibacterial activity of recombinant acc-royalisin from royal jelly of Asian honeybees against Gram-positive and Gram-negative bacteria. The authors reported that both recombinant acc-royalisin and nisin showed antibacterial properties against several Gram-positive bacterial strains, with minimum inhibitory concentrations of 62.5, 125 and 250 μg/mL for *B. subtilis*, *Micrococcus flavus* and *S. aureus* in the microplate assay, respectively. However, the authors also mentioned that recombinant acc-royalisin did not show antimicrobial activity against tested Gram-negative bacteria, such as *E. coli, Salmonella typhimurium* and *Proteus vulgaris*. More recently, Bilikova et al. (2015) analyzed the antibacterial activity of royalisin against Gram-positive and Gram-negative bacteria and found that this peptide produced minimal inhibitory concentration values of 7.5, 9.0, 4.0 and 10.5 μg/mL for Gram-positive *S. aureus*, *Streptococcus*

alactolyticus, *Staphylococcus intermedius B* and *Staphylococcus xylosus*, respectively, and 10.0, 9.0 and 4.0 μg/mL for Gram-negative *Ps. aeruginosa*, *Salmonella cholearasuis* and *Vibro parahaemolyticus*, respectively. Royalisin decreased bacterial cell hydrophobicity and disrupted cell membrane permeability in certain Gram-positive bacteria, inducing disruption and dysfunction of bacterial cell walls and membranes (Shen et al. 2012).

Other important peptides found in RJ with strong antibacterial properties are jelleines (jellein I, jellein II, jellein III and jellein IV). The antimicrobial activity of these peptides has been tested against Gram-positive and Gram-negative bacteria. Fontana et al. (2004) reported that jellein I and II, purified from RJ by reverse phase-HPLC, were active against Gram-positive bacteria as follows: *S. aureus* with minimum inhibitory concentrations of 10.0 and 15.0 μg/mL, respectively; *Staphylococcus saprophyticus* with minimum inhibitory concentrations of 15.0 and 10.0 μg/mL, respectively; and *B. subtilis* with minimum inhibitory concentrations of 10.0 and 30.0 μg/mL, respectively. The authors also reported that these compounds were active against Gram-negative bacteria, as follows: *E. coli* with minimum inhibitory concentrations of 2.5 and 15.0 μg/mL, respectively; *Escherichia cloacae*, *Ps. aeruginosa* and *K. pneumoniae* with minimum inhibitory concentrations of 10.0 and 15.0 μg/mL, respectively. Finally, the authors mentioned that jellein III showed a less broad spectrum of activity, while jellein IV was inactive against all of the tested microorganisms. In a similar study, Romanelli et al. (2011) reported that the peptide jellein I showed total inhibition of bacterial growth for *S. aureus*, *L. monocytogenes* and *S. typhimurium* only at very high concentrations (≥200 μg/mL). At these concentrations, jellein I also exhibited bactericidal activity against these three bacterial species. Jellein II and III, however, were inactive even at 200 μg/mL. Antimicrobial peptides exert their antimicrobial action through one of the following mechanisms: (a) by impairing the synthesis of metabolic enzymes or DNA, e.g. some microbial peptides, which is not completely understood; (b) acting directly on the cell membrane by altering its permeability or by making canals or pores, e.g. Ɩ-helix peptides (Montaño and Vargas Albores 2002).

All of these results provide evidence for the presence of antibacterial compounds in this secretion of *Apis millifera*. These findings clearly demonstrate and confirm the effectiveness of royal jelly in inhibiting microbial activity.

10.3 Anti-inflammatory Activity

Inflammation is a natural defense response of an organism against stimuli such as trauma, infectious agents, toxic and chemical substances, and physical and surgical operations. The inflammatory process is triggered by several chemical and/or biological aspects that include pro-inflammatory enzymes and cytokines, low-molecular-weight compounds such as eicosanoids, or the enzymatic degradation of tissues (Dao et al. 2004). This process, which is the first physiological defense system triggered in the human body, can protect against injuries caused by physical

wounds, poisons, and so on. This defense system, also called short-term inflammation, can destroy infectious microorganisms, eliminate irritants, and maintain normal physiological functions; however, long-term over-inflammation might cause dysfunction of regular physiology, resulting in conditions such as asthma and rheumatic arthritis (Lee et al. 2010).

Royal jelly, or different bioactive compounds such as fatty acids present therein, has been proposed as an anti-inflammatory agent. Fujii et al. (1990) reported that the anti-inflammatory properties of RJ are demonstrated both by inhibitory effects on capillary permeability during the acute phase of inflammation and reduction in granulation tissue in the chronic inflammatory phase in streptozotocin-diabetic rats. Similarly, Taniguchi et al. (2003) indicated that oral administration of RJ prevented the progression of dermatitis-like skin lesions in NC/Nga mice. More recently, Šimúth et al. (2004) reported that apalbumin-1 and apalbumin-2, two major proteins in RJ, stimulate mouse macrophages to release tumor necrosis factor (TNF-α). Kohno et al. (2004) also examined the anti-inflammatory effects of RJ. They reported that when supernatants of RJ suspensions were added to a culture of mouse peritoneal macrophages stimulated with lipopolysaccharide and IFN-gamma, the production of pro-inflammatory cytokines, such as TNF-α, interleukin 6 (IL-6), and interleukin 1 (IL-1), was efficiently inhibited in a dose-dependent manner without having cytotoxic effects on macrophages. This suggests that RJ contains compound(s) responsible for the suppression of proinflammatory cytokine secretion. Karaca et al. (2012) investigated the effects of RJ supplements at doses of 150 mg/kg body weight on acetic acid-induced colitis by monitoring the distribution of CD3+, CD5+, and CD45+ T-cell, and CD68+ cells in rats. The authors reported that colonic CD3+, CD5+ and CD45+ T-cell, and CD68+ macrophage levels were significantly elevated in colitic rats, and no change was induced by RJ. The proliferative response of CD3+ and CD45+ T-cells in rats with colitis was affected by treatment with RJ. No differences were observed for CD5+ T-cells and CD68+ macrophages in colitic rats treated with RJ compared with the control group, indicating that RJ has an anti-inflammatory effect. Recently, Aslan and Aksoy (2015) carried out a study to analyze the anti-inflammatory effects of RJ by inducing renal inflammation in rats with the use of ethylene glycol. At the end of the study, proinflammatory/anti-inflammatory cytokines TNF-α, IL-1β and interleukin 18 (IL-18) levels in blood and renal tissue samples from the rats reflected the anti-inflammatory effects of RJ, possibly due to its antiradical and antioxidative effects. In addition, RJ can have help prevent urolithiasis and reduce inflammation in existing urolithiasis, aiding medical treatment. The anti-inflammatory properties of RJ were also investigated in formalin-induced rat paw edema by Arzi et al. (2015). The authors demonstrated that RJ had a dose-dependent relationship in inhibiting formalin-induced inflammation. Thus, RJ (doses 50 and 100 mg/kg) significantly inhibited formalin-induced rat paw edema as compared to the control group. It was also shown that there was no significant difference between groups treated with doses of 50 and 100 mg/kg and the positive control group (aspirin 300 mg/kg) in inhibiting formalin-induced swelling. A lower dose of 25 mg/kg RJ did not diminish paw swelling in response to formalin, but royal jelly at doses of 50 and 100 mg/kg showed comparable

effects to aspirin (300 mg/kg) in inhibiting paw edema by up to 60%. Yang et al. (2010) investigated *trans*-10-hydroxy-2-decenoic acid (10H2DA), which was purified from RJ, to see if it could inhibit TNF-α induced production of metalloproteinases (MMPs) through the activation of intracellular signaling pathways of mitogen-activated protein kinase (MAPK). The molecular investigation revealed that 10H2DA-mediated suppression was likely to occur through blocking p38 kinase and c-Jun N-terminal kinase-AP-1 signaling pathways. In contrast, 10H2DA had no effect on extracellular signal-regulated kinase activity, nuclear factor-κB DNA-binding activity and IκBα degradation. In a similar work, Sugiyama et al. (2013) had demonstrated that 10-H2DA could inhibit NF-*κ*B activation via suppressing NO production and I*κ*B-*ζ* mRNA expression and transcription stimulated by LPS and IFN-*β*. Also, immunoblotting revealed that 10-H2DA did not inhibit LPS-induced IKK-*α* phosphorylation and I*κ*B*α* degradation. Previously, the effect of 10-hydroxy-*trans*-2-decenoic acid (10H2DA), a major fatty acid component of royal jelly, on LPS-induced cytokine production in murine macrophage cell line RAW264 was investigated by Sugiyama et al. (2012). The authors reported that 10H2DA inhibited LPS-induced IL-6 production dose-dependently, but did not inhibit TNF-α production. 10H2DA inhibited LPS-induced NF-κB activation in a dose-dependent fashion. In addition, NF-κB activation induced by over-expression of either MyD88 or Toll/IL-1 receptor domain-containing adaptor inducing IFN-β (TRIF) was also inhibited by 10H2DA. In another study, Kohno et al. (2015) examined the anti-inflammatory activity of adenosine N1-oxide (ANO), which is found in royal jelly. This molecule is an oxidized product of adenosine at the N1 position of the adenine base moiety. The authors reported that ANO inhibited the secretion of inflammatory mediators at much lower concentrations than adenosine and dipotassium glycyrrhizate when used with peritoneal macrophages and THP-1 cells that were stimulated by lipopolysaccharide (LPS) plus interferon-γ (IFN-γ). The potent anti-inflammatory activity of ANO could not be solely accounted for by its refractoriness to adenosine deaminase. ANO was superior to the synthetic A1 AR-selective agonist, 2-chloro-N6-cyclopentyladenosine (CCPA); A2A AR-selective agonist, 2-[p-(2-carboxyethyl)phenethylamino]-5′-N-ethylcarboxamideadenosine hydrochloride (CGS21680); and A3 AR-selective agonist, N6-(3-iodobenzyl)adenosine-5′-N-methyluronamide (IB-MECA), in suppressing the secretion of a broad spectrum of pro-inflammatory cytokines by peritoneal macrophages. The capacities of ANO to inhibit pro-inflammatory cytokine production by THP-1 cells were comparable to those of CCPA and IB-MECA. Reflecting its potent anti-inflammatory effects in vitro, intravenous administration of ANO significantly reduced the lethality of LPS-induced endotoxin shock. A significant increase in survival rate was also observed by oral administration of ANO. Mechanistic analysis suggested that up-regulation of the anti-inflammatory transcription factor c-Fos was, at least in part, involved in ANO-induced suppression of pro-inflammatory cytokine secretion. Chen et al. (2016) analyzed the anti-inflammatory activity of the three major fatty acids present in RJ: *trans*-10-hydroxy-2-decenoic acid (10-H2DA), 10-hydroxydecanoic acid (10-HDAA), and sebacic acid (SEA). They evaluated and compared the in vitro anti-inflammatory effects of these RJ fatty acids in

lipopolysaccharide-stimulated RAW 264.7 macrophages. The results showed that 10-H2DA, 10-HDAA, and SEA had potent, dose-dependent inhibitory effects on the release of major inflammatory-mediators nitric oxide and interleukin-10, while only SEA decreased TNF-α production. Several key inflammatory genes have also been modulated by these RJ fatty acids, with 10-H2DA showing distinct modulating effects as compared to the other two fatty acids. Additionally, the authors found that these three FAs regulated several proteins involved in MAPK and NF-κB signaling pathways.

10.4 Immunomodulatory Effects

Immunomodulatory effects play an important role in cancer, allergy, and inflammation. They can be activating and deactivating. In the case of RJ the activating effects predominate, as reported in many studies. Hamerlinck (1999) showed that RJ exhibited immunomodulatory properties by stimulating antibody production and immunocompetent cell proliferation in mice, or by depressing humoral immune functions in rats. More recently, Vucevic et al. (2007) studied the effect of 10-hydroxy-2-decanoic acid (10-HDA) and 3,10-dihydroxy-decanoic acid (3,10-DDA), isolated from RJ, on the immune response using a model of rat dendritic cell (DC)-T-cell cocultures. Both fatty acids, at high concentrations, inhibited the proliferation of allogeneic T-cells. The effect of 10-HDA was stronger and was followed by a decrease in interleukin-2 (IL-2) production and down-regulation of IL-2 receptor expression. Spleen DCs, cultivated with 10 μg/mL fatty acids, down-regulated the expression of CD86 and the production of IL-12, but up-regulated the production of IL-10. In contrast, DCs pretreated with 100 μg/mL 3,10-DDA up-regulated the expression of CD86 and augmented the proliferation of allogeneic T-cells. The highest dose (200 μg/mL) of both fatty acids, which was non-apoptotic for both T-cells and DCs, down-regulated the expression of MHC class II and CD86, decreased the production of IL-12, and made the DCs less allostimulatory. In a similar study, Gasic et al. (2007) analyzed the effect of different components isolated from RJ using an in vitro rat T-cell proliferation assay. They found that lower concentrations of a water extract of RJ and 3–10-dihydroxydecanoic acid stimulated T-cell proliferation, triggered by concanavalin A (Con-A), and the process was followed by an increase in the production of interleukin-2 (IL-2). Similarly, higher concentrations of 3–10-dihydroxydecanoic acid, a dry powder of RJ and *trans*-10-hydroxydec-2-enoic acid inhibited T-cell proliferation. The inhibition of T-cell proliferation in the presence of 3–10-dihydroxydecanoic acid was followed by a decrease in IL-2 production, which was partly abrogated by exogenous IL-2, a decrease in nitric oxide (NO) production and increased apoptosis. More recently, Dzopalic et al. (2011) tested the effect of 3,10-dihydroxy-decanoic acid (3,10-DDA), a fatty acid isolated from royal jelly, on the maturation and functions of human monocyte-derived dendritic cells (MoDCs). They showed that 3,10-DDA stimulated maturation of MoDCs by up-regulating the expression of CD40, CD54,

CD86 and CD1a, and increased their allostimulatory potential in co-culture with allogeneic CD4+ T cells. 3,10-DDA-treated MoDCs enhanced the production of IL-12 and IL-18, and stimulated the production of interferon-γ in co-culture with allogeneic CD4+ T cells compared to control MoDCs. In contrast, the production of IL-10 was down-regulated. On the other hand, Majtan et al. (2006) reported that apalbumin 1, the most abundant royal jelly and honey glycoprotein, exerts immunostimulatory and proinflammatory activities by up-regulating the production of tumor necrosis factor α (TNF-α).

10.5 Vasodilative and Hypotensive Activities

High blood pressure (BP) is one of the most prevalent cardiovascular risk factors and the single greatest contributor to cardiovascular disease worldwide (Lim et al. 2012). High BP commonly clusters with other cardiovascular risk factors, such as metabolic syndrome (Malik et al. 2004). Lifestyle factors that may lower blood pressure are sodium restriction, weight reduction or physical activity programs, and reduction of excessive alcohol intake (Watkins 2003).

Some peptides, in particular di- and/or tri-peptides, have been found to reveal a practical in vivo antihypertensive effect in humans by inhibiting the production of pressor active angiotensin (Ang) II or retarding the catalytic action of angiotensin I-converting enzyme (ACE). Matsui et al. (2002) reported that intact RJ and its protein fraction did not retard the action of ACE activity at all. However, the ability of RJ to inhibit ACE was recently observed to occur by pepsin hydrolysis. Thus, peptides derived from major royal jelly protein 1 as a result of gastrointestinal enzyme hydrolysis possessed potent angiotensin I-converting enzyme inhibitory activity in spontaneously hypertensive rats. Tokunaga et al. (2004) reported that Protease N treated Royal Jelly (ProRJ) and peptides from ProRJ (Ile-Tyr (IY), Val-Tyr (VY), Ile-Val-Tyr (IVY)) inhibited ACE activity and have an antihypertensive effect after repeated oral administration for 28 days on spontaneously hypertensive rats. Additionally, they analyzed the contributive ratio of these peptides in ProRJ on antihypertensive effect for single oral administration in SHR. For single oral administration of each peptide and peptide mixture (MIX; IY, VY and IVY) at doses of 0.5, 1 and 10 mg/kg, the systolic blood pressure (SBP) of SHR was reduced dose-dependently. This antihypertensive effect was sustained for 8 h. These results suggest that the peptides contributed to the antihypertensive effect of ProRJ. In a more recent study, Takaki-Doi et al. (2009) investigated the ACE inhibitory and hypotensive effects of seven peptide fractions (Frs) of royal jelly protein hydrolysate (RJPH) in comparison with those of RJPH alone. The authors found that Fr 4 and Fr 5 showed the highest ACE inhibitory activity and yield, respectively. The molecular weights (MWs) of RJPH and Fr 1–7 were distributed between 100 and 5000, increasing from Fr 1 to 7. RJPH, Fr 3 and Fr 4 at doses of 10, 30 and 100 mg/kg, and Fr 5 and Fr 6 at doses of 30 and 100 mg/kg caused transiently significant hypotensive effects in spontaneously hypertensive rats (SHR). Fr 3, 4, 5 and 6 at a

dose of 1000 mg/kg also showed significant hypotensive effects after oral administration in SHR at 3, 4–5, 7–8 and 8 h, respectively. RJPH resulted in long-lasting hypotensive effects in proportion to the magnitude of the MWs of the RJPH fractions. The hypotensive pattern of RJPH was similar to the combined pattern of Fr 3–6.

On the other hand, blood regulatory function is largely dependent on contractibility and migrating ability of vascular smooth muscle cells (VSMCs), which are a major cell type of the arterial wall. Fan et al. (2016) reported that the expression of major royal jelly protein1 (MRJP1) in VSMCs significantly reduced cell contraction, migration, and proliferation, suggesting a potential role in decreasing hypertension via action on VSMCs. These anti-hypertensive activities were further observed in changes in the proteome setting of mouse VSMCs.

10.6 Antioxidant Activity

Imbalanced production and consumption of reactive oxygen species, leading to oxidative stress, is implicated in the pathophysiology of a plethora of genetic and acquired human disorders, such as cancer, arteriosclerosis, malaria and rheumatoid arthritis, as well as neurodegenerative diseases and ageing processes (Vasconcelos et al. 2007). Therefore, attention has been focused on the use of antioxidants, especially natural antioxidants, to protect tissues from damage by free radicals (Khanum et al. 2004). RJ has received particular attention because of studies that have reported it as a highly efficient antioxidant with free radical scavenging capacity and for its ability to decrease the toxic effects of chemical agents.

Jamnik et al. (2007) investigated the antioxidative action of RJ in cells of the yeast *Saccharomyces cerevisiae* as a model organism. Yeast was cultivated in YEPD medium enriched with different concentrations of royal jelly, such as 1, 2 and 5 g/L. The results showed that royal jelly decreased intracellular oxidation in a dose dependent manner. Additionally, it affected growth and cell energy metabolic activity in a growth phase dependent manner. Protein profile analysis showed that royal jelly does not act only as a scavenger of reactive oxygen species, but it also affects protein expression in the cell. Differentially expressed proteins were identified. Türkmen et al. (2009) examined the protective effect of RJ on genotoxicity and lipid peroxidation, induced by petroleum wastewater, in *Allium cepa* L. root-tip cells. The authors reported that RJ treatment caused amelioration in the indices of lipid peroxidation and mitotic index, and in the frequency of micronucleus (MN) and chromosomal aberrations when compared with the group treated with petroleum wastewater alone. RJ application also induced recuperation of anatomical structural damages induced by the petroleum wastewater. Each dose of RJ provided protection against wastewater toxicity, while the strongest protective effect was observed at a dose of 50 microm. In another study, Kanbur et al. (2009) investigated the protective effect of RJ against paracetamol-induced liver damage. The study was conducted on 90 female Swiss Albino mice which were administered 200 mg/kg RJ for 1 day or

200 mg/kg RJ for 7 days. The authors observed considerable changes in the biochemical parameters of the group which was administered with RJ over a 7 day period. Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), glutathione peroxidase (GSH-Px) and liver MDA levels, which were statistically significant, converged towards those of the control group; among these, the statistical differences for MDA level and GSH-Px activity between the trial group (200 mg/kg RJ for 7 days) and the control group disappeared. Guo et al. (2008) reported that RJ peptides isolated from hydrolysates of water-soluble royal jelly proteins prepared with protease P exhibited significantly stronger hydroxyl radical-scavenging activity and antioxidant activity against lipid peroxidation than water-soluble royal jelly protein in vitro. Karadeniz et al. (2011) determined the effects of RJ on oxidative stress caused by Cisplatin (CDDP) injury of the kidneys and liver, by measuring tissue biochemical and antioxidant parameters. Sprague Dawley rats were treated with RJ (300 mg/kg/day) for 15 consecutive days by gavage. Malondialdehyde (MDA) and glutathione (GSH) levels, and glutathione *S*-transferase (GST), glutathione peroxidase (GSH-Px) and superoxide dismutase (SOD) activities were determined. The authors found that RJ exerted a significant protective effect towards the liver and kidneys by decreasing the level of lipid peroxidation (MDA), elevating the level of GSH, and increasing the activities of GST, GSH-Px, and SOD. In a comparable work, Silici et al. (2011) investigated the effects of royal jelly on cisplatin-induced nephrotoxicity and oxidative stress in rats. RJ was administered orally once daily at 50 and 100 mg/kg for 10 days. At the end of the study, it was found that treatment with RJ for 10 days markedly reversed cisplatin-induced increases in serum BUN level, and a dose of 100 mg/kg RJ was more effective. Treatment with RJ also decreased BUN, uric acid, AST, and ALT levels when compared with CP alone. In the RJ treated groups, total protein levels were significantly higher than those in the control group. Uzbekova et al. (2006) administered thyroxine, which is known to cause liver damage, followed by 10 mg/kg RJ for 10 days, demonstrating that RJ exhibited protective effects against thyroxine-induced liver damage. El-Nekeety et al. (2007) reported that administration of 200 mg/kg bw fumonisin and 100 or 150 mg/kg body weight RJ to rats for a period of 3 weeks resulted in significant improvement in serum MDA levels. Cemek et al. (2010) analyzed the hepatoprotective and antioxidant activities of RJ against carbon tetrachloride (CCl(4))-induced acute liver damage. Sprague-Dawley rats which were orally administered RJ at doses of 50, 100, and 200 mg/kg every other day for 20 days were used for the experiment, and malondialdehyde, reduced glutathione in whole blood and tissues, ceruloplasmin, sialic acid and liver enzymes levels in serum were measured. The authors reported that RJ exerted significant protective effects on the liver as well as ameliorating oxidative stress induced by CCl(4), resulting in reduced lipid peroxidation and improved endogenous antioxidant defense systems. It also reduced the elevated levels of liver enzymes. Cihan et al. (2013) investigated the effects of royal jelly against gamma-radiation induced oxidative damage in liver and lung tissue after total body irradiation. Adult male Sprague Dawley rats were administrated with RJ at doses of 25 or 50 mg/kg/day. At the end of the study, it was shown that administration of RJ resulted in a significant

decrease in oxidative stress and biochemical parameters (MDA, GSH-Px, CAT and SOD), and that RJ certainly increased antioxidant activities. Abd El-Monem (2011) revealed that RJ generated significant recovery in the antioxidant status of reduced glutathione (GSH) and inhibition of malondialdehyde (MDA) production, and ameliorated DNA damage and genotoxicity induced by malathion in rat cells. In another study, Yapar et al. (2009) analyzed the protecting effect of RJ on cisplatin (cDDP)-induced nephrotoxicity in adult albino mice. The mice treated with RJ at doses of 100 mg/kg body weight showed attenuation of cDDP-induced nephrotoxicity, which was manifested by preventing elevation in serum creatinine and blood urea nitrogen levels. Moreover, RJ supplementation restored glutathione (GSH) content and malondialdehyde (MDA) production levels in kidney tissue following cDDP treatment. In a similar work, Cavuşoğlu et al. (2009) studied the protective role of RJ on cadmium-induced oxidative damage, which causes a significant decrease in GSH levels and a significant increase in MDA levels in the liver and kidneys. Treatment with two doses (100 and 250 mg/kg body weight) of RJ resulted in significant recovery in the antioxidant status of GSH and significant inhibition of MDA production. More recently, Azab et al. (2011) designed a study to determine the possible protective effects of RJ against radiation induced oxidative stress in male Wister albino rats. In this study, RJ administration to irradiated rats at a dose of 1 g/kg body weight during a period of 28 days decreased serum TBARS levels when compared to irradiated rats. Increases in TBARS levels (28%) were claimed as an important determinant of altered lipid metabolism due to radiation exposure. Cavuşoğlu et al. (2011) investigated the protective effect of RJ against toxicity induced by a synthetic pyrethroid insecticide, lambda-cyhalothrin (LCT), in Swiss albino mice. The authors found that oral treatment with two doses (100 and 250 mg/kg body weight) of RJ significantly ameliorated the indices of hepatotoxicity, nephrotoxicity, lipid peroxidation, and genotoxicity induced by LCT. Both doses of RJ tested provided significant protection against LCT-induced toxicity, with the strongest effect observed for the 250 mg/kg body weight dose. In a similar study, Ahmed et al. (2014) investigated the potential protective effects of RJ against azathioprine-induced toxicity in rats. The authors found that oral administration of RJ at 200 mg/kg body weight was efficient in counteracting azathioprine toxicity; it altered anemic conditions, leucopenia and thrombocytopenia induced by azathioprine. Furthermore, RJ exerted significant protection against liver damage induced by azathioprine by reducing the elevated activities of serum hepatic enzymes. Royal jelly also blocked azathioprine-induced lipid peroxidation by decreasing malondialdehyde formation.

10.7 Antihypercholesterolemic Activity

Plasma low-density lipoprotein (LDL) is the major risk factor for CVD. Increased LDL oxidation is hypothesized to be causally associated with an increasing risk of atherosclerosis (Kong et al. 2010). Atherosclerosis is a chronic disease with a

significant impact on health, since it contributes to mortality and morbidity in the western world more than any other disorder (Viuda-Martos et al. 2010). It is characterized by lesions called atheromas or fibro-fatty plaques which protrude into the lumen, weaken the underlying media and undergo a series of complications as CVD progresses (Albertini et al. 2002). Hypolipidemic agents are a diverse group of drugs used to treat hyperlipidemia. They are used to reduce total cholesterol concentration in blood, LDL cholesterol and triglycerides, especially in patients with a high risk of developing cardiovascular problems (Viuda-Martos et al. 2010). In the scientific literature there are several studies in which the antihypercholesterolemic activity of RJ has been determined. Nakajin et al. (1982) stated that although royal jelly has no effect on lipid levels in blood plasma in normal rabbits, it can reduce the cholesterol content in the blood of animals fed on a diet that induced high levels of blood cholesterol. Vittek (1995) analyzed the effect of RJ on serum total lipid and cholesterol levels in rats and rabbits and reported that RJ significantly decreased the levels of both. The author suggested that at a dose of approximately 50–100 mg per day, RJ decreased serum total cholesterol (TC) levels by about 14% and total serum lipids by about 10% in the group of patients studied. The decrease in TC and LDL levels may result from lowering very low density lipoprotein (VLDL) levels, as reported by Guo et al. (2008), due to the strong hydroxyl radical scavenging ability of RJ. Azab et al. (2011) designed a study to determine the possible protective effects of RJ against radiation induced oxidative stress and biochemical alterations in male Wister albino rats. In this study, RJ administration at a dose of 1 g/kg body weight during a period of 28 days reduced serum cholesterol (14%), triglycerides (30.45%) and LDL (24%) in serum. Moreover, significant amelioration was observed in HDL levels of RJ treated irradiated rats. Silici et al. (2011) investigated the effects of royal jelly on serum triglyceride cholesterol levels in cisplatin-induced nephrotoxicity in rats. The authors found that treatment with RJ at doses of 50 and 100 mg/kg for 10 days markedly decreased serum triglyceride and cholesterol levels when compared with the control group.

The composition of RJ could influence the antihypercholesterolemic activity. Thus, the large number of proteins in RJ may decrease plasma levels of cholesterol. Kamakura et al. (2006) showed that RJ reduces the levels of cholesterol biosynthesis enzyme and influences the activity of hepatic lipoprotein receptors that regulate very low lipoprotein uptake in mice. Similarly, Kashima et al. (2014) reported that purified major royal jelly protein 1 (MRJP1), which is the most abundant protein of the bile acid-binding proteins in RJ, exhibited taurocholate-binding activity in vitro. The micellar solubility of cholesterol was significantly decreased in the presence of MRJP1 compared with casein in vitro. Liver bile acid levels were significantly increased, and cholesterol 7α-hydroxylase (CYP7A1) mRNA and protein levels tended to increase following MRJP1 feeding, compared with the control. CYP7A1 mRNA and protein levels were significantly increased by MRJP1 tryptic hydrolysate treatment compared with that of casein tryptic hydrolysate in hepatocytes. The hypocholesterolemic effect of MRJP1 has been investigated in rats. The cholesterol-lowering action induced by MRJP1 occurs via MRJP1 interaction with bile acids, which induces a significant increase in fecal bile acid excretion and a tendency to

increase fecal cholesterol excretion, while also enhancing hepatic cholesterol catabolism. Interestingly, MRJP1 exhibits greater hypocholesterolemic activity than the medicine β-sitosterol in rats.

In studies with humans, Guo et al. (2007) examined the effects of RJ supplementation on serum lipoprotein metabolism. Fifteen volunteers were divided into a RJ intake group and a control group. The RJ group took 6 g per day for 4 weeks. Their serum total cholesterol (TC) and serum low-density lipoprotein (LDL) levels decreased significantly compared with those of the control group. There were no significant differences in serum high-density lipoprotein (HDL) or triglyceride concentrations. Moreover, the relationship between serum cholesterol and lipoprotein levels was investigated. Among the lipoprotein fractions, small very-low-density lipoprotein was decreased after RJ intake. Our results suggest that dietary RJ decreases TC and LDL levels by lowering small VLDL levels. Lambrinoudaki et al. (2016) examined the effect of RJ on cardiovascular and bone turnover markers in clinically healthy postmenopausal women. Participants received 150 mg RJ daily for three months. The RJ used in this study was particularly rich in medium chain fatty acids, compounds with hypolipidemic properties, which comprised 63% of the dry weight fatty content. At the end of the study, it was shown that RJ treatment resulted in a significant increase in high density lipoprotein-cholesterol levels (HDL-C 60.2 mg/dL versus 64.7 mg/dL, 7.7% increase), as well as a significant decrease in low density lipoprotein-cholesterol (LDL-C, 143.9 mg/dLversus 136.2 mg/dL, 4.1% decrease,) and total cholesterol levels (224.4 compared to 216.1, 3.09% decrease). No statistically significant changes were observed in the other cardiovascular or bone turnover parameters.

10.8 Antitumor Activity

Nutritional factors have been suggested to play an important role in the prevention of chronic diseases, such as cancers. Cancer is the second leading cause of death worldwide (Desai et al. 2008). The increasing proportion of cancer occurrence and lack of anti-cancer drugs has forced researchers to chemically evaluate and investigate the pharmacological activity of anticancer bioactive compounds from natural sources (Dharmalingam et al. 2014). One such substance with anti-carcinogenic properties could be RJ. However, as mentioned by Pavel et al. (2011), there is no clear consensus in the scientific literature regarding the influence of RJ on neoplastic growth. Antitumoral action has been studied in different experimental models. However, to date there have been no human studies with which to compare these results, so claims cannot be substantiated.

Focusing on experimental models, Taniguchi et al. (2003) studied the effect of oral administration of RJ to one of two groups of laboratory mice prior to tumor cell implantation; they observed that the gel had no effect on leukemia cells but that it did on sarcoma cells, and increased the lifespan of the mice by about 20%. In this study, the size of the tumors decreased by about 50%. Similarly, Inoue et al. (2003)

investigated the effect of dietary RJ on tissue DNA oxidative damage and the life span of C3H/HeJ mice. In C3H/HeJ mice that were fed a dietary supplement of RJ, the levels of 8-hydroxy-2-deoxyguanosine, a marker of oxidative stress, were significantly reduced in kidney DNA and serum. Regarding the effect of dietary RJ on the life span of C3H/HeJ mice, the 50% mortality rate for intermediate- (about 6 mg RJ/kg weight) and high-dose groups (about 60 mg RJ/kg weight) was reached at significantly longer times than that of the control group. Orsolic et al. (2005) analyzed the effects of RJ on tumor development and metastasis in murine tumor models, such as spontaneous mammary carcinoma (MCa) and methylcholanthrene induced fibrosarcoma (FS), in albino mice. The authors found that RJ did not affect the formation of metastases when given intraperitoneally or subcutaneously. On the other hand, synchronous intravenous application of tumor cells and RJ significantly inhibited the formation of metastases. For Orsolic et al. (2005), these findings demonstrated that RJ products given orally or systemically may play an important role in controlling tumor growth and metastasis. Bincoletto et al. (2005) examined the role of RJ on the haematopoietic response of Ehrlich ascites tumour (EAT)-bearing mice. The results demonstrated that RJ prevented myelosupression induced by the temporal evolution of the tumor and abrogated splenic haematopoiesis observed in EAT-bearing mice. The stimulating effect of RJ was also observed in vitro on multipotent bone marrow stem cells, evaluated by long-term bone marrow cultures (LTBMCs). A more effective antitumoural response was observed with a more prolonged treatment regimen. In this regard, administration of RJ for 33 days afforded the highest protection, producing extension of survival of 38, 71 and 85% for doses of 500, 1000 and 1500 mg/kg, respectively, whereas 23 and 28 day treatment increased survival rate by 19 and 23%, respectively. The authors suggested that the increased survival rate might be related to the decreased Prostaglandin E2 (PGE2) levels observed in EAT-bearing mice after RJ treatment. Nakaya et al. (2007) reported the anti-environmental estrogen activity of royal jelly. Bisphenol A (BPA) is an environmental estrogen that stimulates the proliferation of human breast cancer MCF-7 cells. Royal jelly inhibited the growth-promoting effect of BPA on MCF-7 cells, even though it did not affect the proliferation of cells in the absence of BPA. In addition, this inhibiting effect of RJ was heat-stable. Cavuşoğlu et al. (2009) studied the protective role of RJ on cadmium-induced genotoxicity in mice. The authors reported that oral administration of RJ at two doses (100 and 250 mg/kg body weight) resulted in significant suppression of the mutagenic effects of cadmium. Han et al. (2011) investigated the hypo-pigmentary mechanism of RJ in a mouse melanocyte cell line, B16F1. Treatment of B16F1 cells with RJ markedly inhibited melanin biosynthesis in a dose-dependent manner. Inhibition of melanin synthesis occurred through a decrease in tyrosinase activity. The mRNA levels of tyrosinase were also reduced by royal jelly. For these authors, the results obtained suggested that RJ reduces melanin synthesis by down-regulation of tyrosinase mRNA transcription and could serve as a new candidate in the design of novel skin-whitening or therapeutic agents. Shirzad et al. (2013) carried out a study to investigate the effect of RJ on the growth of WEHI-164 fibrosarcoma cells in syngenic Balb/c mice. The animals were orally administered 100, 200, and 300 mg/kg

RJ. The tumor size of each individual mouse was measured every 2 days. After 28 days, the results showed that the average tumor size in mice receiving royal jelly was smaller than that of the control group. Additionally, no metastasis was observed in the test groups. Galaly et al. (2014) investigated the anti-mutagenic and anti-histopathologic effects of RJ on valproic acid-induced genotoxicity and nephrotoxicity in male albino mice. They found that in RJ treated mice, at doses of 50 and 100 mg/kg, the kidney sections showed normal histological structure with non-significant changes in chromosomal aberrations (CA) and mitotic index (MI), while a dose of 200 mg/kg resulted in mild inflammatory cell infiltration and hyperemic glomeruli but not highly significant changes in CA and MI. Han et al. (2015) carried out a study to analyze the anti-melanogenic efficacy of water-soluble royal jelly (WSRJ). B16F1 melanoma cells were first treated with 10 nM α-melanocyte stimulating hormone (α-MSH) and then with various doses of WSRJ. In addition, they investigated the mRNA and protein expression of melanogenesis-related genes such as tyrosinase, tyrosinase related protein-1 (TRP-1) and TRP-2. The authors reported that WSRJ directly inhibited tyrosinase and cellular tyrosinase activity, which decreased melanin synthesis in α-MSH stimulated B16F1 melanoma cells at a level comparable to that observed with arbutin. In addition, WSRJ decreased the mRNA and protein expression of tyrosinase, TRP-1, and TRP-2, in a manner comparable to that observed with arbutin. WSRJ has strong anti-melanogenic activity, exerted via direct inhibition of tyrosinase enzyme activity and suppression of expression of melanogenesis related genes.

10.9 Other Physiological Effects

As well as its antibacterial, antioxidant anti-inflammatory, vasodilative, hypotensive, hipocholesterolemiant, hepatoprotective, and antitumor activities, which have been extensively analyzed previously, RJ also shows other physiological effects due to its high content of bioactive compounds.

Morita et al. (2012) reported that six month ingestion of 3 g RJ in humans improved erythropoiesis, glucose tolerance and mental health. Acceleration of conversion from DHEA-S to T by RJ may have been observed among these favorable effects. Furthermore, Kataoka et al. (2001) have demonstrated that a soluble fraction of RJ shows antiallergic activities, including reduced antigen-specific IgE levels in the sera of allergic mice. Honda et al. (2011) reported that RJ and its related substances extend lifespan in *Caenorhabditis elegans*, suggesting that RJ may possess lifespan extending capability. Further analysis and characterization of the lifespan-extending agents in RJ broaden our understanding of the gene network involved in longevity regulation in diverse species and may lead to the development of nutraceutical interventions for aging. Münstedt et al. (2009) reported the antidiabetic effects of RJ: 20 healthy volunteers underwent the standardized oral glucose tolerance test (OGTT) and afterwards a second OGTT after ingestion of 20 g RJ. Serum glucose levels after 2 h and the area under the curve for glucose were significantly lower after

RJ administration. Mishima et al. (2005) provide evidence that RJ has estrogenic activities through interaction with estrogen receptors. The reporter gene expression assays suggested that 0.1–1 mg/mL RJ activated estrogen receptors, leading to enhanced transcription of a reporter gene through an estrogen-responsive element. 1 mg/mL RJ stimulated mRNA expression of estrogen-responsive pS2 and vascular endothelial growth factor (VEGF) by increasing gene transcription in MCF-7 cells. Moradi et al. (2013) investigated the protective effects of RJ supplementation on sperm kinematics and plasma membrane functionality. The authors found that supplementation of sperm with different concentrations (0, 0.5, 1, 1.5 and 2%) of RJ resulted in a significant increase in sperm viability, with the highest increase observed at 1% RJ concentration for 120 h incubation. Significant protective effects of RJ on sperm membrane functionality were observed at lower concentrations (0.5 and 1%) for all incubation periods. The most prominent protective effect of RJ on sperm motility parameters was found to be on the progressive velocity after 72 h storage.

10.10 Conclusions

Royal jelly is an important functional food that possesses several health promoting properties. As a result, it has been widely used in commercial medical products, healthy foods and cosmetics in many countries. However, in order to improve our knowledge of royal jelly production and composition, as well as our understanding of its physiological effects in the human body, collaborative studies are needed involving the participation of researchers from different scientific areas: chemistry, biochemistry, biotechnology, biology, physiology, nutrition, and medicine.

References

Abd El-Monem DD (2011) The ameliorative effect of royal jelly against malathion genotoxicity in bone marrow and liver of rat. J Am Sci 7(12):1251–1256

Agostoni C, Bresson J, Fairweather-Tait S, Flynn A, Golly I et al (2011) EFSA panel on dietetic products, nutrition and allergies (NDA): scientific opinion on the substantiation of health claims related to royal jelly. Eur Food Safety Author J 9(4):2083

Ahmed WMS, Khalaf AA, Moselhy WA, Safwat GM (2014) Royal jelly attenuates azathioprine induced toxicity in rats. Environ Toxicol Pharmacol 37(1):431–437

Albertini R, Moratti R, DeLuca G (2002) Oxidation of low-density lipoprotein in atherosclerosis from basic biochemistry to clinical studies. Curr Mol Med 2:579–592

Alzoreky NS, Nakahara K (2003) Antimicrobial activity of extracts from some edible plants commonly consumed in Asia. Int J Food Microbiol 80:223–230

Antinelli JF, Zeggane S, Davico R, Rognone C, Faucon JP, Lizzani L (2003) Evaluation of (E)-10-hydroxydec-2-enoic acid as a freshness parameter for royal jelly. Food Chem 80:85–89

Arzi A, Olapour S, Yaghooti H, Karampour NS (2015) Effect of royal jelly on formalin induced-inflammation in rat hind paw. Jundishapur J Nat Pharm Prod 10(1):e22466. doi:10.17795/jjnpp-22466

Aslan Z, Aksoy L (2015) Anti-inflammatory effects of royal jelly on ethylene glycol induced renal inflammation in rats. Int Braz J Urol 41(5):1008–1013

Attalla KM, Owayss AA, Mohanny KM (2007) Antibacterial activities of bee venom, propolis, and royal jelly produced by three honey bee, *Apis mellifera* L., hybrids reared in the same environmental conditions. Ann Agric Sci 45:895–902

Azab KS, Bashandy M, Salem M, Ahmed O, Tawfik Z, Helal H (2011) Royal jelly modulates oxidative stress and tissue injury in gamma irradiated male Wister Albino rats. North Am J Med Sci 3:268–276

Bărnuțiu LI, Mărghitaş LA, Dezmirean DS, Mihai CM, Otilia Bobiş O (2011) Chemical composition and antimicrobial activity of royal jelly – review. Anim Sci Biotechnol 44(2):67–72

Bilikova K, Hanes J, Nordhoff E, Saenger W, Klaudiny J, Šimúth J (2002) Apisimin, a new serine valinrich peptide from honeybee (*Apis mellifera* L.) royal jelly: purification and molecular characterization. FEBS Lett 528:125–129

Bilikova K, Huang SC, Lin IP, Simuth J, Peng CC (2015) Structure and antimicrobial activity relationship of royalisin, an antimicrobial peptide from royal jelly of *Apis mellifera*. Peptides 68:190–196

Bincoletto C, Eberlin S, Figueiredo CA, Luengo MB, Queiroz ML (2005) Effects produced by royal jelly on haematopoiesis: relation with host resistance against Ehrlich ascites tumour challenge. Int Immunopharmacol 5(4):679–688

Caparica-Santos C, Marcucci MC (2007) Quantitative determination of trans-10-hydroxy-2-decenoic acid (10-HDA) in Brazilian royal jelly and commercial products containing royal jelly. J Apic Res 46(3):149–153

Cavuşoğlu K, Yapar K, Yalçin E (2009) Royal jelly (honey bee) is a potential antioxidant against cadmium-induced genotoxicity and oxidative stress in albino mice. J Med Food 12(6):1286–1292

Cavuşoğlu K, Yapar K, Yalçin E (2011) The protective effect of royal jelly on chronic lambda-cyhalothrin toxicity: serum biochemical parameters, lipid peroxidation, and genotoxic and histopathological alterations in swiss albino mice. J Med Food 14(10):1229–1237

Cemek M, Aymelek F, Büyükokuroğlu ME, Karaca T, Büyükben A, Yilmaz F (2010) Protective potential of royal jelly against carbon tetrachloride induced-toxicity and changes in the serum sialic acid levels. Food Chem Toxicol 48(10):2827–2832

Chen YF, Wang K, Zhang YZ, Zheng YF, Hu FL (2016) In vitro anti-inflammatory effects of three fatty acids from royal jelly. Mediat Inflamm 2016:Article ID 3583684. doi:10.1155/2016/3583684

Cihan YB, Ozturk A, Gokalp SS (2013) Protective role of royal jelly against radiation induced oxidative stress in rats. Int J Hematol Oncol 23:79–87

Dao TT, Chi YS, Kim J, Kim HP, Kim S, Park H (2004) Synthesis and inhibitory activity against COX-2 catalyzed prostaglandin production of chrysin derivatives. Bioorg Med Chem Lett 14:1165–1167

Desai AG, Qazi GN, Ganju RK, El-Tamer M, Singh J, Saxena AK, Bedi YS, Taneja SC, Bhat HK (2008) Medicinal plants and cancer chemoprevention. Curr Drug Met 9:581–591

Dharmalingam SR, Madhappan R, Ramamurthy S, Meka VS, Shanmugham S, KLS K (2014) Anticarcinogenic effects of nothapodytes nimmoniana against Dalton's lymphoma ascites tumor model. Eur J Exp Biol 4(1):527–533

Dinkov D, Stratev D, Balkanska R, Sergelidis D (2016) Antibacterial activity of royal jelly and rape honey against methicillin-resistant *Staphylococcus aureus* strains. J Food Health Sci 2(2):67–73

Dzopalic T, Vucevic D, Tomic S, Djokic J, Chinou I, Colic M (2011) 3,10-Dihydroxy-decanoic acid, isolated from royal jelly, stimulates Th1 polarising capability of human monocyte-derived dendritic cells. Food Chem 126:1211–1217

El-Nekeety AA, El-Kholy W, Abbas NF, Ebaid A, Amra HA, Abdel-Wahhab MA (2007) Efficacy of royal jelly against the oxidative stress of fumonisin in rats. Toxicon 50:256–269

Eshraghi SS (2005) An evaluation of the potent inhibitory effects of royal jelly fractions against Streptomyces bacteria. Pak J Med Sci 21(1):63–68

Fan P, Bin Han B, Feng M, Fang Y, Zhang L, Hu H, Hao Y, Qi Y, Zhang X, Li J (2016) Functional and proteomic investigations reveal major royal jelly protein 1 associated with antihypertension activity in mouse vascular smooth muscle cells. Sci Rep 6:30230. doi:10.1038/srep30230

Fontana R, Mendes MA, de Souza BM, Konno K, Cesar LM, Malaspina O, Palma MS (2004) Jelleines: a family of antimicrobial peptides from the Royal Jelly of honeybees (*Apis mellifera*). Peptides 25:919–928

Fratini F, Cilia G, Mancini S, Felicioli A (2016) Royal jelly: an ancient remedy with remarkable antibacterial properties. Microbiol Res 192:130–141

Fujii A, Kobayashi S, Kuboyama N, Furukawa Y, Kaneko Y, Ishihama S et al (1990) Augmentation of wound healing by royal jelly (RJ) in streptozotocin-diabetic rats. Jpn J Pharmacol 5:331–337

Galaly SR, Abdella EM, Mohammed HM, Khadrawy SM (2014) Effects of royal jelly on genotoxicity and nephrotoxicity induced by valproic acid in albino mice. J Basic Appl Sci 3:1–15

Garcia MC, Finola MS, Marioli JM (2013) Bioassay directed identification of royal jelly's active compounds against the growth of bacteria capable of infecting cutaneous wounds. Adv Microbiol 3:138–144

Gasic S, Vucevic D, Vasilijic S, Antunovic M, Chinou I, Colic M (2007) Evaluation of the immunomodulatory activities of royal jelly components *in vitro*. Immunopharmacol Immunotoxicol 29:521–536

Guo H, Saiga A, Sato M, Miyazawa I, Shibata M, Takahata Y, Morimatsu F (2007) Royal jelly supplementation improves lipoprotein metabolism in humans. J Nutr Sci Vitaminol (Tokyo) 53(4):345–348

Guo H, Ekusa A, Iwai K, Yonekura M, Takahata Y, Morimatsu F (2008) Royal jelly peptides inhibit lipid peroxidation in vitro and in vivo. J Nutr Sci Vitaminol 54:191–195

Hamerlinck FF (1999) Neopterin: a review. Exp Dermatol 9:167–176

Han SM, Yeo JH, Cho YH, Pak SC (2011) Royal jelly reduces melanin synthesis through down-regulation of tyrosinase expression. Am J Chin Med 39:1253–1260

Han SM, Kim JM, Hong IP, Woo SO, Kim SG, Jang HR, Park KK, Pak SC (2015) Whitening effect of water soluble royal jelly from South Korea. Korean J Food Sci Anim Resour 35(5):707–713

Honda Y, Fujita Y, Maruyama H, Araki Y, Ichihara K, Sato A, Kojima T, Tanaka M, Nozawa Y, Ito M, Honda S (2011) Lifespan-extending effects of royal jelly and its related substances on the nematode *Caenorhabditis elegans*. PLoS One 6(8):e23527. doi:10.1371/journal.pone.0023527

Inoue S, Koya-Miyata S, Ushio S, Iwaki K, Ikeda M, Kurimoto M (2003) Royal jelly prolongs the life span C3H/HeJ mice: correlation with reduced DNA damage. Exp Gerontol 38:965–969

Jamnik P, Goranovic D, Raspor P (2007) Antioxidative action of royal jelly in the yeast cell. Exp Gerontol 42:594–600

Kanbur M, Eraslan G, Beyaz L, Silici S, Atasever A (2009) The effects of royal jelly on liver damage induced by paracetamol in mice. Exp Toxicol Pathol 61:123–132

Kamakura M, Moriyama T, Sakaki T (2006) Changes in hepatic gene expression associated with the hypocholesterolaemic activity of royal jelly. J Pharm Pharmacol 58:1683–4689

Karaca T, Şimşek N, Uslu S, Kalkan Y, Can I, Kara A, Yörük M (2012) The effect of royal jelly on CD3+, CD5+, CD45+ T-cell and CD68+ cell distribution in the colon of rats with acetic acid-induced colitis. Allergol Immunopathol 40:357–361

Karadeniz A, Simsek N, Karakus E, Yildirim S, Kara A, Can I, Kisa F, Emre H, Turkeli M (2011) Royal jelly modulates oxidative stress and apoptosis in liver and kidneys of rats treated with cisplatin. Oxidative Med Cell Longev 2011:981793

Kashima Y, Kanematsu S, Asai S, Kusada M, Watanabe S, Kawashima T, Nakamura T, Shimada M, Goto T, Nagaoka S (2014) Identification of a novel hypocholesterolemic protein, major royal jelly protein 1, derived from royal jelly. PLoS One 9(8):e105073. doi:10.1371/journal.pone.0105073. eCollection 2014

Kataoka M, Arai N, Yoshifumi Y, Kohno K, Iwaki K, Ikeda M, Kurimoto M (2001) Analysis of anti-allergic function of royal jelly. Nat Med 55:174–180

Khanum F, Anilakumar KR, Viswanathan KR (2004) Anticarcinogenic properties of garlic: a review. Crit Rev Food Sci Nutr 4:479–488

Kohno K, Okamoto I, Sano O, Arai N, Iwaki K, Ikeda M, Kurimoto M (2004) Royal jelly inhibits the production of proinflammatory cytokines by activated macrophages. Biosci Biotechnol Biochem 68:138–145

Kohno K, Ohashi E, Sano O, Kusano H, Kunikata T, Arai N, Hanaya T, Kawata T, Nishimoto T, Fukuda S (2015) Anti-inflammatory effects of adenosine N1-oxide. J Inflamm 12:2. doi:10.1186/s12950-014-0045-0

Kong KW, Khoo HE, Prasad KN, Ismail A, Tan CP, Rajab NF (2010) Revealing the power of the natural red pigment lycopene. Molecules 15:959–987

Koya-Miyata S, Okamoto I, Ushio S, Iwaki K, Ikeda M, Kurimoto M (2004) Identification of a collagen production-promoting factor from an extract of royal jelly and its possible mechanism. Biosci Biotechnol Biochem 68:767–773

Lambrinoudaki I, Augoulea A, Rizos D, Politi M, Tsoltos N, Moros M, Chinou I, Graikou K, Kouskouni E, Kambani S, Panoulis K, Moutsatsou P (2016) Greek-origin royal jelly improves the lipid profile of postmenopausal women. Gynecol Endocrinol 26:1–5

Lee CJ, Chen LG, Liang WL, Wanga CC (2010) Anti-inflammatory effects of *Punica granatum* Linne in vitro and in vivo. Food Chem 118:315–322

Lim SS et al (2012) Comparative risk assessment of burden of disease and injury attributable to 67 risk factors and risk factor clusters in 21 regions, 1990–2010: a systematic analysis for the global burden of disease study 2010. Lancet 380:2224–2260. doi:10.1016/S0140-6736(12)61766-8

López-Gutiérrez N, Aguilera-Luiz MM, Romero-González R, Martínez Vidal JL, Garrido Frenich A (2014) Fast analysis of polyphenols in royal jelly products using automated TurboFlow™-liquid chromatography–Orbitrap high resolution mass spectrometry. J Chromatogr B 973(15):17–28

Majtan J, Kovacova E, Bilikova K, Simuth J (2006) The immunostimulatory effect of the recombinant apalbumin 1-major honeybee royal jelly protein-on TNFalpha release. Int Immunopharmacol l6:269–278

Malik S, Wong ND, Franklin SS, Kamath TV, L'Italien GJ, Pio JR (2004) Impact of the metabolic syndrome on mortality from coronary heart disease, cardiovascular disease and all causes in United States adults. Circulation 110:1245–1250

Matsui T, Yukiyoshi A, Doi S, Sugimoto H, Yamada H, Matsumoto K (2002) Gastrointestinal enzyme production of bioactive peptides from royal jelly protein and their antihypertensive ability in SHR. J Nutr Biochem 13:80–86

Melliou E, Chinou I (2005) Chemistry and bioactivity of royal jelly from Greece. J Agric Food Chem 53(23):8987–8992

Mishima S, Suzuki KM, Isohama Y, Kuratsu N, Araki Y, Inoue M, Miyata T (2005) Royal jelly has estrogenic effects in vitro and in vivo. J Ethnopharmacol 101:215–220

Montaño K, Vargas Albores F (2002) Péptidos Antimicrobianos: un mecanismo de defensa ancestral con mucho futuro. Interciencia 27(1):21–27

Moradi AR, Malekinejad H, Farrokhi-Ardabili F, Bernousi I (2013) Royal Jelly improves the sperm parameters of ram semen during liquid storage and serves as an antioxidant source. Small Rum Res 113:346–352

Morita H, Ikeda T, Kajita K, Fujioka K, Mori I, Okada H, Uno Y, Ishizuka T (2012) Effect of royal jelly ingestion for six months on healthy volunteers. Nutr J 11:77–83

Moselhy WA, Fawzy AM, Kamel AA (2013) An evaluation of the potent antimicrobial effects and unsaponifiable matter analysis of the royal jelly. Life Sci J 10:290–296

Münstedt K, Bargello M, Hauenschild A (2009) Royal jelly reduces the serum glucose levels in healthy subjects. J Med Food 12:1170–1172

Nagai T, Inoue R (2004) Preparation and functional properties of water extract and alkaline extract of royal jelly. Food Chem 84:181–186

Nakajin S, Okiyama K, Yamashita S, Akiyama Y, Shinoda M (1982) Effect of royal jelly on experimental hypercholesterolemia in rabbits. Yakugaku Zasshi 36:65–69

Nakaya M, Onda H, Sasaki K, Yukiyoshi A, Tachibana H, Yamada K (2007) Effect of royal jelly on bisphenol a-induced proliferation of human breast cancer cells. Biosci Biotechnol Biochem 71:253–255

Orsolic N, Terzic S, Sver L, Basic I (2005) Honey-bee products in prevention and/or therapy of murine transplantable tumours. J Sci Food Agric 85:363–370

Pavel CI, Mărghitaş LA, Bobiş O, Dezmirean DS, Şapcaliu A, Radoi I, Mădaş MN (2011) Biological activities of royal jelly – review scientific papers. Anim Sci Biotechnol 44:108–118

Ratanavalachai T, Wongchai V (2002) Antibacterial activity of intact royal jelly, its lipid extract and its defatted extract. Thammasat Int J Sci Technol 7:5–12

Rihar K, Exel DG, Božnar M, Filipič B (2013) Effect of royal jelly on IFN-α inhibition of human colon cancer cells proliferation in vitro. Eur Geriatr Med 4(1):S142

Romanelli A, Moggio L, Montella RC, Campiglia P, Iannaccone M, Capuano F, Pedone C, Capparelli R (2011) Peptides from royal jelly: studies on the antimicrobial activity of jelleins, jelleins analogs and synergy with temporins. J Peptide Sci 17:348–352

Sabatini AG, Marcazzan LG, Caboni MF, Bogdanov S, Bicudo de Almeida-Muradian L (2009) Quality and standardization of royal jelly. J ApiPro ApiMed Sci 1(1):16–21

Shen L, Liu D, Li M, Jin F, Din M, Parnell LD et al (2012) Mechanism of action of recombinant acc-royalisin from royal jelly of Asian honeybee against Gram-positive bacteria. PLoS One 7(10):e47194. doi:10.1371/journal.pone.0047194

Shirzad M, Kordyazdi R, Shahinfard N, Nikokar M (2013) Does Royal jelly affect tumor cells? J Herb Med Plarmacol 2(2):45–48

Silici S, Ekmekcioglu O, Kanbur M, Deniz K (2011) The protective effect of royal jelly against cisplatin-induced renal oxidative stress in rats. World J Urol 29(1):127–132

Šimúth J, Bíliková K, Kovácová E, Kuzmová Z, Schroder W (2004) Immunochemical approach to detection of adulteration in honey: physiologically active royal jelly protein stimulating TNF-α release is a regular component of honey. J Agric Food Chem 52:2154–2158

Sugiyama T, Takahashi K, Tokoro S, Gotou T, Neri P, Mori H (2012) Inhibitory effect of 10-hydroxy-trans-2-decenoic acid on LPS-induced IL-6 production via reducing IκB-ζ expression. Innate Immun 18(3):429–437

Sugiyama T, Takahashi K, Kuzumaki A, Tokoro S, Neri P, Mori H (2013) Inhibitory mechanism of 10-hydroxy-trans-2-decenoic acid (royal jelly acid) against lipopolysaccharide- and interferon-β-induced nitric oxide production. Inflammation 36:372–378

Takaki-Doi S, Hashimoto K, Yamamura M, Kamei C (2009) Antihypertensive activities of royal jelly protein hydrolysate and its fractions in spontaneously hypertensive rats. Acta Med Okayama 63:57–64

Taniguchi Y, Kohno K, Inoue S, Koya-Miyata S, Okamoto I, Arai N et al (2003) Oral administration of royal jelly inhibits the development of atopic dermatitis-like skin lesions in NC/Nga mice. Int Immunopharmacol 3:1313–1324

Tokunaga KH, Yoshida C, Suzuki KM, Maruyama H, Futamura Y, Araki Y, Mishima S (2004) Antihypertensive effect of peptides from royal jelly in spontaneously hypertensive rats. Biol Pharm Bull 27(2):189–192

Türkmen Z, Cavuşoğlu K, Cavuşoğlu K, Yapar K, Yalçin E (2009) Protective role of royal jelly (honeybee) on genotoxicity and lipid peroxidation, induced by petroleum wastewater, in *Allium cepa* L. root tips. Environ Technol 30(11):1205–1214

Uzbekova D, Chugunova L, Makarova V, Ryabkov A, Mirgorodskaya L (2006) Efficacy of royal jelly and lactulose on thyroxin-induced liver damage in rats. Hepatology 28:157–162

Vasconcelos SML, Goulart MOF, Moura JBF et al (2007) Reactive oxygen and nitrogen species, antioxidants and markers of oxidative damage in human blood: main analytical methods for their determination. Quím Nova 30:1323–1338

Vittek J (1995) Effect of royal jelly on serum lipids in experimental animals and humans with atherosclerosis. Experientia 51:927–935

Viuda-Martos M, López-Marcos MC, Fernández-López J, Sendra E, López-Vargas JH, Perez-Álvarez JÁ (2010) Role of fiber in cardiovascular diseases: a review. Compr Rev Food Sci Food Safety 9(2):240–258

Vucevic D, Melliou E, Vasilijic S, Gasic S, Ivanovski P, Chinou I, Colic M (2007) Fatty acids isolated from royal jelly modulate dendritic cell-mediated immune response in vitro. Int Immunopharmacol 7(9):1211–1220

Watkins PJ (2003) ABC of diabetes: cardiovascular disease, hypertension and lipids. Br Med J 326:874–876

Yang XY, Yang DS, Zhang W, Wang JM, Li CY, Ye H, Lei KF, Chen XF, Shen NH, Jin LQ, Wang JG (2010) 10-Hydroxy-2-decenoic acid from royal jelly: a potential medicine for RA. J Ethnopharmacol 128:314–321

Yapar K, Cavuşoğlu K, Oruç E, Yalçin E (2009) Protective effect of royal jelly and green tea extracts effect against cisplatin-induced nephrotoxicity in mice: a comparative study. J Med Food 12(5):1136–1142

Part IV
Bee Pollen

Chapter 11
Chemical Composition of Bee Pollen

Adriane Alexandre Machado De-Melo and Ligia Bicudo de Almeida-Muradian

Contents

A.A.M. De-Melo (✉)
Department of Food and Experimental Nutrition, Pharmaceutical Sciences School, University of São Paulo, São Paulo, SP 05580-000, Brazil

Laboratório de Análise de Alimentos da Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, Avenida Professor Lineu Prestes, 580, Bloco 13-A, Cidade Universitária, São Paulo, SP 05580-000, Brazil
e-mail: adriane.melo@usp.br

L.B. de Almeida-Muradian
Department of Food and Experimental Nutrition, Pharmaceutical Sciences School, University of São Paulo, São Paulo, SP 05580-000, Brazil

J.M. Alvarez-Suarez (ed.), *Bee Products - Chemical and Biological Properties*,
DOI 10.1007/978-3-319-59689-1_11

11.1 Introduction

Bee pollen is a food produced by bees to serve as a nutrient source for the development and maintenance of the colony. The insects collect hundreds and even thousands of pollen grains and use salivary secretions, nectar, and/or honey to agglutinate them, making up pollen loads generally varying between 1.4 and 4 mm in size (Brazil 2001; Campos et al. 2008; Saavedra et al. 2013). The bees transport the pollen loads adhered to their legs, specifically to the pollen basket (Pereira et al. 2013); and, once in the hive, store the pollen loads within the alveoli for subsequent consumption. Bee pollen is collected by beekeepers by setting traps at the hive entrance so that the pollen loads are retained at the moment the insects entering the hive.

Approximately 250 substances can be found in this product (Komosinska-Vassev et al. 2015). Amongst the macronutrients, protein levels, which can reach 62 g/100 g, stand out. Vitamins, minerals, and fatty acids are also present, as well as secondary metabolites of plants with an antioxidant, antibacterial, antifungal, anti-inflammatory, antiallergic, hepatoprotective, and antitumor potential (Campos et al. 2008; Abdella et al. 2009; Leblanc et al. 2009; Graikou et al. 2011; Bogdanov 2012b; Kacániová et al. 2012; Nogueira et al. 2012; Fatrcová-Šramková et al. 2013; Pascoal et al. 2014).

The chemical composition varies significantly, according to the botanical and geographical origin of the pollen grains that make up the pollen loads. The composition of the grains, in their turn, suffers the influence of the soil and climate conditions, age and nutritional state of the plant during the development of the grain. In the same plant species there can be variations in pollen composition according to region, season of the year, and even between years (Funari et al. 2003; Almeida-Muradian et al. 2005; Melo et al. 2009; Silveira 2012). Bees visit around 200 flowers to produce one load of bee pollen, which generally contains grains from the same plant species (Campos et al. 1997; Bogdanov 2012a; Komosinska-Vassev et al. 2015).

It is possible to identify the botanical origin of bee pollen. Currently, the most utilized method is the morphological and structural analysis of the grains under a microscope. This method allows the identification of which species, genus, or family the grains belong to and determines the percentage of each pollen type. The frequency of taxa therefore is used to classify monofloral bee pollen, where there is a predominance of one pollen type, or heterofloral or multifloral pollen, when there is no predominance (Barth et al. 2010).

Processing can also influence the physico-chemical, microbiological, and potential biological characteristics of bee pollen. It is common that the product goes through pre-cleaning, freezing, defrosting, dehydration, aeration, final cleaning, packaging, and storing stages (Barreto et al. 2005b). As bee pollen contains a lot of water when is collected from the hive, a process of dehydration is necessary in order to avoid fermentation and spoilage (Estevinho et al. 2012). Drying is an important step in the production of commercial bee pollen and it is responsible for promoting the preservation of the product and for increasing of its shelf life (Moreira et al.

2008). Currently, the great majority of beekeepers carry out this step, and the dehydration conditions vary according to production unit.

The commercialization of bee pollen is regulated in countries such as Argentina, Armenia, Bulgaria, Brazil, China, Cuba, Japan, Poland, Russia, Switzerland, Turkey, and Uraguay with physico-chemical parameters generally being established (Almeida-Muradian 2009). Brazilian legislation, for example, includes moisture limits on the product in natura (30% maximum) and dehydration (4% maximum), as well as minimum protein levels of 8 g/100 g, 1.8 g/100 g of lipids, and 2 g/100 g of brute fiber, maximum levels of 4 g/100 g of ash and sugars total between 14.5 and 55 g/100 g, all on a dry base. Maximum free acidity levels of 300 mEq kg^{-1} and pH of 4–6 were also established (Brazil 2001). Russian legislation established moisture levels of 8 to 10%, a minimum of 21 g/100 g of protein, a maximum of 4 g/100 g of ashes, up to 0.1 g/100 g of mechanical impurities, and up to 0.6 g/100 g of mineral impurities, a minimum flavonoid value of 2.5 g/100 g, pH of 4.3–5.3 and an oxidation index of up to 23 s, beyond sensorial parameters (Russia 1990). Swiss legislation established a maximum moisture of 8% in bee pollen (Switzerland 2005).

Due to the nutritional value and biological potential of bee pollen, a number of studies have been carried out with the aim of characterizing samples from different regions, seeking to identify particularities and those samples with elevated levels of certain compounds. In this chapter the results of this research are presented, with the aim of bringing together the greatest quantity of information regarding the composition of the bee pollen.

11.2 Vitamin Content

Vitamins are a group of organic compounds which are necessary to ensure growth and maintain the health of humans (Ball 1994). These compounds have different chemical compositions and biological functions, and are necessary for the synthesis of essential cofactors and metabolic reactions. Vitamins are essential for the maintenance of biological functions and can appear in nature as a vitamin itself or in the form of precursors such as provitamins (Ball 1998; Lopes et al. 2011).

These compounds can be classified in terms of their solubility. Lipid-soluble vitamins (e.g. vitamins A, D, E and K) are a group of chemical substances with different structure, but they have in common solubility in organic solvents. They are stored in body fat and can be toxic when consumed in excess (hypervitaminosis). Water-soluble vitamins (e.g. vitamins B_1 or thiamine, B_2 or riboflavin, B_3 or niacin, B_5 or pantothenic acid, B_6 or pyridoxine, B_7 or biotin, B_9 or folic acid, B_{12} or cyanocobalamin, and C or ascorbic acid) are not normally stored in significant amounts in the body, so we need a daily supply of these vitamins. These vitamins are involved in the metabolism of fats, carbohydrates, and proteins (Ball 1998). Daily requirements for these vitamins are low, which can be explained by their nonparticipation in body structure formation and the fact that they are not an energy source.

Bee pollen contains hydrosoluble and liposoluble vitamins: B complex, vitamin C, E and carotenes with provitamin A function (α and β-carotene) (Melo et al. 2009; Oliveira et al. 2009; Melo and Almeida-Muradian 2010; Arruda et al. 2013a; b; Souza 2014; Sattler et al. 2015). According to the authors Zafra (1979), Schmidt and Buchmann (1992), Krell (1996), and Mizrahi and Lensky (1997), bee pollen is rich in the B complex vitamins: thiamine, niacin, riboflavin, pyridoxine, pantothenic acid, folic acid, and biotin. B complex vitamins in bee pollen have also been reported by Dutcher (1918), Arruda et al. (2013a, b) and Souza (2014). This food is also rich in provitamin A and contains varying amounts of vitamin C and lipid soluble vitamins. Botanical origin and processing conditions appear to be the main influences on the vitamin content in bee pollen.

The analysis of vitamins naturally present in food can be considered a challenge because they are present in low concentrations. The presence of an interfering matrix complexity further complicates the task, as well as the need for special care due to low stability. One of the main challenges of characterizing vitamin content in food is the development and establishment of simultaneous methods. This is desirable because it significantly reduces time and expenses for analysis, and generates less waste (Macrae 1990; Polesello and Rizzolo 1990). Before vitamin analysis, moisture content must be determined, in order to facilitate the expression of results on the dry base. In the study by Melo and Almeida-Muradian (2011) six methods for moisture content determination in dehydrated bee pollen samples were compared (conventional oven at 100 °C, vacuum oven at 70 °C, desiccator with sulfuric acid, drying out process with infrared light at 85 °C, lyophilization and Karl Fisher's method). Results showed that the methods which use infrared and the lyophilization processes are the most recommended.

11.2.1 Pro-Vitamin A (Carotenoids)

Carotenoids such as provitamin A are considered one of the antioxidant vitamins. Almeida-Muradian et al. (2005) reported carotenoids but not β-carotene (provitamin A) in samples from southern Brazil. The authors argued that the absence of this provitamin could be due to the use of thermal processes for dehydration, which can damage vitamins because of their thermal instability. Barajas et al. (2012) specifically tested the influence of the dehydration process on carotenoid content. The authors analyzed bee pollen from Colombia dried at two different temperatures (35 and 45 °C): one sample showed the values 0.77 (fresh), 0.78 (dried at 35 °C), and 0.51 mg/g (dried at 45 °C) for carotenes; the second sample showed the values of 0.21 (fresh), 0.22 (dried at 35 °C) and 0.17 mg/g (dried at 45 °C).

Oliveira et al. (2009) analyzed beta-carotene as provitamin A of fresh bee pollen samples from southeastern Brazil (São Paulo state). The authors obtained a range of 56.3–198.9 μg/g for beta-carotene. Samples collected in April (autumn in Brazil) can be considered provitamin A sources. A relationship was observed between botanical origin and β-carotene content: *Raphanus* sp., *Macroptilium* sp. and *Mimosa caesalpineafolia*.

Melo et al. (2009) analysed beta-carotene (provitamin A) of dehydrated bee pollen samples collected from southeastern Brazil (São Paulo state). Six batches of samples were analyzed and a variation of 3–78 μg/g for β-carotene was found. The pollen types Arecaceae, *Cecropia*, and Fabaceae were correlated with β-carotene content and a negative correlation of *Mimosa caesalpiniaefolia* and Poaceae types with β-carotene was observed. Sattler et al. (2015) determined α and β-carotene of dehydrated bee pollen samples from southern Brazil, and obtained the following average values: 61.3 μg/g of α-carotene and 17.7 μg/g of β-carotene. The provitamin A value varied from 3.9 to 4945.2 retinol μg/100 g.

In Thailand, a vitamin A—β-carotene value of 1530.4 μg/100 g for monofloral corn bee pollen was reported (Chantarudee et al. 2012). Bee pollen from Spain was studied by Muniategui et al. (1990), who obtained a mean of 46.7 mg of β-carotene/100 g lipids. These samples presented a mean of 6.2% for lipids. Szczesna et al. (1991) found 10.87 for provitamin A (mg%) in bee pollen from Poland. In samples from Romania, the authors observed a range of 1–200 mg/kg for β-carotene (Mărgăoan et al. 2010).

11.2.2 B Complex Vitamins

Vitamins B_1 (thiamine), B_2 (riboflavin), three vitamers of B_6 (pyridoxol, pyridoxal and pyridoxamine) and two vitamers of vitamin PP (nicotinic acid and nicotinamide) were analyzed in Brazilian bee pollen samples. For vitamin B_1 a variation from 0.59 to 1.09 mg/100 g (fresh samples) was obtained and from 0.64 to 1.01 mg/100 g for dried samples. Vitamin B_2 concentrations varied from 1.73 to 2.23 mg/100 g (fresh samples) and from 1.77 to 2.56 mg/100 g (dried samples). Vitamin PP concentrations varied from 6.43 to 15.34 mg/100 g (the fresh samples) and from 7.27 to 14.43 mg/100 g (dried samples). Vitamin B_6 content varied from 0.50 to 0.79 mg/100 g (fresh samples) and from 0.33 to 0.77 mg/100 g (dried samples). The amount of pyridoxol was very low and sometimes below the limit of quantification (0.07 mg/100 g) for either fresh or dried samples. The concentration of pyridoxal and pyridoxamine was also above that limit. Regarding the pollinic analysis, a direct proportional correlation was found between the percentages of *Myrcia* and *Vernonia* pollen types, and the concentration of vitamin B_1. It was also observed that the greater the presence of the *Cestrum* pollen type, the lower the concentration of vitamin B_2. The occurrence of the *Ilex* pollen type was associated in a marginally significant way and inversely proportional to the concentration of vitamin PP. There was no association between the pollen types and vitamin B_6 (Arruda et al. 2013a, b).

Further, in southern Brazil, the B complex vitamins (vitamin B_1, B_2, and the vitamers of vitamins B_3 and B_6) in dehydrated bee pollen were studied (Souza 2014). This region has a differentiated climate, topography, and vegetation and is one of the largest Brazilian producers of bee pollen. The results were expressed on a dry basis and the following was found: vitamin B_1 (0.46–1.83 mg/100 g); vitamin

B_2 (0.40–1.86 mg/100 g); for vitamin B_6, with only the pyridoxal and pyridoxamine vitamers quantified (pyridoxal = from 0.42 to 6.70 mg/100 g; pyridoxamine = from 0.26 to 0.95 mg/100 g). For 25 g of bee pollen (suggested daily intake), 15 out of 28 samples were considered a source and 2 were rich in thiamine; 19 lots were sources and 3 were rich in riboflavin, and, 2 lots were sources of and 26 were rich in pyridoxine in relation to the Daily Intake Reference (DIR) for adults, as established by Brazilian legislation (Brazil 2005).

De-Melo et al. (2016) showed B complex vitamin in heterofloral bee pollen samples from São Paulo State, Brazil, submitted to two different dehydration procedures. The results obtained for thiamine (vitamin B_1) were 0.6–1.3 mg/100 g (electric oven dehydration) and 0.5–0.9 mg/100 g (lyophilized samples); for riboflavin (vitamin B_2) the values reported were 0.4–0.5 mg/100 g (electric oven dehydration) and 0.4–0.5 mg/100 g (lyophilized samples). For niacin the results observed were 1.3–2.6 mg/100 g (electric oven dehydration) and 1.3–1.5 mg/100 g (lyophilized samples); and for niacinamide the values 3.0–3.2 mg/100 g (electric oven dehydration) and 3.7–3.8 mg/100 g (lyophilized samples) were obtained. Regarding vitamin B_6, for pyridoxamine, the values 0.4–0.6 mg/100 g (electric oven dehydration) and the mean of 0.5 mg/100 g (lyophilized samples) were obtained; for pyridoxal 1.9–3.8 mg/100 g (electric oven dehydration) and 3.4–3.6 (lyophilized samples) was reported. For pyridoxol 0.1–0.2 for both dehydration procedures was observed.

In 1980, Loper et al. reported the following vitamins in bee pollen: 0.76 mg/100 g of panthothenic acid, 7.10 mg/100 g of niacin, 0.61 mg/100 g of pyridoxine, and 188 mg/100 g of inositol. In bee pollen from France, Donadieu (1983) presented a range of 16.30–19.20 μg/g for vitamin B_2 and 0–9 μg/g (on a dry basis) for vitamin B_6 (not separating the vitamins of vitamin PP and B6). Thiamine was analyzed in American samples from Beltsville and a maximum value of 6.5 μmol/100 g (dry weight) was obtained (Herbert et al. 1987). Sánchez (2004) found 5 μg/g of vitamin B_6 (without separation of the vitamins) in bee pollen from Mexico. Bee pollen from Romania presented a range of 6–13 mg/kg of thiamine; 6–20 mg/kg of riboflavin; 40–110 mg/kg of niacin; 5–20 mg/kg of pantothenic acid, and 2–7 mg/kg of pyridoxine (Mărgăoan et al. 2010). In Thailand, monofloral bee pollen from corn was evaluated and the authors reported the following values: 0.20 mg/100 g of thiamine; 0.50 mg/100 g for riboflavin; 7.03 mg/100 g of niacin; 0.39 mg/100 g of vitamin B_5 and 1.87 μg/100 g of vitamin B_{12}. Vitamin B_6 was not found (Chantarudee et al. 2012).

Campos et al. (2008), on behalf of the International Honey Commission, proposed a standard for bee pollen composition and established values of 0.6–1.3 mg/100 g of vitamin B_1, 0.6–2 mg/100 g of vitamin B_2, 4–11 mg/100 g of vitamin PP and 0.2–0.7 mg/100 g of vitamin B_6. These values were based on the results of Stanley and Linskens (1974) and Szczesna and Rybak-Chmielewska (1998).

11.2.3 Vitamin C

Loper et al. (1980) reported the value of 20.6 mg/100 g of vitamin C for bee pollen. The first authors who tried to analyze vitamin C in Brazilian bee pollen, found no vitamin C at all (Almeida-Muradian et al. 2005) in samples from southern Brazil. Another study was carried out by Oliveira et al. (2009) which analyzed vitamin C (ascorbic acid) from fresh bee pollen samples from southeast Brazil. A range from 273.9 to 560.3 μg/g of ascorbic acid was observed. Samples collected in October (Brazil's spring) could be considered vitamin C sources. A relationship between the following botanical origins: *Anadenanthera* sp., *Arecaceae* type, and *Philodendron* sp. and vitamin C content was observed.

Other studies using southeastern Brazilian dried bee pollen (Melo et al. 2009; Melo and Almeida-Muradian 2010) obtained values from 114 to 340 μg/g of vitamin C. The pollen types from *Myrtaceae* had a correlation with vitamin C content. Regarding the stability of this vitamin (Melo and Almeida-Muradian 2010), it was observed that on average samples stored at room temperature (exposed and protected from light) presented a 50% loss in ascorbic acid concentration. According to the statistical analysis, there was a significant difference ($p < 0.05$) between these two conditions when compared to vitamin concentrations at time 0. No difference, however, was observed between the periods of analysis (6 and 12 months of storage) using either storage method at room temperature. This suggests that when bee pollen is stored at room temperature, vitamin C is rapidly lost. After this initial sharp loss, however, it becomes stable during the storage process. Samples stored in the freezer presented a lower level of vitamin C loss (22% in 6 months and 26% in 12 months) when compared to losses that occurred in storage at room temperature. Nevertheless, for initial vitamin C concentrations, there was a statistically significant difference ($p < 0.05$) after twelve months of storage in the freezer. These results indicated that the storage of dried bee pollen in the freezer until consumption was advisable.

Ascorbic acid was also evaluated in southern Brazilian bee pollen samples by Sattler et al. (2015) and an average value of 262.3 μg/g for vitamin C was obtained. Bee pollen from Poland presented the values 12.8 and 15.21 mg% of vitamin C (Szczesna et al. 1991). Bee pollen from Romania presented a range of 70–560 mg/kg for ascorbic acid (Mărgăoan et al. 2010).

Barajas et al. (2012) studied vitamin C of dehydrated bee pollen from Colombia (samples dried at 35 and 45 °C). The first samples presented the values 40.22 mg/100 g (fresh), 31.75 mg/100 g (dried at 35 °C), and 27.35 mg/100 g (dried at 45 °C). The second sample showed the values 40.37 mg/100 g (fresh), 32.79 mg/100 g (dried at 35 °C) and 28.75 mg/100 g (dried at 45 °C). Therefore, the content of this vitamin seems to be altered by processing conditions, especially temperature.

11.2.4 Vitamin E

Vitamin E is one of the antioxidant vitamins, with α-tocopherol being the most studied compound. In Brazil, the first attempt to identify α-tocopherol in bee pollen was made by Almeida-Muradian et al. (2005). This vitamin, however, was not found in the commercial samples, probably due to the dehydration process. The second attempt in Brazilian bee pollen was made by Oliveira et al. (2009) and the authors used fresh bee pollen samples from southeastern Brazil. A range of 13.5–42.5 μg/g for vitamin E (α-tocopherol) was observed, and a relationship between vitamin E and the following botanical origins: *Raphanus* sp., *Eucalyptus* sp., *Macroptilium* sp., *Mimosa caesalpineafolia* was also reported. Melo et al. (2009) analyzed α-tocopherol from dehydrated bee pollen samples collected in southeastern Brazil and observed a range of 16–39 μg/g for vitamin E. The four tocopherols (alpha, beta, gamma, and delta-tocopherol) were determined by Sattler et al. (2015), who reported the following mean values for vitamin E: 32.8 μg/g of alpha-tocopherol, 2.1 μg/g of beta-tocopherol, 4.6 μg/g of gama-tocopherol, and 6.3 μg/g of delta-tocopherol.

De-Melo et al. (2016) studied Brazilian heterofloral bee pollen (São Paulo state) using two different dehydration processes. The results obtained for vitamin E (α-tocopherol) were 27.2–27.5 μg/g (bee pollen dehydrated in an electric oven with forced air circulation) which was lower than lyophilized samples (37.5–53.7 μg/g). The same parameters such as light, oxygen, and temperature are responsible for degradation of tocopherols; therefore, processing conditions significantly influence vitamin E content in bee pollen.

Corn monofloral bee pollen from Thailand presented the value 6.21 mg/100 g for vitamin E (alpha-tocopherol) (Chantarudee et al. 2012).

11.3 Protein and Amino Acid Content

Protein levels in dehydrated bee pollen can vary between 2.5 and 62 g/100 g per sample, mainly according to the botanical origin. Elevated levels of this macronutrient make some samples an alternative dietary supplement, especially for vegetarians. The Food and Agriculture Organization of the United Nations (FAO 2011), recommends that adults (>18 years) consume 0.66 g/kg/day of protein, something around 49.5 g for an individual of 75 kg. For these individuals, a 25 g portion of bee pollen (the equivalent of one soupspoon) can provide up to 31% of the recommended intake. Further, according to the FAO, it is important to consider the intake of essential amino acids, which are present in bee pollen as will be subsequently considered.

The protein content of samples collected in different countries, with different botanical origins presented in Table 11.1. Considering monofloral samples from different countries, bee pollen of *Brassica napus* contains protein levels between 23 g/100 g (Brazil) and 27.3 g/100 g (China); of *Cistus* sp. ranging from 12.6 g/100 g

Table 11.1 Protein and lipid content in monofloral and heterofloral bee pollen collected from different countries

Botanical origin	Origin/Country	Proteins Min-Max (g/100 g)	Lipids Min-Max (g/100 g)	References
Alchornea	Brazil	21.2	5.4	Modro et al. (2009)
Alternanthera	Brazil	8.8–10.6	5.1–6.9	De-Melo (2015)
Anadenanthera	Brazil	16–32.2	3.2–9.8	Arruda (2013) and De-Melo (2015)
Arecaceae	Brazil	22.1–22.8	4.7–5.7	Arruda et al. (2013b)
Baccharis	Brazil	19.6	4.1	Modro et al. (2009)
Brassicaceae	Serbia	23.6	6.8	Kostic et al. (2015a)
Brassica	Brazil	14.5–23.2	8.6–14	Souza (2014) and De-Melo (2015)
Brassica kaber	Egypt	29	9.7	Youssef et al. (1978)
Brassica juncea	India	18.2	13.8	Ketkar et al. (2014)
Brassica napus	China	27.3	6.6	Yang et al. (2013)
Brassica napus	Brazil	23–24.5	4.7–6.2	Souza (2014)
Camellia japonica	China	29	5.3	Yang et al. (2013)
Castanea	Italy	26.6	2.1	Gabriele et al. (2015)
Cestrum	Brazil	24.8	4.6	Arruda et al. (2013b)
Cirsium	Brazil	16.2	4.6	Modro et al. (2009)
Cistus	Spain	12.6–22.5	2.9–7.2	Serra-Bonvehí and Escolà-Jordà (1997) and Nogueira et al. (2012)
Cistus	Italy	25.9	1.9	Gabriele et al. (2015)
Cistus	Portugal	23–27.1	2.8–5.2	Féas et al. (2012) and Nogueira et al. (2012)
Citrullus lanatus	China	20.7	3.6	Yang et al. (2013)
Cocos nucifera	Brazil	10.3–21.6	4.6–8.3	Arruda (2013) and De-Melo (2015)
Coffea	Brazil	27.7	1.8	Modro et al. (2009)
Dendranthema indicum	China	14.9	4	Yang et al. (2013)
Encelia canescens	Peru	14.3	0.2	Saavedra et al. (2013)
Eucalyptus	Brazil	12.5–19.5	3.8–4	Souza (2014) and De-Melo (2015)
Fabaceae	Serbia	19.9	2.1	Kostic et al. (2015a)
Fagopyrum esculentum	China	14.3	5.2	Yang et al. (2013)
Helianthus annuus	China	15.3	1.5	Yang et al. (2013)
Helianthus annuus	South Africa	14.2	5.5	Nicolson and Human (2013)

(continued)

Table 11.1 (continued)

Botanical origin	Origin/Country	Proteins Min-Max (g/100 g)	Lipids Min-Max (g/100 g)	References
Mimosa caesalpiniaefolia	Brazil	17.6–21.2	4.9–8.3	Arruda (2013) and De-Melo (2015)
Mimosa scabrella	Brazil	11.7–33.9	2.5–8.6	Arruda (2013), Souza (2014), and De-Melo (2015)
Myrcia	Brazil	12.5–41.2	2.4–7.8	Modro et al. (2009), Arruda et al. (2013b), and De-Melo (2015)
Nelumbo nucifera	China	17	5.2	Yang et al. (2013)
Onagraceae	Poland	21.5	–	Szczesna (2006b)
Papaver rhoeas	China	25	2.2	Yang et al. (2013)
Prosopis pallida	Peru	13.7–16	0.2	Saavedra et al. (2013)
Rosa rugosa	China	22.9	1.4	Yang et al. (2013)
Rubus	Italy	28.4	2.8	Gabriele et al. (2015)
Salix	Serbia	24.8	2.9	Kostic et al. (2015b)
Schisandra chinensis	China	27.4	2.4	Yang et al. (2013)
Trifolium alexandrium	Egypt	35.5	7.1	Youssef et al. (1978)
Vernonia	Brazil	15.4–20.9	3–6.6	Modro et al. (2009)
Vicia faba	China	27.9	0.7	Yang et al. (2013)
Vicia faba	Egypt	29.2	10.6	Youssef et al. (1978)
Zea mays	China	17.9	4	Yang et al. (2013)
Zea mays	Egypt	23.3	9.9	Youssef et al. (1978)
Zea mays	Thailand	19.1	7	Chantarudee et al. (2012)
ND	Argentina	17.2–30.9	1.8–6.8	Baldi-Coronel et al. (2004)
Heterofloral or ND	Brazil	8.4–40.5	0.4–14	Bastos et al. (2003), Funari et al. (2003), Almeida-Muradian et al. (2005), Barreto et al. (2005a), Modro et al. (2007), Carpes et al. (2009), Martins et al. (2011), Arruda et al. (2013b), and Sattler et al. (2015)
Heterofloral or ND	Spain	12.5–20.8	2.8–5.6	Nogueira et al. (2012) and Fuenmayor et al. (2014)
Heterofloral	Portugal	18.8–34.2	2.3–3.3	Estevinho et al. (2012), Féas et al. (2012), and Nogueira et al. (2012)
Heterofloral	Serbia	14.8–27.2	1.3–5.5	Kostic et al. (2015a)

ND, not determined

(Spain) to 27.1 g/100 g (Portugal); of *Helianthus annuus* contains protein ranging from 14.2 (South Africa) to 15.3 g/100 g (China); *Vicia fala* ranging from 27.9 g/100 g (China) to 29.2 g/100 g (Egypt); and of *Zea mays* contains from 17.9 g/100 g (China) to 23.3 g/100 g (Egypt).

A positive correlation between protein content and *Brassica napus*, *Cestrum* and *Anadenanthera* pollen types has been observed, and a negative correlation with the *Astrocaryum*, *Baccharis*, *Gochnatia*, and *Vernonia* types (Modro et al. 2007; Melo et al. 2009; Arruda et al. 2013b; Sattler et al. 2015). However, it is not always possible to establish this type of relation, since variation in pollen composition can occur within the same species, grown under different environmental conditions (Funari et al. 2003; Almeida-Muradian et al. 2005; Melo et al. 2009). De-Melo (2015), who evaluated monofloral *Mimosa scabrella* samples collected in different regions of Brazil, observed protein content varying significantly between 11.7 and 33.9 g/100 g.

Pollen from plants that flower during spring tends to have a higher protein content than those which flower during summer or autumn (Liolios et al. 2016). Therefore, bees can produce bee pollen with a higher level of this compound during this season. The insects seem to be able to identify pollen sources with greater nutritional value (Cook et al. 2003; Barth 2004; Modro et al. 2011). However, other factors, such as the quantity of resource available, the size and form of the pollen grain, the presence of competitors, and the distance between the plant source and the hive, also influence pollen harvesting by bees and in this way, the composition of the final product (Cook et al. 2003; Luz et al. 2010; Silveira 2012).

In terms of amino acid content, interest in this compound's profile in bee pollen goes beyond the question of nutrition, given that the level of certain amino acids could serve as an indicator of the freshness and adequacy of the drying process and storage. Further, it could also indicate species-specific profiles and quantities could be used as a tool to determine the botanical and even geographical origin of samples (González-Paramás et al. 2006). The greater part of the amino acids found in this product are in the bound form, with only about 1/10 of the total protein comes from free amino acids (González-Paramás et al. 2006; Bogdanov 2012b). The composition of the free amino acid profile in bee-pollen is influenced by the botanical origin, but also external factors, such as storage and heating conditions (Serra-Bonvehí and Escolà-Jordà 1997; Domínguez-Valhondo et al. 2011).

Influences from botanical origin on amino acids present in bee pollen seem to be more quantitative than qualitative. With few exceptions, all essential amino acids are found (Human and Nicolson 2006; González-Paramás et al. 2006; Szczesna 2006b; Bogdanov 2012a), which represent from 12 to 43% of the total amino acid level (González-Paramás et al. 2006; Szczesna 2006b; Martins 2010; Yang et al. 2013; Komosinska-Vassev et al. 2015). The quantity of each essential amino acid is variable, since there is variation in pollen grain composition between plant species (Loper and Cohen 1987). It is believed that bees, owing to learning acquired during foraging, seek pollen sources which better suit their needs for these compounds (Cook et al. 2003), and if necessary, they may seek more than one species, with the aim of obtaining a product that meets their nutritional requirements.

The total amino acid content in bee pollen can vary between 108.1 and 287.7 mg/g of product. The following amino acids were already quantified: aspartic acid (10.6–32.3 mg/g), alanine (6.5–14.5 mg/g), glycine (5.5–12.8 mg/g), glutamine (5.0–14.2 mg/g), isoleucine (4.8–16.0 mg/g), phenylalanine (4.6–27.2 mg/g), arginine (4.3–25.8 mg/g), threonine (4.2–12.5 mg/g), proline (3.8–59.5 mg/g), asparagine (3.4–5.7 mg/g), glutamic acid (3.3–29.3 mg/g), histidine (2.9–44.9 mg/g), tyrosine (2.6–37.6 mg/g), serine (2.4–13.3 mg/g), leucine (2.2–23.1 mg/g), tryptophan (1.6–148.0 mg/g), lysine (1.3–37.1 mg/g), valine (1.3–15.8 mg/g), cystine (0.6–3.6 mg/g), methionine (0.2–7.2 mg/g), γ-aminobutyric acid (GABA) (0.16–0.34 mg/g), cysteine, hydroxyproline, ornithine, homoserine (Cook et al. 2003; Baldi-Coronel et al. 2004; González-Paramás et al. 2006; Szczesna 2006b; You et al. 2007; Martins 2010; Bogdanov 2012b; Yang et al. 2013).

Proline was the main amino acid found in samples produced in Spain, with an average of 21.8 mg/g per sample (Serra-Bonvehí and Escolà-Jordà 1997; González-Paramás et al. 2006; Domínguez-Valhondo et al. 2011). It was also the main amino acid found in Brazilian samples, with average concentrations of 10.7 mg/g (Martins 2010) and in samples produced in China, in which average content was between 11.8 mg/g and 23.3 mg/g (You et al. 2007; Yang et al. 2013). In samples produced in Korea, glutamic acid (23.2 mg/g average), aspartic acid (22.5 mg/g average), and proline (21.9 mg/g average) made up 36% of total amino acids (Szczesna 2006b).

Bees directly contribute to the proline content (Louveaux et al. 1978; González-Paramás et al. 2006). The level of this amino acid tends to increase over time with storage. This is because the presence of the enzyme glutamate dehydrogenase leads to the biosynthesis of proline from glutamic acid (Serra-Bonvehí and Escolà-Jordà 1997; Baldi-Coronel et al. 2004). When, during the dehydration of bee pollen, beekeepers use high temperatures over extended periods of time, there is a reduction in the level of free amino acids, and consequently, a proportional increase in proline content (Serra-Bonvehí and Escolà-Jordà 1997).

Amongst essential amino acids, leucine and lysine show the greatest quantities, varying between 0.4 and 23.1 mg/g and from 0.2 to 21.1 mg/g, respectively. Valine (0.06–15.7 mg/g), isoleucine (0.02–13.3 mg/g), and methionine (ND-9.1 mg/g) occur at lower quantities (González-Paramás et al. 2006; Szczesna 2006b; You et al. 2007; Martins 2010; Bogdanov 2012b; Yang et al. 2013). Usually, tryptophan cannot be determined due to the process of hydrolysis employed during the analyses (Nicolson and Human 2013), however, quantities varying between 7 and 148 mg/g were already found in monofloral bee pollen from *Helianthus annuus* and from *Camellia japonica*, respectively (Yang et al. 2013).

In terms of the solubility of proteins present in bee pollen, at pH 7, this parameter can vary between 2.8 and 25.9 g/100 g, which could be compared with low solubility commercial soy protein products (concentrated and isolated flours) (Kostic et al. 2015a). In food technology, protein solubility should be considered, since this property is particularly important because it can affect emulsification, foaming, and gelation (Kinsella 1976). In bee pollen, there seems to be a positive correlation between protein solubility and carbohydrate levels, and a negative correlation between this parameter and lipid and ash content. In a complex matrix, such as is the

case with this product, this parameter can be determined by protein composition and conformation as well as by interaction amongst proteins and constituents (Kostic et al. 2015a).

11.4 Lipid and Fatty Acid Content

Lipid content in this food source can reach 22 g/100 g (Table 11.1). Certain steroids, carotenoids, and squalene are found in the lipid fraction, beyond a variety of fatty acids (Xu et al. 2011; Komosinska-Vassev et al. 2015). Given that the type of compound can influence the techno-functional properties of the food source, Kostic et al. (2015a) observe that bee pollen suspensions do not produce foams in assayed conditions, maybe due to the surface-active lipids present in bee pollen samples. The results of this study indicate that bee pollen can be used as a foam depressant or as a food ingredient where foaming properties are not desirable.

Lipid levels in bee pollen generally vary according to the botanical origin. However, at the time of analysis, the extraction of compounds can be influenced by the processing adopted by the beekeeper. De-Melo et al. (2016) observed that the dehydration conditions influenced the result, which corroborated the hypothesis of Domínguez-Valhondo et al. (2011) that processing can affect the structure of the grains, facilitating or not the extraction of compounds.

Studies of correlations between the botanical origins and lipid content of bee pollen suggest a positive association of this parameter with the pollen types *Astrocaryum* and *Brassica napus* and a negative correlation with the pollen types *Mimosa caesalpiniaefolia* and *Cestrum*, in samples produced in the south and southeast of Brazil (Melo et al. 2009; Arruda et al. 2013b; Sattler et al. 2015). These results indicate that greater or lesser percentages of these pollen types can be associated, respectively, with greater or lesser concentrations of lipids in bee pollen. Modro et al. (2007) tested, in samples collected in the southeast of Brazil, the correlation between lipid levels and the pollen types *Anadenanthera*, *Baccharis*, *Cecropia*, *Coffea*, *Elephantopus*, *Eucalyptus*, *Gochnatia*, *Myrcia*, *Ricinus*, *Senecio*, *Trema* and *Vernonia* where they observed no association via the statistical analysis employed.

Considering monofloral samples collected in different countries, bee pollen of *Brassica napus* contains lipid content between 4.7 g/100 g (Brazil) and 6.6 g/100 g (China); of *Cistus* sp. contains values between 1.9 g/100 g (Italy) and 7.2 g/100 g (Spain); of *Helianthus annuus* ranging from 1.5 g/100 g (China) to 5.5 g/100 g (South Africa); of *Vicia faba* contains from 0.7 g/100 g (China) to 10.6 g/100 g (Egypt); and *Zea mays* contains levels varying from 4 g/100 g (China) to 9.9 g/100 g (Egypt) (Table 11.1).

Variable levels of fatty acids are present in the lipid fraction. Nicolson and Human (2013), analyzing samples collected in South Africa, found that half of the lipid content of monofloral bee pollen from *Helianthus annuus* consisted of long-chain fatty acids. In samples produced in Israel, the total fatty acid content in the bee

pollen averaged 3.8%, ranging from a minimum 2.3% to a maximum 6.6% (Avni et al. 2014). In all the studies consulted, linoleic and α-linolenic acids, considered strictly essential for the human organism, were quantified. Essential fatty acids have a significant impact from the nutritional point of view, due to their being membrane components and playing an important role in metabolic processes (Perini et al. 2010). Beyond this, the adequate ingestion of these compounds helps in the prevention of cardiovascular and degenerative diseases, and certain types of cancer (Calviello et al. 2007; Cheatham et al. 2006; Carl et al. 2009). Certain fatty acids, such as linoleic, linolenic, myristic, and lauric acids, have bactericidal and antifungal properties (Manning 2001; Yang et al. 2013).

In samples collected in Poland, China, and South Korea α-linolenic, palmitic, and linoleic acids predominate, and myristic, stearic, oleic, arachidonic, behenic, and lignoceric acids were also quantified, all at levels that varied according to the botanical origin. In this same study, the author observed unsaturated-to-saturated fatty acid ratio (UFA/SFA ratio), which ranged from 1.87 to 2.16 for the samples from Poland, from 1.21 to 2.35 for the samples from South Korea, and from 1.50 to 2.09 for the samples from China (Szczesna 2006a). This was similar to what was observed by Serra-Bonvehí and Escolà-Jordà (1997) in samples collected from Spain (1.96).

In bee pollen collected in Romania, α-linolenic, palmitic, and linoleic acids also predominated, and as well as these, caproic, caprylic, capric, lauric, myristic, stearic, oleic, elaidic, arachidonic, 11-eicosenoic, behenic acids were also identified (Mărgăoan et al. 2014). In samples from Colombia, with a mean lipids value of 6.9 g/100 g, the most abundant fatty acids were also α-linolenic (6.8–42.0 mg/100 mg lipids), palmitic (6.3–16.6 mg/100 mg lipids) and linoleic (5.7–12.5 mg/100 mg lipids). The other fatty acids determined were: lauric (0.8–9.1 mg/100 mg lipids), myristic (0.5–2.6 mg/100 mg lipids), stearic (1.1–3.0 mg/100 mg lipids), oleic (1.3–3.9 mg/100 mg lipids), arachidic (0.6–1.5 mg/100 mg lipids), homo-γ-linolenic (0.2–5.3 mg/100 mg lipids) and tricosanoic (0.2–2.3 mg/100 mg lipids) (Fuenmayor et al. 2014).

In bee pollen produced in Brazil, Bastos et al. (2004) observed that the unsaturated compounds represented from 14 to 56% of the total fatty acids. The percentage of linoleic acid varied from 9 to 50% and that of linolenic acid from traces to 4% and the UFA/SFA ratio was <1.0 in almost all samples. In bee pollen collected in Portugal, caprylic, capric, lauric, palmitic, oleic, arachidonic, eicosenoic, linoleic, and α-linolenic acids were quantified, with the last representing between 26 and 57% of the total fatty acids. The polyunsaturated-to-saturated fatty acid ratio (PUFA/SFA ratio) ranged between 1.40 and 4.51 and the linoleic acid:α-linolenic acid proportion varied between 0.11 and 0.96 (Estevinho et al. 2012; Féas et al. 2012). Linoleic and α-linolenic acids are key compounds for cell membranes, neurotransmission, and brain function (Féas et al. 2012).

It is possible that bees seek out species whose pollen grains contain greater unsaturated than saturated fatty acid levels (Szczesna 2006a), and that this perception has a relationship with aromatic substances present in the lipid fraction which ends up related, by the bees, to pollen grain composition (Manning 2001). The

UFA/SFA ratio also shows the influence of dehydration and storing processes, during which time decomposition of unsaturated compounds can occur, explaining the values of ratios below 1 in certain samples (Bastos et al. 2004; Human and Nicolson 2006).

The level of fatty acids in the pollen grain can vary between plant species, or within the same species according to the cultivar. Environmental factors can also influence the composition of pollen grains and, consequently, bee pollen; and different extraction and analysis methods for fatty acids can reach different results. In monofloral samples of *Zea Mays* produced in China α-linolenic (52%) and palmitic (25%) acids predominated, while in samples from the same botanical origin but produced in Egypt, oleic (42%) and myristic (40%) acid predominated (Shawer et al. 1987; Yang et al. 2013). In *Helianthus annuus* monofloral bee pollen lauric (33%) and palmitic (23%) acids predominated when the samples originated from South Africa, and α-linolenic (42%) and palmitic (29%) when the samples originated from China (Nicolson and Human 2013; Yang et al. 2013). In monofloral *Vicia faba* samples, produced in China and Egypt, the same fatty acids predominated, however in different percentages in relation to the total: α-linolenic (52%: China; 22%: Egypt) and palmitic (36%: China; 28%; Egypt) (Shawer et al. 1987; Yang et al. 2013).

In monofloral bee pollen from *Aloe greatheadii* var. davyana, collected in South Africa, the main fatty acids found were gadoleic (42%; 12.9 mg/g) and palmitic (14%; 4.4 mg/g). As well as these, the following saturated fatty acids were identified and quantified: myristic, palmitic, stearic, arachidic, behenic, and lignoceric; and the monounsaturated fatty acids: oleic and gadoleic; and the polysaturated fatty acids: ricinoleic, linoleic, α-linolenic, γ-linolenic, eicosadienoic, homo-g-linolenic, timnodonic, and brassic (Human and Nicolson 2006). Xu et al. (2011) analyzed monofloral bee pollen from *Nelumbo nucifera* produced in China, in which palmitic (45%) and oleic (19%) acids predominated, and other compounds were identified: myristic, pentadecanoic, palmitoleic, margaric, stearic, linnoleic, α-linolenic, and behenic acids. In *Cistus ladanifer* monofloral bee pollen from Spain, the predominant fatty acids were oleic (28%) and palmitic (12%). Lauric, myristic, myristoleic, palmitoleic, margaroleic, stearic, linoleic, linolenic, arachidic, gadoleic, heneicosanoic and lignoceric acids were also identified (Domínguez-Valhondo et al. 2011).

Myristic and linolenic acids were the major acids in monofloral bee pollen of *Trifolium alexandrinum* and *Brassica kaber*, and myristic and oleic acids were the dominant acids in monofloral bee pollen of *Melilotus siculus* from Egypt (Shawer et al. 1987). In monofloral *Brassica campestres* samples, collected in China, the predominant compounds were α-linolenic and palmitic acids (Xu et al. 2009). Yang et al. (2013), who also analyzed samples produced in China, identified that in monofloral bee pollen from *Citrullus lanatus*, *Camellia japonica* and *Schisandra chinensis* α-linolenic and palmitic acids predominated; from *Fagopyrum esculentum* and *Rosa rugosa* α-linolenic and oleic acids predominated; from *Brassica napus* α-linolenic and myristic acids predominated; from *Dendranthema indicum* oleic and linoleic predominated, from *Nelumbo nucifera* palmitic and oleic acids

predominated, and from *Papaver rhoeas* α-linolenic and oleic acids were dominant. Generally speaking, fatty acids present in different bee pollen samples are the same, however, the proportion of these compounds is variable.

11.5 Phenolic Compounds

These compounds are present in all plant structures, including pollen grains (Simões et al. 2004; Cunha and Roque 2009a). Some compounds are widely found, such as benzoic acid derivatives, while others have a smaller scale distribution, such as caffeic acid esters found only in plants from the Lamiaceae family. Freire et al. (2012) researched the occurrence of certain flavonoids in 25 bee pollen samples collected in northeastern Brazil, and concluded that the phenolics: isoquercetin, quercetin, and isorhamnetin were present in more than 90% of the samples from different botanical origins. The importance of identifying specific compounds is that they can be used as taxonomic markers (Simões et al. 2004).

Phenolics are considered bioactive substances, in other words, not synthesized by the human organism, having a specific physiological or metabolic action, which, when regularly present in significant quantities in the diet, aid in the prevention of disease (Carratu and Sanzini 2005; Campos et al. 2008; Horst and Lajolo 2011; Oliveira and Bastos 2011). The action of these compounds seems to be related to an intrinsic capacity for reduction, though other mechanisms are indicated, such as the modulation of the activity of certain enzymes, interactions with receptors, and modes of signal transduction and regulation of the cellular cycles (Menezes et al. 2010; Oliveira and Bastos 2011; Bogdanov 2012b). The biological activity of phenolics should also be considered in terms of the rate of intestinal absorption, metabolization, and excretion of these substances by the organism (Oliveira and Bastos 2011).

Over the years, researchers have studied the antioxidant, antimicrobial, anti-inflammatory, anxiolytic, antiarterosclerosis, antimutagenic, and cardioprotective action of different compounds (Angelo and Jorge 2007; Halliwell and Gutteridge 2007; Hamalainen et al. 2007; Campos et al. 2008; Yang et al. 2008; Aguirre-Hernández et al. 2010; Lopes et al. 2011; Cabrera and Montenegro 2013; Fatrcová-Šramková et al. 2013; Komosinska-Vassev et al. 2015). Aguirre-Hernández et al. (2010) attributed anxiolytic action to the flavonoids quercetin and kaempferol, that resulted in a significant reduction in the activity of male rats. Yang et al. (2008), in studies performed on rutin in different *in vitro* systems, concluded that it was a powerful antioxidant agent whose response is dose dependent. Hamalainen et al. (2007) and Komosinska-Vassev et al. (2015) noted that flavonoids, phenolic acids, fatty acids, and phytosterols are determined as the main agents responsible for the anti-inflammatory capacity of bee pollen.

To determine the level of these substances in bee pollen certain methods were adopted. The colorimetric method with Folin-Ciocalteu reagent was frequently used to indicate the levels of phenolic compounds in gallic acid equivalents (GAE), even

if it was not specifically intended to quantify these compounds. Certain substances, such as ascorbic acid, amines, carbohydrates, and sulfur-containing compounds, can also react with phosphomolybdic and phosphotungstic acids of the reagent used (Huang et al. 2005). To determine the total flavonoid level, a colorimetric method based on quantifying the formation of complexes between flavonoids and the aluminium present in the reagent was frequently utilized, with the result being expressed in equivalents of quercetin (EQ) or epicatechin (EE). Currently, the colorimetric methods tend to be carried out together with other more specific methods, such as high efficiency liquid chromatography in conjunction with mass spectrometry (HPLC-MS), which allows the individual identification and quantification of compounds. According to Cunha and Roque (2009b), the results of the mass spectrometry have a higher precision, even in complex mixtures.

The total phenolic and flavonoid content and the compounds identified and quantified in the bee pollen are presented in Table 11.2, together with the botanical origin and precedence of the samples. The total phenolic content varied from 0.5 to 213.2 mg GAE/g and the flavonoid content can represent up to 92% of the total phenolic content (Leja et al. 2007; Carpes et al. 2008; Leblanc et al. 2009; Mărghitas et al. 2009; Menezes et al. 2010; Morais et al. 2011; Freire et al. 2012; Mejías and Montenegro 2012; Nogueira et al. 2012; Rebiai and Lanez 2012; Fatrcová-Šramková et al. 2013; Kaškonienė et al. 2015; Ketkar et al. 2014; Ulusoy and Kolayli 2014; De-Melo 2015; De-Melo et al. 2016).

In monofloral samples collected in Romania, total phenolic content was differentiated and decreasing in the following order: *Salix* sp. > *Taraxacum officinale* > *Centaurea cyanus* > *Crataegus monogyna* > *Capsella bursa pastoris* > *Matricaria chamomilla* > *Carex* sp. > *Carduus* sp. > *Helianthus annuus* > *Onobrychis viciifolia* > *Pinus* sp. > *Knautia arvensis,* while total flavonoid content diminished in the following order: *Salix* sp. > *M. chamomilla* > *C. cyanus* > *C. monogyna* > *H. annuus* > *Carduus* sp. > *C. bursa pastoris* > *O. viciifolia* > *Carex* sp. > *T. officinale* > *K. arvensis* > *Pinus* sp. (Mărghitas et al. 2009). In Chile, total phenolic content of monofloral bee pollen of *Lotus pedunculatus* was 0.5 mg GAE/g, while in the monofloral sample of *Escallonia rubra* it was 1.2 mg GAE/g (Mejías and Montenegro 2012). In bee pollen from Brazil, total phenolic and flavonoid contents were determined in a monofloral *Myrica* sample (29.2 mg GAE/g e 17.5 mg QE/g, respectively), in a monofloral *Alternanthera* sample (13.8 mg GAE/g e 1.0 mg QE/g), and in a monofloral *Cocos nucifera* sample (11.6 mg GAE/g e 3.4 mg QE/g) (De-Melo 2015).

The variation in total phenolic and flavonoid contents of bee pollen is related to the botanical origin and the type of processing employed by the beekeeper. De-Melo et al. (2016), when studying samples collected in the southeast of Brazil, observed total phenolic content of on average 14.4 mg GAE/g of product submitted to dehydration in an electric oven with forced air circulation; and 16.1 mg GAE/g in the same product when submitted to freeze-drying. The authors attributed this result to the action of enzymes such as polyphenol oxidase and peroxidase liberated during the freezing and defrosting processes that preceded dehydration in the electric oven.

Table 11.2 Total phenolic (TP) and flavonoid contents (FC) and compounds identified and quantified (CIQ) in monofloral and heterofloral bee pollen collected from different countries.

Botanical origin	Origin/ country	TP (mg GAE/g)	FC (mg QE/g)	CIQ (μg/g)	References
Eucalyptus globulus	Portugal	–	–	Quercetin-3-sophoroside, myricetin, tricetin, luteolin, 3-*O*-methylquercetin	Campos et al. (1997)
Washingtonia	USA	15.9	–	2,6-Dihydroxy-6-methylbenzaldehyde (38.0), 2-formyloxy-1-phenylethanone (20.0), 4′,5-dihydroxy-7-methoxyflavanone (58.0), anthraquinone derivative (85.0), 5-methoxy-7-methyl-1,2-naphthoquinone (70.0), 7-hydroxy-1-indanone (38.0), naringenin (13.0), methyl benzoate (10.0)	LeBlanc et al. (2009)
Yucca	USA	19.5	–	Naringenin (580.0), 2-methyl-5-hydroxybenzofuran (92.0), 4′,5-dihydroxy-7-methoxyflavanone (90.0), anthraquinone derivative (62.0), 5-methoxy-7-methyl-1,2-naphthoquinone (41.0), 1,2,3,4-tetrahydro-2-(2-hydroxy-3-phenoxypropyl)-6,7-dimethoxyisoquinoline (34.0), 1-)2-methoxy phenyl)-9,10-anthracenedione (14.0)	LeBlanc et al. (2009)
Cecropia	Brazil	122.1	–	Isoquercetin, tricetin, quercetin, kaempferol	Freire et al. (2012)
Elaeis	Brazil	213.2	–	Isoquercetin, myricetin, tricetin, quercetin, luteolin, selagin, isorhamnetin, kaempferol	Freire et al. (2012)
Eupatorium	Brazil	140.1	–	Isoquercetin, quercetin, kaempferol	Freire et al. (2012)
Mimosa pudica	Brazil	77.6	–	Isoquercetin, quercetin, isorhamnetin, kaempferol	Freire et al. (2012)

Table 11.2 (continued)

Botanical origin	Origin/ country	TP (mg GAE/g)	FC (mg QE/g)	CIQ (μg/g)	References
Scoparia	Brazil	134.1	–	Myricetin	Freire et al. (2012)
Brassica juncea	Brazil	18.3	1.2	Kaempferol (65.4), quercetin (51.4)	Ketkar et al. (2014)
Brassica	Brazil	10.3	4.0	Rutin (292.5), kaempferol (19.2), β-resorcylic acid (3815.0), *p*-coumaric acid (16.1), ferulic acid (189.8)	De-Melo (2015)
Mimosa verrucosa	Brazil	7.7	5.5	Rutin (97.6), naringenin (72.8), kaempferol (157.9), chlorogenic acid (42.7), vanillic acid (16.4)	De-Melo (2015)
Astrocaryum	Brazil	16.9	1.2	Cinnamic acid (117.7)	De-Melo (2015)
Anadenanthera	Brazil	11.2	1.0	Catechin (6.9), vanillic acid, ampeloptin, β-resorcylic acid (125.4), ferulic acid (10.4), quercetin (74.2), quercetin diglucoside, quercetin-3-*O*-arabinoside, quercetin-3-*O*-rutinoside, quercetin rutinoside (isomer), isorhamnetin-3-*O*-rhamnosyl-glucoside, isorhamnetin-3-*O*-rhamnosyl-glucoside (isomer), eriodictyol, rutin (36.6), 1-sinapoyl-2-feruloyl gentiobiose, naringenin hexoside, cinnamic acid (130.5), tri-*p*-coumaroyl spermidine, tri-*p*-coumaroyl spermidine (isomer), ellagic acid, naringenin (45.7), N′,N″,N‴-tris-*p*-feruloylspermidine	De-Melo (2015)

(continued)

Table 11.2 (continued)

Botanical origin	Origin/ country	TP (mg GAE/g)	FC (mg QE/g)	CIQ (μg/g)	References
Mimosa caesalpiniaefolia	Brazil	12.7	1.3	Catechin (7.5), chlorogenic acid (45.3), caffeic acid (1.5), siringeic acid (53.6), 4-methylsulfinylbutyl glucosinolate (glucoraphanin), kaempferol-3-*O*-rhamnosyl-glucoside, isorhamnetin-3-*O*-diglucoside, isorhamnetin-3-*O*-rutinoside-7-*O*-glucoside, isorhamnetin-3-*O*-rhamnosyl-glucoside, rutin (83.9), kaempferol-3-*O*-glucoside, 1-sinapoyl-2-feruloyl gentiobiose, isorhamnetin-3-*O*-glucoside, quercetin (29.1), kaempferol-3-*O*-acetulglucoside, kaempferol, quercetin-3-methyl ether, pinobanksin-5-methylether-3-*O*-acetate, bis-methylated quercetin, isorhamnetin −3-*O*-methyl hexuronide, dihydroquercetin-3-*O*-rhamnoside	De-Melo (2015)
Brassica campestris	China	–	–	Quercetin (1370.0) kaempferol (23,440.0)	Lv et al. (2015)
Zea mays	Egypt	–	–	Gallic acid, vanillic acid, synringic acid, *p*-coumaric acid, ferulic acid, caffeic acid, quercitin, rutin, catechin, epicatechin, α-Catechin, kaempferol, apigenin, 3,4-dimethoxycinnamic acid, naringenin, luteolin	Mohdaly et al. (2015)
Heterofloral	Portugal	–	–	Kaempferol-3-neohesperidoside, quercetin-3-rhamnoside	Campos et al. (1997)

Table 11.2 (continued)

Botanical origin	Origin/ country	TP (mg GAE/g)	FC (mg QE/g)	CIQ (μg/g)	References
Heterofloral	New Zealand	–	–	Kaempferol-3-neohesperidoside, kaempferol-3-sophoroside, 7-*O*-methylherbacetin-3-diglycoside, 8-*O*-methylherbacetin-3-diglycoside, 7-*O*-methylherbacetin-3-sophoroside, quercetin-3-diglycoside, highly derivatized herbacetin glycoside, isorhamnetin-3-sophoroside-diglycoside, 8-*O*-methylherbacetin-3-sophoroside, 8-*O*-methylherbacetin-3-sophoroside, 7-*O*-methylkaempferol-3-neohesperidoside, 8-*O*-methylherbacetin-3-sophoroside, 8-*O*-methylherbacetin-glycoside	Campos et al. (1997)
Heterofloral	Brazil	–	–	Rosmarinic acid dihexoside derivative, myricetin-3-*O*-rhamnosyl-glucoside, quercetin-3-*O*-arabinoside, isorhamnetin-3-*O*-diglucoside, kaempferol-3-*O*-rhamnosyl-glucoside, isorhamnetin-3-*O*-(2″,3″-dirhamnosyl)glucoside, rutin, isorhamnetin-3-*O*-rhamnosyl-glucoside, kaempferol-3-*O*-glucoside, patuletin-3-*O*-rhamnosylglucoside, quercitrin, chalcone, kaempferol-7-*O*-rhamnosyl-3-*O*-galloyl glucuronide, *N*′,*N*″,*N*′″-tris-*p*-coumaroylspermidine, *N*′,*N*″,*N*′″-tris-*p*-feruloylspermidine	Negri et al. (2011)

(continued)

Table 11.2 (continued)

Botanical origin	Origin/ country	TP (mg GAE/g)	FC (mg QE/g)	CIQ (μg/g)	References
Heterofloral	Greece	–	–	Ferulic acid (149.1), *o*-coumaric acid (36.7), quercetin (29.6), cinnamic acid (23.5), naringenin (21.9), hesperetin (3.0), kaempferol (7.8)	Fanali et al. (2013)
No information	Egypt	–	–	Gallic acid (38.5–173), pyrogallol (527.9–635.5), 4-Aminobenzoic acid (40–49.2), 3-Hydroxytyrosol (62–117.0), protocatechuic acid (257.1–525.7), chlorogenic acid (153.5–193.5), epicatechin (250.4–772.7), catechin (63.8–137.2), catechol (73–211.8), caffeine (1805.0), p-OH-benzoic acid (242.7–304.9), caffeic acid (86–124.5), vanillic acid (16.1–114.9), *p*-coumaric acid (83.9–218.1), ferulic acid (9.8–53), isoferulic acid (21.5–147), resveratrol (20.5–112.6), ellagic acid (37.6–555.6), e-vanillic acid (3197.4–6909.8), α-coumaric (42.1–1058.3), rosmarinic acid (267.1–1670.7), benzoic acid (50.4–3617.3), 3,4,5-methoxy-cinnamic acid (32.2), coumarin (19.7–345.4), salicylic acid (160.4–2176.6), cinnamic acid (8.4–31.5)	Shady et al. (2016)

Flavonoids and phenolic acids are the main phenolic compounds present in bee-pollen. In samples collected in Turkey, the main compounds quantified were rutin (25.6–692.9 μg/100 g), abscisic acid (21.0–288.7 μg/100 g), syringic acid (10.6–259.5 μg/100 g), ferulic acid (36.8–230.6 μg/100 g) and *p*-coumaric acid (34.2–184.2 μg/100 g). The compounds: benzoic acid (46.9–1077.6 μg/100 g), epicatechin (39.2–520.0 μg/100 g), and quercetin (55.9–499.2 μg/100 g) were observed in significant quantities, however they were not present in all the samples (Ulusoy and Kolayli 2014).

Eight phenolic acids (gallic, chlorogenic, caffeic, ferulic, syringic, synapic, 3,4-dihydrobenzoic, and 2-hydroxycinnamic acid), and three flavonoids (rutin, naringenin, and quercetin) were identified in the bee pollen produced in Latvia and Spain and, with the exception of ferulic acid, they were also present in the samples collected in Lithuania and China (Kaškonienė et al. 2015). In *Zea mays* monofloral bee pollen from Egypt, 3,4-dimethoxycinnamic acid was the major phenolic component in the pollen extract (45.8 mg/mL) (Mohdaly et al. 2015).

Some researchers have put forward the hypothesis that each plant species had a profile of specific phenolic compounds (Campos et al. 2003; Ulusoy and Kolayli 2014). Campos et al. (2015) carried out exhaustive research into pollen from *Zea mays* (including corn hybrids and genetic modified samples), collected on plantations located in Portugal, Brazil, and Mexico between the years 2000 and 2012, and concluded that the flavonoid/phenolic profile does neither change over the time of sampling nor in terms of the harvesting region. Bee pollen characterization studies were undertaken, attempting to identify, based on a specific profile, the botanical species from which the pollen grain was harvested, which would also allow an association between the biological activity and the botanical origin of the bee pollen (Campos et al. 2003, 2008; Ulusoy and Kolayli 2014). Zhou et al. (2015) analyzed monofloral bee pollen samples produced in China and proposed, based on the presence or not and on content, the quercetin-3-*O*-β-D-glucosyl-(2 → l)-β-glucoside compounds as a marker that identifies bee pollen samples from seed watermelon, rape, camellia, corn poppy, corn, buckwheat and rose; kaempferol-3,4′-di-*O*-β-D-glucoside as a marker to evaluate the floral origins of bee pollen from sesame, broad bean, rape, motherwort and corn; and kaempferol-3-*O*-β-D-glucosyl-(2 → l)-β-D-glucoside as a marker to classify bee pollen samples from sesame, broad bean, rape, corn poppy, buckwheat, camellia, and corn.

One should also consider, however, that the pollen of some species can present different phenolic compound profiles due to the agricultural and genotypic differences, or climatic conditions (temperature, hydrological stress, and light intensity) and soil, which can influence plant growth and maintenance (Daoud et al. 2015). Monofloral bee pollen from *Mimosa scabrella* collected in different regions of Brazil was submitted to HPLC-MS analysis and the results were different profiles for each sample (De-Melo 2015). In monofloral bee pollen from *Mimosa pudica* and *Elaeis* collected monthly from the same apiary located in Brazil, between February and November, total phenolic content and the compounds identified varied significantly throughout the period (Freire et al. 2012). In another study with Brazilian samples, this time monofloral from *Eucalyptus*, a considerable variation in total phenolic content (27.1–132.4 mg GAE/g) and total flavonoids (1.8–2.5 mg EE/g) was observed throughout one year (Menezes et al. 2010).

Another variable that should be considered is the processing employed by the beekeeper, beyond storage, as previously mentioned. In monofloral bee pollen from *Cistus ladanifer* collected in Spain, quercetin-3-rutinoside content, the compound observed in greater quantities in the samples, was 7.3 mg/100 g in the product in natura and 3.3 mg/100 g in the lyophilized product (Domínguez-Valhondo et al. 2011). The influence of storage was observed by Tavdidishvili et al. (2014) in samples collected in the USA.

11.6 Bioelement Content

Bee pollen contains elements that are indispensable for the development of bees and humans, which makes this food an alternative nutritional supplement. Potassium (1.4–38 g/kg), phosphorus (0.2–9.6 mg/kg), calcium (0.2–5.8 g/kg), manganese (5–430 mg/kg), zinc (5.1–340 mg/kg), copper (3–42 mg/kg), chromium (0.03–42 mg/kg), boron (8.2–14 mg/kg), molybdenum (0.1–4.6 mg/kg) and selenium (<0.01–4.5 mg/kg) were found (Youssef et al. 1978; Somerville and Nicol 2002; Funari et al. 2003; Carpes et al. 2009; Bogdanov 2012a; Formicki et al. 2013; Morgano et al. 2012; Yang et al. 2013; Fuenmayor et al. 2014; Sattler et al. 2015).

The content of these nutrients varies according to the botanical origin and geographical region, and season during the year. The characteristics of the region, especially the soil type where the plant developed, are determinant for the bioelement content of bee pollen (Formicki et al. 2013; Yang et al. 2013; De-Melo 2015). Beyond this, a part of these elements can come from the nectar added by the bees at the time of pollen harvesting (Kostic et al. 2015b). Somerville and Nicol (2002) published a study comparing the mineral content of monofloral samples from a single plant species, but collected in different regions of Australia between 1991 and 1998, and the results indicated variations in bioelement concentration. On the other hand, processing and storage conditions did not influence mineral content (Youssef et al. 1978; De-Melo et al. 2016).

Researchers from the international Honey Commission, who proposed quality criteria for bee pollen, suggested parameters for the contents: potassium (4000–20,000 mg/kg), phosphorus (800–6000 mg/kg), magnesium (200–3000 mg/kg), calcium (200–3000), zinc (30–250 mg/kg), manganese (20–110 mg/kg), iron (11–170 mg/kg) and copper (2–16 mg/kg) (Campos et al. 2008).

11.6.1 Potassium (K)

Independent of the origin of bee pollen, this mineral occurs in elevated concentrations, reaching 38 g/kg in monofloral bee pollen from *Asphodelus fistulosus* collected in Australia (Somerville and Nicol 2002). According to the World Health Organization, the ideal intake of K should be 3.51 g/day (WHO 2012a); therefore, a 25 g portion of bee pollen provides up to 27% of the daily intake recommended by WHO.

In South American countries (Argentina, Brazil, and Colombia) K content in bee pollen varies between 1.4 and 9.9 g/kg (Funari et al. 2003; Baldi-Coronel et al. 2004; Carpes et al. 2009; Morgano et al. 2012; Fuenmayor et al. 2014; De-Melo 2015; De-Melo et al. 2016). Some of the highest values were observed in monofloral samples from *Mimosa* or heterofloral samples where species from this genus were present (De-Melo 2015). In bee pollen collected in China the content of this mineral was between 2.4 and 9.5 g/kg (Szczesna 2007; Yang et al. 2013; Fuenmayor et al. 2014), with the lowest value found being in a monofloral sample from *Dendranthema indicum* and the highest value in a heterofloral product.

In samples from Saudi Arabia, K content varied from 6.2 g/kg, in monofloral bee pollen from *Helianthus annuus,* to 8.3 g/kg, and in monofloral bee pollen from *Brassica napus* (Taha 2015). In monofloral samples from *Brassica napus* collected in China and Australia the average K content was lower, at 4.7 and 5.4 g/kg, respectively (Somerville and Nicol 2002; Yang et al. 2013). In harvesting undertaken in Serbia and Poland, samples considered to be monofloral from an unidentified pollen type from the Brassicaceae family presented a K content of 3.2 and 3.6 g/kg, respectively (Szczesna 2007; Kostic et al. 2015b). In a monofloral sample from another species from the genus *Brassica* (*B. kaber*), collected in Egypt, K content was 3.8 g/kg (Youssef et al. 1978).

In Spain, a K content in bee pollen of between 2.5 and 6.4 g/kg was found (Serra-Bonvehí and Escolà-Jordà 1997; Fuenmayor et al. 2014), something close to that observed in samples produced in Poland (2.8–5.8 g/kg) (Szczesna 2007; Fuenmayor et al. 2014) and Serbia (2.5–4.2 g/kg) (Kostic et al. 2015b). In the later country, monofloral bee pollen from *Salix* had a content of 3.4 g of potassium/kg (Kostic et al. 2015b); close to that observed in bee pollen with the same botanical origin collected in Australia (3.7 g/kg) (Somerville and Nicol 2002).

The content of this bioelement in bee pollen in South Korea varied from 3.5 to 5.5 g/kg (Szczesna 2007) and similar values were identified in samples from Solvenia (5.2 g/kg), Romania (4.9 g/kg), India (4.8 g/kg), Bulgaria (4.6 g/kg), and Hungary (3.6 g/kg) (Fuenmayor et al. 2014).

11.6.2 Sodium (Na)

Currently, a preoccupation with the excessive intake of sodium by the population exists and is frequently related to the consumption of processed and mass produced foods. For this reason, it is interesting that a natural food, which can be utilized as a food supplement, as is the case with bee pollen, does not contain elevated levels of Na. The results so far indicate that a 25 g portion of this food provides, at the maximum, 10% of the maximum intake of 2 g/day of sodium, recommended by the World Health Organization (WHO 2012b). The K/Na ratio is also considered relevant, since it is important to maintain an equilibrium of these bioelements in the organism. The high K/Na ratio found in bee pollen makes this food useful for diets with a defined electrolytic balance (Serra-Bonvehí and Escolà-Jordà 1997; Carpes et al. 2009).

In general, the Na content in bee pollen is below 2 g/kg, but in Saudi Arabia this value reached 8.4 g/kg in monofloral bee pollen from *Brassica napus* (Taha 2015). This value was far above that observed in monofloral samples of this same pollen type collected in China (459.1 mg/kg) and Australia (30.5 mg/kg) (Somerville and Nicol 2002; Yang et al. 2013).

In samples from China, the content of this mineral varied from 125 to 2447 mg/kg (Szczesna 2007; Yang et al. 2013; Fuenmayor et al. 2014). In the product sourced from Spain, the variation was from 198 to 2375 mg/kg (Serra-Bonvehí and Escolà-Jordà 1997; Fuenmayor et al. 2014), very similar to that found in samples produced in Poland (236–2191 mg/kg) (Szczesna 2007). In bee pollen produced in Brazil Na

content was between <0.004 and 1466 mg/kg (Carpes et al. 2009; Morgano et al. 2012; De-Melo 2015; De-Melo et al. 2016), though 75% of the samples from this country had a Na content below 400 mg/kg. Szczesna (2007) found a maximum Na content in bee pollen from South Korea (1113 mg/kg) similar to that observed in Brazilian samples.

A content of 46–554 mg of sodium/kg of product was found in bee pollen from Argentina (Baldi-Coronel et al. 2004). Somerville and Nicol (2002) encountered, in samples from Australia, a content for this mineral varying between 16 and 480 mg/kg, with an average of 82 mg/kg. Amongst the Australian samples, values far above the average were identified in monofloral bee pollen from *Banksia ericifolia* (480 mg/kg), *Eucalyptus punctata* (340 mg/kg), *Casuarina littoralis* (290 mg/kg), and *Hypochoeris radicata* (220 mg/kg).

Youssef et al. (1978) observed an average Na content of 426 mg/kg. Fuenmayor et al. (2014) found a Na content of 8.9–206 mg/kg in samples from Colombia, of 2140 mg/kg in samples from Slovenia, 219 mg/kg in samples from Hungary, 199 mg/kg in bee pollen produced in Bulgaria, 113 mg/kg in a sample collected in India, and 84 mg/kg in product from Romania. In Serbia, the content of this mineral varied from 4.9 to 54.9 mg/kg (Kostic et al. 2015b).

11.6.3 Phosphorus (P)

In bee pollen produced in China, Yang et al. (2013) observed phosphorus content of 2.1 g/kg, in bee pollen from *Dendranthema indicum*, and of 9.6 g/kg, in bee pollen from *Nelumbo nucifera*. In Brazil, samples had a phosphorus content between 2.2 and 8.9 g/kg (Funari et al. 2003; Carpes et al. 2009; Morgano et al. 2012; Sattler et al. 2016), similar to that found in Australia (1.4–8.0 g/kg) (Somerville and Nicol 2002). On the other hand, a lower P content occurred in bee pollen from Spain (294.7–854.4 mg/kg) (Serra-Bonvehí and Escolà-Jordà 1997; Fuenmayor et al. 2014) and Saudi Arabia (234.4–468.1 mg/kg) (Taha 2015).

Bee pollen from *Echium plantagineum* contains a P content between 7.1 and 8.0 g/kg, from *Brassica napus* between 0.4 and 6.8 g/kg, from *Zea mays* between 3.7 and 4.7 g/kg, and from *Helianthus annuus* between 0.2 and 2.6 g/kg (Somerville and Nicol 2002; Yang et al. 2013; Taha 2015). These values are related to the region where they were collected, especially to the characteristics of the soil that influenced the composition of the pollen grains of each plant.

11.6.4 Calcium (Ca)

Calcium is a mineral of great importance for the human organism. Amongst its functions are: constituting bones and tissues, acting in the contraction of muscular fibers, in nerve conduction, and in the liberation of hormones, beyond being an

enzyme component (Forsén and Kördel 1994). A moderate lack of this bioelement is common, with the recommended intake being 1000 mg/day for adult individuals (19–50 years) (USDA 2011).

Taha (2015) identified the highest levels of Ca in bee pollen samples (2.1–5.8 g/kg). The authors evaluated products from Saudi Arabia. The bee pollen collected in Brazil had a Ca content between 0.3 and 4.7 g/kg (Funari et al. 2003; Carpes et al. 2009; Morgano et al. 2012; De-Melo 2015; De-Melo et al. 2016; Sattler et al. 2016). De-Melo (2015) observed, in Brazilian products, a tendency towards greater Ca content in monofloral samples from *Cocos nucifera* or heterofloral samples with a strong participation of this pollen type, when compared to monofloral samples from *Brassica* or heterofloral samples with a significant participation of this pollen type.

Somerville and Nicol (2002) found Ca content of 0.4–3.1 g/kg in bee pollen produced in Australia. In that country, the content of this element in monofloral samples from *Eucalyptus* was from 0.8 to 3.1 g/kg, while in Brazil a value of 1.6 g/kg was observed (Somerville and Nicol 2002; De-Melo 2015). Each eucalypt species produced pollen with a characteristic composition, beyond this, the content of this biolement in the soil is determinant in the composition of the grain for all plant species.

In China, the content of this mineral varied from 0.2 to 3.1 g/kg (Szczesna 2007; Yang et al. 2013; Fuenmayor et al. 2014), while in Argentina and Colombia it did not reach 2.5 g/kg (Baldi-Coronel et al. 2004; Fuenmayor et al. 2014), in Serbia it varied between 0.9 and 2.0 g/kg (Kostic et al. 2015b) and in Egypt it was between 0.7 and 2.1 g/kg (Youssef et al. 1978). In samples collected in Europe (Spain, Poland, Hungary, Slovenia, Romania, and Bulgaria) Ca content varied between 0.3 and 1.7 (Serra-Bonvehí and Escolà-Jordà 1997; Szczesna 2007; Fuenmayor et al. 2014). A lower Ca content was found in samples collected in South Korea (105–694 mg/kg) (Szczesna 2007).

11.6.5 Magnesium (Mg)

The worldwide preoccupation with calcium intake is well known, however, there needs to be an equilibrium in calcium and magnesium intake for the perfect functioning of the organism. According to Rosanoff et al. (2012), in the United States almost half the population consumes less than the recommended magnesium intake. Research into bee pollen indicated that a 25 g portion would provide from 2 to 45% of the recommended magnesium intake for an adult individual whose requirement would be 260 mg/day.

In bee pollen produced in Saudi Arabia, Mg content was between 2.4 g/kg, in monofloral samples of *Cucurbita pepo*, and 4.7 g/kg, in monofloral samples of *Phoenix dactylifera* (Taha 2015). In the product collected in Brazil, Mg content was between 0.4 and 3.6 g/kg (Funari et al. 2003; Carpes et al. 2009; Morgano et al. 2012; De-Melo 2015; De-Melo et al. 2016). In this country, De-Melo (2015) observed that samples with a significant presence of the *Cocos nucifera* pollen type

had a higher content of this mineral (2.1–2.3 g/kg) compared to those in which the *Brassica* type predominated (1.3–1.8 g/kg). In monofloral samples from *Brassica kaber* from Egypt, Youssef et al. (1978) found a Mg content of 1.3 g/kg, while in samples from *Brassica napus* from Australia and Saudi Arabia the content of this mineral was 1.4 and 3.9 g/kg, respectively (Somerville and Nicol 2002; Yang et al. 2013). In monofloral bee pollen from an unidentified pollen type from Brassicaceae, the values encountered for this mineral were 832 mg/kg (in samples from Serbia) and 1.1 g/kg (in samples from Poland) (Szczesna 2007; Kostic et al. 2015b).

Szczesna (2007), Yang et al. (2013), and Fuenmayor et al. (2014) found an Mg content in samples collected in China of 0.3–2.8 g/kg. In Australia and Argentina, a similar range was observed: 0.2–2.7 g of Mg/kg and 0.4–2.2 g of Mg/kg, respectively (Somerville and Nicol 2002; Baldi-Coronel et al. 2004). In bee pollen from South Korea, the content of this mineral was between 1.0 and 1.8 g/kg (Szczesna 2007). In a monofloral sample from *Artemisia* a lower Mg value (0.7 g/kg) was found, amongst the products evaluated from Poland on the other hand, the highest value (1.7 g/kg) was found in a sample with an undetermined botanical origin (Szczesna 2007; Fuenmayor et al. 2014). In Colombia, between 343 and 1.5 g of magnesium/kg were quantified in the samples.

Fuenmayor et al. (2014) studied a sample from each country: India (1.4 g/kg), Slovenia (1.1 g/kg), Romania (865 mg/kg), Hungary (635 mg/kg) and Bulgaria (577 mg/kg). In Spain, Mg content varied between 273 and 816 mg/kg (Serra-Bonvehí and Escolà-Jordà 1997; Fuenmayor et al. 2014) and in Serbia it was between 503 and 964 mg/kg, with lower and higher values identified in heterofloral samples (Kostic et al. 2015b).

11.6.6 Iron (Fe)

According to the World Health Organization, iron deficiency is a widespread and common public health condition that mainly affects women and children, as much in developing and developed nations (WHO 2015). This mineral is an essential component of hemoglobin and enzymes and deficiencies lead to anemia, persistent cognitive and developmental impairment, and depressed immune function (USDA 2015). Based on results presented in the following, considering an individual whose necessary daily iron intake would be 14 mg/day, a 25 g portion of bee pollen would provide from 2 to 220% of the recommended intake.

In Egypt, elevated content for this mineral was observed in monofloral bee pollen samples: *Vicia faba* (1290 mg/kg), *Trifolium alexandrium* (1265 mg/kg), *Brassica kaber* (820 mg/kg) and *Zea mays* (555 mg/kg) (Youssef et al. 1978). Yang et al. (2013) also evaluated Fe content in bee pollen from *Vicia faba* (158.3 mg/kg) and *Zea mays* (75.2 mg/kg), collected this time in China. Somerville and Nicol (2002) determined Fe content from *Zea mays* bee pollen (26.0 mg/kg) produced in Australia.

In bee pollen from Brazil, Fe content reached 1179 mg/kg (Funari et al. 2003; Carpes et al. 2009; Morgano et al. 2012; De-Melo 2015; Sattler et al. 2016). In a

monofloral sample from *Anadenanthera* collected in southeastern Brazil, the content was 466.3 mg/kg, while in a monofloral sample from this same species, this time produced in the south, it was 143 mg/kg. In monofloral samples from *Alternanthera*, collected from the same apiary in different months, Fe content was 821.0 mg/kg (harvested in autumn: the end of May and start of June), 1017.5 mg/kg (harvested during winter: end of June and start of July) and 1179 mg/kg (harvested during winter: August) (De-Melo 2015).

In Saudi Arabia, considerable Fe values were also observed (338.1–562.1 mg/kg) (Taha 2015), just as in samples collected in Australia (14.0–520.0 mg/kg) (Somerville and Nicol 2002) and South Korea (74.3–365.9 mg/kg) (Szczesna 2007). In Saudi Arabia, the greatest value was observed in a monofloral sample from *Cucurbita pepo* and the lowest value in a monofloral sample form *Phoenix dactylifera*, while in Australia the greatest and lowest values were observed in monofloral samples from *Eucalyptus punctata* and *Lavandula*, respectively.

Bee pollen produced in China contained between 59.0 and 207.8 mg/kg of Fe/kg of product (Szczesna 2007; Yang et al. 2013). In monofloral samples from *Fagopyrum esculentum*, *Brassica napus*, and *Helianthus annuus* from the same country, Fe content was significantly higher (207.8, 89.4 and 88.3 mg/kg, respectively) than that found in samples from the same botanical origins produced in Australia (51, 27.5 and 40 mg/kg, respectively). In Saudi Arabia, the content in monofloral bee pollen from *Brassica napus* and *Helianthus annuus* was greater still (361.3 and 562.1 mg/kg, respectively).

In Argentina, Fe content in bee pollen was between 23 and 229 mg/kg (Baldi-Coronel et al. 2004), in India, where only one sample was analyzed, it was 197.7 mg/kg (Fuenmayor et al. 2014), in European countries is was between 11.1 and 169 mg/kg (Serra-Bonvehí and Escolà-Jordà 1997; Szczesna 2007; Formicki et al. 2013; Fuenmayor et al. 2014; Kostic et al. 2015b), and in Colombia it varied between 23.3 and 126.6 mg/kg (Fuenmayor et al. 2014). The iron content in bee pollen produced in Chile varied between 146.7 and 181.8 mg/kg, with one monofloral sample from *Lotus pedunculatus* having 175.4 mg/kg and a monofloral sample from *Escallonia rubra* having 181.8 mg/kg (Mejías and Montenegro 2012).

11.6.7 Zinc (Zn)

Bee pollen contains significant zinc levels. Based on the results presented in the following, this food source provides up to 120% of the recommended intake of 7 mg/day for an individual adult. In Brazil, products of animal origin, mainly red meat, are the main source of zinc in the diet (Pereira and Hessel 2009), such that insufficient intake of this mineral can occur in vegetarians and vegans or in populations in which red meat is not a constant part of the menu (USDA 1998). In these cases, the consumption of bee pollen can contribute as an important source of this bioelement, whose deficiency is prevalent throughout the world (Caulfield and Black 2004).

In bee pollen collected in Australia the average content of this mineral was 58.3 mg/kg, however in a monofloral sample from *Echium plantagineum* the value was 340 mg/kg (Somerville and Nicol 2002). The authors analyzed eight other samples from the same species and the average content was 68 mg/kg. Though these nine bee pollen samples have been collected in different regions of Australia over different years, it was not clear why there was such a high value in only one sample.

In research carried out in Brazil, zinc levels observed varied between 5.1 and 118 mg/kg (Funari et al. 2003; Carpes et al. 2009; Morgano et al. 2012; De-Melo 2015; Sattler et al. 2016). According to Morgano et al. (2012) the levels of this mineral tend to be high in samples from northeastern Brazil. Bee pollen with a significant quantity of pollen types from the genus *Brassica* tends to have a Zn content between 30 and 80 mg/kg, when produced in southern Brazil (De-Melo 2015). A positive correlation between zinc and copper content and antioxidant capacity, measured by DPPH and ORAC methods, was observed in heterofloral bee pollen collected in southeastern Brazil. The authors reported that these minerals were cofactors for copper, zinc superoxide dismutase (CueZn SOD), an important antioxidant enzyme already reported in plants; therefore, Cu and Zn might be indicators of the presence of the Cu/ZnSOD in these bee pollen samples (De-Melo et al. 2016).

Baldi-Coronel et al. (2004) found a Zn content of 23–106 mg/kg in samples from Argentina with an undetermined botanical origin. In Spain, values between 18.8 and 81.1 mg/kg were observed (Serra-Bonvehí and Escolà-Jordà 1997; Fuenmayor et al. 2014), close to that quantified in samples produced in Serbia (28.8–75.9 mg/kg), Colombia (19.8–70.6 mg/kg) (Fuenmayor et al. 2014), and China (23.9–65.3 mg/kg) (Szczesna 2007; Yang et al. 2013; Fuenmayor et al. 2014). In monofloral samples from *Zea mays* collected in China, Australia, and Egypt, Zn content was, respectively, 54.6, 48, and 80 mg/kg (Youssef et al. 1978; Somerville and Nicol 2002; Yang et al. 2013).

In samples collected in Chile, Zn content was similar, being between 62.7 and 63.9 mg/kg (Mejías and Montenegro 2012). In South Korea, values between 23.7 and 60.7 mg of Zn/kg were found (Szczesna 2007). In European countries (Poland, Hungary, Slovenia, Romania, and Bulgaria) levels of this mineral between 25.6 and 53.6 mg/kg were encountered (Szczesna 2007; Fuenmayor et al. 2014), while in Saudi Arabia Zn content in bee pollen samples varied from 31.9 to 44.2 mg/kg (Taha 2015).

11.6.8 Copper (Cu)

Levels of copper in bee pollen are elevated. In research carried out by Somerville and Nicol (2002) on samples collected in Australia, Cu levels varied between 3 and 42 mg/kg. These authors observed little variation when comparing monofloral products from the same botanical origin: *Hypochoeris radicata* (4–5 mg/kg), *Eucalyptus bridgesiana* (16–17 mg/kg), *Echium plantagineum* (5–7 mg/kg),

Corymbia maculata (39–42 mg/kg) and *Brassica napus* (6–7 mg/kg). In bee pollen from *Brassica napus* collected in Saudi Arabia Cu content was similar to that observed in Australian samples (6.3 mg/kg), however in samples collected in China, the value was greater (15.7 mg/kg) (Yang et al. 2013; Taha 2015).

In Brazil, Cu content in samples varied between 3.2 and 25.4 mg/kg (Funari et al. 2003; Carpes et al. 2009; Morgano et al. 2012; De-Melo 2015; Sattler et al. 2016), similar to that observed by Szczesna (2007) and Yang et al. (2013) in samples from China (3.2–25.1 mg/kg), by Szczesna (2007) and Fuenmayor et al. (2014) in samples from Poland (5.6–23.9 mg/kg) and by Baldi-Coronel et al. (2004) in bee pollen produced in Argentina (6–20 mg/kg). Similar Cu levels were also observed in samples collected in Spain (4.1–15.7 mg/kg), in South Korea (5.3–14.7 mg/kg), Chile (8.8–12.5 mg/kg), Serbia (4.4–10.7 mg/kg), Egypt (6.0–10.0 mg/kg), and in Saudi Arabia (4.3–7.0 mg/kg) (Youssef et al. 1978; Serra-Bonvehí and Escolà-Jordà 1997; Szczesna 2007; Mejías and Montenegro 2012; Fuenmayor et al. 2014; Kostic et al. 2015b; Taha 2015).

11.6.9 Manganese (Mn)

In bee pollen from China, manganese content varied between 8.7 and 429.8 mg/kg, with the highest level being found in a sample of undetermined botanical origin (Szczesna 2007; Yang et al. 2013). In a product collected in Brazil, Mn levels were between 12 and 314 mg/kg (Funari et al. 2003; Carpes et al. 2009; Morgano et al. 2012; De-Melo 2015; Sattler et al. 2016). De-Melo (2015) observed that in samples from the same apiary, located in the southeast of Brazil, collected during the winter, spring, and summer, Mn levels were high (157–215 mg/kg) when compared to the other results (21–162 mg/kg), independent of the predominant botanical origin at the time of harvesting and the season of the year. This was only seen at this apiary.

In monofloral samples collected in Egypt, the content of this mineral was 210 mg/kg (*Vicia faba* and *Trifolium alexandrium*), 205 mg/kg (*Brassica kaber*) and 189 mg/kg (*Zea mays*) (Youssef et al. 1978). Research into monofloral products from China indicated values in samples of *Camellia japonica* (357.4 mg/kg), in *Rosa rugosa* (156.1 mg/kg), in *Nelumbo nucifera* (99.4 mg/kg), in *Zea mays* (24.1 mg/kg), in *Brassica napus* (24.2 mg/kg) and in *Helianthus annuus* (16.7 mg/kg) (Yang et al. 2013). Monofloral samples from *Zea mays* (14 mg/kg), *Brassica napus* (30–42 mg/kg), and *Helianthus annuus* (12 mg/kg) were also studied in Australia. Generally, in bee pollen collected in Australia, the values were between 5 and 110 mg/kg (Somerville and Nicol 2002).

In samples from South Korea and Serbia, similar Mn levels were found: 25.6–99.4 and 13.5–92.2 mg/kg, respectively (Szczesna 2007; Kostic et al. 2015b). The same was found in bee pollen analyzed from Poland (13.3–59.7 mg/kg) by Szczesna (2007), and from Argentina (14–57 mg/kg) by Baldi-Coronel et al. (2004). In Chile, Mn content varied from 52.1–62.9 mg/kg (Mejías and Montenegro 2012), while the lowest values occurred in samples produced in Saudi Arabia (16.6–38.6 mg/kg)

(Taha 2015) and Spain (7.4–18.3 mg/kg) (Serra-Bonvehí and Escolà-Jordà 1997; Fuenmayor et al. 2014).

Manganese levels in bee pollen indicated that 25 g of a sample provided up to four times more than the daily requirement of, for example, 2.3 mg/day. Manganese deficiencies can occur, especially in women (Panziera et al. 2011) however, the inclusion of bee pollen in the diet can contribute as an important supplementary source of this bioelement.

11.6.10 Other Bioelements

Chrome and molybdenum content in bee pollen from Brazil was 2.0–42 mg/kg and from 0.2 to 4.6 mg/kg, respectively (Sattler et al. 2016); while in samples produced in China molybdenum content varied from 0.1 to 0.4 mg/kg, and chrome was not detected (Yang et al. 2013). In bee pollen produced in Chile and Serbia, a lower chrome content was observed, when compared with Brazilian samples: 0.03–0.5 and 0.2–0.5 mg/kg, respectively (Mejías and Montenegro 2012; Kostic et al. 2015b).

Funari et al. (2003) encountered boron levels varying between 8.2 and 14 mg/kg in bee pollen from southeastern Brazil. Adequate boron intake is necessary to promote bone and brain health (Nielsen 2008). Morgano et al. (2012) identified selenium content of <0.01–4.45 mg/kg in 154 samples across all regions of Brazil. In samples collected in China, this bioelement was not detected (Yang et al. 2013). Selenium is a component of proteins that play a critical role in reproduction, thyroid hormone metabolism, DNA synthesis, and protection from oxidative damage and infection (Sunde et al. 2012; NIH 2016).

References

Abdella EM, Tohamy A, Ahmad RR (2009) Antimutagenic activity of egyptian propolis and bee pollen water extracts against cisplatin-induced chromosomal abnormalities in bone marrow cells of mice. Iran J Cancer Prev 2:175–181

Aguirre-Hernández E, González-Trujano E, Martínez AL, Moreno J, Kite G, Terrazas T, Soto-Hernández M (2010) HPLC/MS analysis and anxiolytic-like effect of quercetin and kaempferol flavonoids from *Tilia americana* var. mexicana. J Ethnopharmacol 127:91–97

Almeida-Muradian LB (2009) Qualidade dos produtos apícolas e otimização quimiométrica dos métodos de análise do mel por espectroscopia no infravermelho (FT-IR ATR)

Almeida-Muradian LB, Pamplona LC, Coimbra S, Barth OM (2005) Chemical composition and botanical evaluation of dried bee pollen pellets. J Food Compos Anal 18:105–111

Angelo PM, Jorge N (2007) Compostos fenólicos em alimentos – uma breve revisão. Rev Inst Adolfo Lutz 66:232–240

Arruda VAS (2013) Pólen apícola desidratado: composição físico-química, qualidade microbiológica, compostos fenólicos e flavonoides, atividade antioxidante e origem botânica. http://www.teses.usp.br/teses/disponiveis/9/9131/tde-22062015-172352/es.php. Accessed 26 Feb 2017

Arruda VAS, Estevinho MLF, Almeida-Muradian LB (2013a) Presence and stability of B complex vitamins in bee pollen using different storage conditions. Food Chem Toxicol 51:143–148
Arruda VAS, Santos-Pereira AA, Freitas AS, Barth OM, Almeida-Muradian LB (2013b) Dried bee pollen: B complex vitamins, physicochemical and botanical composition. J Food Compos Anal 29:100–105
Avni D, Hendriksma HP, Dag A, Uni Z, Shafir S (2014) Nutritional aspects of honey bee-collected pollen and constraints on colony development in the eastern Mediterranean. J Insect Physiol 69:65–73
Baldi-Coronel B, Grasso D, Pereira SC, Fernández G (2004) Caracterización bromatológica del polen apícola argentino. Cienc Docencia Tecnol 29:145–181
Ball GFM (1994) Water-soluble vitamin assays in human nutrition. Chapman & Hall, London
Ball GFM (1998) Bioavailability and analysis of vitamins in foods. Chapman & Hall, London
Barajas J, Cortes-Rodriguez M, Rodríguez-Sandoval E (2012) Effect of temperature on the drying process of bee pollen from two zones of Colombia. J Food Process Eng 35:134–148
Barreto LMRC, Funari SRC, Orsi RO (2005a) Composição e qualidade do pólen apícola proveniente de sete Estados brasileiros e do Distrito Federal. Bol Ind Anim 62:167–175
Barreto LMRC, Funari SRC, Orsi RO (2005b) Pólen apícola: perfil da produção no Brasil. In: Annals of Congresso De Apicultura Del Mercosur 1. Punta Del Este, Sociedad de Apicultores Uruguaya, p 20
Barth OM (2004) Melissopalynology in Brazil: a review of pollen analysis of honeys, propolis and pollen loads of bees. Sci Agric 61:342–350
Barth OM, Freitas AS, Oliveira ES, Silva RA, Maester FM, Andrella RR, Cardozo GM (2010) Evaluation of the botanical origin of commercial dry bee pollen load batches using pollen analysis: a proposal for technical standardization. An Acad Bras Cienc 82:893–902
Bastos DHM, Barth OM, Rocha CI, Cunha IBS, Carvalho PO, Torres EAS (2004) Fatty acid composition and palynological analysis of bee (*Apis*) pollen loads in the states of Sao Paulo and Minas Gerais, Brazil. J Apicult Res 43:35–39
Bastos DHM, Rocha CI, Cunha IBS, Carvalho PO, Torres EAS (2003) Composition and quality of bee-collected pollen commercialized in some country towns in São Paulo and Minas Gerais - Brazil. Rev Inst Adolfo Lutz 62:239–244
Bogdanov S (2012a) The bee pollen book: chapter 1. http://www.bee-hexagon.net. Accessed 12 Nov 2014
Bogdanov S (2012b) The bee pollen book: chapter 2. http://www.bee-hexagon.net. Accessed 12 Nov 2014
Brazil (2001) Technical regulations of identity and quality of bee venom, beeswax, royal jelly, lyophilized royal jelly, bee pollen, propolis and propolis extract. http://www.agricultura.gov.br/legislacao/sislegis. Accessed 24 Apr 2015
Brazil (2005) Technical regulation on the Recommended Dietary Allowance (RDA) of proteins, vitamins and minerals. http://bvsms.saude.gov.br/bvs/saudelegis/anvisa/2005/rdc0269_22_09_2005.html. Accessed 01 Jul 2017
Cabrera C, Montenegro G (2013) Pathogen control using a natural Chilean bee pollen extract of known botanical origin. Cienc Investig Agrar 40:223–230
Calviello G, Serini S, Piccioni E (2007) n-3 polyunsaturated fatty acids and the prevention of colorectal cancer: molecular mechanisms involved. Curr Med Chem 14:81–89
Campos MGR, Markham KR, Mitchell KA, Cunha AP (1997) An approach to the characterization of bee pollens via their flavonoid/phenolic profiles. Phytochem Anal 8:181–185
Campos MGR, Webby RF, Markham KR, Mitchell KA, Da Cunha AP (2003) Age-induced diminution of free radical scavenging capacity in bee pollens and the contribution of constituent flavonoids. J Agric Food Chem 51:742–745
Campos MGR, Bogdanov S, Almeida-Muradian LB, Szczesna T, Mancebo Y, Frigerio C (2008) Pollen composition and standardisation of analytical methods. J Apicult Res Bee World 47:156–163

Campos MGR, Almaraz-Abarca N, Matos MP, Gomes NM, Arruda VA, Barth OM (2015) *Zea mays* L. pollen: an approach to its quality control. J Agric Sci Technol B 5:513–522

Carl JL, Richard VM, Mandeep RM, Hector OV (2009) Omega-3 polyunsaturated fatty acids and cardiovascular diseases. J Am Coll Cardiol 54:585–594

Carpes ST, Prado A, Moreno IAM, Mourão GB, Alencar SM, Masson ML (2008) Avaliação do potencial antioxidante do pólen apícola produzido na Região Sul do Brasil. Quím Nova 31:1660–1664

Carpes ST, Mourão GB, Alencar SM, Masson ML (2009) Chemical composition and free radical scavenging activity of *Apis mellifera* bee pollen from Southern Brazil. Braz J Food Technol 12:220–229

Carratu E, Sanzini E (2005) Sostanze biologicamente attive presenti negli alimenti di origine vegetable. Ann Ist Super Sanità 41:7–16

Caulfield LE, Black RE (2004) Zinc deficiency. In: Ezzati M, Lopez AD, Rodgers A, CJL M (eds) Comparative quantification of health risks. World Health Organization, Geneva, pp 257–280

Chantarudee A, Phuwapraisirisan P, Kimura K, Okuyama M, Mori H, Kimura A (2012) Chemical constituents and free radical scavenging activity of corn pollen collected from *Apis mellifera* hives compared to floral corn pollen at Nan, Thailand. BMC Complement Altern Med 12:1–12

Cheatham CL, Colombo J, Carlson SE (2006) n-3 fatty acids and cognitive and visual acuity development: methodologic and conceptual considerations. Am J Clin Nutr 83: 1458S–1466S

Cook SM, Awmack CS, Murray DA, Williams IH (2003) Are honey bees' foraging preferences affected by pollen amino acid composition? Ecol Ent 28:622–627

Cunha AP, Roque OR (2009a) Compostos fenólicos: características e origem biossintética. In: Cunha AP (ed) Farmacognosia e fitoquímica. Fundação Calouste Benkian, Lisboa, pp 211–224

Cunha AP, Roque OR (2009b) Obtenção de moléculas com atividade farmacológica a partir de material vegetal e sua transformação em medicamento. In: Cunha AP (ed) Farmacognosia e fitoquímica. Lisboa, Fundação Calouste Benkian, pp 109–123

Daoud A, Malika D, Bakari S, Hfaiedh N, Mnafgui K, Kadri A (2015) Assessment of polyphenol composition, antioxidant and antimicrobial properties of various extracts of Date Palm Pollen (DPP) from two Tunisian cultivars. Arab J Chem. doi:10.1016/j.arabjc.2015.07.014

De-Melo AAM (2015) Perfil químico e microbiológico, cor, análise polínica e propriedades biológicas do pólen apícola desidratado. http://www.teses.usp.br/teses/disponiveis/9/9131/tde-18122015-142742/pt-br.php. Accessed 26 Feb 2017

De-Melo AAM, Estevinho MLMF, Sattler JAG, Souza BR, Silva-Freitas A, Barth OM (2016) Effect of processing conditions on characteristics of dehydrated bee-pollen and correlation between quality parameters. Food Sci Technol 65:808–815

Domínguez-Valhondo D, Gil DB, Hernández MT, González-Gómez D (2011) Influence of the commercial processing and floral origin on bioactive and nutritional properties of honeybee-collected pollen. Int J Food Sci Tech 46:2204–2211

Donadieu Y (1983) Le pollen: thérapeutique naturelle, 6th edn. Maloine, Paris

Dutcher RA (1918) Observations on the curative properties of honey, nectar, and corn pollen in avian polyneuritis. J Biol Chem 36:551–555

Estevinho ML, Rodrigues S, Pereira AP, Feás X (2012) Portuguese bee pollen: palynological study, nutritional and microbiological evaluation. Int J Food Sci Technol 47:429–435

FAO Food and Agriculture Organization of the United Nations (2011) Dietary protein quality evaluation in human nutrition. http://www.fao.org/ag/humannutrition/35978-02317b979a686a57aa4593304ffc17f06.pdf. Accessed 12 Dec 2016

Fanali C, Dugo L, Rocco A (2013) Nano-liquid chromatography in nutraceutical analysis: Determination of polyphenols in bee pollen. J Chromatogr A 1313:270–274

Fatrcová-Šramková K, Nôžková J, Kačániová M, Máriássyová M, Rovná K, Stričík M (2013) Antioxidant and antimicrobial properties of monofloral bee pollen. J Environ Sci Health B 48:133–138

Féas X, Vázquez-Tato MP, Estevinho L, Seijas JA, Iglesias A (2012) Organic bee pollen: botanical origin, nutritional value, bioactive compounds, antioxidant activity and microbiological quality. Molecules 17:8359–8377

Forsén S, Kördel J (1994) Calcium in biological systems. In: Bertini I, Gray HB, Lippard SJ, Valentine JS (eds) Bioinorganic chemistry. University Science Books, Mill Valley, CA, pp 107–166

Formicki G, Gren A, Stawarz R, Zysk B, Gal A (2013) Metal content in honey, propolis, wax, and bee pollen and implications for metal pollution monitoring. Pol J Environ Stud 22: 99–106

Freire KRL, Lins ACS, Dórea MC, Santos FAR, Camara CA, Silva TMS (2012) Palynological origin, phenolic content, and antioxidant properties of honeybee-collected pollen from Bahia, Brazil. Molecules 17:1652–1664

Fuenmayor C, Zuluaga C, Díaz C, Quicazán M, Cosio M, Mannino S (2014) Evaluation of the physicochemical and functional properties of Colombian bee pollen. Rev MVZ Córdoba 19:4003–4014

Funari SRC, Rocha HC, Sforcin JM, Filho HG, Curi PR, Gomes Dierckx SMA (2003) Composições bromatológica e mineral do pólen coletado por abelhas africanizadas (*Apis mellifera* L.) em Botucatu, Estado de São Paulo. Arch Latinoam Prod Anim 11:88–93

Gabriele M, Parri E, Felicioli A, Sagona S, Pozzo L, Biondi C (2015) Phytochemical composition and antioxidant activity of tuscan bee pollen of different botanic origins. Ital J Food Sci 27:248–259

González-Paramás AMG, Bárez JAG, Marcos CC, García-Villanova RJ, Sánchez JS (2006) HPLC-fluorimetric method for analysis of amino acids in products of the hive (honey and bee-pollen). Food Chem 95:148–156

Graikou K, Kapeta S, Aligiannis N, Sotiroudis G, Chondrogianni N, Gonos E (2011) Chemical analysis of Greek pollen – antioxidant, antimicrobial and proteasome activation properties. Chem Cent J 5:1–9

Halliwell B, Gutteridge J (2007) Free radicals in biology and Medicine, 4rd edn. Oxford University Press Inc., New York, NY

Hamalainen M, Nieminen R, Vuorela P, Heinonen M, Moilanen E (2007) Anti-inflammatory effects of flavonoids: genistein, kaempferol, quercetin, and daidzein inhibit STAT-1 and NF-κB activations, whereas flavone, isorhamnetin, naringenin, and pelargonidin inhibit only NF-κB activation along with their inhibitory effect on iNOS expression and NO production in activated macrophages. Mediat Inflamm 2007:1–10

Herbert JREW, Vanderslice JT, Huang M, Higgs DJ (1987) Levels of thiamine and its esters in bee collected pollen using liquid chromatography and robotics. Apidologie 18:129–136

Horst MA, Lajolo FM (2011) Biodisponibilidade de compostos bioativos de alimentos. http://wwwfcfuspbr. Accessed 15 Jun 2013

Huang D, Ou B, Prior RL (2005) The chemistry behind antioxidant capacity assays. J Agric Food Chem 53:1841–1856

Human H, Nicolson SW (2006) Nutritional content of fresh, bee-collected and stored pollen of *Aloe greatheadii* var. davyana (Asphodelaceae). Phytochemistry 67:1486–1492

Kacániová M, Vukovic N, Chlebo R, Haščík P, Rovná K, Cubon J (2012) The antimicrobial activity of honey, bee pollen loads and beeswax from Slovakia. Arch Biol Sci 64:927–934

Kaškonienė V, Ruočkuvienė G, Kaškonas P, Akuneca I, Maruška A (2015) Chemometric analysis of bee pollen based on volatile and phenolic compound compositions and antioxidant properties. Food Anal Method 8:1150–1163

Ketkar SS, Rathore AS, Lohidasan S, Rao L, Paradkar AR, Mahadik KR (2014) Investigation of the nutraceutical potential of monofloral Indian mustard bee pollen. J Integr Med 12:379–389

Kinsella JE (1976) Functional properties of protein foods. Crit Rev Food Sci Nutr 1:219–229

Komosinska-Vassev K, Olczyk P, Kafmierczak J, Mencner L, Olczyk K (2015) Bee pollen: chemical composition and therapeutic application. Evid Based Complement Alternat Med 2015:1–6

Kostic AZ, Barac MB, Stanojevic SP, Milojkovic-Opsenica DM, Tesic ZL, Sikoparija B, Radisic P, Prentovic M (2015a) Physicochemical composition and techno-functional properties of bee pollen collected in Serbia. Food Sci Technol 62:301–309

Kostic AZ, Pešić MB, Mosić MD, Dojčinović BP, Natić MM, Trifković JD (2015b) Mineral content of bee pollen from Serbia. Arh Hig Rada Toksikol 66:251–258

Krell R (1996) Value-added Products from Beekeeping. FAO Agric Serv Bull 124:87–113

Leblanc BW, Davis OK, Boue S, Delucca A, Deeby T (2009) Antioxidant activity of Sonoran Desert bee pollen. Food Chem 115:1299–1305

Leja M, Mareczek A, Wyzgolik G, Klepacz-Baniak J, Czekonska K (2007) Antioxidative properties of bee pollen in selected plant species. Food Chem 100:237–240

Liolios V, Tananaki V, Dimou M, Kanelis D, Goras G, Karazafiris E (2016) Ranking pollen from bee plants according to their protein contribution to honey bees. J Apic Res 54:582–592

Loper GM, Cohen AC (1987) Amino acid content of dandelion pollen, a honey bee (Hymenoptera: Apidae) nutritional evaluation. J Econ Entomol 80:14–17

Loper GM, Standifer LN, Thompson MJ, Gilliam M (1980) Biochemistry and microbiology of bee collected almond (*Prumus dulcis*) pollen and bee bread. I. Fatty acids, vitamins and minerals. Apidologie 11:63–73

Lopes J, Stanciu OG, Campos MG, Almaraz-Abarca N, Almeida-Muradian LB, Marghitas LA (2011) Bee pollen antioxidant activity – a review: achievements and further challenges. J Pharmacol 2:25–38

Louveaux J, Maurizio M, Vorvohl G (1978) Methods of melissopalynology. Bee World 59: 139–157

Luz CFP, Bacha-Junior GL, Fonseca RLS, Sousa PR (2010) Comparative pollen preferences by africanized honeybees *Apis mellifera* L. of two colonies in Pará de Minas, Minas Gerais, Brazil. Ann Acad Bras Ciênc 82:293–304

Lv H, Wang X, He Y, Wang H, Suo Y (2015) Identification and quantification of flavonoid aglycones in rape bee pollen from Qinghai-Tibetan Plateau by HPLC-DAD-APCI/MS. J Food Compost Anal 38:49–54

Macrae R (1990) HPLC determination of vitamins. J Micronutr Anal 7:247–260

Manning R (2001) Fatty acids in pollen: a review of their importance for honey bees. Bee World 82:60–75

Mărgăoan R, Mărghitas LA, Dezmirean DS, Mihai CM, Bobis O (2014) Bull UASVM Anim Sci Biotechnol 67:254–259

Mărghitas LA, Stanciu OG, Dezmirean DS, Bobis O, Popescu O, Bogdanov S (2009) *In vitro* antioxidant capacity of honeybee-collected pollen of selected floral origin harvested from Romania. Food Chem 115:878–883

Martins MCT (2010) Pólen apícola brasileiro: valor nutritivo e funcional, qualidade e contaminantes inorgânicos. http://www.bibliotecadigital.unicamp.br/document/?code=000477526. Accessed 26 Feb 2017

Mejías E, Montenegro G (2012) The antioxidant activity of chilean honey and bee pollen produced in the Llaima volcano's zones. J Food Qual 35:315–322

Melo ILP, Almeida-Muradian LB (2010) Stability of antioxidants vitamins in bee pollen samples. Quím Nova 33:514–518

Melo ILP, Almeida-Muradian LB (2011) Comparison of methodologies for moisture determination on dried bee pollen samples. Ciênc Tecnol Aliment 31:194–197

Melo ILP, Freitas AS, Barth OM, Almeida-Muradian LB (2009) Relação entre a composição nutricional e a origem floral de pólen apícola desidratado. Rev Inst Adolfo Lutz 68:346–353

Menezes JDS, Maciel LF, Miranda MS, Druzian JI (2010) Compostos bioativos e potencial antioxidante do pólen apícola produzido por abelhas africanizadas (*Apis mellifera* L.) Rev Inst Adolfo Lutz 69:233–242

Mizrahi A, Lensky Y (1997) Bee products: properties, applications and apitherapy. Springer, New York, NY

Modro AFH, Message D, Luz CFP, Meira-Neto JAA (2007) Composição e qualidade de pólen apícola coletado em Minas Gerais. Pesq Agropec Bras 42:1057–1065
Modro AFH, Silva IC, Luz CFP, Message D (2009) Analysis of pollen load based on color, physicochemical composition and botanical source. Ann Acad Bras Ciênc 81:281–285
Modro AFH, Message D, Luz CFP, Meira-Neto JAA (2011) Flora de importância polinífera para *Apis mellifera* (L.) na região de Viçosa, MG. Rev Árvore 35:1145–1153
Mohdaly AAA, Mahmoud AA, Roby MHH, Smetanska I, Ramadan MF (2015) Phenolic extract from propolis and bee pollen: composition, antioxidant and antibacterial activities. J Food Biochem 39:538–547
Morais M, Moreira L, Feás X, Estevinho LM (2011) Honeybee-collected pollen from five Portuguese Natural Parks: palynological origin, phenolic content, antioxidant properties and antimicrobial activity. Food Chem Toxicol 49:1096–1101
Moreira L, Dias LG, Pereira JA, Estevinho L (2008) Antioxidant properties, total phenols and pollen analysis of propolis samples from Portugal. Food Chem Toxicol 46:3482–3485
Morgano MA, Martins MCT, Rabonato LC, Milani RF, Yotsuyanagi K, Rodriguez-Amaya DB (2012) A comprehensive investigation of the mineral composition of brazilian bee pollen: geographic and seasonal variations and contribution to human diet. J Braz Chem Soc 23: 727–736
Muniategui S, Sancho MT, López-Hernández J, Simal-Lozano J (1990) Determination of carotenes from bee-collected pollen by high performance liquid chromatography. J Apicult Res 29:147–150
Negri G, Teixeira EW, Alves MLTMF, Moreti ACCC, Otsuk IP, Borguini RG, Salatino A (2011) Hydroxycinnamic acid amide derivatives, phenolic compounds and antioxidant activities of extracts of pollen samples from Southeast Brazil. J Agric Food Chem 59:5516–5522
Nicolson SW, Human H (2013) Chemical composition of the 'low quality' pollen of sunflower (*Helianthus annuus*, Asteraceae). Apidologie 44:144–152
Nielsen FH (2008) Is boron nutritionally relevant? Nutr Rev 66:183–191
NIH (2016) Selenium. https://ods.od.nih.gov/factsheets/Selenium-HealthProfessional/. Accessed 12 Dec 2016
Nogueira C, Iglesias A, Feás X, Estevinho LM (2012) Commercial bee pollen with different geographical origins: a comprehensive approach. Int J Mol Sci 13:11173–11187
Oliveira DM, Bastos DHM (2011) Biodisponibilidade de ácidos fenólicos. Quím Nova 34:1051–1056
Oliveira KCLS, Moriya M, Azedo RAB, Almeida-Muradian LB, Teixeira EW, Alves MLTMF (2009) Relationship between botanical origin and antioxidants vitamins of bee-collected pollen. Quím Nova 32:1099–1102
Panziera FB, Dorneles MM, Durgante PC, Silva VL (2011) Avaliação da ingestão de minerais antioxidantes em idosos. Rev Bras Geriatr Gerontol 14:49–58
Pascoal A, Rodrigues S, Teixeira A, Féas X, Estevinho LM (2014) Biological activities of commercial bee pollens: antimicrobial, antimutagenic, antioxidant and anti-inflammatory. Food Chem Toxicol 63:233–239
Pereira TC, Hessel G (2009) Deficiência de zinco em crianças e adolescentes com doenças hepáticas crônicas. Rev Paul Pediatr 27:322–328
Pereira FM, Lopes MTR, Camargo RCR, Vilela SLO (2013) Produção de mel. http://www.sistemasdeproducao.cnptia.embrapa.br. Accessed 2 Mar 2014
Perini JÂL, Stevanato FB, Sargi SC, Visentainer JEL, Dalalio MMO, Matshushita MS (2010) Ácidos graxos poli-insaturados n-3 e n-6: metabolismo em mamíferos e resposta imune. Rev Nutr 23:1075–1086
Polesello S, Rizzolo A (1990) Application of HPLC to the determination of water soluble vitamins in foods: 2 (a review 1985–1989). J Micronutr Anal 8:105–158
Rebiai A, Lanez T (2012) Chemical composition and antioxidant activity of *Apis mellifera* bee pollen from northwest Algeria. J Fundam Appl Sci 4:155–163

Rosanoff A, Weaver CM, Rude RK (2012) Suboptimal magnesium status in the United States: are the health consequences underestimated? Nutr Rev 70:153–164

Russia (1990) Flower pollen (beebread) – specifications. http://gostexpert.ru/data/files/28887-90/9ab039cee5dcc6668687b174db81e5f8.pdf. Accessed 12 Mar 2015

Saavedra KIC, Rojas CI, Delgado GEP (2013) Características polínicas y composición química del polen apícola colectado en Cayaltí (Lambayeque – Perú). Rev Chil Nutr 40:71–78

Sánchez ET (2004) Polen: producción envasado y comercialización. Diproansa, Morelos

Sattler JAG, Melo ILP, Granato D, Araújo E, Silva-Freitas A, Barth OM (2015) Impact of origin on bioactive compounds and nutritional composition of bee pollen from southern Brazil. Food Res Int 77:82–91

Sattler JAG, De-Melo AAM, Nascimento KS, Melo ILP, Mancini-Filho J, Sattler A (2016) Essential minerals and inorganic contaminants (barium, cadmium, lithium, lead and vanadium) in dried bee pollen produced in Rio Grande do Sul State, Brazil. Food Sci Technol 36:505–509

Schmidt JO, Buchmann SL (1992) Other products of hive. In: Graham JM, Amgrose JT, Langstroth LL (eds) The hive and the honey bee: a new book on beekeeping which continues the tradition of "langstroth on the hive and the honeybee". Hamilton, Dadant and Sons, pp 928–977

Shady HMA, Mohamed WF, Sayed-Ahmed EF, Amer SA (2016) A comparative study on propolis and pollen extracts: chemical profile analysis, antioxidant and anticancer activity. Int J Curr Microbiol Appl Sci 5:397–414

Serra-Bonvehí J, Escolà-Jordà R (1997) Nutrient composition and microbiological quality of honeybee-collected pollen in Spain. J Agric Food Chem 45:725–732

Shawer MB, Ali SM, Abdellatif MA, El-Refai AA (1987) Biochemical studies of bee-collected pollen in Egypt 2. Fatty acids and non-saponifiables. J Apic Res 26:133–136

Silveira TA (2012) Caracterização sazonal do pólen apícola quanto à origem botânica, aspectos físico-químicos e elementos traços como bioindicadora de poluição ambiental. http://www.teses.usp.br/teses/disponiveis/11/11146/tde-20032012-090016/pt-br.php. Accessed 26 Feb 2017

Simões CMO, Guerra MP, Schenkel EP, Zannin M, Mentz LA, Bordignon SAL (2004) Farmacognosia: da planta ao medicamento, 5th edn. UFRGS, Porto Alegre

Somerville DC, Nicol HI (2002) Mineral content of honeybee-collected pollen from southern New South Wales. Aust J Exp Agric 42:1131–1136

Souza BR (2014) Quantificação das vitaminas do complexo B (B1, B2) e vitâmeros das vitaminas B3 e B6 em amostras de pólen apícola desidratado provenientes da Região Sul do Brasil. http://www.teses.usp.br/teses/disponiveis/9/9131/tde-27052015-141055/pt-br.php. Accessed 26 Feb 2017

Stanley R, Linskens H (1974) Pollen: biology biochemistry management. Springer, Berlin

Sunde RA (2012) Selenium. In: Ross AC, Caballero B, Cousins RJ, Tucker KL, Ziegler TR. Modern nutrition in health and disease. 11th edn. Philadelphia, PA: Lippincott Williams & Wilkins; p. 225–237.

Swiss (2005) Ordinance of the DFI on foodstuffs of animal origin: chapter 11 – honey, royal jelly and pollen. https://www.admin.ch/opc/fr/classified-compilation/20050164/index.html. Accessed 12 Mar 2015

Szczesna T (2006a) Long-chain fatty acids composition of honeybee-collected pollen. J Apic Sci 50:81–90

Szczesna T (2006b) Protein content and amino acid composition of bee-collected pollen from selected botanical origins. J Apic Sci 50:81–90

Szczesna T (2007) Concentration of selected elements in honeybee-collected pollen. J Apic Sci 51:5–13

Szczesna T, Rybak-Chmielewska H (1998) Some properties of honey bee collected pollen. Pszczelnicze Zeszyty Naukowe 42:79–80

Szczesna T, Rybak-Chimielewska H, Bornus L (1991) Effect of storage on variation of contents vitamin C and A in pollen collected bees. Apiacta 2:1–3

Taha EA (2015) Chemical composition and amounts of mineral elements in honeybee-collected pollen in relation to botanical origin. J Apic Sci 59:75–81
Tavdidishvili D, Khutsidze T, Pkhakadze M, Vanidze M, Kalandia A (2014) Flavonoids in georgian bee bread and bee pollen. J Chem Chem Eng 8:676–681
Ulusoy E, Kolayli S (2014) Phenolic composition and antioxidant properties of anzer bee pollen. J Food Biochem 38:73–82
USDA (1998) Vegetarians, watch your zinc! https://agresearchmag.ars.usda.gov/1998/mar/zinc/. Accessed 12 Mar 2015
USDA (2011) Dri dietary reference intakes: calcium vitamin D. https://www.nap.edu/read/13050/chapter/1. Accessed 12 Mar 2015
USDA (2015) Nutrient vitamins and minerals. https://www.ams.usda.gov/sites/default/files/media/Nutrient%20Vitamins%20TR%202015.pdf. Accessed 12 Dec 2016
WHO World Health Organization (2012a) Guideline: potassium intake for adults and children. World Health Organization, Geneva
WHO World Health Organization (2012b) Guideline: sodium intake for adults and children. World Health Organization, Geneva
WHO World Health Organization (2015). Micronutrient deficiencies: iron deficiency anaemia. http://www.who.int/nutrition/topics/ida/en/. Accessed 12 Dec 2016
Xu X, Sun L, Dong J, Zhang H (2009) Breaking the cells of rape bee pollen and consecutive extraction of functional oil with supercritical carbon dioxide. Innov Food Sci Emerg Technol 10:42–46
Xu X, Dong J, Mu X, Sun L (2011) Supercritical CO_2 extraction of oil, carotenoids, squalene and sterols from lotus (*Nelumbo nucifera* Gaertn) bee pollen. Food and Bioprod Process 89:47–52
Yang J, Guo J, Yuan J (2008) In vitro antioxidant properties of rutin. Food Sci Technol 41:1060–1066
Yang K, Wu D, Ye X, Liu D, Chen J, Sun P (2013) Characterization of chemical composition of bee pollen in China. J Agric Food Chem 61:708–718
You J, Liu L, Zhao W, Zhao X, Suo Y, Wang H (2007) Study of a new derivatizing reagent that improves the analysis of amino acids by HPLC with fluorescence detection: application to hydrolyzed rape bee pollen. Anal Bioanal Chem 387:2705–2718
Youssef AM, Farag RS, Ewies MA, El-Shakaa SMA (1978) Chemical studies on pollen collected by honeybees in Giza region, Egypt. J Apic Res 17:110–113
Zafra AO (1979) El polen y su salud. Puebla, Pue, Florimiel
Zhou J, Qi Y, Ritho J, Zhang Y, Zheng X, Wu L (2015) Flavonoid glycosides as floral origin markers to discriminate of unifloral bee pollen by LC-MS/MS. Food Cont 57:54–62

Chapter 12
Health Benefits and Uses in Medicine of Bee Pollen

Adriane Alexandre Machado De-Melo and Ligia Bicudo de Almeida-Muradian

Contents

12.1 Introduction

Bee pollen is the result of the agglomeration of pollen harvested by *Apis mellifera* L. bees, which use their saliva secretions or nectar to agglutinate the grains (Campos et al. 2008). There is evidence that bee pollen has been intentionally consumed by people for centuries. In Ancient Egypt, it was called the "life-giving dust". Hippocrates and Pythagoras recommended the consumption to their patients because they believed in its therapeutic potential (Campos et al. 2008). In North America, there are records of intentional consumption between 1400 BC and 200 AD by

A.A.M. De-Melo (✉)
Department of Food and Experimental Nutrition, Pharmaceutical Sciences School, University of São Paulo, São Paulo, SP 05580-000, Brazil

Laboratório de Análise de Alimentos da Faculdade de Ciências Farmacêuticas, University of São Paulo, Avenida Professor Lineu Prestes, 580, Bloco 13-A, Cidade Universitária, São Paulo, SP 05580-000, Brazil
e-mail: adriane.melo@usp.br

L.B. de Almeida-Muradian
Department of Food and Experimental Nutrition, Pharmaceutical Sciences School, University of São Paulo, São Paulo, SP 05580-000, Brazil

J.M. Alvarez-Suarez (ed.), *Bee Products - Chemical and Biological Properties*,
DOI 10.1007/978-3-319-59689-1_12

indigenous peoples (Reinhard et al. 1991; Linskens and Jorde 1997). In recent years, consumption was driven by demand from the population for natural products, complementary to diet or with therapeutic effects (Barreto et al. 2005).

The health benefits associated with bee pollen are related to the presence of compounds with the biological potential to inhibit the growth of microorganisms and aid in the prevention of oxidative stress, identified as one of the causes of the development of chronic degenerative diseases such as cancer, cardiovascular diseases, and neuronal degeneration (Uttara et al. 2009). Commonly, this food is consumed as a rejuvenator or tonic, to increase physical resistance, and to help in the treatment of respiratory infirmities, hormonal disturbances, and in the prevention of cancer, though current research is insufficient to validate all of these indications (Ulbricht et al. 2009).

Research was carried out with the aim of characterizing samples from various countries, seeking to identify those with greater biological potential and the varieties which influence this potential. Recently, some researchers have focused on *in vivo* studies with the inclusion of this food source in the diet of animals and in the formulation of medications with a bee pollen base. In this chapter the results of *in vitro* and *in vivo* studies of the antibacterial, antioxidant and antitumor activity of bee pollen are presented, as well as factors that influence the biological potential of this product.

12.2 Antibacterial Activity

Antibacterials can be defined as compounds that, at low concentrations, are capable of selectively inhibiting the development of bacteria or deactivating them (Cunha and Roque 2009). These compounds can be utilized in the treatment or prevention of infections (Levin et al. 2014) and as supplements in alimentary formulations (Melo et al. 2005). In commercial production, they are added to improve growth rate and/or alimentary conversion of animals, and function as growth stimulants in this context. In agricultural cultivation, they are part of the measures adopted to control bacterial illnesses (Maia et al. 2009).

Bee pollen contains compounds that inhibit the growth of Gram-positive and Gram-negative bacteria. The activity varies according to the potential of compounds present in each product (Morais et al. 2011; Nogueira et al. 2012; Cabrera and Montenegro 2013; Fatrcová-Šramková et al. 2013). Substances responsible for the antibacterial capacity of this food act by deactivating enzymes, promoting alterations in the permeability of membranes and loss of cellular material, or further, by deactivating or destroying genetic material of bacterial cells (Borguini 2006; Almeida 2007).

Phenolic compounds are the main antibacterial agents of bee pollen (Carpes et al. 2008), but are not the only ones. Spermidine derivatives have considerable antibacterial potential and have been identified in bee pollen (Bassard et al. 2010; Mihajlovic et al. 2015; De-Melo 2015). Some fatty acids also possess antimicrobial action (Barbosa et al. 2006), and glucose oxidase, an enzyme produced by bees and added during the formation of the grain, which can also be responsible for part of the antibacterial effect of this food (Denisow and Denisow-Pietrzyk 2016).

In Brazilian bee pollen, there was a positive correlation between the content of total phenolic compounds and the inhibition of Gram-negative and Gram-positive bacterial growth (De-Melo 2015). As far as can be seen, the action is not related to the totality, but rather, to specific phenolic compounds (Campos et al. 2008; Carpes et al. 2008; Morais et al. 2011; Pascoal et al. 2014; De-Melo 2015). Graikou et al. (2011) tested the action of isolated flavonoids from bee pollen extracts through liquid chromatography. The flavonoids exercised a strong activity against Gram-positive bacteria, which led the authors to associate a significant part of this action to the presence of these compounds. According to Almeida (2007), the greater the degree of hydroxylation, the greater their toxicity.

Processing has impact on antibacterial activity of bee pollen. In a study with heterofloral samples from Brazil, it was observed that the dehydration conditions influenced the antibacterial activity, perhaps because the processing also influenced the phenolic content. The MIC values for *S. aureus*, *S. pyogenes*, *E. coli* and *Klebsiella* were lower in the lyophilized samples than those observed for samples treated in an electric oven (De-Melo et al. 2016).

The antibacterial capacity of bee pollen varies according to the microorganism. Generally, Gram-positive bacteria are more sensitive to antibacterials than Gram-negatives, with some studies highlighting *Staphylococcus aureus* as the microorganism most sensitive to bee pollen (Graikou et al. 2011; Khider et al. 2013; Pascoal et al. 2014; De-Melo 2015; Borycka et al. 2016). The greater resistance of Gram-negative bacteria seems related to the external membrane that covers the cell, whose chemical structure can restrict the passage of certain substances (Morais et al. 2011).

The solvent/dilution method utilized in the extraction of compounds also influences the results. With methanolic extracts (99.9% methanol) a greater inhibitory effect on *E. coli* was observed, while with ethanolic extracts (70% ethanol), the greatest effect was seen against a fungus (Kacániová et al. 2012). These variations can be related to compounds extracted by each solvent/dilution, a fact which was also observed by Carpes et al. (2007) and Graikou et al. (2011) in previous studies. Fatrcová-Šramková et al. (2013) evaluated ethanolic and methanolic extracts from bee pollen and concluded that those with an ethanol base showed greater activity.

The antibacterial capacity of a food or isolated compound can be determined *in vitro* by bioautographic tests, diffusion tests, and dilution tests. The bioautographic tests involved the use of thin-layer chromatography for the separation of the compounds, which are then placed in contact with, or immersed in wells previously inoculated with the test microorganism. The diffusion tests are based on the diffusion of the extract in a solid culture medium previously inoculated. In the dilution test the addition of aliquots of test solution in the culture medium previously inoculated with the microorganism is carried out, and after the period of incubation, the growth is compared with a negative control (Almeida 2007). The effectiveness of the compound mixture or isolated compound is frequently presented as minimum inhibitory concentration (MIC), i.e. the lowest extract concentration capable of ceasing or slowing the growth of the test microorganism.

Pascoal et al. (2014) tested, using the microdilution method on microplates, eight methanolic bee pollen extracts from Portugal and Spain on the inhibition of

Staphylococcus aureus, *Pseudomonas aeruginosa* e *Escherichia coli*. The *S. aureus* strains were the most vulnerable, with MIC values between 1.81 and 4.28 mg/mL. For the other microorganisms, the authors observed between 3.71–6.96 mg/mL (*P. aeruginosa*) and 4.08–9.42 mg/mL (*E. coli*). In samples collected in five Portuguese Nature Reserves, Morais et al. (2011) identified that the methanolic extracts were more effective against Gram-positive bacteria (*S. aureus* and *Bacillus cereus*) than against Gram-negative bacteria (*Salmonella typhi* and *E. coli*). The method used was microdilution on microplates.

Monofloral samples collected in Montesinho Nature Reserve in Portugal were extracted in *n*-hexane and tested using the agar well diffusion method. The lipophilic extract of *Cistus ladanifer* bee pollen was the most effective at inhibiting bacterial growth, followed by the *Castanea sativa* bee pollen extract and then by lipophilic extract of *Rubus* sp. bee pollen. The extracts were essentially composed of fatty acids, sterols, long chain aliphatic alcohols as well as smaller amounts of alkanes and alkenes. Fatty acids, such as linoleic acid, are notably antimicrobial compounds (Barbosa et al. 2006).

In Egypt, monofloral bee pollen was extracted with methanol. Extracts were analyzed using agar plate diffusion and the MIC was determined by dilution. Different results were obtained depending on the botanical origin of the samples of *Trifolium alexandrinum* bee pollen (*E. coli*, *S. aureus* and *Listeria monocytogenes*: 320 μg/mL; *Salmonella enteritidis*: 640 μg/mL; *P. aeruginosa*: 1280 μg/mL) and *Zea mays* bee pollen (*S. enteritidis*, *E. coli*, *S. aureus* and *L. monocytogenes*: 320 μg/mL; *P. aeruginosa*: 640 μg/mL) (Khider et al. 2013). Mohdaly et al. (2015) also evaluated the methanol extracts of the monofloral bee pollen from *Zea mays* produced in Egypt, however they encountered greater values for MIC: 0.30 mg/mL (*L. monocytogenes*), 0.78 mg/mL (*S. aureus*), 1.25 mg/mL (*E. coli*) and 1.35 mg/mL (*Salmonella enterica*).

Fatrcová-Šramková et al. (2013) analyzed different extracts of fresh (in natura) monofloral samples from Slovakia using the agar well diffusion method. After 24 h of incubation, the sensitivity of the microorganisms to the ethanolic extract (ethanol 70%) from the bee pollen from *Papaver somniferum* followed the sequence: *S. aureus* (*Sa*) > *E. coli* (*Ec*) > *L. monocytogenes* (*Lm*) > *Salmonella enterica* (*Se*) > *P. aeruginosa* (*Pa*); to the ethanolic extract of *Brassica napus subsp. napus*: *Lm* > *Pa* and *Se* > *Sa* and *Ec*; and to the extract of *Helianthus annuus*: *Se* > *Sa* > *Lm* and *Ec* > *Pa.*

De-Melo (2015) evaluated methanol extracts from monofloral and heterofloral bee pollen extracts produced in Brazil by the microplate and microdilution methods. With the Gram-positive strains the author observed the following MIC values: MIC: 1.1–6.5 mg/mL (*Streptococcus pyogenes*) and 1.6–7.2 mg/mL (*S. aureus*). In relation to the Gram-negative strains, the MIC values were the following: 1.9–8.4 mg/mL (*E. coli*) and 2.4–9.4 mg/mL (*Klebsiella* sp.). Generally, a monofloral sample from *Mimosa scabrella* produced in south Brazil had greater antibacterial activity against all the microorganisms tested, and the heterofloral sample with predominance of pollen from *Mimosa caesalpiniaefolia* and *Myrcia*, produced in the southeast region, showed less antibacterial action.

In another study with Brazilian samples, all collected in south Brazil, there was no inhibitory or bactericidal activity of the ethanolic extracts (ethanol 70%) against the bacteria tested (Carpes et al. 2009). In Turkey, methanolic extracts from bee pollen at concentrations between 0.02% and 2.5% had no antimicrobial effect on different spoilage and pathogenic microorganisms (Erkmen and Özcan 2008).

In Tunisia, different extracts of monofloral bee pollen of *Phoenix dactylifera* were effective in inhibiting the growth of *Bacillus subtilis*, *B. cereus*, *S. aureus, Enterococcus faecalis*, *Micrococcus luteus*, *L. monocytogenes*, *Salmonella enteric serotype* Enteritidis, *Salmonella enteric serotype* Typhimurium, *E. coli* and *Klebsiella pneumoniae,* by agar well diffusion (Daoud et al. 2015).

Samples collected in Morocco showed a potential activity against the growth of *S. aureus, B. cereus, P. aeruginosa*, and *E. coli* strains, using the agar well diffusion method. These strains were isolated from human samples and were resistant to antibiotics. There was no extraction of compounds and the samples were only diluted (Abouda et al. 2011). Cabrera and Montenegro (2013) tested, by agar diffusion method, aqueous extracts from bee pollen produced in Chile. The MIC for *S. aureus* and *S. pyogenes* was 21 mg/mL, and the MIC for *P. aeruginosa* and *E. coli* was 41 mg/mL and 82 mg/mL, respectively.

In Greece, Graikou et al. (2011) evaluated methanolic extracts from bee pollen by agar dilution technique. The MIC varied from 0.74 mg/mL (*S. aureus*) to >10 mg/mL (*E. coli*). These extracts were rich in sugars, fatty acids, fatty acid esters, and phenolic acids (*p*-coumaric acid, ferulic acid, their glycerol esters and glycerol ester of caffeic acid). The flavonoids isolated from extracts were more effective against Gram-positive bacteria (*S. aureus* and *S. epidermidis*) than against Gram-negative bacteria (*E. coli*, *Enterobacter cloacae*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*).

Kačániová et al. (2014) determined the antibacterial activity of ethanolic extracts (unknown dilution) of bee pollen from Slovakia, by using agar disc diffusion method, against the *Clostridium* genus. Extracts with pollen concentrations of 25% and 50% were the best to inhibit the growth of the five species (*C. butyricum, C. histolyticumm C. intestinale*, *C. perfringens* and *C. ramosum*).

Basim et al. (2006) observed that methanolic extracts (bee pollen from Turkey) inhibited the growth of thirteen different species of agricultural bacterial pathogens, with *Agrobacterium tumefaciens* being the most sensitive and *Pseudomonas syringae* the most resistant. These results suggest that the bee pollen extract can be an alternative to protect seeds and in this way avoid the transmission of these pathogens.

The antibacterial action of bee pollen has been tested in *in vivo*. Kročko et al. (2012) evaluated the antibacterial action of bee pollen in the gastrointestinal tract of broiler chickens. The product was included in the food of the birds, seeking to improve feed conversion, performance, growth and health of broiler chickens through mechanisms associated with gastrointestinal tract and bacteria colonization. There was a desirable reduction in the bacterial count for the Enterobacteriaceae family in the gastrointestinal tract.

In the search for medications with a natural compound base, Olczyk et al. (2016) tested preparations based on bee pollen extracts and pharmaceutical products in the

treatment of burns inflicted on swine. The use of preparations with a bee pollen base not only reduced the time for scarring and affected positively the general state of the animals, but it also prevented infection caused by microorganisms of recently formed tissue.

12.3 Antioxidant Activity

A considerable number of studies were carried out to evaluate the benefits associated with the inclusion of natural antioxidants in the diet. Daily intake of these compounds could help prevent oxidative stress, and, consequently, diseases and cellular aging (Halliwell and Gutteridge 2007; Lopes et al. 2011). Another possible benefit of natural antioxidants is an increase in the oxidative stability in alimentary formulations, and their use in medicines and cosmetics.

Bee pollen contains compounds of significant antioxidant capacity in variable quantities. The potential of this product is greater than that found in honey (MORAIS et al. 2011), and similar to that found in red fruits, that are recognized as foods with an elevated antioxidant capacity (Sousa et al. 2011; Huang et al. 2012).

Phenolic compounds, especially phenolic acids and flavonoids, seem to be the main agents of the antioxidant capacity of bee pollen (Campos et al. 2003; Carpes et al. 2008; LeBlanc et al. 2009; Mărghitas et al. 2009; Negri et al. 2011; Freire et al. 2012). These substances are capable of donating hydrogen atoms to the reactive species, therefore making them stable (Angelo and Jorge 2007). Campos et al. (2003) evaluated ethanolic extracts from *E. globulus* bee pollen and found that the phenolic fraction was the most active, though it did not account alone for the total action. Other antioxidants, such as β-carotene (provitamin A) and vitamins E and C, are responsible for part of the antioxidant action of bee pollen (Campos et al. 2003; Oliveira et al. 2009; Melo and Almeida-Muradian 2010).

The type and content of each antioxidant is associated with its botanical origin (Carpes et al. 2008; Campos et al. 2008; De-Melo 2015). Variation even occurs between monofloral bee pollen from the same botanical species (De-Melo 2015), indicating that agricultural or genotypic differences, climatic conditions, such as temperature, hydrological stress, and light intensity, as well as soil conditions, can affect the composition of the pollen grain by the plant (Daoud et al. 2015).

Processing conditions and storage should be considered. De-Melo et al. (2016) analyzed bee pollen from the state of São Paulo, Brazil, after dehydration under different conditions, and concluded that antioxidant capacity was greater in lyophilized samples than in those dehydrated in an electric oven with forced air circulation. Domínguez-Valhondo et al. (2011) also observed a negative impact due to dehydration process on the antioxidant capacity of monofloral bee pollen from *Cistus ladanifer* collected in Chile. In terms of storage, Campos et al. (2003) observed a reduction in the antioxidant capacity of bee pollen from Portugal of up to 50% over three years.

The determination of the antioxidant capacity involves two steps: the extraction of the compounds and the activity test. The conditions of compound extraction,

especially the solvent and its dilution, influenced the results. De-Melo (2015) tested the efficiency of the three methods in the extraction of 64 bee pollen samples from Brazil: in 70% of them, an extraction based on 50% methanol and 70% acetone was more efficient, while in 27% of the samples it was with an extraction based on 70% ethanol in which the highest values for antioxidant capacity were observed. Results can be related to the characteristics of the compounds, and, consequently, with the affinity between these and the solvents. LeBlanc et al. (2009) compared eight solvents of different polarities and concluded that methanol and dimethylformamide were the most efficient with samples from North Tuscon, USA. Pérez-Pérez et al. (2012) found better results in ethanolic extracts than in methanolic and aqueous extracts, in bee pollen from Mérida, Venezuela.

Various methods exist for determining, *in vitro*, the antioxidant capacity of a sample. Examples of methods adopted in studies with bee pollen are: 1,1- diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging activity assay; inhibition of lipid peroxidation using thiobarbituric acid reactive substances (TBARS); ferric reducing/antioxidant power (FRAP) assay; 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) assay; cupric reducing power (CUPRAC) assay; β-carotene bleaching (BCB) assay; and oxigen-radical absorbancy capacity (ORAC). Generally, the studies are carried out with more than one trial for a better understanding of the antioxidant potential (Castelo-Branco and Torres 2011), expecting that different methods arrive at different conclusions. The results are frequently presented as EC_{50} (*effective concentration* 50), i.e. the sample concentration (mg/mL of extract) necessary to reduce 50% of the radical; or in equivalents of some compound, commonly Trolox (TE), per g of bee pollen.

The antioxidant capacity of bee pollen collected in Brazil varies from 0.35 to 13.4 mg/mL (extraction with 70% ethanol; analysis by DPPH method) (Carpes et al. 2009; Arruda 2013; De-Melo 2015) and from 133 to 576 μmol TE/g (70% ethanol; ORAC method) (Arruda 2013; De-Melo 2015). Similar values were found in ethanolic extracts (95% ethanol) from samples produced in Italy. Analyses using DPPH and ORAC methods indicate an antioxidant capacity of, respectively, 215 μg/mL and 544 μmol TE/g (*Castanea* sp. bee pollen), 224 μg/mL and 540 μmol TE/g (*Cistus* sp. bee pollen) and 641 μg/mL and 519 μmol TE/g (*Rubus* sp. bee pollen) (Gabriele et al. 2015).

In Portugal, samples collected in five Portuguese nature reserves and commercially acquired, had similar EC_{50} values using the DPPH method (2.0–6.7 mg/mL) and the BCB method (3.1–6.5 mg/mL) (Morais et al. 2011; Féas et al. 2012; Pascoal et al. 2014). In Spain, antioxidant capacity of the samples was from 3.3 to 5.1 mg/mL (DPPH trial) and from 1.1 to 2.2 mg/mL (TBARS trial). In all the experiments, methanolic extracts were analyzed.

Campos et al. (2003) found in ethanolic extracts (50% ethanol) from monofloral bee pollen samples (Portugal) the following values for antioxidant capacity in EC_{50}: 40 μg/mL (*Eucalyptus globulus* bee pollen), and over 500 μg/mL (*Cistus ladanifer, Echium plantagineum* and *Erica australis* bee pollen). In Brazil, the study undertaken by De-Melo (2015) suggested that, the greater the participation of the *Eucalyptus* genus in bee pollen, the greater the antioxidant capacity. Freire et al. (2012)

found the greatest antioxidant potential in samples with 50–70% of the *Eucalyptus* genus. Menezes et al. (2010) identified the greatest antioxidant capacity values in samples with a predominance of this genus, beyond a positive correlation between this pollen type and total flavonoid and phenolic content. On the other hand, Arruda (2013) found a negative correlation between antioxidant activity and this genus. It is possible that the geographical origin and different species of *Eucalyptus* in the sample were related to these results.

In Northwest Algeria, the greatest antioxidant capacity was encountered in monofloral bee pollen from wild carrot (scientific name not given), followed by bee pollen from rosemary (scientific name not given) and by bee pollen from *Eucalyptus* (Rebiai and Lanez 2012). In monofloral bee pollen from Sonoran Desert (Tucson, USA), LeBlanc et al. (2009) reported that the antioxidant capacity was greater in samples from *Yucca* bee pollen than in samples from *Washingtonia* bee pollen. In Chile, the antioxidant capacity of monofloral bee pollen from *Escallonia rubra* was greater than in the monofloral product from *Lotus pedunculatus*. The authors found that the geographical origin of the samples had an impact on the biological potential and that, in general, the antioxidant capacity of monofloral bee pollen from these species was much greater than that of the honey with the same origin (Mejías and Montenegro 2012).

Leja et al. (2007) analyzed methanolic extracts (80% methanol) of monofloral samples from bee pollen from the Krakow area in Poland, and classified them into three groups: high (*Lupinus polyphyllus*, *Phacelia tanacetifolia*, *Trifolium* sp., *Sinapis alba, Robinia pseudoacacia* and *Aesculus hippocastanum* bee pollen), medium (*Zea mays*, *Chamerion angustifolium* and *Pyrus communis* bee pollen) and low antioxidant capacity (*Lamium purpureum*, *Taraxacum officinale* and *Malus domestica* bee pollen).

In Egypt, Khider et al. (2013) encountered EC_{50} of 0.6 μg/mL in monofloral bee pollen from *Z. mays*. In Nan, Thailand, Chantarudee et al. (2012) found a value of 7.4 μg/mL in monofloral samples of this pollen type, which signified an antioxidant potential almost 29 times higher in bee pollen than in the pollen grains of the plant (212 μg/mL). It was not clear how the bees combine the pollen with other substances and the alterations that occur, which forms a product with greater biological potential.

Mărghitas et al. (2009), using the DPPH method, observed that in the Transylvania area of Romania, the antioxidant capacity of monofloral samples increased in the following order: *Pinus* sp. < *Knautia arvensis* < *Taraxacum officinale* < *Helianthus annuus* < *Onobrychis viciifolia* < *Crataegus monogyna* < *Capsella bursa pastoris* < *Matricaria chamomilla* < *Carduus* sp. < *Carex* sp. < *Centaurea cyanus* < *Salix* sp. Variations in this sequence were observed with ABTS and FRAP methods, and correlations between the DPPH and ABTS methods suggested that the same compounds act in both systems. Methanolic extracts were analyzed.

Fatrcová-Šramková et al. (2013) tested, using the DPPH method, the antioxidant capacity of bee pollen collected in the Nitra region of Slovakia. In the ethanolic extracts (90% ethanol) the values indicated that the sample from *Brassica napus subsp. napus* had greater biological potential than the product with a predominance

of the *Papaver somniferum* pollen type, which, in its turn, had greater potential than that observed in bee pollen from *Helianthus annuus.* The authors also used two other methods to determine the antioxidant capacity of the samples, based on the reduction of Mo (VI) to Mo (V), and another in which the protective effect of antioxidants against oxidative DNA damage was measured, with both showing the activity of the samples.

While Mărghitas et al. (2009), in Romania, observed values of 0.45 mmol TE/g in monofloral bee pollen from *Helianthus annuus*, Zhang et al. (2015) encountered 0.04 mmol TE/g in samples from this botanical origin collected in China. Beyond the possible influence of geographical origin, or of distinct varieties of plants, the different solvents utilized in the extraction could have a relationship to these results. These variables could also have influenced the different values of antioxidant capacity found in monofloral bee pollen from *Phoenix dactylifera.* In Tunisia, EC_{50} reached 46.6 μg/mL (Daoud et al. 2015), while in Egypt the value was 0.8 μg/mL (Khider et al. 2013).

In bee pollen produced in Rize, Turkey, EC_{50} methanolic extracts varied from 0.7 to 8.2 mg/mL (DPPH assay), from 33.1 to 91.8 μmol TE/g bee pollen (CUPRAC assay), and from 11.8 to 105.1 μmol TE/g (FRAP assay); and, of the nine phenolics identified (*p*-OH benzoic acid, vanillic acid, *trans*-cinnamic acid, syringic acid, *p*-coumaric acid, ferulic acid, rutin, *cis*, *trans*-abscisic acid and caffeic acid), only *p*-coumaric acid had some correlation with the biological activity, which was negative (Ulusoy and Kolayli 2014).

In samples collected in Brazil, of the eighteen phenolic compounds identified (gallic acid, protocatechic acid, vanillic acid, siringeic acid, β-resorcylic acid, ferulic acid, synapic acid, cinnamic acid, caffeic acid, *p*-coumaric acid, chlorogenic acid, catechin, epicatechin, naringenin, naringin, rutin, kaempferol and quercetin), only quercetin had a positive correlation with the antioxidant capacity, found using the ORAC and DPPH methods (De-Melo 2015). The antioxidant capacity of a compound depends on physico-chemical characteristics, such as polarity, solubility, capacity to form hydrogen bridges, and potential oxidation-reduction (Borguini 2006; Campos et al. 2008; Oliveira and Bastos 2011).

In 1997, Bevzo and Grygor'eva observed that byintroduction of bee pollen into the diet of mice, their livers normalized the activity of glutathione system enzymes, preventing damage that was expected from the exposure of the animals to radiation. Eraslan et al. (2008) tested the effects of daily administration of a water-solubilized extract of monofloral bee pollen from *Brassica napus* (Kayseri, Turkey), at doses of 50 or 100 mg/kg, to female Wistar albino rats, hours after receiving 225 mg/kg of carbaryl. At the end of the experiment, the inclusion of bee pollen in the diet of Wistar rats seems to alleviate the toxic effects of oxidative stress caused by a concomitantly administered pesticide. The results of the study released by Saríc et al. (2009), in which bee pollen from Croatia was included in the diet of rats, suggested that this product is a source of compounds with health protection potential.

Tohamy et al. (2014) investigated the antioxidant activity of water extracts from Egyptian bee pollen on cisplatin induced hepatic, renal, testicular, and genotoxicity in male albino mice (*Mus musculus*). The inclusion of extracts into the diet, at doses

140 mg/kg for 14 days, resulted in a significant reduction in the level of lipid peroxidation and the elevation in catalase activity and glutathione concentration. This effect was most pronounced in the testes. Saral et al. (2016) observed that the inclusion of bee pollen in the diet of rats effectively reduced liver damage caused by the injection of carbon tetrachloride, and that this effect seems related to the antioxidant potential of this food.

12.4 Antitumor Activity

Bee pollen has shown greater or lesser antitumor potential against some types of cancer. This potential can be related to the antioxidant capacity of the compounds that act by removing or deactivating reactive species. There may also be a relation with the induction of apoptosis of carcinogenic cells and stimulation of the secretion of tumor necrosis factor-alpha (TNF-α), or further still, with the strengthening of the immunological system (Denisow and Denisow-Pietrzyk 2016). Beyond this, this product seems to be able to reduce the toxic effects associated with the use of pharmaceuticals utilized in the treatment of cancer (Furusawa et al. 1995; Pinto et al. 2010).

The antitumor potential of bee pollen is related to the presence of certain compounds. Quercetin, a flavonoid of great prevalence in this matrix, is capable of blocking the expression of specific genes in tumor cells. Rutin and chyrisin can also act in the prevention of cancer. Kaempferol, another fairly common flavonoid, caused a reversible growth inhibition of PC-3 cancer cells. There is evidence suggesting that phytosterols, as well as β-carotene, can reduce the risk of some prostate carcinoma (Bogdanov 2012).

Apigenin, a flavonoid of the flavone class, was identified by De-Melo (2015) in monofloral bee pollen from *Myrcia* from the State of Rio Grande do Sul, Brazil. This compound is commonly found in fruits and vegetables and has an antitumor action. The author also reported the presence of 4-methylsulfonyl-butyl glycosinolate (glucoraphanine) in monofloral samples of *Alternanthera* (São Paulo State, Brazil), *Mimosa caesalpiniaefolia* (Sergipe State, Braszil) and *Mimosa scabrella* (Santa Catarina State, Brazil). Traka et al. (2013) suggested an anticarcinogenic potential of glucoraphanine (Shukla and Gupta 2010).

Wang et al. (2013) extracted, fractionated, and purified polysaccharides of monofloral bee pollen from *Rosa rugosa* in Beijing, China. In this study, which was carried out *in vitro*, the authors found anti-proliferative activity in human colon cancer HT-29 and HCT116 cells. The extracts also showed a concentration-dependent proliferation-inhibitory effect. Aliyazicioglu et al. (2005), in research with heterofloral samples collected in Turkey, observed that bee pollen extracts (dimethyl sulfoxide extracts at concentrations of 50, 25, and 12.5 mg/mL) suppress respiratory burst within cancer cell lines (K-562).

In a study carried out on bee pollen from Spain and Portugal, Pascoal et al. (2014) tested the anti-genotoxic effects of methanolic extracts on *Saccharomyces cerevisiae* exposed to ethyl methanesulfonate, which produces random mutations in genetic material by nucleotide substitution. All samples showed antigenotoxic

activity at concentrations of up to 0.75 mg/mL, although some were more efficient at reducing the number of gene conversion colonies and only two samples, one from Portugal and the other from Spain, significantly reverted the mutations.

Aqueous extracts of bee pollen from Beni-Suef, Egypt, were tested as antimutagenic agents against cisplatin-induced chromosomal abnormalities in bone marrow cells of mice (*Mus musculus*). The animals received, for one week, injections of cisplatin [*cis*-diammine-dichloro-platinum (II)] (CDDP), a cytotoxic antineoplastic drug, that causes oxidative stress and a series of adverse effects. Subsequently, they received daily doses of pollen extract (140 mg/kg, orally) for 14 days. There was a significant reduction in the incidence of chromosomal damage induced by CDDP in bone marrow cells and an increase in the frequency of the mitotic indices of bone marrow cells, showing a chemoprotective effect of bee pollen against CDDP induced genotoxicity (Abdella et al. 2009). In research carried out by Tohamy et al. (2014), also with samples of bee pollen from Egypt, similar results were found.

The effects of the administration of the water phase of extracts from bee pollen were studied in syngeneic mice with Lewis lung carcinoma implanted intraperitoneally. The extracts were not cytotoxic in cell cultures at concentrations of up to 2.5 mg/mL, and significantly prolonged the life expectancy of mice carrying the tumor without any apparent side effects at 0.5 g/kg. When administration occurred in conjunction with standard cytotoxic antitumor drugs, there was a significant increase in the survival rate of the animals, with some managing to survive in a tumor free state until the end of the experiment. The antitumor action of the extracts seems related to the immune-stimulation of the macrophages (Furusawa et al. 1995).

The ethanolic extract (95% ethanol) from monofloral bee pollen from *Cistus* (*C. ladanifer* and *Cistus albidus*), produced in Spain, at a concentration of 300 μg/mL, showed suppressive effects against VEGF-induced angiogenesis in a study with human umbilical vein endothelial cells. Vascular endothelial growth factor (VEGF) is a key regulator of pathogenic angiogenesis in diseases such as cancer (Izuta et al. 2009).

In other studies undertaken with monofloral bee pollen from *Cistus* (*C. ladaniferus*) (origin unknown), the administration of aqueous extracts (10 mg/100 g of body weight, administered orally) on rats submitted to ovariectomy resulted in a smaller decrease in mineral content, mineral density, and polar strength strain index in the femoral-metaphyseal tissues (Yamaguchi et al. 2007a); and the administration of these extracts (10 or 20 mg/100 g of body weight, administered orally) for streptozotocin-diabetic rats had a preventive effects on bone loss, beyond reducing other negative effects associated with the use of streptozotocin, such as the increase in calcium levels and a reduction of inorganic phosphorus levels in the serum (Yamaguchi et al. 2007b).

Murakami et al. (2008) utilized ethanolic extracts (95% ethanol) from bee pollen with a predominance of the genus *Cistus* (origin unknown) to investigate the efficacy and safety of 12-week intake of honeybee-collected pollen lump extract (HPLE)-supplemented food in 44 patients with benign prostatic hyperplasia. Especially in patients who received a larger dose (320 mg/day) there was improvement in some symptoms associated with benign prostatic hyperplasia.

Ethanolic (70% ethanol) and aqueous extracts from bee pollen produced in Egypt show greater anticancer activity against hepatocellular carcinoma (HerpG2) cells and less activity against breast adenocarcinoma (MCF-7) cells. The authors also analyzed propolis extracts and in these, the activity was greater (Shady et al. 2016).

Wu and Lou (2007) tested *in vitro* the activity of steroid fraction of a chloroform extract from bee pollen of *Brassica campestris* (China) against nine human cancer cell lines: androgen-independent prostate cancer PC-3, estrogen-responsive breast adenocarcinoma MCF-7, androgen-sensitive prostate cancer LNCaP, human cervix carcinoma Hela, gastric adenocarcinoma BCG-823, hepatocarcinoma BEL-7402, squamous carcinoma KB, lung carcinoma A549 and ovarian carcinoma HO8910. The PC-3 cells were more sensitive than the others, which led the authors to recommend that the bee pollen extract from *B. campestris* should be further studied as a possible treatment for prostate cancer.

Yang et al. (2014) induced, using testosterone injections, a benign prostatic hyperplasia in Sprague-Dawley rats to evaluate the effects of the administration of *B. campestris* bee pollen supercritical CO_2 fluid extract (SFE-CO_2) at concentrations of 21.3 or 88.7 mg/kg. Treated animals showed a significant reduction in prostatic index, as well as the DHT, 5α-reductase and COX-2 expression levels, showing that the extracts inhibited the development of the benign prostatic hyperplasia.

In another experiment with bee pollen from *B. campestris* produced in China, Chen et al. (2016) identified, in the blood of male ICR strain mice feed on the samples, two microRNAs (miR-166a and miR-159); it was further observed that some miRNAs present in the plants could have antitumor effects. Chin et al. (2016) concluded that the abundance of miR-159 in human serum was inversely correlated with breast cancer incidence and progression in patients.

References

Abdella EM, Tohamy A, Ahmad RR (2009) Antimutagenic activity of egyptian propolis and bee pollen water extracts against cisplatin-induced chromosomal abnormalities in bone marrow cells of mice. Iran J Cancer Prev 2:175–181

Abouda Z, Zerdani I, Kalalou I, Faid M, Ahami MT (2011) The antibacterial activity of moroccan bee bread and bee-pollen (fresh and dried) against pathogenic bacteria. Res J Microbiol 6:376–384

Aliyazicioglu Y, Deger O, Ovali E, Barlak Y, Hosver I, Tekelioglu Y (2005) Effects of Turkish pollen and propolis extracts on respiratory burst for K-562 cell lines. Int Immunopharmacol 5:1652–1657

Almeida AAP (2007) Atividade antimicrobiana de extratos e de compostos fenólicos e nitrogenados do café: avaliação *in vitro* e em modelo alimentar. http://www.bibliotecadigital.ufmg.br/dspace/handle/1843/MBSA-739HY4. Accessed 26 Feb 2017

Angelo PM, Jorge N (2007) Compostos fenólicos em alimentos – uma breve revisão. Rev Inst Adolfo Lutz 66:232–240

Arruda VAS (2013) Pólen apícola desidratado: composição físico-química, qualidade microbiológica, compostos fenólicos e flavonoides, atividade antioxidante e origem botânica. http://www.teses.usp.br/teses/disponiveis/9/9131/tde-22062015-172352/es.php. Accessed 26 Feb 2017

Barbosa SITR, Silvestre AJD, Simões MMQ, Estevinho MLMF (2006) Composition and antibacterial activity of the lipophilic fraction of honeybee pollen from native species of Montesinho Natural Park. Int J Agr Res 1:471–479

Barreto LMRC, Funari SRC, Orsi RO (2005) Composição e qualidade do pólen apícola proveniente de sete Estados brasileiros e do Distrito Federal. Bol Ind Anim 62:167–175

Basim E, Basim H, Özcan M (2006) Antibacterial activities of Turkish pollen and propolis extracts against plant bacterial pathogens. J Food Eng 77:992–996

Bassard JE, Ullmann P, Bernier F, Werck-Reichhart D (2010) Phenolamides: bridging polyamines to the phenolic metabolism. Phytochemistry 71:1808–1824

Bevzo VV, Grygor'eva NP (1997) Effect of bee pollen extract on glutathione system activity in mice liver under X-ray irradiation. Ukr Biokhim Zh 69:115–117

Bogdanov S (2012) The bee pollen book: chapter 2. http://wwwbee-hexagonnet. Accessed 12 Nov 2014

Borguini RG (2006) Avaliação do potencial antioxidante e de algumas características físico-químicas do tomate (Lycopersicon esculentum) orgânico em comparação ao convencional. http://www.teses.usp.br/teses/disponiveis/6/6133/tde-14082006-153722/pt-br.php. Accessed 26 Feb 2017

Borycka K, Grabek-Lejko D, Kasprzyk I (2016) Antioxidant and antibacterial properties of commercial bee pollen products. J Apicul Res 54:491–502

Cabrera C, Montenegro G (2013) Pathogen control using a natural Chilean bee pollen extract of known botanical origin. Cien Investig Agrar 40:223–230

Campos MGR, Webby RF, Markham KR, Mitchell KA, Da Cunha AP (2003) Age-induced diminution of free radical scavenging capacity in bee pollens and the contribution of constituent flavonoids. J Agric Food Chem 51:742–745

Campos MGR, Bogdanov S, Almeida-Muradian LB, Szczesna T, Mancebo Y, Frigerio C (2008) Pollen composition and standardisation of analytical methods. J Apicult Res Bee World 47:156–163

Carpes ST, Begnini R, Alencar SM, Masson ML (2007) Study of preparations of bee pollen extracts, antioxidant and antibacterial activity. Cien Agrotecnol 31:1818–1825

Carpes ST, Prado A, Moreno IAM, Mourão GB, Alencar SM, Masson ML (2008) Avaliação do potencial antioxidante do pólen apícola produzido na Região Sul do Brasil. Quím Nova 31:1660–1664

Carpes ST, Mourão GB, Alencar SM, Masson ML (2009) Chemical composition and free radical scavenging activity of *Apis mellifera* bee pollen from Southern Brazil. Braz J Food Technol 12:220–229

Castelo-Branco VN, Torres AG (2011) Capacidade antioxidante total de óleos vegetais comestíveis: determinantes químicos e sua relação com a qualidade dos óleos. Rev Nutr 24:173–187

Chantarudee A, Phuwapraisirisan P, Kimura K, Okuyama M, Mori H, Kimura A (2012) Chemical constituents and free radical scavenging activity of corn pollen collected from *Apis mellifera* hives compared to floral corn pollen at Nan, Thailand. BMC Complement Altern Med 12:1–12

Chen X, Dai G, Ren Z, Tong Y, Yang F, Zhu Y (2016) Identification of dietetically absorbed rapeseed (*Brassica campestris* L.) bee pollen micrornas in serum of mice. Biomed Res Int 2016:1–5

Chin AR, Fong MY, Somlo G, Wu J, Swiderski P, Wu X (2016) Cross-kingdom inhibition of breast cancer growth by plant miR159. Cell Res 26:1–12

Cunha AP, Roque OR (2009) Antibióticos de origem natural, compostos semi-sintéticos e sintéticos com actividade antibacteriana e antibióticos citotóxicos. In: Cunha AP (ed) Farmacognosia e fitoquímica. Lisboa, Fundação Calouste Benkian, pp 608–642

Daoud A, Malika D, Bakari S, Hfaiedh N, Mnafgui K, Kadri A (2015) Assessment of polyphenol composition, antioxidant and antimicrobial properties of various extracts of Date Palm Pollen (DPP) from two Tunisian cultivars. Arab J Chem. doi:10.1016/j.arabjc.2015.07.014

De-Melo AAM (2015) Perfil químico e microbiológico, cor, análise polínica e propriedades biológicas do pólen apícola desidratado. http://www.teses.usp.br/teses/disponiveis/9/9131/tde-18122015-142742/pt-br.php. Accessed 26 Feb 2017

De-Melo AAM, Estevinho MLMF, Sattler JAG, Souza BR, Silva-Freitas A, Barth OM (2016) Effect of processing conditions on characteristics of dehydrated bee-pollen and correlation between quality parameters. Food Sci Technol 65:808–815

Denisow B, Denisow-Pietrzyk M (2016) Biological and therapeutic properties of bee pollen: a review. J Sci Food Agric 96:4303–4309

Domínguez-Valhondo D, Gil DB, Hernández MT, González-Gómez D (2011) Influence of the commercial processing and floral origin on bioactive and nutritional properties of honeybee-collected pollen. Int J Food Sci Tech 46:2204–2211

Eraslan G, Kanbur M, Silici S, Liman B, Altinordulu S, Karabacak M (2008) Evaluation of protective effect of bee pollen against propoxur toxicity in rat. Ecotoxol Environ Saf 72:931–937

Erkmen O, Özcan MM (2008) Antimicrobial effects of turkish propolis, pollen, and laurel on spoilage and pathogenic food-related microorganisms. J Med Food 11:587–592

Fatrcová-Šramková K, Nôžková J, Kačániová M, Máriássyová M, Rovná K, Stričík M (2013) Antioxidant and antimicrobial properties of monofloral bee pollen. J Environ Sci Health B 48:133–138

Féas X, Vázquez-Tato MP, Estevinho L, Seijas JA, Iglesias A (2012) Organic bee pollen: botanical origin, nutritional value, bioactive compounds, antioxidant activity and microbiological quality. Molecules 17:8359–8377

Freire KRL, Lins ACS, Dórea MC, Santos FAR, Camara CA, Silva TMS (2012) Palynological origin, phenolic content, and antioxidant properties of honeybee-collected pollen from Bahia, Brazil. Molecules 17:1652–1664

Furusawa E, Chou SC, Hirazumi A (1995) Antitumour potential of pollen extract on lewis lung carcinoma implanted intraperitoneally in syngeneic mice. Phytother Res 9:255–259

Gabriele M, Parri E, Felicioli A, Sagona S, Pozzo L, Biondi C (2015) Phytochemical composition and antioxidant activity of tuscan bee pollen of different botanic origins. Ital J Food Sci 27:248–259

Graikou K, Kapeta S, Aligiannis N, Sotiroudis G, Chondrogianni N, Gonos E (2011) Chemical analysis of Greek pollen – antioxidant, antimicrobial and proteasome activation properties. Chem Cent J 5:1–9

Halliwell B, Gutteridge J (2007) Free radicals in biology and medicine, 4rd edn. Oxford University Press Inc., New York, NY

Huang WY, Zhang HC, Liu WX, Li CY (2012) Survey of antioxidant capacity and phenolic composition of blueberry, blackberry, and strawberry in Nanjing. J Zhejiang Univ 13:94–102

Izuta H, Shimazawa M, Tsuruma K, Araki Y, Mishima S, Hara H (2009) Bee products prevent VEGF-induced angiogenesis in human umbilical vein endothelial cells. BMC Complement Altern Med 9:1–10

Kacániová M, Vukovic N, Chlebo R, Hascík P, Rovná K, Cubon J, Dzugan M, Pasternakiewicz A (2012) The antimicrobial activity of honey, bee pollen loads and beeswax from Slovakia. Arch Biol Sci 64:927–934

Kačániová M, Vatľák A, Vuković N, Petrová J, Brindza J, Nôžková J (2014) Antimicrobial activity of bee collected pollen against clostridia. Anim Sci Biotechnol 47:362–365

Khider M, Elbanna K, Mahmoud A, Owayss A (2013) Egyptian honeybee pollen as antimicrobial, antioxidant agents, and dietary food supplements. Food Sci Biotechnol 22:1–9

Kročko M, Čanigová M, Bezeková J, Lavová M, Haščík P, Ducková V (2012) Effect of nutrition with propolis and bee pollen supplements on bacteria colonization pattern in gastrointestinal tract of broiler chickens. Anim Sci Biotechnol 45:63–67

Leblanc BW, Davis OK, Boue S, Delucca A, Deeby T (2009) Antioxidant activity of Sonoran Desert bee pollen. Food Chem 115:1299–1305

Leja M, Mareczek A, Wyzgolik G, Klepacz-Baniak J, Czekonska K (2007) Antioxidative properties of bee pollen in selected plant species. Food Chem 100:237–240

Levin ASS, Kobata CHP, Litvoc MN (2014) Princípios do uso de antimicrobianos: perguntas e respostas. Rev Med 93:63–68

Linskens HF, Jorde W (1997) Pollen as food and medicine – a review. Econ Bot 51:78–87

Lopes J, Stanciu OG, Campos MG, Almaraz-Abarca N, Almeida-Muradian LB, Marghitas LA (2011) Bee pollen antioxidant activity – a review: achievements and further challenges. J Pharmacog 2:25–38

Maia PP, Rath S, Reyes FGR (2009) Antimicrobianos em alimentos de origem vegetal – uma revisão. Segur Aliment Nutr 16:49–64

Mărghitas LA, Stanciu OG, Dezmirean DS, Bobis O, Popescu O, Bogdanov S (2009) *In vitro* antioxidant capacity of honeybee-collected pollen of selected floral origin harvested from Romania. Food Chem 115:878–883

Mejías E, Montenegro G (2012) The antioxidant activity of chilean honey and bee pollen produced in the Llaima volcano's zones. J Food Qual 35:315–322

Melo ILP, Almeida-Muradian LB (2010) Stability of antioxidants vitamins in bee pollen samples. Quím Nova 33:514–518

Melo NR, Soares NFF, Gonçalves MPJC (2005) Nisina: um conservante natural para alimentos. Rev Ceres 52:921–938

Menezes JDS, Maciel LF, Miranda MS, Druzian JI (2010) Compostos bioativos e potencial antioxidante do pólen apícola produzido por abelhas africanizadas (*Apis mellifera* L.) Rev Inst Adolfo Lutz 69:233–242

Mihajlovic L, Radosavljevic J, Burazer L, Smiljanic K, Velickovic TC (2015) Composition of polyphenol and polyamide compounds in common ragweed (*Ambrosia artemisiifolia* L.) pollen and sub-pollen particles. Phytochemistry 109:125–132

Mohdaly AAA, Mahmoud AA, Roby MHH, Smetanska I, Ramadan MF (2015) Phenolic extract from propolis and bee pollen: composition, antioxidant and antibacterial activities. J Food Biochem 39:538–547

Morais M, Moreira L, Feás X, Estevinho LM (2011) Honeybee-collected pollen from five Portuguese Natural Parks: palynological origin, phenolic content, antioxidant properties and antimicrobial activity. Food Chem Toxicol 49:1096–1101

Murakami M, Tsukada O, Okihara K, Hashimoto K, Yamada H, Yamaguchi H (2008) Beneficial effect of honeybee-collected pollen lump extract on benign prostatic hyperplasia (BPH) – a double-blind, placebo-controlled clinical trial. Food Sci Technol Res 14:306–310

Negri G, Teixeira EW, Alves MLTMF, Moreti ACCC, Otsuk IP, Borguini RG (2011) Hydroxycinnamic acid amide derivatives, phenolic compounds and antioxidant activities of extracts of pollen samples from Southeast Brazil. J Agric Food Chem 59:5516–5522

Nogueira C, Iglesias A, Feás X, Estevinho LM (2012) Commercial bee pollen with different geographical origins: a comprehensive approach. Int J Mol Sci 13:11173–11187

Olczyk P, Koprowski R, Kaźmierczak J, Mencner L, Wojtyczka R, Stojko J (2016) Bee pollen as a promising agent in the burn wounds treatment. Evid Based Complement Alternat Med 2016:1–12

Oliveira DM, Bastos DHM (2011) Biodisponibilidade de ácidos fenólicos. Quím Nova 34:1051–1056

Oliveira KCLS, Moriya M, Azedo RAB, Almeida-Muradian LB, Teixeira EW, Alves MLTMF (2009) Relationship between botanical origin and antioxidants vitamins of bee-collected pollen. Quím Nova 32:1099–1102

Pascoal A, Rodrigues S, Teixeira A, Féas X, Estevinho LM (2014) Biological activities of commercial bee pollens: antimicrobial, antimutagenic, antioxidant and anti-inflammatory. Food Chem Toxicol 63:233–239

Pérez-Pérez EM, Vit P, Rivas E, Sciortino R, Sosa A, Tejada D (2012) Antioxidant activity of four color fractions of bee pollen from Merida, Venezuela. Arch Latinoam Nutr 62:375–380

Pinto B, Caciagli F, Riccio E, Reali D, Saric A, Balog T (2010) Antiestrogenic and antigenotoxic activity of bee pollen from *Cystus incanus* and *Salix alba* as evaluated by the yeast estrogen screen and the micronucleus assay in human lymphocytes. Eur J Med Chem 45:4122–4128

Rebiai A, Lanez T (2012) Chemical composition and antioxidant activity of *Apis mellifera* bee pollen from northwest Algeria. J Fundam Appl Sci 4:155–163

Reinhard KJ, Hamilton DL, Hevly RH (1991) Use of pollen concentration in palaeopharmacology: coprolite evidence in medical plants. J Ethnobiol 11:117–132

Saral Ö, Yildiz O, Aliyazicioğlu R, Yuluğ E, Canpolat S, Öztürk F (2016) Apitherapy products enhance the recovery of CCL_4-induced hepatic damages in rats. Turk J Med Sci 46:194–202

Saríc A, Balog T, Sobocanec S, Kusic B, Sverko V, Rusak G (2009) Antioxidant effects of flavonoid from Croatian *Cystus incanus* L. rich bee pollen. Food Chem Toxicol 47:547–554

Shady HMA, Mohamed WF, Sayed-Ahmed EF, Amer SA (2016) A comparative study on propolis and pollen extracts: chemical profile analysis, antioxidant and anticancer activity. Int J Curr Microbiol Appl Sci 5:397–414

Shukla S, Gupta S (2010) Apigenin: a promising molecule for cancer prevention. Pharm Res 27:962–978

Sousa MSB, Vieira LM, Lima A (2011) Fenólicos totais e capacidade antioxidante *in vitro* de resíduos de polpas de frutas tropicais. Braz J Food Technol 14:202–210

Tohamy AA, Abdella EM, Ahmed RR, Ahmed YK (2014) Assessment of anti-mutagenic, antihistopathologic and antioxidant capacities of Egyptian bee pollen and propolis extracts. Cytotechnology 66:283–297

Traka MH, Saha S, Huseby S, Kopriva S, Walley PG, Barker GC (2013) Genetic regulation of glucoraphanin accumulation in Beneforté broccoli. New Phytol 198:1085–1095

Ulbricht C, Conquer J, Giese N, Khalsa KPS, Sklar J, Weissner W (2009) An evidence-based systematic review of bee pollen by the natural standard research collaboration. J Diet Suppl 6:290–312

Ulusoy E, Kolayli S (2014) Phenolic composition and antioxidant properties of anzer bee pollen. J Food Biochem 38:73–82

Uttara B, Singh AV, Zamboni P, Mahajan RT (2009) Oxidative stress and neurodegenerative diseases: a review of upstream and downstream antioxidant therapeutic options. Curr Neuropharmacol 7:65–74

Wang B, Diao Q, Zhang Z, Liu Y, Gao Q, Zhou Y (2013) Antitumor activity of bee pollen polysaccharides from *Rosa rugosa*. Mol Med Rep 7:1555–1558

Wu YD, Lou YJ (2007) A steroid fraction of chloroform extract from bee pollen of *Brassica campestris* induces apoptosis in human prostate cancer PC-3 cells. Phytother Res 21:1087–1091

Yamaguchi M, Uchiyama S, Nakagawa T (2007a) Preventive effects of bee pollen *Cistus ladaniferus* extract on bone loss in ovariectomized rats *in vivo*. J Health Sci 53:571–575

Yamaguchi M, Hamamoto R, Uchiyama S, Ishiyama K, Hashimoto K (2007b) Preventive effects of bee pollen *Cistus ladaniferus* extract on bone loss in streptozotocin-diabetic rats in vivo. J Health Sci 53:190–195

Yang B, Jin L, Yang Y, Li K, Peng D (2014) Inhibitory effect of rape pollen supercritical CO_2 fluid extract against testosterone-induced benign prostatic hyperplasia in rats. Exp Ther Med 8:31–37

Zhang H, Wang X, Wang K, Li C (2015) Antioxidant and tyrosinase inhibitory properties of aqueous ethanol extracts from monofloral bee pollen. J Apic Sci 59:119–128

Part V
Honey Bee Venom

Chapter 13
Chemical Composition of Bee Venom

Sok Cheon Pak

Contents

13.1 Chemical Composition

The common honey bee belongs to the family *Apidae* in the *Apoidea* superfamily under the hymenopteran venom system (Moreno and Giralt 2015). Hymenopteran sting, claimed to be a marvel of biological engineering, causes a systemic allergic reaction when it is injected by bees into prey or predators. Honey bee venom is a liquid blend of biologically active substances comprising proteins, peptides, enzymes and other small molecules.

13.1.1 Enzymes

Enzymes in honey bee venom include phospholipase A_2 (PLA_2), phospholipase B (PLB), hyaluronidase which show cytotoxicity, and further nontoxic hydrolytic enzymes such as phosphatase and α-glucosidase.

S.C. Pak
School of Biomedical Sciences, Charles Sturt University, Bathurst, NSW 2795, Australia
e-mail: spak@csu.edu.au

J.M. Alvarez-Suarez (ed.), *Bee Products - Chemical and Biological Properties*,
DOI 10.1007/978-3-319-59689-1_13

13.1.1.1 Phospholipase A_2

PLA_2 plays an important role in diverse cellular processes due to its capacity to metabolize phospholipids and produce fatty acids and lysophospholipids. According to a recent review (Dennis et al. 2011), PLA_2 has been classified into three broad classes based on the cellular distribution: secreted ($sPLA_2$), cytosolic ($cPLA_2$) and Ca^{2+}-independent PLA_2 ($iPLA_2$). $sPLA_2$ originally isolated from human synovial fluid of arthritic knee joints was involved in diverse inflammatory processes such as arthritis (Boilard et al. 2010) and allergic bronchitis (Mruwat et al. 2013). Thus, bee venom PLA_2 conferred protective immunity by inducing a T helper cell type 2 response and additional IgE response (Palm et al. 2013). Furthermore, bee venom PLA_2 was able to modulate regulatory T cells to exert an anti-inflammatory effect (Kim et al. 2014, 2015), ameliorate neuropathic pain (Li et al. 2016) and slow down the progression of brain disease (Ye et al. 2016).

13.1.1.2 Phospholipase B

PLB, first characterized to be extremely thermostable (Doery and Pearson 1964), is present from bacteria to mammals. PLB catalyzes deacylation of both sn-1 and sn-2 ester linkage of diacylphospholipids, remodeling the fatty acids. With a relatively large molecular mass, PLB is known to have broader roles than PLA_2 *in vivo* (Saito 2014).

13.1.1.3 Hyaluronidase

Hyaluronidase induces the cleavage of hyaluronan, the major glycosaminoglycan of connective tissue, and facilitates the diffusion of venom components into the tissues and blood circulation, causing systemic envenomation (Wiezel et al. 2015).

13.1.1.4 Phosphatase

Phosphatase as the prominent hydrolase in the process of dephosphorylation removes a phosphate group from an organic compound by hydrolysis. Thus, acid phosphatase containing bee venom inhibited tumor angiogenesis and metastasis by blocking phosphorylation of a certain growth factor such as vascular endothelial growth factor (Huh et al. 2010), implying the involvement of phosphatase in some medical conditions. Furthermore, bee venom acid phosphatase is known to be a potent releaser of histamine from sensitized human basophils (Grunwald et al. 2006).

13.1.1.5 α-Glucosidase

α-Glucosidase possesses starch-hydrolyzing activity by releasing D-glucose from the non-reducing end side of substrate (Chiba 1997). For the entomopathogenic bacterium *Lysinibacillus sphaericus* to elicit high larvicidal activity, subunits of Binary protoxin have to bind to α-glucosidases on the surface of the midgut microvilli of larvae in order to perform larvicidal activity against some major vectors of a number of human pathogens (Ferreira et al. 2014).

13.1.2 Peptides

In addition to the two major peptides, melittin and apamin, bee venom contains many other peptides such as mast cell degranulating (MCD) peptide, adolapin, tertiapin, secapin, melittin F and cardiopep. Some of them are cell-penetrating peptides and elicit cell lysis, while others are neurotoxins affecting the nervous system.

13.1.2.1 Melittin

Melittin is the main bioactive component of bee venom. It is a small peptide containing 26 amino acid residues; the first 20 are predominantly hydrophobic while the C-terminal part of the molecule is positively charged. In the crystalline state, each polypeptide chain of melittin is composed of two α-helices aligned approximately 120° to each other and its overall shape is that of a bent rod (Terwilliger and Eisenberg 1982). Using such conformation, melittin can cause the breakdown of lipid bilayers upon binding to the membrane, acting like a detergent. It is known to act synergistically through a peptide-enzyme complex with PLA_2 by activating it (Mingarro et al. 1995).

13.1.2.2 Apamin

As an octadecapeptide, apamin is a centrally acting neurotoxin that produces motor hyperexcitability by crossing the blood-brain barrier (Strong 1990). This high penetration and specific distribution to the CNS make the design of an apamin-based drug delivery system feasible. Recently, researchers constructed an efficient CNS-targeting drug delivery system specific for spinal cord injury using apamin as a targeting ligand which showed high efficacy in tissue repair following spinal cord injury (Wu et al. 2014). Apamin is a remarkably stable molecule and insensitive to

its environment which is attributed to the structure with two intramolecular disulphide bridges and an amidated C-terminal carboxyl group (Strong 1990). In addition, apamin possesses an α-helical core with arginine-13 located at the centre of the helical region and adjacent arginine-14, and these two residues are essential for the maintenance of neurotoxic activity (Hider and Ragnarsson 1980). Apamin has long been known as a selective inhibitor of small conductance calcium-activated potassium (SK) channels. It inhibits exclusively a few cloned SK channel subtypes with different affinity. The cloning of the SK channels opened the avenue for the molecular knowledge of the interaction of apamin with the pore region which gives some insight into the development of subunit specific inhibitors of apamin (Nolting et al. 2007).

13.1.2.3 Mast Cell Degranulating (MCD) Peptide

Like apamin, MCD peptide is also extremely basic with similar structure, having two intramolecular disulphide bridges and an amidated C-terminal carboxyl group (Strong 1990). According to the high-resolution two dimensional nuclear magnetic resonance (NMR), MCD peptide has a conformation with an N-terminal β-turn and a C-terminal α-helix which are connected by two tight turns (Kumar et al. 1988). Since bee venom itself contains only a small amount of histamine, MCD peptide contributes additional histamine by releasing it from mast cell granules by fusion of the granule membranes with the mast cell membrane and exocytosis of the granule contents without lysis of mast cells (Karalliedde 1995). Moreover, PLA_2 and melittin cause histamine release from skin mast cells as a result of cytolytic effects in response to bee stings. Thus, MCD peptide is a principal bee venom allergen that reacts with cell-bound specific IgE to induce massive release of a myriad of chemicals for allergic reaction.

13.1.2.4 Adolapin

Adolapin was isolated and named by Shkenderov and Koburova (1982). They reported that adolapin has anti-inflammatory and analgesic effects through local inhibition of prostaglandin production. Further investigation (Koburova et al. 1985) on adolapin revealed that it inhibited the activity of PLA_2 and lipoxygenase from human platelets. In addition, adolapin displayed an antipyretic effect by inhibiting the mean temperature rise similar to other nonsteroid analgetics.

13.1.2.5 Tertiapin

Tertiapin is a small peptide (21 amino acids) and comprises about 0.1% of total honey bee venom. The three-dimensional NMR spectroscopy study of tertiapin in solution indicates that tertiapin is a highly compact molecule resulting from side

chain interactions. It consists of a type 4 reverse turn and an α-helix which are connected by a loop formed by an extended β sheet. Four cysteines within the polypeptide chain form two disulfide bonds (Xu and Nelson 1993). It was found that tertiapin blocked the inward-rectifier potassium channels (Jin and Lu 1998). In this respect, tertiapin prevented acetylcholine-induced complete heart block by inhibiting inward-rectifier potassium channels (Drici et al. 2000). These papers proved the role of tertiapin as a powerful ligand for purifying functional channels as well as for screening pharmaceutical agents against these channels. scapin, melittin F and cardiopep.

13.1.2.6 Secapin

Secapin is a 24-residue peptide containing a large proportion of proline and one disulphide bridge (Gauldie et al. 1976). It is basically non-toxic, but produces marked hypothermia and signs of sedation at high dose in mice (Gauldie et al. 1976).

13.1.2.7 Melittin F

A large scale fractionation of crude bee venom has resulted in the isolation of melittin F, a 19-amino acid residue peptide which is a fragment of melittin consisting of residues of 8–26 (Gauldie et al. 1976).

13.1.2.8 Cardiopep

Cardiopep comprises about 0.7% of the whole bee venom when isolated on a chromatographic column (Shipman and Cole 1969). Cardiopep is an acronym for cardioactive polypeptide because it has a potent, nontoxic beta-adrenergic-like character and possesses anti-arrhythmic property (Vick et al. 1974).

References

Boilard E, Lai Y, Larabee K, Balestrieri B, Ghomashchi F, Fujioka D, Gobezie R, Coblyn JS, Weinblatt ME, Massarotti EM et al (2010) A novel anti-inflammatory role for secretory phospholipase A2 in immune complex-mediated arthritis. EMBO Mol Med 2:172–187

Chiba S (1997) Molecular mechanism in alpha-glucosidase and glucoamylase. Biosci Biotechnol Biochem 61:1233–1239

Dennis EA, Cao J, Hsu YH, Magrioti V, Kokotos G (2011) Phospholipase A2 enzymes: physical structure, biological function, disease implication, chemical inhibition, and therapeutic intervention. Chem Rev 111:6130–6185

Doery HM, Pearson JE (1964) Phospholipase B in snake venoms and bee venom. Biochem J 92:599–602

Drici MD, Diochot S, Terrenoire C, Romey G, Lazdunski M (2000) The bee venom peptide tertiapin underlines the role of I(KACh) in acetylcholine-induced atrioventricular blocks. Br J Pharmacol 131:569–577

Ferreira LM, Romão TP, Nascimento NA, Costa Mda C, Rezende AM, de-Melo-Neto OP, Silva-Filha MH (2014) Non conserved residues between Cqm1 and Aam1 mosquito α-glucosidases are critical for the capacity of Cqm1 to bind the Binary toxin from Lysinibacillus sphaericus. Insect Biochem Mol Biol 50:34–42

Gauldie J, Hanson JM, Rumjanek FD, Shipolini RA, Vernon CA (1976) The peptide components of bee venom. Eur J Biochem 61:369–376

Grunwald T, Bockisch B, Spillner E, Ring J, Bredehorst R, Ollert MW (2006) Molecular cloning and expression in insect cells of honeybee venom allergen acid phosphatase (Api m 3). J Allergy Clin Immunol 117:848–854

Hider RC, Ragnarsson U (1980) A proposal for the structure of apamin. FEBS Lett 111:189–193

Huh JE, Baek YH, Lee MH, Choi DY, Park DS, Lee JD (2010) Bee venom inhibits tumor angiogenesis and metastasis by inhibiting tyrosine phosphorylation of VEGFR-2 in LLC-tumor-bearing mice. Cancer Lett 292:98–110

Jin W, Lu Z (1998) A novel high-affinity inhibitor for inward-rectifier K+ channels. Biochemistry 37:13291–13299

Karalliedde L (1995) Animal toxins. Br J Anaesth 74:319–327

Kim H, Keum DJ, won Kwak J, Chung HS, Bae H (2014) Bee venom phospholipase A_2 protects against acetaminophen-induced acute liver injury by modulating regulatory T cells and IL-10 in mice. PLoS One 9:27

Kim H, Lee H, Lee G, Jang H, Kim SS, Yoon H, Kang GH, Hwang DS, Kim SK, Chung HS et al (2015) Phospholipase A_2 inhibits cisplatin-induced acute kidney injury by modulating regulatory T cells by the CD206 mannose receptor. Kidney Int 88:550–559

Koburova KL, Michailova SG, Shkenderov SV (1985) Further investigation on the antiinflammatory properties of adolapin-bee venom polypeptide. Acta Physiol Pharmacol Bulg 11:50–55

Kumar NV, Wemmer DE, Kallenbach NR (1988) Structure of P401 (mast cell degranulating peptide) in solution. Biophys Chem 31:113–119

Li D, Kim W, Shin D, Jung Y, Bae H, Kim SK (2016) Preventive effects of bee venom derived phospholipase A_2 on oxaliplatin-induced neuropathic pain in mice. Toxins 8:27

Mingarro I, Pérez-Payá E, Pinilla C, Appel JR, Houghten RA, Blondelle SE (1995) Activation of bee venom phospholipase A2 through a peptide-enzyme complex. FEBS Lett 372:131–134

Moreno M, Giralt E (2015) Three valuable peptides from bee and wasp venoms for therapeutic and biotechnological use: melittin, apamin and mastoparan. Toxins 7:1126–1150

Mruwat R, Yedgar S, Lavon I, Ariel A, Krimsky M, Shoseyov D (2013) Phospholipase A2 in experimental allergic bronchitis: a lesson from mouse and rat models. PLoS One 8:e76641

Nolting A, Ferraro T, D'hoedt D, Stocker M (2007) An amino acid outside the pore region influences apamin sensitivity in small conductance Ca2+-activated K+ channels. J Biol Chem 282:3478–3486

Palm NW, Rosenstein RK, Yu S, Schenten DD, Florsheim E, Medzhitov R (2013) Bee venom phospholipase A2 induces a primary type 2 response that is dependent on the receptor ST2 and confers protective immunity. Immunity 39:976–985

Saito K (2014) Reminiscence of phospholipase B in Penicillium notatum. Proc Jpn Acad Ser B Phys Biol Sci 90:333–346

Shipman WH, Cole LJ (1969) A surfactant bee venom fraction: separation on a newly devised constant flow rate chromatographic column and detection by changes in effluent drop volume. Anal Biochem 29:490–497

Shkenderov S, Koburova K (1982) Adolapin—a newly isolated analgetic and anti-inflammatory polypeptide from bee venom. Toxicon 20:317–321

Strong PN (1990) Potassium channel toxins. Pharmacol Ther 46:137–162

Terwilliger TC, Eisenberg D (1982) The structure of melittin. II. Interpretation of the structure. J Biol Chem 257:6016–6022

Vick JA, Shipman WH, Brooks R Jr (1974) Beta adrenergic and anti-arrhythmic effects of cardiopep, a newly isolated substance from whole bee venom. Toxicon 12:139–144

Wiezel GA, Dos Santos PK, Cordeiro FA, Bordon KC, Selistre-de-Araújo HS, Ueberheide B, Arantes EC (2015) Identification of hyaluronidase and phospholipase B in Lachesis muta rhombeata venom. Toxicon 107(Pt B):359–368

Wu J, Jiang H, Bi Q, Luo Q, Li J, Zhang Y, Chen Z, Li C (2014) Apamin-mediated actively targeted drug delivery for treatment of spinal cord injury: more than just a concept. Mol Pharm 11:3210–3222

Xu X, Nelson JW (1993) Solution structure of tertiapin determined using nuclear magnetic resonance and distance geometry. Proteins 17:124–137

Ye M, Chung HS, Lee C, Yoon MS, Yu AR, Kim JS, Hwang DS, Shim I, Bae H (2016) Neuroprotective effects of bee venom phospholipase A2 in the 3xTg AD mouse model of Alzheimer's disease. J Neuroinflammation 13:10

Chapter 14
Health Benefits and Uses in Medicine of Bee Venom

Sok Cheon Pak

Contents

14.1 Introduction

The therapeutic value of honey bee venom to improve the quality of life of patients is acknowledged since more than 100 years. Modern approaches of venomics have allowed the discovery of venom constituents, which were proven to be of pharmacological significance and have opened the way to optimization of therapeutic strategies through the use of active components such as melittin and apamin. Subsequently, the application scope of honey bee venom has been expanding from conventional antinociceptive effect to degenerative diseases of the nervous system.

S.C. Pak
School of Biomedical Sciences, Charles Sturt University, Bathurst, NSW 2795, Australia
e-mail: spak@csu.edu.au

J.M. Alvarez-Suarez (ed.), *Bee Products - Chemical and Biological Properties*,
DOI 10.1007/978-3-319-59689-1_14

This seems to be due to properties of venom enzymes and peptides for their natural stability as injectable solutes, their effectiveness in reaching targeted tissues and their ability to synergize their actions by enhancing cell-cell interactions. This chapter appraises the current evidence of the major biological functions of honey bee venom. Our discussion focuses upon the latest approaches of venom studies for health benefits and uses in medicine (Table 14.1). This contributes to the knowledge base for honey bee venom from the perspective of applied science and should inform future research that seeks to establish the role for valuable peptides in the relief of human diseases.

Table 14.1 Application of honey bee venom components in different disease types

Disease type	Component	References
Parkinson's disease	BV	Kim et al. (2011a), Chung et al. (2012), Alvarez-Fischer et al. (2013)
	Apamin	Salthun-Lassalle et al. (2004), Toulorge et al. (2011), Alvarez-Fischer et al. (2013)
	BV acupuncture	Doo et al. (2010), Khalil et al. (2015)
Amyotrophic lateral sclerosis	BV	Lee et al. (2015)
	Melittin	Lee et al. (2014a), Yang et al. (2011)
	BV acupuncture	Lee et al. (2015), Cai et al. (2015), Yang and Choi (2013)
Multiple sclerosis	BV	Castro et al. (2005), Karimi et al. (2012)
Cancer	BV	Choi et al. (2014), Park et al. (2011a), Jo et al. (2012), Park et al. (2010a), Huh et al. (2010)
	Melittin	Park et al. (2011a, 2010a), Jo et al. (2012)
Liver fibrosis	BV	Kim et al. (2010), (Park et al. 2010b, c)
	Apamin	Lee et al. (2014b)
	Melittin	Lee et al. (2011a), Park et al. (2011b, 2012, 2014)
	PLA_2	Kim et al. (2014)
Atherosclerosis	BV	Son et al. (2006)
	Apamin	Kim et al. (2012a, b, 2015a)
	Melittin	Son et al. (2006), Jeong et al. (2012), Kim et al. (2011b)
Skin disease (acne vulgaris)	BV	Kim et al. (2015b), An et al. (2014)
	Melittin	Lee et al. (2014c)
Skin disease (atopic dermatitis)	BV	Kim et al. (2013)
Learning deficit	Apamin	Messier et al. (1991), Deschaux et al. (1997), Deschaux and Bizot (2005), Ikonen et al. (1998), Ikonen and Riekkinen (1999), Inan et al. (2000), Fournier et al. (2001), Stackman et al. (2002), Brennan et al. (2008), Kallarackal et al. (2013)
Pain	BV	Kang et al. (2012b)
	BV acupuncture	Roh et al. (2004), Yoon et al. (2009), Kang et al. (2011, 2012a), Kang et al. (2015)
Lupus nephritis	BV	Lee et al. (2011b)

14.2 Uses in Medicine of Bee Venom

14.2.1 Parkinson's Disease

Parkinson's disease (PD) is one of the most disabling chronic neurodegenerative diseases with major impact on older adults' lives and the society as a whole (Ellis et al. 2016). Despite advances in therapeutic interventions, patients with PD experience typical motor symptoms such as tremor at rest, rigidity, akinesia, bradykinesia and hypokinesia (Mazzoni et al. 2012) and various nonmotor symptoms including neuropsychiatric symptoms, autonomic dysfunctions, abnormal sense, sleep disorders and fatigue (Zuo et al. 2016).

A study performed in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced mouse model of acute PD showed that bee venom acupuncture once every 3 days for 2 weeks prevented MPTP-induced loss of tyrosine hydroxylase immunoreactivity in the substantia nigra and striatum (Doo et al. 2010). Moreover, bee venom acupuncture attenuated MPTP-induced phospho-Jun immunoreactivity in the substantia nigra, which was attributed to the protection of dopaminergic neurons against MPTP toxicity. A subsequent study in this experimental PD mouse model showed that bee venom suppressed severe dopaminergic cell loss after MPTP injection (Kim et al. 2011a). In addition, bee venom reduced MPTP-induced microglial activation which was associated with increased MAC-1 and iNOS expression in the substantia nigra as the mechanism of neuroinflammation. Based on these findings, it has been suggested that bee venom acupuncture may confer dopaminergic neuroprotective effect against MPTP-related PD model in mice through the suppression of pro-inflammatory factors. A recent bee venom acupuncture therapy against rotenone-induced oxidative stress, neuroinflammation and apoptosis in PD mouse model was conducted (Khalil et al. 2015). Findings suggest that bee venom compared to L-dopa therapy normalized all the neuroinflammatory and apoptotic markers and restored brain neurochemistry following rotenone injury. Interestingly, bee venom did not prevent MPTP neurotoxicity in mice depleted of Tregs by anti-CD25 antibody injection, suggesting the role of regulatory T cells in the modulation of peripheral immune tolerance (Chung et al. 2012).

In vitro tests show that apamin can protect dopaminergic neurons in a model system of midbrain cultures that favors the selective and spontaneous degeneration of dopamine neurons in PD (Salthun-Lassalle et al. 2004; Toulorge et al. 2011). This neuroprotective effect of apamin in experimental PD is attributed to an increased neuronal excitability that is closely associated with increased intracellular calcium involving T-type calcium channel activation. These findings are in accordance with the known pharmacological property of apamin as an inhibitor of SK channels which ensure intracellular calcium transient to changes of membrane potential by promoting potassium efflux. A recent *in vivo* study concluded that bee venom administered by i.p. injection provides substantial protection to dopamine neurons in an animal model that mimics the chronic degenerative process of PD

(Alvarez-Fischer et al. 2013). Apamin, on the other hand, did not provide sufficient protective effects, implying that other components of bee venom might enhance the neuroprotective action of apamin.

14.2.2 Amyotrophic Lateral Sclerosis

Amyotrophic lateral sclerosis (ALS) is a devastating adult-onset neurodegenerative disorder characterized by a selective loss of motor neurons in the brainstem and spinal cord. The most common clinical presentation consists of weakness, fasciculations and hyperreflexivity of facial muscles or limbs (Redler and Dokholyan 2012).

In hSOD1^{G93A} transgenic mice as an ALS animal model, immune dysfunction of organs caused by neuroinflammation is a consistent hallmark. Several studies with ALS experimental animal model have been conducted. In one study, melittin reduced the expression of Iba-1 and CD14 in the lungs and CD14 and COX2 in the spleen that is related to inflammation (Lee et al. 2014a). This finding was supported in a subsequent study, which found that bee venom acupuncture reduced proinflammatory proteins in the liver, spleen and kidney, and increased immune response (Lee et al. 2015). It was noted that bee venom treatment through an acupoint was more effective than an i.p. administration of bee venom. Moreover, bee venom acupuncture enhanced motor function and decreased motor neuron death in the spinal cord with engagement of endogenous immune modulatory system in the CNS (Cai et al. 2015).

α-Synuclein is a synaptic protein that has been implicated in neurodegenerative disorders including ALS (Doherty et al. 2004). Increased expression of α-synuclein phosphorylation and nitration in the brainstem and spinal cord observed in symptomatic hSOD1^{G93A} transgenic mice were reduced with melittin or bee venom administration through acupoint stimulation (Yang et al. 2011; Yang and Choi 2013). A polypeptide called ubiquitin was found to be co-localized in motor neurons of the lumbar spinal cord in ALS animal model involving misfolded protein aggregation, and both melittin and bee venom treatments reduced ubiquitinated proteins in the brainstem and spinal cord.

14.2.3 Multiple Sclerosis

Multiple sclerosis (MS) is a progressive autoimmune disease associated with chronic inflammatory demyelination of the central nervous system. Although immunomodulatory and immunosuppressive treatments lower the number of relapses, they do not cure or reverse existing deficits or improve long-term disability in MS patients due to its complexity and heterogeneity (Wootla et al. 2016).

A clinical trial involving MR imaging and 26 patients with relapsing-remitting or relapsing secondary progressive MS receiving bee sting therapy for 24 weeks found

that there was no significant reduction in the cumulative number of new gadolinium-enhancing lesions which is an indicator of the age of MS lesions (Wesselius et al. 2005). The T2 weighted lesion load further progressed, and there was no significant reduction in relapse rate. There was no improvement of disability, fatigue and health-related quality of life.

Preliminary evidence of bee venom suggests some improvement of symptoms in patients with progressive forms of MS (Castro et al. 2005). It is important to underscore the possible neurological symptoms for some patients, probably through allergic properties of bee venom.

In a rat model of MS called experimental allergic encephalomyelitis, administration of bee venom decreased the symptoms of clinical disorder, pathological changes, inflammatory cell infiltration, demyelination in the central nervous system, serum levels of TNF-α, and nitrates, illustrating the combined effects of bee venom on MS symptoms through anti-inflammatory, immunomodulatory and antioxidant properties (Karimi et al. 2012).

Collectively, the different results reported above for bee venom on MS symptoms could be dependent on the therapeutic protocols including dose used and type of subject model.

14.2.4 Cancer

Bee venom and its components have been widely used in the treatment of tumors with multiple possible molecular mechanisms. Two recent reviews on cancer treatment using either bee venom (Oršolić 2012) or melittin (Gajski and Garaj-Vrhovac 2013) concluded that bee venom and its components are promising agents against cancer by demonstrating cytotoxicity to a broader spectrum of tumor cells.

Apoptosis plays an important role in anti-cancer action of bee venom and its components. Stimulation of death receptor (DR) expression is implicated in the induction of apoptosis in cancer cells. The nuclear factor kappa B (NF-κB) family is a crucial player in several human cancer cell growths and its inactivation is inversely related to the enhanced therapeutic effect.

Choi et al. (2014) showed that bee venom inhibited growth of lung cancer cells with an IC_{50} value of 2–3 μg/mL through the induction of apoptosis via increase of DR expression and inhibition of NF-kB pathway. Bee venom also repressed the expression of anti-apoptotic proteins (Bcl-2), whereas it increased the expression of pro-apoptotic proteins (Bax, cleaved caspase-3, cleaved caspase-9) which are regulated by NF-kB. Apoptotic induction of cancer cell death via activation of caspase was further proven. Park et al. (2011a) reported varying levels of evidence from animal and *in vitro* experiments for actions of bee venom and melittin affecting human prostate cancer cells. Both melittin and bee venom inhibited growth of the late stage and early stage prostate cancer cells through apoptosis which was analyzed by tunnel assay and apoptotic gene expression. This anti-cancer effect was correlated with the down-regulation of various proliferative and anti-apoptotic gene

products which are regulated by NF-kB. In addition, findings demonstrated that bee venom and melittin inhibited DNA binding and transcriptional activity of NF-kB. In another study, ovarian cancer cells were treated with several doses of bee venom and melittin which resulted in a concentration-dependent inhibition of cell proliferation by the induction of apoptotic cell death (Jo et al. 2012). Consistent with apoptotic cell death, expression of DRs was increased which was further linked to increased ratio of pro-apoptotic to anti-apoptotic proteins. Moreover, ovarian cancer cells were transfected with siRNA of DRs for 24 h to target their mRNA for degradation, and then treated with bee venom and melittin for 24 h. Interestingly, deletion of DRs by small interfering RNA significantly reversed bee venom and melittin-induced cell growth inhibitory effect as well as down-regulation of STAT3 which contributes to tumor development.

Matrix metalloproteinases (MMPs) have been implicated in multiple stages of cancer progression because they are capable of degrading various components of extracellular matrix (Yang et al. 2016). Among MMP family members, MMP-9 is a potential biomarker for cancer invasion and metastasis due to its high expression in malignant tumors. The induction of MMP-9 is affected by various factors including phorbol myristate acetate (PMA), activator protein-1 (AP-1) and NF-κB (Shin et al. 2007). In a study evaluating the ability of bee venom and melittin to act on PMA-induced MMP-9 expression, human renal carcinoma cells were used (Park et al. 2010a). The results showed that PMA-induced cell invasion and migration were inhibited by bee venom and melittin which was due to the suppression of MMP-9 expression. Furthermore, cells were transiently transfected with a luciferase reporter gene linked to the MMP-9 promoter with mutations at the AP-1 and NF-κB site. The melittin treatment in the presence of PMA decreased the transcriptional activity of the reporter gene containing mutated AP-1 and NF-κB, implying these two sites are the molecular target of melittin. In addition, melittin suppressed the PMA-induced phosphorylations of ERK and JNK mitogen-activated protein kinases, which are the upstream modulators of AP-1 and NF-κB.

Tumor growth and metastatic dissemination depend on angiogenesis triggered by up- and down-regulation of angiogenic activators and inhibitors, respectively, from tumor cells (Nishida et al. 2006). It is well established that tumor-induced angiogenesis is initiated by angiogenic cytokines including vascular endothelial growth factor (VEGF). As such, targeting VEGF signaling pathways is an attractive treatment strategy for cancer therapy. VEGF signaling is mediated through its binding to the receptors, especially VEGFR2. One study which evaluated the effect of bee venom on tumor angiogenesis and metastasis demonstrated that bee venom inhibited the viability of Lewis lung carcinoma (LLC) cells without exhibiting cytotoxicity (Huh et al. 2010). Bee venom also downregulated the expression of VEGFR2 and blocked the VEGFR2 signaling pathways by interfering with the activation of AKT and p42/44 MAPK that appear to play the most prominent roles in regulating VEGF-induced proliferation, migration and capillary-like tube formation of endothelial cells. These findings were further supported by an in vivo animal study wherein LLC cells were inoculated subcutaneously to initiate primary tumor and subsequent pulmonary metastasis. When given subcutaneously, bee venom suppressed tumor angiogenesis through inhibition of VEGF and VEGFR2 in mice.

14.2.5 Liver Fibrosis

Liver fibrosis occurs with hepatic damage in a variety of liver diseases in conjunction with the accumulation of extracellular matrix proteins including collagen. Once hepatocytes are damaged, they trigger the release of reactive oxygen species and cytokines to cause further release of TNF-α and fibroblast growth factor which promote the activation of hepatic stellate cells (HSCs) and collagen deposition. Activated HSCs, in turn, secrete pro-inflammatory cytokines, up-regulate adhesion molecules and accelerate recruitment of inflammatory cells (Kisseleva and Brenner 2007).

A study investigated the effects of bee venom on apoptosis after ethanol-induced injury to hepatocytes and evaluated mitochondrial intracellular stress signals as the apoptotic mechanism of bee venom action (Kim et al. 2010). An optimal dose of bee venom inhibited the apoptotic cell morphology and increased the cell viability in ethanol-induced hepatocyte apoptosis. In addition, bee venom treated hepatocytes reduced cleavage of caspases and poly ADP-ribose polymerase (PARP). A potent anti-apoptotic property of bee venom (Park et al. 2010b, c) or melittin (Lee et al. 2011a) on hepatocytes was further illustrated when cells were exposed to TNF-α and actinomycin D (Park et al. 2010b) or transforming growth factor (TGF-β1) (Park et al. 2010c; Lee et al. 2011a). Bee venom or melittin suppressed TNF-α/actinomycin D or TGF-β1 induced activation of Bcl-2 family and caspase family, which resulted in inhibition of cytochrome c release and PARP cleavage. As such, these papers found that bee venom or melittin exerts its anti-apoptotic effect on hepatocytes through the regulation of the Bcl family with subsequent inactivation of caspase and PARP.

The NF-κB pathway is one of the main signaling pathways activated in response to pro-inflammatory cytokines. In addition, activation of this pathway plays a central role in inflammation through the regulation of genes encoding various growth factors (De Martin et al. 2000). In a model of thioacetamide-induced liver fibrosis, melittin was shown to suppress the expression of pro-inflammatory cytokines (Park et al. 2011b). It was further supported by *in vitro* data showing that melittin reduced the activity of HSCs and decreased the expression of fibrotic gene responses through interruption of the NF-κB signaling pathway. The authors then investigated the mechanism for suppression of NF-κB transcription by melittin in TNF-α/actinomycin D treated hepatocytes, examining the effect of melittin on NF-κB promoter activity by transiently transfected luciferase reporter plasmid containing the NF-κB promoter sequence (Park et al. 2014). Melittin inhibited NF-κB promoter activity and NF-κB DNA binding activity in TNF-α/actinomycin D treated hepatocytes. These results suggest that melittin suppresses NF-κB activation, leading to an inhibition of hepatocyte apoptosis.

Acute hepatic failure can be induced by an administration of D-galactosamine (GalN) and lipopolysaccharide (LPS) which is accompanied by secretion of various pro-inflammatory cytokines and enhancement of sensitivity of hepatocytes to inflammatory injury (Gong et al. 2010). Acute liver injury was induced with GalN/LPS to determine *in vivo* efficacy of melittin (Park et al. 2012). The increased

expression of TNF-α and IL-1β in GalN/LPS injected mice was attenuated by melittin treatment. Melittin also inhibited the expression of caspase and Bax protein levels as well as cytochrome c release. In addition, melittin prevented the activation of NF-κB induced by GalN/LPS.

It has been suggested that an important source of myofibroblasts is hepatocytes which differentiate into myofibroblasts by epithelial-to-mesenchymal transition (EMT) and subsequently contribute to the development of liver fibrosis (Copple 2010). A recent study has investigated the anti-fibrosis or anti-EMT mechanism by examining the effect of apamin on TGF-β1-treated hepatocytes or carbon tetrachloride-induced liver fibrosis animal model (Lee et al. 2014b). Hepatocytes treated concurrently with TGF-β1 and apamin retained a high expression level of epithelial marker E-cadherin and showed no increase in mesenchymal marker vimentin, demonstrating the potential of apamin for the prevention of EMT progression induced by TGF-β1. In particular, apamin suppressed the expression of Smad-independent and Smad-dependent signaling pathways in cells. Moreover, apamin exhibited prevention of liver pathological changes and fibrotic cytokine expression in mice.

Regulatory T cells (Tregs) have been implicated in acquired immunity by suppressing responses of effector T cells (Zhang et al. 2014). It has been demonstrated that Tregs mediated therapeutic potential against immune-medicated hepatic injury (Stross et al. 2012). Acetaminophen as antipyretic and analgesic is an effective alternative to aspirin. High doses of acetaminophen can be associated with liver damage. *In vivo* research conducted by Kim et al. (2014) showed that PLA_2 given to test acetaminophen-injected mice resulted in reduced levels of serum aspartate aminotransferase, alanine aminotransferase, pro-inflammatory cytokines and nitric oxide compared with PBS-injected mice. These effects were abolished in Treg-depleted or IL-10-deficient mice, suggesting that the hepatoprotective effects of PLA_2 were Treg-dependent or IL-10-dependent.

14.2.6 Atherosclerosis

Atherosclerosis is a chronic inflammatory disease of the arteries, characterized by the accumulation of lipids and the proliferation of arterial wall cells, inflammatory cells and fibrous materials (Hai and Zuo 2016). In the progression of atherosclerosis, the proliferation and migration of vascular smooth muscle cells (VSMC) play an important role in causing stenosis or intimal thickening (Chistiakov et al. 2015). It is well known that VSMC can initiate highly conserved signaling events in response to a platelet-derived growth factor (PDGF) that is released from aggregating platelets at sites of endothelial injury.

Son et al. (2006) investigated the anti-proliferative activity of bee venom and melittin in cultured rat VSMC. Bee venom and melittin effectively inhibited fetal bovine serum- and PDGF-induced VSMC proliferation. Given the nature of VSMC

in atherosclerosis, eliminating excessively proliferating VSMC through the regulation of apoptosis is beneficial. The treatment with of bee venom and melittin induced apoptosis of VSMC. It has been well established that NF-κB activity is regulated by IκB proteins, and that the phosphorylation and degradation of IκB result in the activation of NF-κB. PDGF-induced IκB phosphorylation and degradation were inhibited by melittin. Melittin also attenuated the nuclear translocation of NF-κB p50 subunit in response to the action of PDGF. In further investigations, melittin inhibited upstream signals of NF-κB and increased the ratio of proapoptotic to antiapoptotic proteins.

The proliferation of VSMC is governed by the cell cycle, a common convergent point for the mitogenic signaling cascades (Dong et al. 2010). The cell cycle is tightly regulated by multiple protein kinases, each of which contains a regulatory cyclin component and a catalytic cyclin-dependent kinase (CDK). A recent study examined the cellular mechanisms by which apamin inhibited cell cycle progression of VSMC exposed to PDGF (Kim et al. 2015a). The findings showed that apamin suppressed PDGF-induced VSMC proliferation and migration through the regulation of cyclin D1, CDK4, cyclin E and CDK2.

MMP-9 is also involved in the pathogenesis of atherosclerosis by boosting migration of VSMC after an arterial injury. A study in human aortic smooth muscle cells showed that melittin inhibited TNF-α-induced MMP-9 protein expression that was associated with the inhibition of MMP-9 transcription levels by blocking the activation of NF-κB via IκB signaling pathway (Jeong et al. 2012). Moreover, melittin reduced TNF-α-induced migration of human aortic smooth muscle cells.

Kim et al. (2011b) and Kim et al. (2012a) investigated the protective effects of melittin or apamin, respectively, on serum lipid profiles, pro-inflammatory cytokines, pro-atherosclerotic proteins and adhesion molecule levels in an LPS/high fat-induced mouse model of atherosclerosis and macrophages. The major findings are that melittin or apamin inhibited LPS/high fat-induced expression levels of inflammatory cytokines and adhesion molecules such as TNF-α, IL-1β, vascular cell adhesion molecule, intercellular adhesion molecule, fibronectin and transforming growth factor-β1. The anti-atherogenic effect of melittin or apamin is partly attributable to inhibition of the NF-κB signaling pathway in LPS-treated macrophages.

Several studies have confirmed that some calcium channel blockers can decrease the area of atherosclerotic lesions, production of oxidative stress and expression of inflammatory cytokines without conspicuously affecting blood lipid levels (Mancini 2002). Kim et al. (2012b) evaluated the anti-atherosclerotic or anti-apoptotic mechanisms of apamin, a selective inhibitor of the SK(Ca)-channel, in THP-1-derived macrophages. Treatment of cells with oxidized low-density lipoprotein promoted the accumulation of lipids and expression of apoptotic proteins. However, the administration of apamin into macrophages inhibited apoptosis through regulation of the Bcl-2 family, caspase-3 and PARP apoptotic pathway. *In vivo*, apamin attenuated apoptotic cell death in atherosclerotic mice.

14.2.7 Skin Disease

Acne vulgaris is a chronic skin disease involving inflammation of the pilosebaceous follicles, and *Propionibacterium acnes* is a major factor contributing to the inflammatory component of acne (Eichenfield et al. 2015). *P. acnes* contributes to the inflammatory reaction of acne by inducing monocytes and keratinocytes to produce pro-inflammatory cytokines. The induction of these cytokines by *P. acnes* is mediated by Toll-like receptor (TLR) 2 (Kim 2005).

Kim et al. (2015b) investigated the anti-inflammatory effects of bee venom in heat-killed *P. acnes*-treated keratinocytes (HaCaT) and monocytes (THP-1) by measuring the pro-inflammatory cytokines and chemokines. Heat-killed *P. acnes* increased the secretion of interferon-γ, IL-1β, IL-8 and TNF-α in HaCaT and THP-1 cells. However, bee venom treatment decreased the secretion of those cytokines. In addition, bee venom inhibited heat-killed *P. acnes*-induced TLR2 expression in HaCaT cells.

Another recent study conducted by An et al. (2014) reported that bee venom has a potential anti-bacterial effect against inflammatory skin disease. In this context, *P. acnes* was intradermally injected into ears of mice. Following the injection, bee venom mixed with vaseline was applied to the skin surface of the ear. Histological observation revealed that the *P. acnes* injection induced a considerable increase in the number of infiltrated inflammatory cells and inflammatory cytokines. By contrast, the bee venom treated ears showed noticeably reduced ear thickness. Additionally, bee venom inhibited the number of TNF-α and IL-1β positive cells, and the expression of TLR2 and CD14 in the tissue.

During an inflammatory response, the activation of the TLR-mediated signaling pathways leads to the activation of MAPK and NF-κB signaling pathways. These pathways then modulate inflammatory gene expression, which is crucial in shaping the innate immune response within the inflammatory skin disease (Grange et al. 2009). Lee et al. (2014c) investigated the effects of melittin in the production of inflammatory cytokines in heat-killed *P. acnes*-treated HaCaT cells. Additionally, the molecular mechanism of anti-inflammatory effects of melittin was investigated in living *P. acnes*-induced inflammatory skin disease animal model. Administration of heat-killed *P. acnes* increased expression of IKK, IκB and NF-κB in HaCaT cells. However, the addition of melittin reduced IKK, IκB and NF-κB phosphorylation. These results indicate that treatment with melittin abrogated the effect of *P. acnes* by altering the expression through NF-κB signaling. The same study examined whether melittin modulates MAPK signaling in heat-killed *P. acnes*-treated HaCaT cells. The findings showed that melittin decreased phosphorylated p38 that was increased after treatment with heat-killed *P. acnes*. These results underscore the theory that melittin inhibited pro-inflammatory cytokine expression by suppression of p38 MAPK phosphorylation in heat-killed *P. acnes*-treated HaCaT cells.

Atopic dermatitis (AD) is a chronically relapsing inflammatory skin disease that is accompanied by severe itching. Although the underlying mechanism for AD is still not clearly known, histamine derived from skin mast cells seems to be an

important mediator of itchiness (Eichenfield et al. 2012). A 2013 study reported that bee venom attenuated compound 48/80-induced scratching behaviors in mice (Kim et al. 2013). Intraperitoneal administration of bee venom inhibited the degranulation of mast cells and the production of pro-inflammatory cytokines in compound 48/80-treated skin tissues. Bee venom also inhibited the compound 48/80-induced activation of NF-κB which regulates pro-inflammatory cytokine expression.

The skin sensitization study of bee venom carried out in guinea pigs and rats showed that bee venom was well tolerated and exhibited no dermal irritation potential in animals (Han et al. 2013). The authors concluded that the findings may provide a developmental basis of bee venom for a cosmetic ingredient or external application for topical uses. In fact, the beneficial effects of bee venom serum on the clinical signs of aging skin were assessed (Han et al. 2015). Bee venom serum treatment clinically improved facial wrinkles by decreasing total wrinkle area, total wrinkle count and average wrinkle depth. In this regard, bee venom has been claimed to be the new Botox.

14.2.8 Learning Deficit

Learning is the result of processes by which experiences produce long-term and lasting changes in the nervous system, and subsequent memory formation is derived from these changes which are accompanied by synaptic plasticity. Activation of SK(Ca)-channels being present throughout the CNS contributes to maintenance of the neuronal membrane potential and was shown to underlie the after hyperpolarization that regulates action potential firing and limits the firing frequency of repetitive action potentials (Kuiper et al. 2012). Among different subtypes of SK(Ca)-channels, only $K_{ca}2$ channels can be differentially blocked by apamin through the reduction of synaptic plasticity. Thus, it can be expected that inhibition of $K_{ca}2$ channels improves learning. Several animal studies underscore the relevance of $K_{ca}2$ channels in information processing and storage in the brain.

In the first known study to investigate the effect of apamin on the learning and memory process, administration of apamin to mice before the training at a dose of 0.2 mg/kg increased the acquisition of a bar-pressing response and bar-pressing rates (Messier et al. 1991). Administration of apamin at the same dose after the training also facilitated the memory process, confirming the consolidation processes that took place shortly after acquisition.

Another study using male rats investigated the effect of apamin on memory as assessed in an object recognition task which measures innate exploratory behavior (Deschaux et al. 1997). Apamin improved learning in an object recognition task in animals at a dose of 0.4 mg/kg. Interestingly, doses of 0.1 and 0.2 mg/kg were not effective, and apamin was also ineffective when administered after the training, showing that apamin had no effects on the other stages of memory following acqui-

sition. Further support for a role of apamin in facilitating cognitive performance of tasks requiring the processing of non-spatial information was provided by a subsequent rat study (Deschaux and Bizot 2005).

In a study involving water maze and Y-maze which employ a spatial working memory component, apamin injections before or after the training at doses of 0.02, 0.06 or 0.2 mg/kg did not affect the performance of intact mice (Ikonen et al. 1998). However, apamin at a dose of 0.02 mg/kg administered before the training increased passive avoidance entry latency in intact mice. In an additional experiment using medial septal-lesioned mice, pretraining injection of apamin at doses of 0.02 and 0.06 mg/kg stimulated water maze behavior. In accord with this alleviating effect of apamin in a brain defect animal model, mice with partial hippocampal lesions showed a favorable effect of apamin on water maze spatial navigation and radial arm maze performance (Ikonen and Riekkinen 1999). These observations suggest that apamin can alleviate the symptoms of a damaged septo-hippocampal axis, irrespective of whether the damage is in the septum or in the hippocampus.

Both scopolamine, an anticholinergic drug, and electroconvulsive shock induced amnesia by the reduction of retention latency during passive avoidance test in mice (Inan et al. 2000). Apamin injected immediately after the acquisition trial reversed the amnestic effect of scopolamine or electroconvulsive shock, probably through the cholinergic system.

Fournier et al. (2001) conducted a different type of trial using an olfactory associated task in an attempt to see the effect of an intracerebroventricular apamin injection at a dose of 0.3 ng on learning and memory in rats. The results showed that apamin improved the consolidation of the valence of a new odor pair in a successive odor-pair training task without affecting the odor-reward learning or the long-term retrieval of information.

An *in vitro* and *in vivo* study of apamin demonstrated that blockade of synaptically activated SK channels with apamin increased excitability and decreased the threshold for the induction of hippocampal synaptic plasticity (Stackman et al. 2002). The reduced threshold for induction of synaptic plasticity was further associated with a facilitated early stage of learning called memory encoding in mice.

The cognitive process requires the working memory function of the prefrontal cortex, thus disruption in prefrontal neuronal activity is associated with working memory impairment including learning deficit. Using the medial prefrontal cortex-dependent spatial delayed alternation task, apamin when infused directly into the medial prefrontal cortex improved spatial working memory performance (Brennan et al. 2008).

Neurofibromatosis 1 (NF1) is a genetic disorder due to a single mutation of the Nf1 gene located on chromosome 17, and is characterized by a number of physiological symptoms including learning deficit (Vranceanu et al. 2015). A current animal model for NF1 is a heterozygous $Nf1^{+/-}$ mouse and inherent learning deficits in $Nf1^{+/-}$ mice are similar to those seen in humans (Silva et al. 1997). A recent study found a significant deficit in water maze performance in $Nf1^{+/-}$ mice treated with saline (Kallarackal et al. 2013). This lack of spatial cognitive function seen early in training in $Nf1^{+/-}$ mice was reversed by administration of apamin (0.2 or 0.4 mg/kg)

either through acute interperitoneal injection or chronic micro-osmotic pump delivery. Apamin appeared to produce its ameliorating effects early in training at the time when $Nf1^{+/-}$ mice generally exhibit their visuospatial deficits.

14.2.9 Pain

Neuropathic pain caused by dysfunction either in peripheral nerves or in CNS is associated with loss of sensation or an increase in sensation such as hyperalgesia (an increased response to a stimulus that is normally painful), allodynia (pain as a result of a stimulus that does not normally provoke pain) and an excessive sensitivity to painful stimulus called hyperpathia (Baron 2009).

In a neuropathic condition induced by sciatic nerve chronic constriction injury (CCI), the efficacy of bee venom acupuncture for hyperalgesic and allodynic response to thermal and mechanical stimuli in rats was investigated (Roh et al. 2004). Subcutaneous single injection of bee venom at a dose of 0.25 mg/kg into the Zusanli acupoint reduced CCI-induced thermal hyperalgesia, but failed to reduce mechanical allodynia. This antihyperalgesic effect of bee venom acupuncture was reversed by idazoxan, an α_2-adrenoceptor antagonist, but not by naloxone, an opioid receptor antagonist. These findings suggest that bee venom acupuncture reduced the thermal hyperalgesia generated by CCI and this antihyperalgesic effect was dependent on the activation of α_2-adrenoceptors, not opioid receptors, in the spinal cord.

A subsequent study examined whether bee venom acupuncture can enhance the analgesic effect of clonidine, an α_2-adrenoceptor agonist (Yoon et al. 2009). In a rat neuropathic pain model, intrathecal treatment with clonidine suppressed sciatic nerve CCI-induced mechanical allodynia and thermal hyperalgesia in a dose dependent manner, and this clonidine-induced analgesic effect was potentiated by bee venom acupuncture. Bee venom acupuncture on Zusanli alone or in combination with a low dose of clonidine produced an analgesic effect similar to that of the high dose of clonidine. This synergistic analgesic interaction between clonidine and bee venom acupuncture was blocked with an α_2-adrenoceptor antagonist. In a separate mouse formalin test, bee venom acupuncture was found to suppress formalin-induced licking behavior during the late phase, but not the early phase.

Another study examined whether single or repetitive administration of bee venom on Zusanli acupoint reduced cold allodynia in sciatic nerve CCI rats (Kang et al. 2012a). Single injection of bee venom at a dose of 0.25 mg/kg into an acupoint did not produce any anticold allodynic effect, while a higher dose of 2.5 mg/kg reduced cold allodynia. This anticold allodynic effect with high dose of bee venom was blocked by intrathecal treatment of idazoxan. Interestingly, co-administration of bee venom at a dose of 0.25 mg/kg with α_2-adrenoceptor agonist (clonidine) synergistically reduced cold allodynia. Moreover, repetitive bee venom injections at a dose of 0.25 mg/kg twice a day from day 15 after CCI surgery for 2 consecutive weeks suppressed cold allodynia from 7 days after bee venom treatment.

Fos protein is a useful marker of neuronal activity that can be used to map functionally related neural pathways (Presley et al. 1990). The expression of Fos proteins can be evoked by a variety of stimuli including bee venom. Since peripheral bee venom injection increased Fos expression in brainstem catecholaminergic neurons including many neurons in the locus coeruleus (LC), it was suggested that the analgesic effect of bee venom is mediated by the activation of descending bulbospinal noradrenergic pathways which in turn activate α_2-adrenoceptors in the spinal cord (Kang et al. 2012b). On the other hand, neuropathic pain is associated with some pathophysiological changes including peripheral and central sensitization. The N-methyl-D-aspartate (NMDA) receptor in the spinal cord plays a critical role in the central sensitization for pain perception and it is well established that phosphorylation of the NMDA receptor NR1 subunit (pNR1) is related to increased neuronal responsiveness (Petrenko et al. 2003). In fact, CCI to the sciatic nerve increased the number of pNR1-immunoreactive neurons in the spinal cord dorsal horn (Roh et al. 2008a) and intrathecal clonidine suppressed phosphorylation of pNR1 in the spinal dorsal horn neurons of rats with neuropathic pain (Roh et al. 2008b).

Kang et al. (2012b) investigated the analgesic effect of repetitive injection of bee venom on neuropathy-induced nociception and its relevant mechanism. Subcutaneous injection of bee venom twice a day for 2 weeks reduced mechanical allodynia as well as thermal hyperalgesia. This potent analgesic effect of bee venom on neuropathic pain was associated with the increased activation of LC noradrenergic neurons and decreased expression of spinal pNR1. The overall suppressive effects of repetitive bee venom treatment on neuropathic pain and spinal pNR1 were hindered by intrathecal injection of an α_2-adrenoceptor antagonist.

Glial cells, including astrocytes and microglia, have been recognized as an additional player in the initiation and maintenance of nociception. These glial cells have close interactions with neurons and thus modulate pain transmission during pathological situations (Zhuo et al. 2011). As such, either metabolic inhibition of glia activation (Gwak et al. 2008) or blockade of astrocyte gap junctions (Roh et al. 2010) has shown to have an anti-nociceptive effect in spinal cord injury in rats.

As an analgesic behavioral observation assessment method, the formalin test was used (Kang et al. 2011). Bee venom acupuncture at a dose of 0.8 mg/kg into the Zusanli acupoint attenuated the nociceptive response in the late phase of the formalin test and blocked the formalin-induced increase in spinal expression of the astrocyte activation marker, glial fibrillary acidic protein (GFAP). This glial modulation was potentiated by intrathecal administration of the glial metabolic inhibitor called fluorocitrate. These analgesic effects of bee venom acupuncture were prevented by intrathecal treatment with selective α_{2A}- and α_{2C}-adrenoceptor antagonists.

Experimental spinal cord injury can also be developed by spinal hemisection. Hemisection of the rat spinal cord at thoracic level 13 produced prominent mechanical allodynia and thermal hyperalgesia (Kang et al. 2015). Repetitive bee venom acupuncture into the Zusanli acupoint on the same side as the spinal hemisection (ipsilateral side) twice a day from 15 to 20 days post-surgery suppressed pain behavior in the ipsilateral hind paw. Spinal hemisection-induced increase in spinal

glia expression in terms of GFAP and glial ionized calcium-binding adaptor protein 1 was also hindered by repetitive bee venom acupuncture in the ipsilateral dorsal spinal cord. Moreover, bee venom acupuncture facilitated motor function recovery of the affected hindlimb.

14.2.10 Lupus Nephritis

As an inflammation of the kidneys, lupus nephritis is caused by systemic lupus erythematosus, an autoimmune disease. Lupus nephritis resulting from a multidimensional interplay of autoantibodies in association with nucleosomes, histones, inflammatory cells, stimulated resident cells, complement and cytokines triggers glomerular deposition of immune complexes that promote a cascade of inflammatory events leading to renal damage (Tang et al. 2005).

Data from an animal experiment suggest that bee venom may be of benefit in controlling symptoms of lupus nephritis (Lee et al. 2011b). Authors used New Zealand Black/White F1 female mice which spontaneously develop autoimmune disease as they age, which is characterized by lethal immune complex glomerulonephritis, proteinuria and renal dysfunction. Bee venom at a dose of 3.0 mg/kg was injected subcutaneously once a week from 18 to 32 weeks of age. Bee venom administration delayed the development of proteinuria to a significant extent. According to renal histology, the majority of glomeruli from the control group had lupus membranous nephropathy with subepithelial spike formation combined with global endocapillary and mesangial hypercellularity, early crescent formation, and beginning mesangial and capillary sclerosis. In contrast, many glomeruli from the bee venom group showed mildly abnormal histology. These changes were correlated with reduced glomerular damage in terms of deposition of proteinaceous hyaline material, fibrinoid necrosis and glomerulosclerosis from bee venom treatment. The protective action of bee venom against lupus nephritis was further illustrated by reduced immune deposits in the glomeruli through the immune-modulating effect on $CD4^{+}CD25^{+}$ regulatory T cells.

References

Alvarez-Fischer D, Noelker C, Vulinović F, Grünewald A, Chevarin C, Klein C, Oertel WH, Hirsch EC, Michel PP, Hartmann A (2013) Bee venom and its component apamin as neuroprotective agents in a Parkinson disease mouse model. PLoS One 8:e61700

An HJ, Lee WR, Kim KH, Kim JY, Lee SJ, Han SM, Lee KG, Lee CK, Park KK (2014) Inhibitory effects of bee venom on Propionibacterium acnes-induced inflammatory skin disease in an animal model. Int J Mol Med 34:1341–1348

Baron R (2009) Neuropathic pain: a clinical perspective. Handb Exp Pharmacol 194:3–30

Brennan AR, Dolinsky B, Vu MA, Stanley M, Yeckel MF, Arnsten AF (2008) Blockade of IP3-mediated SK channel signaling in the rat medial prefrontal cortex improves spatial working memory. Learn Mem 15:93–96

Cai M, Choi SM, Yang EJ (2015) The effects of bee venom acupuncture on the central nervous system and muscle in an animal hSOD1G93A mutant. Toxins 7:846–858

Castro HJ, Mendez-Lnocencio JI, Omidvar B, Omidvar J, Santilli J, Nielsen HS Jr, Pavot AP, Richert JR, Bellanti JA (2005) A phase I study of the safety of honeybee venom extract as a possible treatment for patients with progressive forms of multiple sclerosis. Allergy Asthma Proc 26:470–476

Chistiakov DA, Orekhov AN, Bobryshev YV (2015) Vascular smooth muscle cell in atherosclerosis. Acta Physiol (Oxf) 214:33–50

Choi KE, Hwang CJ, Gu SM, Park MH, Kim JH, Park JH, Ahn YJ, Kim JY, Song MJ, Song HS, Han SB, Hong JT (2014) Cancer cell growth inhibitory effect of bee venom via increase of death receptor 3 expression and inactivation of NF-kappa B in NSCLC cells. Toxins 6:2210–2228

Chung ES, Kim H, Lee G, Park S, Kim H, Bae H (2012) Neuro-protective effects of bee venom by suppression of neuroinflammatory responses in a mouse model of Parkinson's disease: role of regulatory T cells. Brain Behav Immun 26:1322–1330

Copple BL (2010) Hypoxia stimulates hepatocyte epithelial to mesenchymal transition by hypoxia-inducible factor and transforming growth factor-beta-dependent mechanisms. Liver Int 30:669–682

De Martin R, Hoeth M, Hofer-Warbinek R, Schmid JA (2000) The transcription factor NF-kappa B and the regulation of vascular cell function. Arterioscler Thromb Vasc Biol 20:E83–E88

Deschaux O, Bizot JC (2005) Apamin produces selective improvements of learning in rats. Neurosci Lett 386:5–8

Deschaux O, Bizot JC, Goyffon M (1997) Apamin improves learning in an object recognition task in rats. Neurosci Lett 222:159–162

Doherty MJ, Bird TD, Leverenz JB (2004) Alpha-synuclein in motor neuron disease: an immunohistologic study. Acta Neuropathol 107:169–175

Dong LH, Wen JK, Miao SB, Jia Z, Hu HJ, Sun RH, Wu Y, Han M (2010) Baicalin inhibits PDGF-BB-stimulated vascular smooth muscle cell proliferation through suppressing PDGFRβ-ERK signaling and increase in p27 accumulation and prevents injury-induced neointimal hyperplasia. Cell Res 20:1252–1262

Doo AR, Kim ST, Kim SN, Moon W, Yin CS, Chae Y, Park HK, Lee H, Park HJ (2010) Neuroprotective effects of bee venom pharmaceutical acupuncture in acute 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced mouse model of Parkinson's disease. Neurol Res 32(Suppl 1):88–91

Eichenfield LF, Ellis CN, Mancini AJ, Paller AS, Simpson EL (2012) Atopic dermatitis: epidemiology and pathogenesis update. Semin Cutan Med Surg 31(3 Suppl):S3–S5

Eichenfield LF, Del Rosso JQ, Mancini AJ, Cook-Bolden F, Stein Gold L, Desai S, Weiss J, Pariser D, Zeichner J, Bhatia N, Kircik L (2015) Evolving perspectives on the etiology and pathogenesis of acne vulgaris. J Drugs Dermatol 14:263–272

Ellis TD, Cavanaugh JT, Earhart GM, Ford MP, Foreman KB, Thackeray A, Thiese MS, Dibble LE (2016) Identifying clinical measures that most accurately reflect the progression of disability in Parkinson disease. Parkinsonism Relat Disord 25:65–71

Fournier C, Kourrich S, Soumireu-Mourat B, Mourre C (2001) Apamin improves reference memory but not procedural memory in rats by blocking small conductance Ca(2+)-activated K(+) channels in an olfactory discrimination task. Behav Brain Res 121:81–93

Gajski G, Garaj-Vrhovac V (2013) Melittin: a lytic peptide with anticancer properties. Environ Toxicol Pharmacol 36:697–705

Gong X, Luo FL, Zhang L, Li HZ, Wu MJ, Li XH, Wang B, Hu N, Wang CD, Yang JQ, Wan JY (2010) Tetrandrine attenuates lipopolysaccharide-induced fulminant hepatic failure in D-galactosamine-sensitized mice. Int Immunopharmacol 10:357–363

Grange PA, Raingeaud J, Calvez V, Dupin N (2009) Nicotinamide inhibits Propionibacterium acnes-induced IL-8 production in keratinocytes through the NF-kappaB and MAPK pathways. J Dermatol Sci 56:106–112

Gwak YS, Crown ED, Unabia GC, Hulsebosch CE (2008) Propentofylline attenuates allodynia, glial activation and modulates GABAergic tone after spinal cord injury in the rat. Pain 138:410–422

Hai Z, Zuo W (2016) Aberrant DNA methylation in the pathogenesis of atherosclerosis. Clin Chim Acta 456:69–74

Han SM, Lee KG, Park KK, Pak SC (2013) Skin sensitization study of bee venom (Apis mellifera L.) in guinea pigs and rats. Cutan Ocul Toxicol 32:27–30

Han SM, Hong IP, Woo SO, Chun SN, Park KK, Nicholls YM, Pak SC (2015) The beneficial effects of honeybee-venom serum on facial wrinkles in humans. Clin Interv Aging 10:1587–1592

Huh JE, Baek YH, Lee MH, Choi DY, Park DS, Lee JD (2010) Bee venom inhibits tumor angiogenesis and metastasis by inhibiting tyrosine phosphorylation of VEGFR-2 in LLC-tumor-bearing mice. Cancer Lett 292:98–110

Ikonen S, Riekkinen P Jr (1999) Effects of apamin on memory processing of hippocampal-lesioned mice. Eur J Pharmacol 382:151–156

Ikonen S, Schmidt B, Riekkinen P Jr (1998) Apamin improves spatial navigation in medial septal-lesioned mice. Eur J Pharmacol 347:13–21

Inan SY, Aksu F, Baysal F (2000) The effects of some K(+) channel blockers on scopolamine- or electroconvulsive shock-induced amnesia in mice. Eur J Pharmacol 407:159–164

Jeong YJ, Cho HJ, Whang K, Lee IS, Park KK, Choe JY, Han SM, Kim CH, Chang HW, Moon SK, Kim WJ, Choi YH, Chang YC (2012) Melittin has an inhibitory effect on TNF-α-induced migration of human aortic smooth muscle cells by blocking the MMP-9 expression. Food Chem Toxicol 50:3996–4002

Jo M, Park MH, Kollipara PS, An BJ, Song HS, Han SB, Kim JH, Song MJ, Hong JT (2012) Anti-cancer effect of bee venom toxin and melittin in ovarian cancer cells through induction of death receptors and inhibition of JAK2/STAT3 pathway. Toxicol Appl Pharmacol 258:72–81

Kallarackal AJ, Simard JM, Bailey AM (2013) The effect of apamin, a small conductance calcium activated potassium (SK) channel blocker, on a mouse model of neurofibromatosis 1. Behav Brain Res 237:71–75

Kang SY, Kim CY, Roh DH, Yoon SY, Park JH, Lee HJ, Beitz AJ, Lee JH (2011) Chemical stimulation of the ST36 acupoint reduces both formalin-induced nociceptive behaviors and spinal astrocyte activation via spinal alpha-2 adrenoceptors. Brain Res Bull 86:412–421

Kang SY, Roh DH, Park JH, Lee HJ, Lee JH (2012a) Activation of spinal α2-adrenoceptors using diluted bee venom stimulation reduces cold allodynia in neuropathic pain rats. Evid Based Complement Alternat Med 2012:784713

Kang SY, Roh DH, Yoon SY, Moon JY, Kim HW, Lee HJ, Beitz AJ, Lee JH (2012b) Repetitive treatment with diluted bee venom reduces neuropathic pain via potentiation of locus coeruleus noradrenergic neuronal activity and modulation of spinal NR1 phosphorylation in rats. J Pain 13:155–166

Kang SY, Roh DH, Choi JW, Ryu Y, Lee JH (2015) Repetitive treatment with diluted bee venom attenuates the induction of below-level neuropathic pain behaviors in a rat spinal cord injury model. Toxins 7:2571–2585

Karimi A, Ahmadi F, Parivar K, Nabiuni M, Haghighi S, Imani S, Afrouzi H (2012) Effect of honey bee venom on lewis rats with experimental allergic encephalomyelitis, a model for multiple sclerosis. Iran J Pharm Res 11:671–678

Khalil WK, Assaf N, ElShebiney SA, Salem NA (2015) Neuroprotective effects of bee venom acupuncture therapy against rotenone-induced oxidative stress and apoptosis. Neurochem Int 80:79–86

Kim J (2005) Review of the innate immune response in acne vulgaris: activation of Toll-like receptor 2 in acne triggers inflammatory cytokine responses. Dermatology 211:193–198

Kim KH, Kum YS, Park YY, Park JH, Kim SJ, Lee WR, Lee KG, Han SM, Park KK (2010) The protective effect of bee venom against ethanol-induced hepatic injury via regulation of the mitochondria-related apoptotic pathway. Basic Clin Pharmacol Toxicol 107:619–624

Kim JI, Yang EJ, Lee MS, Kim YS, Huh Y, Cho IH, Kang S, Koh HK (2011a) Bee venom reduces neuroinflammation in the MPTP-induced model of Parkinson's disease. Int J Neurosci 121:209–217

Kim SJ, Park JH, Kim KH, Lee WR, Kim KS, Park KK (2011b) Melittin inhibits atherosclerosis in LPS/high-fat treated mice through atheroprotective actions. J Atheroscler Thromb 18:1117–1126

Kim SJ, Park JH, Kim KH, Lee WR, Pak SC, Han SM, Park KK (2012a) The protective effect of apamin on LPS/fat-induced atherosclerotic mice. Evid Based Complement Alternat Med 2012:305454

Kim SJ, Park JH, Kim KH, Lee WR, An HJ, Min BK, Han SM, Kim KS, Park KK (2012b) Apamin inhibits THP-1-derived macrophage apoptosis via mitochondria-related apoptotic pathway. Exp Mol Pathol 93:129–134

Kim KH, Lee WR, An HJ, Kim JY, Chung H, Han SM, Lee ML, Lee KG, Pak SC, Park KK (2013) Bee venom ameliorates compound 48/80-induced atopic dermatitis-related symptoms. Int J Clin Exp Pathol 6:2896–2903

Kim H, Keum DJ, won Kwak J, Chung HS, Bae H (2014) Bee venom phospholipase A_2 protects against acetaminophen-induced acute liver injury by modulating regulatory T cells and IL-10 in mice. PLoS One 9:27

Kim JY, Kim KH, Lee WR, An HJ, Lee SJ, Han SM, Lee KG, Park YY, Kim KS, Lee YS, Park KK (2015a) Apamin inhibits PDGF-BB-induced vascular smooth muscle cell proliferation and migration through suppressions of activated Akt and Erk signaling pathway. Vascul Pharmacol 70:8–14

Kim JY, Lee WR, Kim KH, An HJ, Chang YC, Han SM, Park YY, Pak SC, Park KK (2015b) Effects of bee venom against Propionibacterium acnes-induced inflammation in human keratinocytes and monocytes. Int J Mol Med 35:1651–1656

Kisseleva T, Brenner DA (2007) Role of hepatic stellate cells in fibrogenesis and the reversal of fibrosis. J Gastroenterol Hepatol 22(Suppl 1):S73–S78

Kuiper EF, Nelemans A, Luiten P, Nijholt I, Dolga A, Eisel U (2012) K(Ca)2 and k(ca)3 channels in learning and memory processes, and neurodegeneration. Front Pharmacol 3:107

Lee WR, Park JH, Kim KH, Park YY, Han SM, Park KK (2011a) Protective effects of melittin on transforming growth factor-β1 injury to hepatocytes via anti-apoptotic mechanism. Toxicol Appl Pharmacol 256:209–215

Lee H, Lee EJ, Kim H, Lee G, Um EJ, Kim Y, Lee BY, Bae H (2011b) Bee venom-associated Th1/Th2 immunoglobulin class switching results in immune tolerance of NZB/W F1 murine lupus nephritis. Am J Nephrol 34:163–172

Lee SH, Choi SM, Yang EJ (2014a) Melittin ameliorates the inflammation of organs in an amyotrophic lateral sclerosis animal model. Exp Neurobiol 23:86–92

Lee WR, Kim KH, An HJ, Kim JY, Lee SJ, Han SM, Pak SC, Park KK (2014b) Apamin inhibits hepatic fibrosis through suppression of transforming growth factor β1-induced hepatocyte epithelial-mesenchymal transition. Biochem Biophys Res Commun 450:195–201

Lee WR, Kim KH, An HJ, Kim JY, Chang YC, Chung H, Park YY, Lee ML, Park KK (2014c) The protective effects of melittin on Propionibacterium acnes-induced inflammatory responses in vitro and in vivo. J Invest Dermatol 134:1922–1930

Lee SH, Choi SM, Yang EJ (2015) Bee venom acupuncture augments anti-inflammation in the peripheral organs of hSOD1G93A transgenic mice. Toxins 7:2835–2844

Mancini GB (2002) Antiatherosclerotic effects of calcium channel blockers. Prog Cardiovasc Dis 45:1–20

Mazzoni P, Shabbott B, Cortés JC (2012) Motor control abnormalities in Parkinson's disease. Cold Spring Harb Perspect Med 2:a009282

Messier C, Mourre C, Bontempi B, Sif J, Lazdunski M, Destrade C (1991) Effect of apamin, a toxin that inhibits Ca(2+)-dependent K+ channels, on learning and memory processes. Brain Res 551:322–326

Nishida N, Yano H, Nishida T, Kamura T, Kojiro M (2006) Angiogenesis in cancer. Vasc Health Risk Manag 2:213–219

Oršolić N (2012) Bee venom in cancer therapy. Cancer Metastasis Rev 31:173–194
Park JH, Jeong YJ, Park KK, Cho HJ, Chung IK, Min KS, Kim M, Lee KG, Yeo JH, Park KK, Chang YC (2010a) Melittin suppresses PMA-induced tumor cell invasion by inhibiting NF-kappaB and AP-1-dependent MMP-9 expression. Mol Cells 29:209–215
Park JH, Kim KH, Kim SJ, Lee WR, Lee KG, Park KK (2010b) Bee venom protects hepatocytes from tumor necrosis factor-alpha and actinomycin D. Arch Pharm Res 33:215–223
Park JH, Kim KH, Kim SJ, Lee WR, Lee KG, Park JH, Park KK (2010c) Effect of bee venom on transforming growth factor-beta1-treated hepatocytes. Int J Toxicol 29:49–56
Park MH, Choi MS, Kwak DH, Oh KW, Yoon do Y, Han SB, Song HS, Song MJ, Hong JT (2011a) Anti-cancer effect of bee venom in prostate cancer cells through activation of caspase pathway via inactivation of NF-κB. Prostate 71:801–812
Park JH, Kum YS, Lee TI, Kim SJ, Lee WR, Kim BI, Kim HS, Kim KH, Park KK (2011b) Melittin attenuates liver injury in thioacetamide-treated mice through modulating inflammation and fibrogenesis. Exp Biol Med (Maywood) 236:1306–1313
Park JH, Kim KH, Lee WR, Han SM, Park KK (2012) Protective effect of melittin on inflammation and apoptosis in acute liver failure. Apoptosis 17:61–69
Park JH, Lee WR, Kim HS, Han SM, Chang YC, Park KK (2014) Protective effects of melittin on tumor necrosis factor-α induced hepatic damage through suppression of apoptotic pathway and nuclear factor-kappa B activation. Exp Biol Med (Maywood) 239:1705–1714
Petrenko AB, Yamakura T, Baba H, Shimoji K (2003) The role of N-methyl-D-aspartate (NMDA) receptors in pain: a review. Anesth Analg 97:1108–1116
Presley RW, Menétrey D, Levine JD, Basbaum AI (1990) Systemic morphine suppresses noxious stimulus-evoked Fos protein-like immunoreactivity in the rat spinal cord. J Neurosci 10:323–335
Redler RL, Dokholyan NV (2012) The complex molecular biology of amyotrophic lateral sclerosis (ALS). Prog Mol Biol Transl Sci 107:215–262
Roh DH, Kwon YB, Kim HW, Ham TW, Yoon SY, Kang SY, Han HJ, Lee HJ, Beitz AJ, Lee JH (2004) Acupoint stimulation with diluted bee venom (apipuncture) alleviates thermal hyperalgesia in a rodent neuropathic pain model: involvement of spinal alpha 2-adrenoceptors. J Pain 5:297–303
Roh DH, Kim HW, Yoon SY, Seo HS, Kwon YB, Han HJ, Beitz AJ, Lee JH (2008a) Depletion of capsaicin-sensitive afferents prevents lamina-dependent increases in spinal N-methyl-D-aspartate receptor subunit 1 expression and phosphorylation associated with thermal hyperalgesia in neuropathic rats. Eur J Pain 12:552–563
Roh DH, Kim HW, Yoon SY, Seo HS, Kwon YB, Han HJ, Beitz AJ, Lee JH (2008b) Intrathecal clonidine suppresses phosphorylation of the N-methyl-D-aspartate receptor NR1 subunit in spinal dorsal horn neurons of rats with neuropathic pain. Anesth Analg 107:693–700
Roh DH, Yoon SY, Seo HS, Kang SY, Han HJ, Beitz AJ, Lee JH (2010) Intrathecal injection of carbenoxolone, a gap junction decoupler, attenuates the induction of below-level neuropathic pain after spinal cord injury in rats. Exp Neurol 224:123–132
Salthun-Lassalle B, Hirsch EC, Wolfart J, Ruberg M, Michel PP (2004) Rescue of mesencephalic dopaminergic neurons in culture by low-level stimulation of voltage-gated sodium channels. J Neurosci 24:5922–5930
Shin Y, Yoon SH, Choe EY, Cho SH, Woo CH, Rho JY, Kim JH (2007) PMA-induced up-regulation of MMP-9 is regulated by a PKCalpha-NF-kappaB cascade in human lung epithelial cells. Exp Mol Med 39:97–105
Silva AJ, Frankland PW, Marowitz Z, Friedman E, Laszlo GS, Cioffi D, Jacks T, Bourtchuladze R (1997) A mouse model for the learning and memory deficits associated with neurofibromatosis type I. Nat Genet 15:281–284
Son DJ, Ha SJ, Song HS, Lim Y, Yun YP, Lee JW, Moon DC, Park YH, Park BS, Song MJ, Hong JT (2006) Melittin inhibits vascular smooth muscle cell proliferation through induction of apoptosis via suppression of nuclear factor-kappaB and Akt activation and enhancement of apoptotic protein expression. J Pharmacol Exp Ther 317:627–634

Stackman RW, Hammond RS, Linardatos E, Gerlach A, Maylie J, Adelman JP, Tzounopoulos T (2002) Small conductance Ca2+-activated K+ channels modulate synaptic plasticity and memory encoding. J Neurosci 22:10163–10171

Stross L, Günther J, Gasteiger G, Asen T, Graf S, Aichler M, Esposito I, Busch DH, Knolle P, Sparwasser T, Protzer U (2012) Foxp3+ regulatory T cells protect the liver from immune damage and compromise virus control during acute experimental hepatitis B virus infection in mice. Hepatology 56:873–883

Tang S, Lui SL, Lai KN (2005) Pathogenesis of lupus nephritis: an update. Nephrology (Carlton) 10:174–179

Toulorge D, Guerreiro S, Hild A, Maskos U, Hirsch EC, Michel PP (2011) Neuroprotection of midbrain dopamine neurons by nicotine is gated by cytoplasmic Ca2+. FASEB J 25:2563–2573

Vranceanu AM, Merker VL, Park ER, Plotkin SR (2015) Quality of life among children and adolescents with neurofibromatosis 1: a systematic review of the literature. J Neurooncol 122:219–228

Wesselius T, Heersema DJ, Mostert JP, Heerings M, Admiraal-Behloul F, Talebian A, van Buchem MA, De Keyser J (2005) A randomized crossover study of bee sting therapy for multiple sclerosis. Neurology 65:1764–1768

Wootla B, Watzlawik JO, Stavropoulos N, Wittenberg NJ, Dasari H, Abdelrahim MA, Henley JR, Oh SH, Warrington AE, Rodriguez M (2016) Recent advances in monoclonal antibody therapies for multiple sclerosis. Expert Opin Biol Ther 16:827–839

Yang EJ, Choi SM (2013) α-Synuclein modification in an ALS animal model. Evid Based Complement Alternat Med 2013:259381

Yang EJ, Kim SH, Yang SC, Lee SM, Choi SM (2011) Melittin restores proteasome function in an animal model of ALS. J Neuroinflammation 8:69

Yang JS, Lin CW, Su SC, Yang SF (2016) Pharmacodynamic considerations in the use of matrix metalloproteinase inhibitors in cancer treatment. Expert Opin Drug Metab Toxicol 12:191–200

Yoon SY, Roh DH, Kwon YB, Kim HW, Seo HS, Han HJ, Lee HJ, Beitz AJ, Lee JH (2009) Acupoint stimulation with diluted bee venom (apipuncture) potentiates the analgesic effect of intrathecal clonidine in the rodent formalin test and in a neuropathic pain model. J Pain 10:253–263

Zhang H, Kong H, Zeng X, Guo L, Sun X, He S (2014) Subsets of regulatory T cells and their roles in allergy. J Transl Med 12:125

Zhuo M, Wu G, Wu LJ (2011) Neuronal and microglial mechanisms of neuropathic pain. Mol Brain 4:31

Zuo LJ, Yu SY, Wang F, Hu Y, Piao YS, Du Y, Lian TH, Wang RD, Yu QJ, Wang YJ, Wang XM, Chan P, Chen SD, Wang Y, Zhang W (2016) Parkinson's disease with fatigue: clinical characteristics and potential mechanisms relevant to α-synuclein oligomer. J Clin Neurol 12:172–180