8. THE DEVELOPMENT AND APPLICATION OF PERILLA EXTRACT AS AN ANTI-ALLERGIC SUBSTANCE

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INTRODUCTION

In our laboratory we have been seeking out and researching natural substances contained in plants and food which are beneficial to the human body. Perilla extract was discovered and developed partially because of this research.

Recently there has been a marked increase in the number of people who have allergies. To find effective treatments for allergies, a great effort has been made in the medical field. However, a satisfactory treatment has yet to be established. It is particularly difficult because most allergies are caused by living habits and conditions, environmental factors, eating habits, individual predispositions including genetic causes, and stress related factors. The treatment is often lengthy.

In order to prevent allergies, possible allergens have been removed from our diet. However, this has resulted in an unbalanced diet and diseases which have been caused by a loss of tolerance to allergens, especially in children. For treatment and prevention, we thought of using those physiologically active substances contained in foods which would be beneficial to the human body. Therefore, they should be harmless in large amounts and familiar to the diet. We started by analysing the effects of various vegetables on the human immune system and observed the reactions. As a result of this research, Perilla extract was found to be an effective anti-allergy substance (Oyanagi *et al.*, 1992).

SCREENING OF FOODS FOR ANTI-ALLERGY PROPERTIES

The effects of food on biophylaxis, that is the immune system of the body, is now receiving much attention. Cytokines, a bacterial formulation of BRM (biological response modifier) and certain foods (vegetables, mushrooms, fruit) boost the immune system and enhance the body's self-defence system (Ueda *et al.* 1991a, b; Yamazaki, 1992; Yamazaki and Nishimura 1992) whereas some steroids and other foods inhibit the self-defence system. The self-defence system needs to be regulated according to the body's condition. BRMs and steroids are used in order to intentionally control it. BRMs are used for enhancement of cellular immunity in cancer treatment. Steroids are used as anti-allergy agents. With this consideration, we have focused on the fact that food and food substances have an immunoregulatory influence.

Dr. M. Yamazaki of Teikyo University in Japan, adopted the TNF (Tumour Necrosis Factor) which is produced by the leukocyte, as a measure of the activity of the body's

Table 1 Neutrophil accumulation by vegetable juice

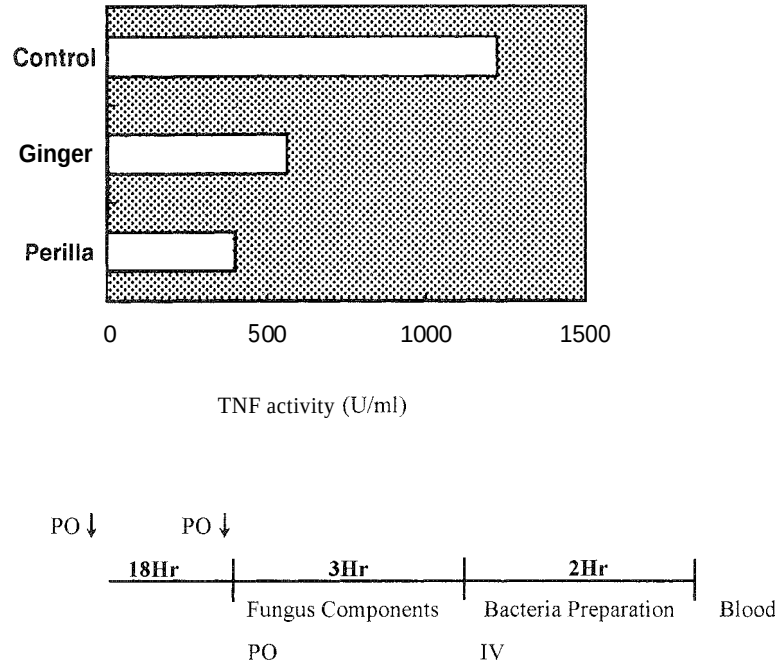
Sample	*% of Neutrophil*	ED_{50} *(ml/mouse)*
Control	2 ± 1	
Turnip	42 ± 7	1.4
Japanese radish	45 ± 8	0.7
Cucumber	51 2 5	0.6
Green pepper	62 ± 9	0.4
Eggplant	59 ± 3	0.4
Parsley	57 ± 7	0.3
Carrot	71 ± 8	0.2
Spinach	94 ± 3	0.18
Spring onion	78 ± 5	0.15
Cabbage	74 ± 8	0.09
Ginger	76 ± 4	0.08
Onion	82 ± 7	0.07
Perilla	89 ± 6	0.02
Garlic	72 ± 7	0.02

Control : Saline
E D : the effective dose when Neutrophil accounts for 50% of the all cells

Table 2 Nutritional value of green and purple Perilla leaves (100 g)

Moisture			87.5 g
Protein			3.8 g
Lipid			0.1 g
Carbohydrate	Carbohydrate		5.5 g
	Fibre		1.5 g
Mineral	Calcium		220 mg
	Phosphorous		65 mg
	Iron		1.6 mg
	Sodium		1 mg
Ash			1.6 g
Vitamin	A	Retinol	0 μg
		Carotene	8700* μg
		A-effect	4800 IU
	B1		0.12 mg
	B2		0.32 mg
	Niacin		1.0 mg
	C		55 mg

*Purple Perilla 7200



U/ml : Unit per ml (1 Unit is the 50% concentration that TNF affects L929 cells)
PO : per os
IV : intravenous

Figure 1 Inhibition of TNF production by food.

self-defence system (Ueda *et al.*, 1991a,b). We have focused on foods which inhibit excessive TNF production so as to reduce the adverse effects of allergies.

For screening for BRM, accumulation of blood neutrophil and TNF production activity were used and Perilla's inhibition of TNF production was found from this screening (Yamazaki *et al.*, 1992; Yamazaki and Ueda, 1995). Neutrophil accumulation of various vegetable components was examined and some of them showed high activity (Table 1). Perilla and ginger showed a higher TNF inhibiting activity as it showed in Figure 1 (Yamazaki, 1992). Dr. Yamazaki and our laboratory staff have been collaborating in discovering food substances which regulate the functioning of the immune system and Perilla was chosen for further studies.

Green and purple Perilla leaves have been in the diet of the Japanese people for a long time. The nutritional value of the leaves is given in Table 2. However, the leaves are ingested in small amount due to their unique odour and flavour which is mainly caused by perillaldehyde in essential oil of the leaves. The development of a Perdla extract has been focused on obtaining one with anti-allergic effects and the perillaldehyde has been excluded.

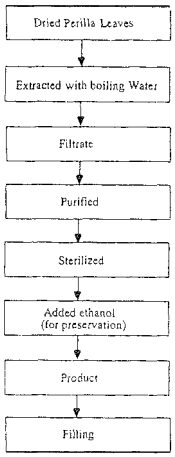


Figure *2* Manufacturing process of anti-allergic Perilla extract

MANUFACTURING PROCESS FOR PERILLA PRODUCTS

Material

The material used was limited to Perdla leaf from Hokkaido (Japan) from the view point of its quality, its anti-allergic effects and the control of its production. The variety used in the manufacturing process was *Perilla frutescens* var. *acuta* Kudo forma *viridis* Makino.

Extraction of Perilla Leaf

The decoction of the dried leaves afforded by boiling water gave an extract (Figure 2) with greater and-allergy activity than that obtained using aqueous ethanol and avoided the precipitation problems caused by chlorophyll etc., in the aqueous ethanol extract (Kosuna *et al.*, 1994).

Perillaldehyde is considered to be die main cause of contact dermatitis experienced by Perdla farmers (Okazaki and Matsunaga, 1981). During our research, perillaldehyde was proved not to inhibit TNF production as it showed in Figure 3 (Yamazaki, 1994). The extraction and purification process yielded an extract free of perillaldehyde, protein and lipids all of which can be allergens. The resulting extract was a light reddish brown in colour and has a slight characteristic odour.

Spray Drying

By the addition of dextrin to the aqueous Perilla extract, followed by spray drying, a stable powdered form was achieved, namely Perdla extract powders with 1 g equivalent to 1 ml of Perilla extract. This powder was available for capsule and tablet formulations and for use in a range of health products.

	TNF (U/ml)	Inhibition (%)
Perillaldehyde	2047	- 32
Perilla Juice	732	54
Control	1562	0

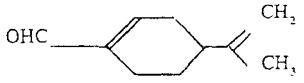


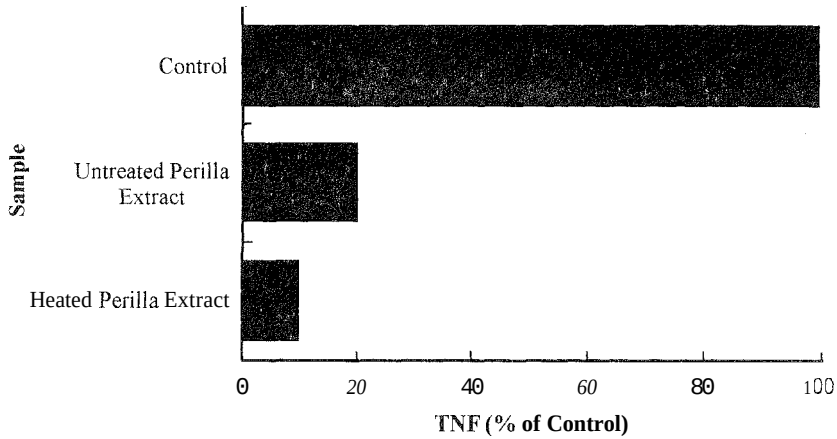
Figure 3 Inhibition of TNF activity by perillaldehyde.

Application of Perilla Extract to Various Products

A special feature of the aqueous extract was that it did not contain excessive colouring matter, flavour, nor stimulating substance. Hence it could be used in various products for daily use (Kosuna, 1993). For example, it was available for use in foods (drinks, breads, sweets etc.), cosmetics (hair and body shampoo, skin cream, skin lotion, soap etc.), and in medicines (external and internal medicines for allergic dermatitis) (Figure 4).

In recent years, as people consider health care more and more, Perilla extract has been increasingly used in various products. On the Japanese market Perilla is now used in 80 products (including the toiletries and cosmetic range) and in 15 other kinds (including health foods such as encapsulated products) and the range is still growing (March, 1995).

Figure 4 Some products developed from Perilla extract.



Control. Saline

Figure 5 Heat resistance of Perilla extract,

For the product of Perilla extract it was highly concentrated and remained sterile when in a small bottle. It was effective in a small dose and it was not injurious to health even if taken in excess (Kosuna, 1992). For practical use, 0.4 to 0.5 ml for adults and 0.1 to 0.3 ml for children could be added to water, juice, milk, tea, or soup. The effectivity did not change after adding to hot drinks.

INVESTIGATION OF PERILLA EXTRACT

Product Specification

Analysis showed that Perilla extract contained 0.4% of carbohydrates, 0.1% of ash, and 99.4% of water. Only 0.01 mg of vitamin B was present in 100 g. Formaldehyde, arsenic, lead, and residue of organochlorine pesticides were not detected. As microelements, 6 mg of phosphorus, and 43 mg of sodium were present in 100 g of Perilla extract.

Factors Affecting Product

Heat Resistance Examination

Perilla extract was heated for 10 minutes at 100° C. The TNF inhibiting activity of the heated sample was similar to that of the unheated one (Kosuna, 1992; Yamazaki, 1992). The active component has not been identified yet. However, it is presumed that the active component is contained in a saccharide which is stable for the time and temperature used as it showed in Figure 5 (Kosuna, 1993; Ueda and Yamazaki, 1993).

Effects of pH

When using Perilla extract in products, there was concern that the pH (hydrogen ion) of the mixture would have some influence on the Perilla extract. The influence of pH on the Perilla extract was studied. One month after adjustment of pH there was no precipitation at pH 5.0 to 11.0 but precipitation occurred at other values. The colour darkened between pH 2.0 and pH 12.0.

effect of Ion

These salts, NaCl, NH_4Cl and Na_2SO_4, when added to the Perilla extract caused no precipitation whereas precipitation occurred with $CaCl_2$ and $FeCl_2$ and with a high concentration of Na_2CO_3 and Na_2HPO_4.

Effect of Surfactants

When these surfactants were mixed with Perilla extract no precipitation occurred: sodium lauroylmethylalanine, 2-alkyl-N-carboxymethyl-N-hydroxyethyl imidazolynium betaine, polyoxyethylene-20 sorbitanmonostearate, polyoxyethylene-20 hydrogenatedcastoroil. Precipitation occurred with benzalkonium chloride.

Effects of Solvent

Solvents such as ethanol and isopropanol when mixed with the Perilla extract caused precipitation whereas propylene glycol and glycerol did not do so.

Preservation Stability Test

Perilla extract was obtained by using hot boiling water as mentioned previously. Heat sterilised bottles were filled aseptically. For use as an ingredient, 10% of ethanol was added as a preservative agent. Chemical additives were not used for disinfection nor stabilisation. For confirmation of stability and sterilisation, the variation of colour (O.D. at 400 nm) and contamination by bacteria were checked. Storage was carried out for 8 months. There was no variation in colour at 5°C but the colour darkened at 25°C and 37°C. After 8 months the fructose spot test showed the absence of bacteria.

Toxicity and **Safety**

Safety of Perilla extract was examined.

Acute Toxicity Test

At Teikyo University the Perilla extract was tested for acute toxicity by using 8 weeks old C3H/He inbred mice from Shizuoka Experimental Animal Farm (10/group) and intraperitoneal dosage of 10.0 ml, 5.0 ml, 1.0 ml, and 0.2 ml. The activity of the 10 ml

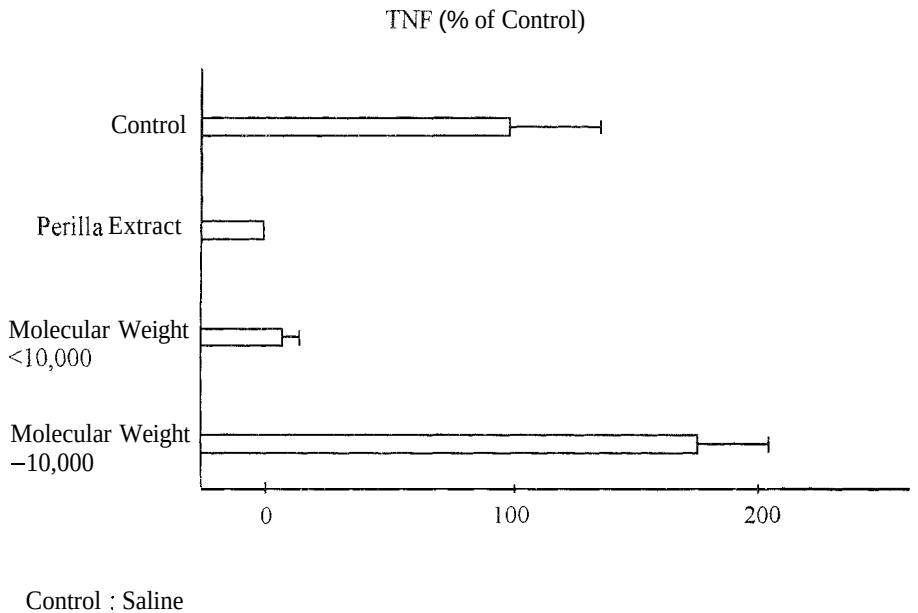


Figure 6 Inhibition of TNF production (with demarcation through ultrafilter membrane)

group slowed down for a few hours after receiving the Perilla extract, recovering to normal on the next day Observation was continued for one month and there was no weight loss or death.

Influence Of Continuous Administration

Also at Teikyo University two groups of 8 week old C3H/He inbred mice were allowed to take tap water containing 1.0% and 0.1% Perilla extract, respectively, freely for 31 days. The mice showed no aversion to this water. There was no difference in weight variation compared with a control group. The amount of feed taken was virtually the same also. The body condition and behaviour of the two test groups were normal and no different from the control group (Kosuna, 1992; Kosuna, 1994).

The internal organs, liver, spleen, kidney and thymus are closely associated with the immune system, such as the production of macrophages, B cells, T cells and cytolunes. After the administration of the Perilla extract there was no difference in the weight and appearance (before and after dissection) of these organs when compared with those from the control group. The administered amount used for mice (1.0% group) is equivalent to approximately 100 ml of Perilla extract given to a human. However, it is considered that this high dosage would have no adverse influence on the normal functions of the immune and metabolic system.

Table 3 Inhibition of TNF production of components in Perilla extract

Sample	*E D (mg/ml, mean ± SD)*
Perilla Extract	0.42 ± 0.27
Protocatechualdehyde	0.11 ± 0.04
Caffeic acid	0.13 ± 0.05
Rosmarinic acid	0.13 ± 0.04

E D : the effective dose of 50% inhibition of control TNF production from Macrophage by different compounds

The Active Principles

The inhibition of TNF production was measured using mice. Perilla extract was administered simultaneously with a priming agent (fungus components) normally giving rise to intense inflammation, TNF production was inhibited by 70–80%. It was fully inhibited (99.5%) by steroids. It can be said that it is preferable not to inhibit TNF production totally, because TNF is an important substance for the body's defence system.

In order to detect key compounds with anti-allergy properties, inhibition of TNF production was examined (Kosuna *et al.*, 1995) with selective demarcation through an ultrafilter membrane with a cut-off of molecular weight 10,000 (Figure6). Perilla extract was found to be effective for mice endoabdominal macrophage cultured in *vitro.* The examination results were similar to that of in *vivo* (Yamazaki, 1995). Thus, this in *vitro* examination was adopted because it was economical and simple. As a result, inhibiting TNF production was confirmed with material of molecular weight under 10,000.

So far, by chromatographic separation etc., ten fractions from the Perilla extract have been shown to inhibit TNF production. The main compounds identified have been protocatechualdehyde, rosmarinic acid, and caffeic acid. However, the natural plants contain a lot of compounds which are in a tiny amount and difficult to be isolated. It could be thought that some other compounds altogether inhibit TNF production with these three compounds. Their inhibition of TNF production is shown in Table 3, where the E D is the effective dose of 50% inhibition of control TNF production from macrophage by different compounds (Kosuna *et al.*, 1995).

SUMMARY

Perilla extract inhibits TNF production and has value as an anti-allergy agent. The extract is now used in various products such as foods, cosmetics, and other items and for these the demand is increasing. In a preliminary clinical trial, a perillaldehyde-free cream applied to the skin has been shown to be beneficial in the treatment of atopic dermatitis. Research is continuing on the anti-allergy principles of the extract and it is hoped to develop a more active extract in the future.

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