Published in final edited form as:
Phytother Res. 2009 March ; 23(3): 373–384. doi:10.1002/ptr.2635.

Complement-fixing Activity of Fulvic Acid from Shilajit and Other Natural Sources

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Abstract

Shilajit has been used traditionally in folk medicine for treatment of a variety of disorders, including syndromes involving excessive complement activation. Extracts of Shilajit contain significant amounts of fulvic acid (FA), and it has been suggested that FA is responsible for many therapeutic properties of Shilajit. However, little is known regarding physical and chemical properties of Shilajit extracts, and nothing is known about their effects on the complement system. To address this issue, we fractionated extracts of commercial Shilajit using anion exchange and size-exclusion chromatography. One neutral (S-I) and two acidic (S-II and S-III) fractions were isolated, characterized, and compared with standardized FA samples. The most abundant fraction (S-II) was further fractionated into three sub-fractions (S-II-1 to S-II-3). The van Krevelen diagram showed that the Shilajit fractions are products of polysaccharide degradation, and all fractions, except S-II-3, contained type II arabinogalactan. All Shilajit fractions exhibited dose-dependent complement-fixing activity *in vitro* with high potency. Furthermore, we found a strong correlation between complement-fixing activity and carboxylic group content in the Shilajit fractions and other FA sources. These data provide a molecular basis to explain at least part of the beneficial therapeutic properties of Shilajit and other humic extracts.

Keywords

Shilajit; humic substances; fulvic acid; complement-fixing activity; carbohydrates

Introduction

Over the past three decades, research on medicinal properties of natural products has increased significantly, and a large body of evidence suggests extracts from peat, sapropel, and shilajit humus may represent a source of novel compounds with medicinal properties [reviewed in (Schepetkin et al., 2002)]. Shilajit (common names: mumie, vegetable asphalt, mineral pitch) is a semi-hard brownish black resin formed through long-term humification of several plant types, mainly bryophytes, present in the vicinity of shilajit-exuding rocks (Ghosal et al., 1991b;Agarwal et al., 2007). Shilajit is found in specific mountain regions of the world at altitudes between 0.6 and 5 km (Ghosal et al., 1991b;Agarwal et al., 2007), and has been used therapeutically for centuries as part of traditional systems of medicine in many countries [reviewed in (Schepetkin et al., 2002;Agarwal et al., 2007)]. For example, Shilajit has been used as a treatment for genitourinary diseases, diabetes, digestive disorders, nervous diseases, tuberculosis, chronic bronchitis, asthma, anemia, eczema, bone fractures, and other diseases (Acharya et al., 1988;Goel et al., 1990).

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Although Shilajit samples from different regions of the world have similar physical properties and qualitative chemical composition, they differ in the ratio of individual components (Galimov et al., 1986). Shilajit humus consists of organic matter (60-80%), mineral matter (20-40%), and ~5% trace elements (Ghosal et al., 1991a;Frolova and Kiseleva, 1996). For therapeutic applications, Shilajit has been used in the form of an aqueous extract, and extracts of Shilajit have been shown to activate phagocytosis and cytokine release by murine peritoneal macrophages (Bhaumik et al., 1993), stimulate osteoblastic differentiation of mesenchymal stem cells (Jung et al., 2002), and induce proliferation of lymphocytes in the cortical thymus layer and increased migration of these cells into thymus-dependent zones of the lymph nodes and spleen (Agzamov et al., 1988).

The primary organic substance in aqueous extracts of Shilajit humus is fulvic acid (FA), and it has been suggested that FA may account for many biological and medicinal properties of Shalajit (Ghosal et al., 1988;Schepetkin et al., 2002). Indeed, FA has been used externally to treat hematoma, phlebitis, desmorrhexis, myogelosis, arthrosis, polyarthritis, osteoarthritis, and osteochondrosis. Likewise, FA has been taken orally as a therapy for gastritis, diarrhea, stomach ulcers, dysentery, colitis, and diabetes mellitus [reviewed in (Schepetkin et al., 2002;Agarwal et al., 2007)]. Despite the broad spectrum use of FA for a variety of medical conditions, far less is known regarding the mechanisms of action of FA. The few reports available have shown that humic substances can stimulate osteoclastic resorption of transplanted bones as well as hydroxyapatite (Schlickewei et al., 1993), and FA/humic substances isolated from soil and water reservoirs have been reported to stimulate neutrophil and lymphocyte immune function (Joone et al., 2003;Schepetkin et al., 2003).

Since the complement system is involved in many disease syndromes that have been traditionally been treated with extracts of Shilajit and other humic substances containing high levels of FA (e.g., arthritis (Mizuno, 2006), asthma (Wills-Karp, 2007), eczema (Ferguson and Salinas, 1984), and vascular disease (Acosta et al., 2004), we hypothesized that part of the beneficial effects of these natural products might relate to their ability to modulate complement. However, very little is known regarding the effects of FA/humic substances on the complement system *in vitro* or *in vivo*. Thus, we performed studies to fractionate and characterize physiochemical properties of humic substances extracted from Shilajit and then examined their complement-fixing activity in comparison with standard FA samples obtained from the International Humic Substances Society (IHSS).

Materials and Methods

Reagents

β-glucosyl Yariv reagent [1,3,5-tri-(4-β-D-glucosopyranosyloxyphenyl-azo)-2,4,6-trihydroxybenzene] was purchased from Biosupplies Australia (Parkville, Australia). Gum arabic was purchased from Fluka BioChemica (Buchs, Switzerland). Cetyltrimethylammonium bromide (CTABr), diethylaminoethyl (DEAE) cellulose, Sephadex G-50, galacturonic acid, galactose, arabinose, rhamnose, glucose, diphenylamine, aniline, anthrone, thiourea, trifluoroacetic acid (TFA), lipopolysaccharide (LPS) from *Escherichia coli* K-235, o-phenylene diamine, antibody-sensitized sheep erythrocytes, and gelatin veronal buffer (GVB) were purchased from Sigma Chemical Co. (St. Louis, MO). Heparin sodium salt from bovine lung was purchased from Calbiochem (San Diego, CA). The following fulvic acid (FA) standards were purchased from IHSS: Suwannee river FA (SRFA; IHSS code, 1S101F), Nordic Aquatic FA (NAFA; IHSS code, 1R105F), Florida (Pahokee) Peat FA (FPFA; IHSS code, 2S103F), Pony Lake FA (PLFA; IHSS code, 1R109F), and Waskish Peat FA (WPFA; IHSS code, 1R107F).

Fractionation of Shilajit Humus

Crude Shilajit was obtained from Agada Herbs (St. Joseph, MI). This product is a water extract of the raw resinous substance (Shilajit humus), collected in the Himalaya Mountains of Nepal. The Shilajit from this company has been used successfully for medicinal purposes in for many years. The isolation of humic substances from Shilajit was performed using the sequential of precipitation by ethanol and adsorption on DEAE cellulose (Hejzlar et al., 1994). Briefly, 1 kg of raw Shilajit extract was shaken for 2 hr at room temperature in 5 L of distilled H_2O, and any insoluble residue was separated from the supernatant by centrifugation. The supernatant was precipitated by addition of a 4-fold volume of ethanol and incubation overnight at 4°C. The precipitate was pelleted by centrifugation, re-dissolved in distilled H_2O, sonicated, and filtered through 0.2 μm membrane filters. The filtrate was concentrated in an Amicon concentrator with a 5 kDa cut-off polyethersulfone membrane. The concentrate was diluted by addition of a 10-fold volume of distilled H_2O and ultra-filtered again. This procedure was repeated at least 4 times to remove ethanol and H_2O-soluble low-molecular weight compounds.

One aliquot of the final concentrated filtrate was lyophilized to give a crude extract of Shilajit humic substances (designated as SHS), and the remainder of the extract was applied to a DEAE cellulose column (500 ml) equilibrated with 50 mM Tris-HCl, pH 7.0. The column was washed with 2 L of equilibration buffer to obtain the neutral, unbound fraction and then sequentially eluted with 2 L of equilibration buffer containing 2 M NaCl and 2 L of 0.2 N NaOH. The fractions were filtered through 0.2 μm membrane filters, concentrated in an Amicon concentrator, and subjected to 6 rounds of dilution and concentration, as described above. The final concentrated filtrates were lyophilized to give three fractions, designated as S-I (neutral fraction, eluted by equilibration buffer), S-II (acid fraction eluted by 2 M NaCl), and S-III (acid fraction eluted by 0.2 N NaOH).

Fraction S-II was further fractionated using size exclusion chromatography (SEC) on a Sephadex G-50 column (2.5×92 cm) equilibrated with 10 mM Tris-HCl buffer (pH 7.4) containing 150 mM NaCl at flow rate of 22 ml/hr. The elution profile was monitored by: (1) measuring absorbance at 254 nm, (2) measuring fluorescence (λ_{ex}=340 nm; λ_{em}=460 nm); and (3) determining carbohydrate content, as described below. The three fractions obtained, designated as S-II-1, S-II-2, and S-II-3, were pooled and concentrated using ultrafiltration (for fraction S-II-1 and fraction S-II-2) or ion exchange chromatography on DEAE cellulose column, followed by elution with 0.2 N NaOH and ethanol precipitation (for fraction S-II-3). For analysis of biological activity, the fractions were diluted in Hanks balanced salt solution (HBSS) to a concentration of 5 mg/ml and filtered through sterile 0.22 μm filters.

To evaluate the role of endotoxin, samples were applied to a column containing Detoxi-Gel Endotoxin Removing Gel (Pierce, St. Louis, MO) and eluted with 0.05 M phosphate buffer containing 0.5 M NaCl to decrease ionic interactions of sample molecules with the affinity ligand. Concentrations of eluted samples were adjusted using absorbance at 254 nm, and the samples were analyzed for biological activity as described below.

High Performance SEC (HP-SEC)

The homogeneity and average molecular weight of the polysaccharide fractions were determined by HP-SEC using a Shimadzu Class VP HPLC and TSK-GEL G3000W_{XL} column (7.8 mm × 300 mm) eluted with 50 mM sodium citrate buffer, pH 7.5, containing 0.15 M NaCl and 0.01% NaN_3 at a flow rate of 0.3 ml/min. Peaks were detected using a refractive index (RID-10A) detector (Shimadzu, Torrance, CA). The molecular weights of the fractions were estimated by comparison with retention times of pullulan standards (P-800, 400, 200, 100, 50, 20, and 10; Phenomenex, Torrance, CA) or polyethylene glycol standards (PEG-11000, 5000, 3600, 1000, and 600; Pressure Chemical Co., Pittsburg, PA).

Physical Characterization of Shilajit Fractions

For ^{1}H-nuclear magnetic resonance (^{1}H-NMR) analysis, samples (6 mg) were dissolved in 0.6 ml D_2O, filtered through 0.2 μm filters, and spectra were recorded on a Bruker DRX-600 spectrometer (Bruker BioSpin, Billerica, MA) at 20°C using 3-(trimethylsilyl)-propionic 2,2,3,3,-d_4 acid sodium salt as an internal reference. For ^{13}C-NMR, samples (50 mg) were dissolved in 1 ml D_2O, filtered through 0.2 μm filters, and spectra were recorded on a Bruker DRX-500 spectrometer (Bruker BioSpin, Billerica, MA) at 20°C.

UV–Vis spectra of samples dissolved in $NaHCO_3$ (25 mM, pH 8.5) were recorded on a SpectraMax Plus spectrophotometer (Molecular Devices, Palo Alto, CA) in a 1 cm quartz cuvette by scanning from 200 to 800 nm. The E4:E6 ratio was determined at 465 and 665 nm, as described by (Chen et al., 1977).

Fluorescence measurements were performed using an LS50 luminescence spectrometer (Perkin Elmer). Samples were dissolved in $NaHCO_3$ (25 mM, pH 8.5). Slit width for emission and excitation wavelengths was 10 nm. For determination of the humification index (HIX), we used the formula: $HIX=(\Sigma I_{435\rightarrow 480})/(\Sigma I_{300\rightarrow 345})$, where I is the fluorescence emission intensity with excitation at $\lambda_{ex} = 254$ nm (Ohno, 2002). Since fluorescence intensity can be attenuated by the solution itself (i.e., inner-filtering effect), we corrected for both primary and secondary fluorescence inner-filtering effects in order to obtain an accurate measurement of the fluorescence emission intensity (Ohno, 2002). For calculation of HIX values corrected for inner-filter effects, we performed linear extrapolation on plots of HIX versus transmittance at 254 nm for 6-7 different concentrations of each fraction. The corrected HIX values correspond to infinite dilution (i.e., approximating 100% transmittance) (Ohno, 2002). Synchronous fluorescence spectra were recorded from 250 to 600 nm at a scan rate of 240 nm/min. The excitation–emission wavelength difference ($\Delta\lambda$) was 20 nm (Chen et al., 2002).

Chemical Analysis of Shilajit Fractions

For elemental analysis, lyophilized samples were submitted to Desert Analytics (Tucson, AZ) for analysis. Carbon, hydrogen, nitrogen, phosphorous, sulfur, halogens and metals were measured by inductively coupled plasma atomic emission spectroscopy (ICP-AES). Oxygen content was taken as the difference from 100%.

The Bradford micro-protein assay was used to determine protein content, which bovine serum albumin as the standard (BioRad, Hercules, CA).

Carbohydrate content was determined by the phenol-H_2SO_4 method [Dubois et al., 1956], modified to a microplate format. Sample of 400 μl (500 μg/ml) were mixed with 200 μl 6% phenol solution and 1 ml concentrated H_2SO_4. D-glucose was used as standard. The reactions were incubated for 20 min at room temperature, and absorbance was measured at 488 nm.

The presence of arabinogalactan in the samples was detected by single radial gel diffusion in a 1% agarose gel containing 100 μg/ml β-glucosyl Yariv reagent, which selectively interacts with and precipitates compounds containing type II arabinogalactan structures (van Holst and Clarke, 1985). Four μl of each Shilajit fraction (10 mg/ml) were loaded into the wells, and the samples were incubated at room temperature for 24 hr in a humid atmosphere. A positive reaction was identified by a reddish circle around the well, and arabic gum (4 mg/ml) served as a positive control.

CTABr, a cationic detergent, was used to analyze carboxylic acid groups in Shilajit fractions and standard FA (Denobili et al., 1990). Stock solution of the sample (1 mg/ml, pH 7.1) was added to different amounts of 0.1% CTABr to produced 20 different CTA^+/sample ratios. Suspensions were left standing for 18 hr at 25°C in the dark before centrifugation at 17400 ×

g for 30 min. Absorbance was measured at 400 nm, and the number of carboxyl groups was determined to be at the minimum absorbance that coincides with quantitative precipitation with the same number of CTA^+ ions.

For monosaccharide composition analysis, samples were hydrolyzed at 100°C for 6 hr with 3 M TFA, and the resulting samples were separated by thin-layer chromatography (TLC) on Whatman silica gel 60 plates with monosaccharide standards for reference (Dogsa et al., 2005). The TLC plates were developed with butanol/acetic acid/water (3:1:1), and bands were visualized by spraying the plates with aniline-diphenylamine reagent (2% aniline, 2% diphenylamine, and 8.5% H_3PO_4 acid in acetone) and heating at 100°C for 10 min. Individual monosaccharide bands were scraped from the plate, extracted with H_2O, and quantified using a colorimetric method with monosaccharide standards. Briefly, the extracts were mixed with anthrone reagent (0.2% anthrone and 1% thiourea in H_2SO_4). After heating at 100°C for 10 min, absorbance was measured at 620 nm.

Complement-fixing Assay

The complement-fixing assay was performed as described (Diallo et al., 2001). Antibody-sensitized sheep erythrocytes were washed three times with GVB containing 0.5 mM Mg^{2+} and 0.15 mM Ca^{2+} (GVB^{2+}) before use. The erythrocytes were resuspended in GVB^{2+} at a concentration of 2×10^8 cells/ml, and human serum was diluted with GVB^{2+} to a concentration giving about 50% hemolysis. Triplicate samples containing 50 μl of each serially-diluted polysaccharide fraction were mixed with 50 μl diluted serum and added to microplate wells and incubated at 37°C. After 30 min, sensitized sheep erythrocytes (50 μl) were added to each well, and the samples were incubated for an additional 30 min at 37°C. After centrifugation (900×g for 5 min), 50 μl of the supernatants were mixed with 200 μl distilled H_2O in flat-bottom microplates, and absorbance was measured at 405 nm. 100% lysis was obtained by adding distilled H_2O to sensitized sheep erythrocytes. Samples containing GVB^{2+}, serum, and sensitized sheep erythrocytes were used as background controls ($A_{control}$), while heparin served as a positive control. Inhibition of hemolysis induced by the test samples was calculated by the formula: $[(A_{control} - A_{sample})/A_{control}]\times100\%$. A dose–response curve (6-7 points) was constructed to calculate the concentration of test sample able to give 50% inhibition of hemolysis (ICH_{50}). Low ICH_{50} means high complement fixing activity. Heparin, a highly sulfated glycosaminoglycan, was used as a positive control.

Statistical Analysis

Linear regression analysis was performed on the indicated sets of data to obtain correlation coefficients, 95% confidence intervals, and statistical significance (GraphPad Prism Software, San Diego, CA). Differences at $P<0.05$ were considered to be statistically significant.

Results and Discussion

Preparation and Partial Characterization of Shilajit Humic Substances

Shilajit humic substances (SHS) obtained from crude Shilajit humus were fractionated by ion exchange chromatography, resulting one neutral fraction (S-I) and two acidic fractions (S-II and S-III). The size distribution of molecules in these fractions and crude Shilajit humus was characterized by HP-SEC, and the elution profiles are shown in Figure 1. Crude Shilajit humus eluted over a broad range of molecular weights, extending from 1 to ~1,000 kDa. The small peak present at ~1,000 kDa likely represents stable macromolecular aggregates in the sample. The SHS elution profile contained four peaks, including three small, broad peaks with modes corresponding to M_r of ~800, 100, and 15 kDa and a major peak corresponding to ~1.1 kDa. The neutral fraction (S-I) elution profile had a bimodal profile, with peak modes corresponding to M_r of ~700 and 15 kDa. The elution profiles of acidic fractions, S-II and S-III, contained three peaks (modes corresponding to M_r of ~700, 100, and 2 kDa) and two peaks (modes corresponding to M_r of ~700 and 2 kDa), respectively.

Fraction S-II, the most abundant fraction isolated by DEAE cellulose chromatography (>90% of total yield), was further fractionated using Sephadex G-50 chromatography. Three sub-fractions were obtained and designated as S-II-1, S-II-2, and S-II-3, based on total carbohydrate, UV absorbance (254 nm), and fluorescence (λ_{ex}=355 nm, λ_{em}=450 nm) elution profiles (Figure 2A). The HP-SEC refractive index elution profile of sub-fraction S-II-1 was similar to that of its parent fraction (S-II) in the M_r region of 20 to ~800 kDa (Figure 2B). In contrast, both fractions S-II-2 and S-II-3 eluted primarily as a single peaks, with modes corresponding to M_r of ~1.8 and 3.1 kDa, respectively (Figure 2B). Note that the average M_r of sub-fractions S-II-2 and S-II-3 is less than the nominal M_r cutoff of the membrane used for concentrating the crude SHS. Thus, it is likely that fraction S-II consisted of non-covalent complexes or micelles that were dissociated under the buffer/salt/mechanical conditions of the final Sephadex G-50 chromatography step. Indeed, it has been previously reported that concentrated solutions of humic substances can form micelles that cannot be filtered even through 100 kDa membranes (Benedetti et al., 2002;Brown et al., 2004). Furthermore, charge effects, solution conditions, and membrane surface characteristics have also been shown to impact ultrafiltration and SEC fractionation, with various organic components being affected differently (Buffle and Leppard, 1995;Schafer et al., 2002;Benedetti et al., 2002).

Lyophilization of the fractions resulted in powders differing in color from white (S-I) to black (S-III), and analysis of carbohydrate and protein content indicated a wide range in composition between fractions (Table 1). In general, the primary fractions and sub-fractions with the lowest carbohydrate content contained the highest levels of protein (e.g., fractions S-III and S-II-3). Sugar composition analysis revealed that polysaccharides in all Shilajit fractions, except for fraction S-II-3, consisted primarily of glucose (Glc), galactose (Gal), xylose (Xyl), and rhamnose (Rha), with Glc and Gal being the dominant monosaccharides. In contrast, fraction S-II-3 contained a minimal amount of Gal, but had a much higher level of glucosamine (GlcA) in mol % than all other fractions (Table 2). Analysis of the Shilajit fractions using the Yariv test showed that all fractions, except for fraction S-II-3, contained type II arabinogalactan (Table 1). This finding supports the current hypothesis that Shilajit originates from a vegetative source (Agarwal et al., 2007). Indeed, latex bearing plants (*Euphorbia royleana* Boiss, *Trifoleum repens*) and bryophytes present in the vicinity of Shilajit-exuding rocks contain a large amount of arabinogalactan (Saare-Surminski et al., 2000;Popper and Fry, 2003).

Fluorescence spectrometry was used to determine the extent of humification in the Shilajit fractions and standard FA samples (Zsolnay et al., 1999;Ohno, 2002). We found that corrected values of HIX and the E4:E6 ratios were lower for the Shilajit fractions, as compared to standard FA obtained from IHSS (Figure 3). These lower HIX values indicate that the Shilajit fractions are enriched in polysaccharides and probably other weakly chromophoric biomolecules (Ohno et al., 2007).

Elemental Composition

The elemental composition of the primary Shilajit fractions (S-I, S-II, and S-III) is shown in Table 3. We calculated the atomic ratios of H/C, O/C, and N/C, which have been commonly used as indicators of structural characteristics of humic substances and their diagenetic history (Kim et al., 2003). The van Krevelen diagram, created by plotting H/C vs. O/C, showed that the humic substances from Shilajit were located clustered near the carbohydrate region, suggesting that they could be products of polysaccharide degradation and/or contain native polysaccharides (Figure 4). The average O/C ratio, which is indicative of carbohydrate content, carboxylic groups, and the degree of oxidation, was higher in the Shilajit fractions than in standard FA and humic acid samples (Figure 4). Conversely, the Shilajit fractions had lower

C/N ratios than the standard FA and humic acid samples, except for Pony Lake FA, which is derived primarily from carbohydrates and proteins of algae and cyanobacteria (Brown et al., 2004;McKnight et al., 1994).

NMR Analysis

The ^{1}H-NMR spectrum of crude Shilajit was close to that of Shilajit obtained from other mountain regions (e.g., see Jung et al., 2002]) and contained much stronger signals in the aliphatic (0.5-2.8 ppm) and aromatic (6-8 ppm) regions, as compared to spectra of the isolated Shalajit fractions. In general, the spectra of crude SHS and primary fractions S-II and S-III were similar to each other, and proton signals in the region from 0.0 to 5.5 ppm were more separated than in the same region of spectra from fraction S-I (Figure 5A).

The chemical shifts of anomeric protons were evaluated according to data previously reported for humic substances and polysaccharides (Gane et al., 1995;Dong and Fang, 2001). ^{1}H-NMR spectra of the Shilajit fractions indicated the presence of alkyl components (0.5-2.3 ppm), including methylene groups from methylenic chains (0.94-1.38 ppm) and terminal methyl groups (0.0-0.94 ppm). Spectra of the fractions indicated a significant amount of methylene and methyl groups α to carbonyl groups and/or attached to aromatic rings, typically resonating in the 1.8-2.7 ppm region. The spectrum of fraction S-II contained strong sharp signals at 1.8-1.9 ppm and 3.2 ppm, which can arise from protons belonging to repetitive chemical fragments, such as protons on CH_3–CO– and CH_3–O– groups that are formed during humification, possibly by oxidative degradation (Ruggiero et al., 1980). In comparison, these peaks were absent in fraction S-I, which contains much more native arabinogalactan. The signal at 2.1 ppm in the spectra of fractions S-II and S-III indicates the presence of α-methyl protons in ketones. Fraction S-III exhibited small, unique proton signals in the region of 2.1-3.1 ppm. For example, a pair small doublets at 2.55-2.68 ppm is consistent with the presence of methylene protons (–CH_2–) in acyl groups (R-CH_2-C=O), such as in free or esterified carboxylic groups (C=O). Spectra of all Shilajit fractions and the parent Shilajit sample showed a broad proton resonance between 3.3 and 4.2 ppm, with a maximum near 3.6-3.7 ppm. Resonances in this region derive from protons belonging to methyl and methylene groups connected to electronegative atoms, primarily oxygen, which are present in carbohydrates, methoxy compounds, carboxylic acids, and organic amines (Sciacovelli et al., 1977;Wilson et al., 1983;Grasso et al., 1990;Yamauchi et al., 2004). The signals in the region of 4.0-5.5 ppm are partially due to protons on alcoholic OH groups. Signals for aromatic protons at 5.6-6.1 ppm were barely visible in the ^{1}H-NMR spectra and appeared as weak resonances in all three samples. However, the spectrum of fraction S-III showed a broad resonance in the 5.8-6.9 and 6.9-8.6 ppm regions, which are normally attributed to protons in olefinic and aromatic moieties, respectively. Spectra of fractions S-I and S-II showed very small broad signal at these regions, possibly because the aromatic groups in these samples was highly oxidized. The sharp peak at 8.35 ppm in the spectrum of fraction S-III suggests the presence of formyl groups covalently bonded the macromolecules of humic substances (Jokic et al., 1995).

The ^{1}H-NMR spectra of sub-fractions S-II-1 and S-II-2 were similar to that of parent fraction S-II, but with less prominent peaks at 1.9 and 3.2 ppm (data not shown). The spectrum of fraction S-II-3 indicated the presence of aromatic protons and much stronger signals at 1.1 and 3.2 ppm (data not shown).

^{13}C-NMR (500 MHz) spectra of fractions S-II and S-III are shown in Figure 5B. The chemical shifts were evaluated according to data previously reported for humic substances and native polysaccharides (Baddi et al., 2004;Jokic et al., 1995;Polle et al., 2002). These spectra were characterized by the presence of many signals in the area of aliphatic carbons (0-50 ppm), carbohydrate carbons (60-96 ppm), anomeric carbons (96-108 ppm), aromatic carbons (108-145 ppm), and carboxyl and carbonyl carbons (163-190 ppm). However, signals for methoxyl carbons (50-60 ppm), phenolic carbons (145-163 ppm), and ketone carbons (190-220 ppm) were absent. The alkyl region (0-50 ppm) of fraction S-II showed a maximum at 18 ppm, which can be attributed to acetate groups in carbohydrates and corresponds to the CH_3–CO– groups with sharp signals at 1.8 ppm in the ^{1}H-NMR spectrum of the fraction. The intensity of the peak at 160-200 ppm, attributed to carboxylic, amidic, and ester carbons, was greater in fraction S-III, as compared to fraction S-II. This feature is likely due to an increase in carboxylic groups and correlates with the strong anionic properties of this fraction. Signals in the region of 60-108 ppm are usually assigned to O- and N-substituted carbons. Since the nitrogen content of these fractions is relatively low and the C/N ratio is relatively high (Table 2), it is likely that most carbon resonances this region arise from carbohydrates and carboxylic groups. Sharp peaks at approximately 62 and 73 ppm could arise from carbon C_6 in carbohydrates and protonated ring carbons (C_2-C_5) of carbohydrates (Jokic et al., 1995).

In comparison with ^{13}C-NMR spectra of natural FA from different sources (for example, see (Baddi et al., 2004), fractions S-II and S-III contained lower levels of aromatic carbon. Thus, ^{13}C- and ^{1}H-NMR data suggest predominantly carbohydrate-derived material in isolated Shilajit fractions with low contribution of aromatic carbons.

Complement-fixing Activity of Shilajit Fractions and FA from IHSS

Crude SHS and all fractions isolated from the Shilajit showed dose-dependent fixation of human complement *in vitro* with ICH_{50} values ranging from 15.4 to 273 μg/ml (Table 4). The most potent complement-fixing ability was found in the relatively low-molecular weight fraction S-II-3, which contained the lowest amount of carbohydrate. In contrast, the neutral fraction S-I failed to fix complement, even at the maximal the concentration tested (500 μg/ml) (data not shown). A plot of carbohydrate content in the Shilajit fractions versus the reciprocal values of ICH_{50} ($1/ICH_{50}$) demonstrated a good negative linear correlation (r= -0.848; n=7; $P<0.02$) between these values (Figure 6).

Five FA standards from IHSS were also tested for complement-fixing activity. All samples exhibited complement-fixing activity (ICH_{50}) (Table 4). It is interesting to note that the least active standard (Pony Lake FA) is formed in an Antartic lake without lignin sources and presumably consists of diagenetic products from algae and bryophyte polysaccharides (McKnight et al., 1994).

Plots of HIX versus ICH_{50} did not demonstrated any correlation when the plots contained the Shilajit fractions together with FA standards (r= 0.201, n=11 for E4:E6 *vs.* ICH_{50}; r=0.314, n=11 for HIX *vs.* ICH_{50}). However, plots of HIX versus ICH_{50} did show some correlation when the plots contained the Shilajit fractions only (r=0.659). Furthermore, the integrated emission in area of the red-shift (435-480 nm) of synchronous fluorescence spectra and $1/ICH_{50}$ were significantly correlated in separated plots contained the Shilajit fractions and FA standards, r=0.838 ($P<0.02$) and r=0.962 ($P<0.01$), respectively (Figure 7). Thus, preferential complement-fixing activity is found in the material with higher levels of humification.

Since endotoxin can be a contaminant of isolated organic materials, we determined whether endotoxin could be contributing to the biological activity of the Shilajit fractions. First, LPS from *E. coli* was evaluated for complement-fixing activity; however, no activity was found over the concentrations tested (2-250 μg/ml) (data not shown). To further verify that endotoxin contamination of the samples did not contribute to complement-fixing activity, we applied fraction S-II-3 to a column of endotoxin-removing gel and analyzed the eluted sample. As shown in Figure 8, complement-fixing activity of fraction S-II-3 after removal of possible endotoxin was essentially the same as that of control fraction S-II-3. Thus, these data clearly demonstrate that endotoxin was not responsible for biological activity of the Shilajit fractions.

Content of Carboxylic Groups in Shilajit Fractions and FA

As noted above, the highest complement-fixing ability was found in the relatively low-molecular weight fraction S-II-3, which contains the lowest amount of carbohydrate. During humification, a progressive transformation of polysaccharides into other oxygenated compounds, particularly carboxylic groups, takes place (Castaldi et al., 2005). Indeed, (poly) carboxylic acids with a very limited number of hydroxyl groups are the major compound class in FA (Reemtsma et al., 2006). Thus, we estimated content of carboxylic groups in the Shilajit fractions and FA from HISS. The content of carboxylic groups in fraction S-II-3 was close to the content in FA from other natural sources, such as Suwannee River, Waskish Peat, and Nordic Aquatic FA (Table 4). Plots of carboxylic group content versus the reciprocal of ICH_{50} ($1/ICH_{50}$) demonstrated a good positive linear correlation r=0.880 (n=12; $P<0.001$) between these values (Figure 9). Thus, these results suggest that carboxylic groups are important for complement-fixing activity of the Shilajit fractions and FA from other natural sources. One possibility is that carboxylic groups play a role in interaction with complement components. For example, carboxylic groups have been reported to be the main molecular fragments required for interaction of humic substances with cell membranes (Muscolo et al., 2007), complexation with cationic species (Livens, 1991;Prado et al., 2006;Esteves da Silva and Oliveira, 2002), and association with inorganic surfaces (Fu and Quan, 2006). Additionally, fraction S-II-3 fraction contains the highest level of glucosamine among the Shilajit fractions (Table 3), suggesting the possibility that glucosamine residues may also be involved in the biological activity observed. Indeed, previous studies have shown that various antigens with complement-fixing capacity were also enriched in glucosamine (Shinagawa and Yanagawa, 1972;Hammerberg et al., 1980).

The potent complement-fixing activity of humic substances isolated from Shilajit may contribute to the therapeutic potential of Shilajit extracts. The complement system plays an essential role in innate immunity, contributing to inflammatory responses and the destruction and removal of pathogens [reviewed in (Gasque, 2004)]. However, excessive or uncontrolled complement activation can also contribute to host tissue damage, and therapeutic strategies have been developed to inhibit this process (Mollnes and Kirschfink, 2006). Likewise, the removal of complement by fixation has also been proposed to be a potential therapeutic strategy for treating inflammatory diseases (Nergard et al., 2004). A number of reports have shown polysaccharides from different plants can enhance wound healing, and some of these polysaccharides also have potent complement-fixing activity (Table 5). For example, Samuelsen et al. (Samuelsen et al., 1995) reported that the wound healing properties of *Plantago major* L. polysaccharides were at least partly due to their ability to fix complement. Similarly, wound healing properties of polysaccharides from *Biophytum petersianum* Klotzsch were reported to be related to their effects on the complement system (Inngjerdingen et al., 2006). Indeed, Wagner (Wagner, 1990) suggested that the anti-complement properties of plant-derived polysaccharides significantly contribute to their anti-inflammatory properties. Our studies suggest that products of oxygenated degradation of plant polysaccharides also have potent complement-fixing properties *in vitro* and are among the most active of the natural products reported to date.

Acknowledgements

We would like to thank Dr. Andrei Khlebnikov (Department of Chemistry, Altai State Technical University, Barnaul, Russia) for reviewing this manuscript and providing helpful suggestions. We would also like to thank Dr. Scott Busse (Montana State University, Bozeman, MT) for help in running NMR samples. This work was supported in part by Department of Defense grant W9113M-04-1-0001, National Institutes of Health grant RR020185 and contract HHSN266200400009C, an Equipment grant from the M.J. Murdock Charitable Trust, and the Montana State University Agricultural Experimental Station. The U.S. Army Space and Missile Defense Command, 64 Thomas Drive, Frederick, MD 21702 is the awarding and administering acquisition office. The content of this report does not necessarily reflect the position or policy of the U.S. Government.

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Abbreviations

FA	fulvic acid
IHSS	International Humic Substances Society
SEC	size exclusion chromatography
HP-SEC	high performance SEC
HIX	humification index
NMR	nuclear magnetic resonance
CTABr	cetyltrimethylammonium bromide
DEAE	diethylaminoethyl
GVB	gelatin veronal buffer
TFA	trifluoroacetic acid
LPS	lipopolysaccharide
SRFA	Suwannee river FA
NAFA	Nordic Aquatic FA
PLFA	Pony Lake FA
FPFA	Florida Peat FA

WPFA	Waskish Peat FA

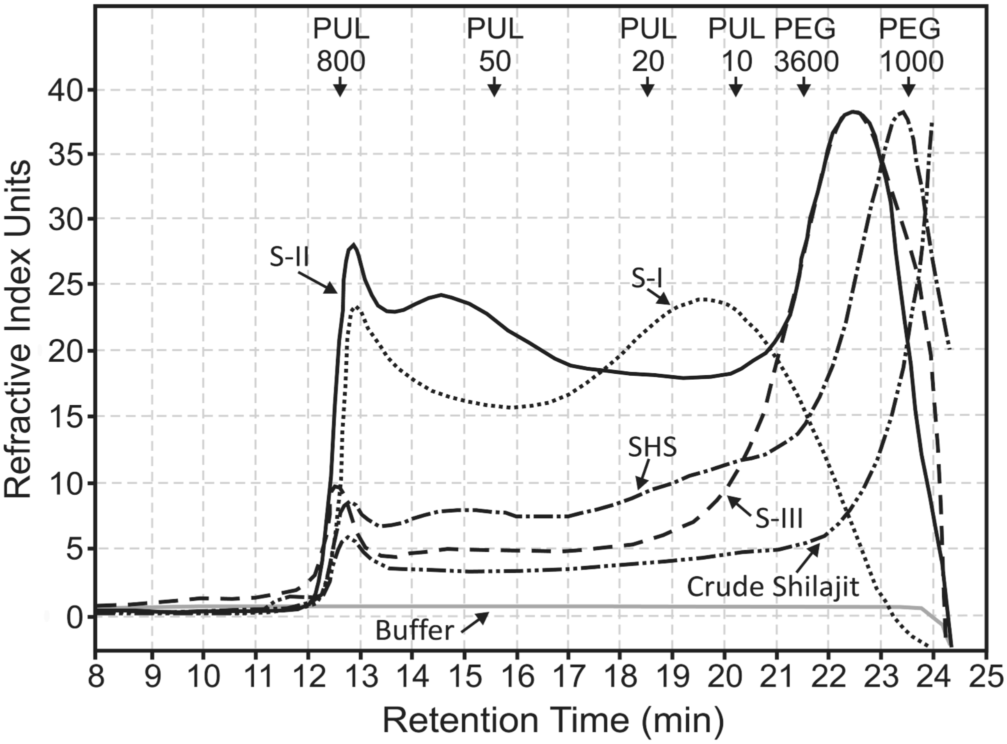


Figure 1.

Analysis of Shilajit fractions by size-exclusion chromatography. Crude Shilajit, total humic substances from the crude Shilajit (SHS), and the three primary fractions (S-I, S-II, and S-III) isolated by ion exchange chromatography were analyzed by HP-SEC and monitored with a refractive index detector, as described. Peak retention times of the indicated pullulan (PUL) and polyethylene glycol (PEG) standards are shown for reference.

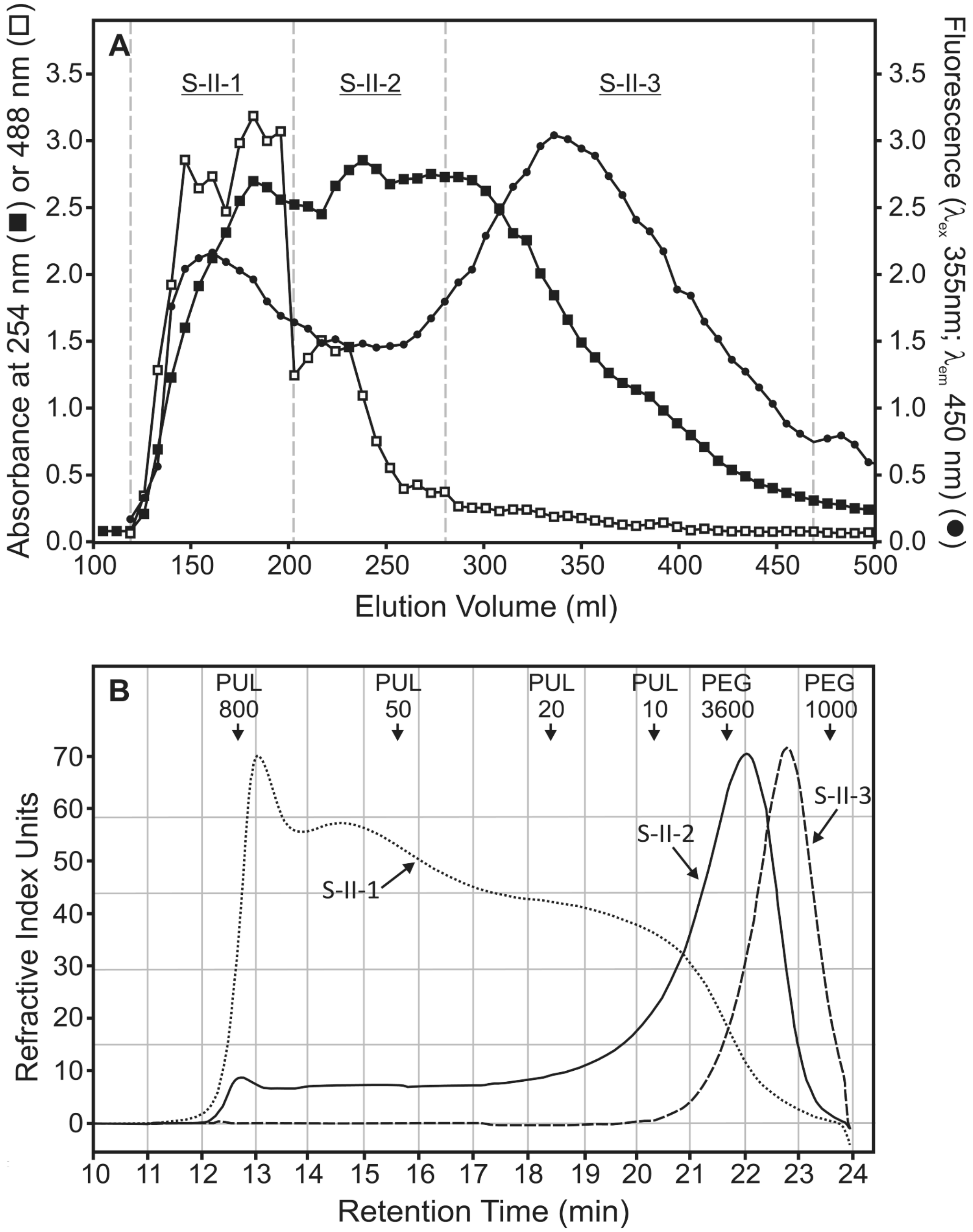


Figure 2.

Chromatographic separation of Shilajit fraction S-II. **Panel A.** Shilajit fraction S-II was separated by SEC on Sephadex G-50 and monitored for absorbance at 254 nm (■) and fluorescence (●). Total carbohydrate content in each fraction was determined by the phenol-H_2SO_2 method (detected at 488 nm) (□). Fractions were combined as indicated to obtain the S-II sub-fractions selected for further analysis (designated S-II-1, S-II-2, and S-II-3). **Panel B.** S-II sub-fractions were analyzed by HP-SEC and monitored with a refractive index detector, as described. Peak retention times of the indicated pullulan (PUL) and polyethylene glycol (PEG) standards are shown for reference.

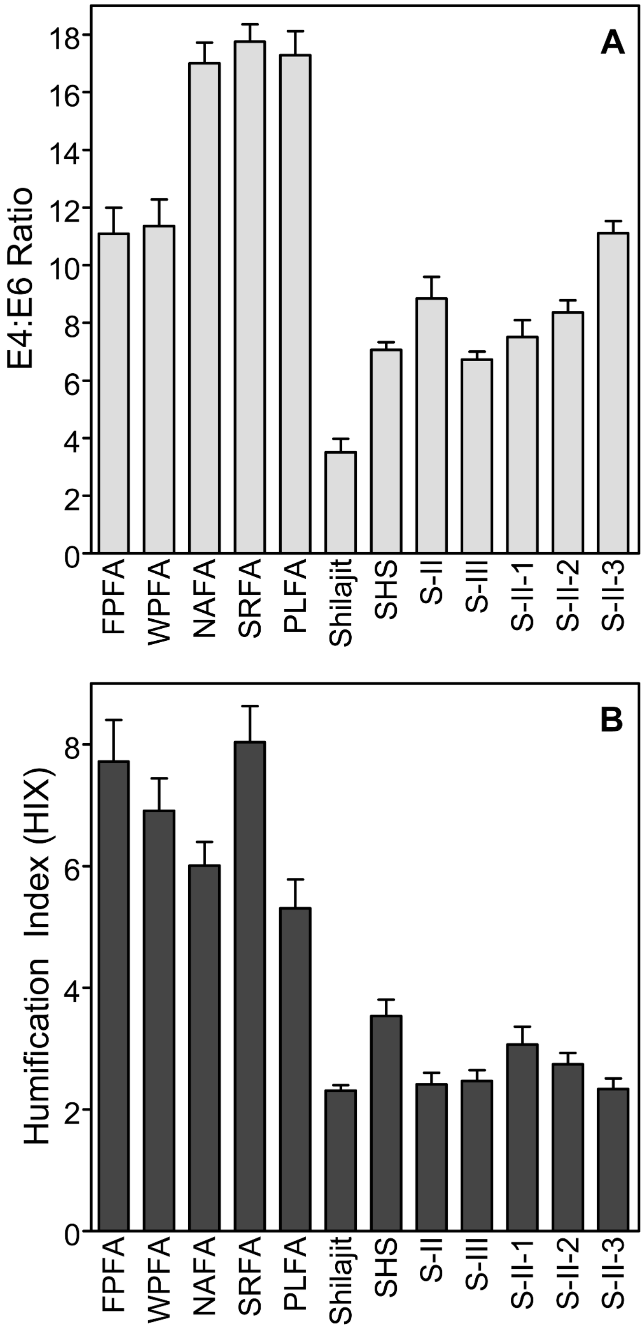


Figure 3.

Humification index (HIX) and E4:E6 ratio of Shilajit fractions and standard FA. Values were determined for each sample, as described under Materials and Methods. The data are presented as the mean ± SEM of 3 independent experiments.

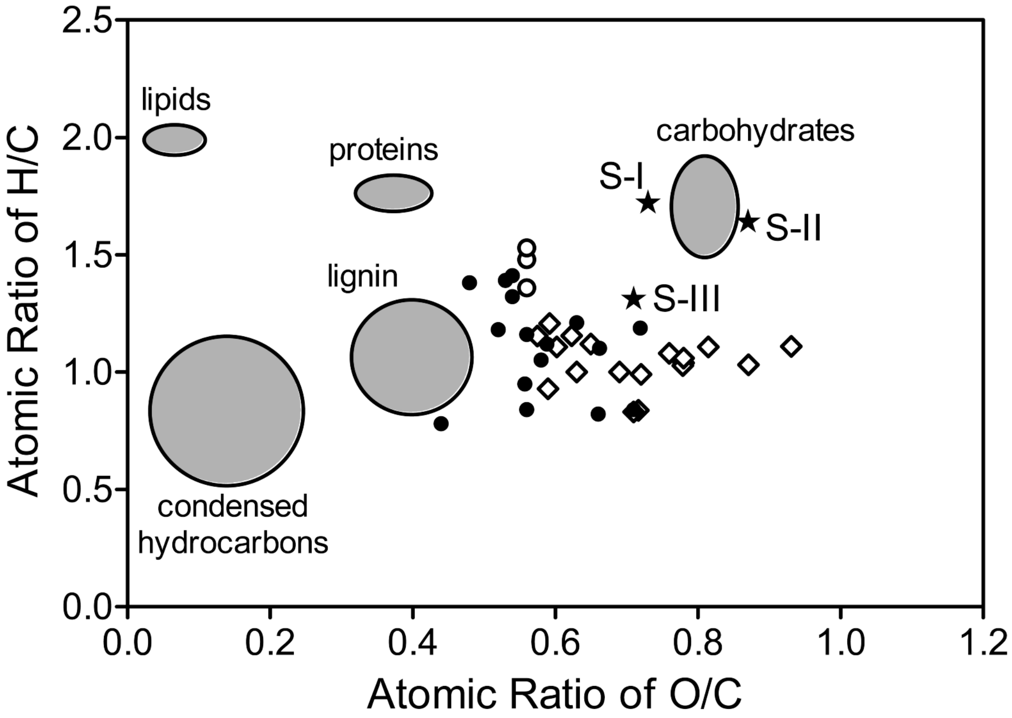
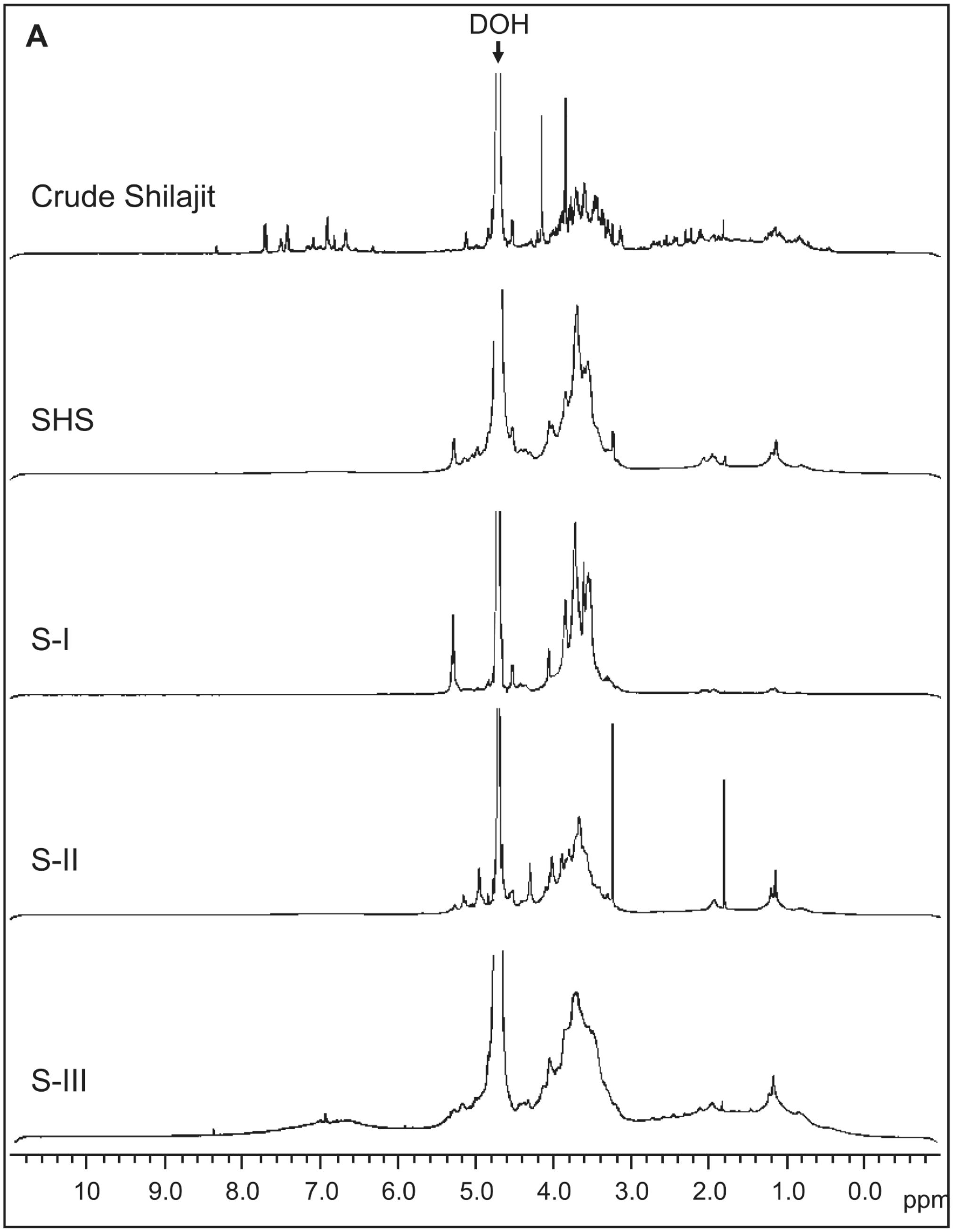


Figure 4.

The van Krevelen diagram of atomic ratios of H/C versus O/C for the Shilajit fractions. Plots for humic acids are shown as solid circles (●), FA (except for Pony Lake FA) are shown as open diamonds (◇), Pony Lake FA are shown as open circles (○), and the Shilajit fractions are shown as stars (⋆). The positions of the Shilajit fractions were determined from data of element analysis (Table 3). The positions of FA and humic acids from other natural sources were using previously published data (Lawrence, 1989;Provenzano and Senesi, 1999;Ma et al., 2001;Brown et al., 2004). The locations of regional plots for primary organic substances on the diagram (lipids, proteins, carbohydrates, condensed hydrocarbons and lignin) were reproduced from (Kim et al., 2003).


A
DOH
Crude Shilajit
SHS
S-I
S-II
S-III
10
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
ppm

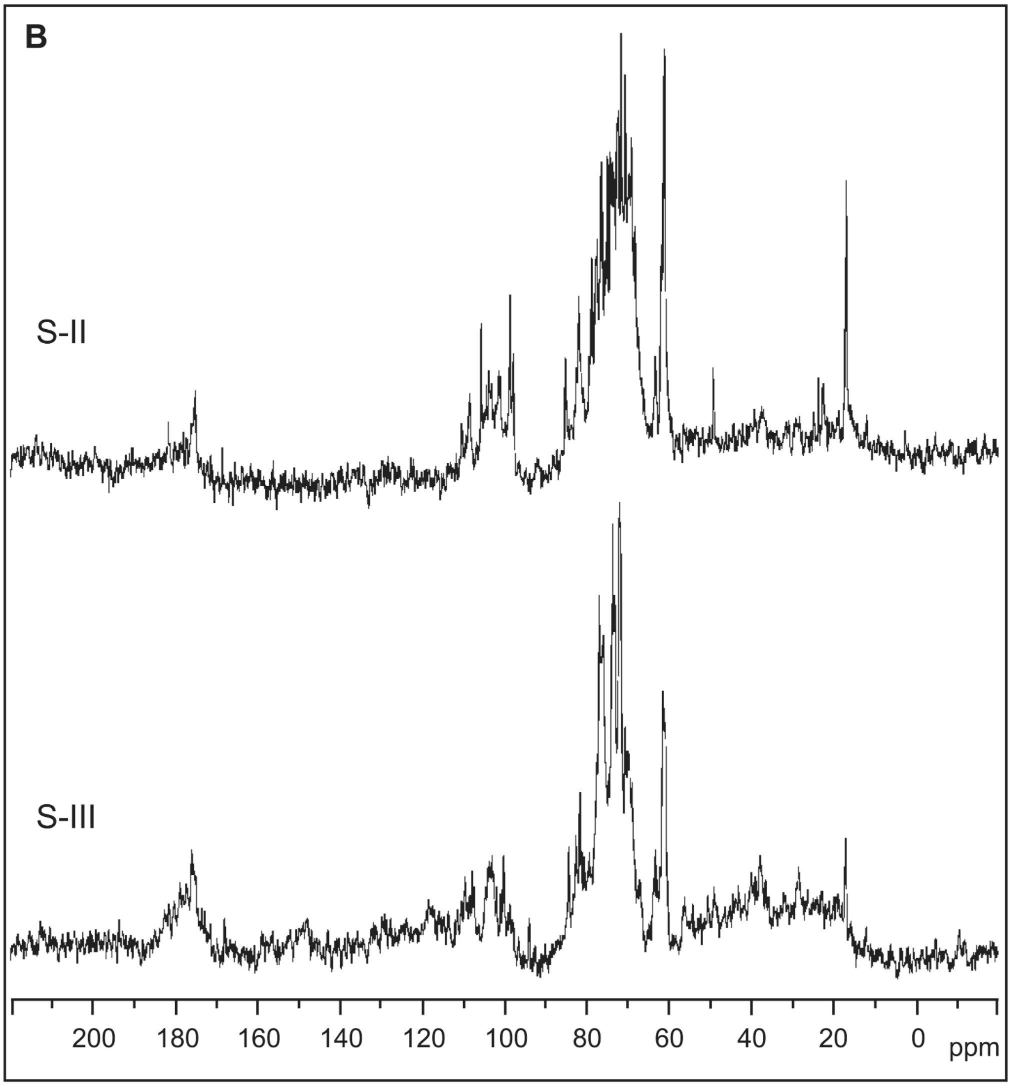


Figure 5.
^{1}H-NMR and ^{13}C-NMR spectra of primary Shilajit fractions. ^{1}H-NMR spectra of crude Shilajit extract and SHS are shown for comparison.

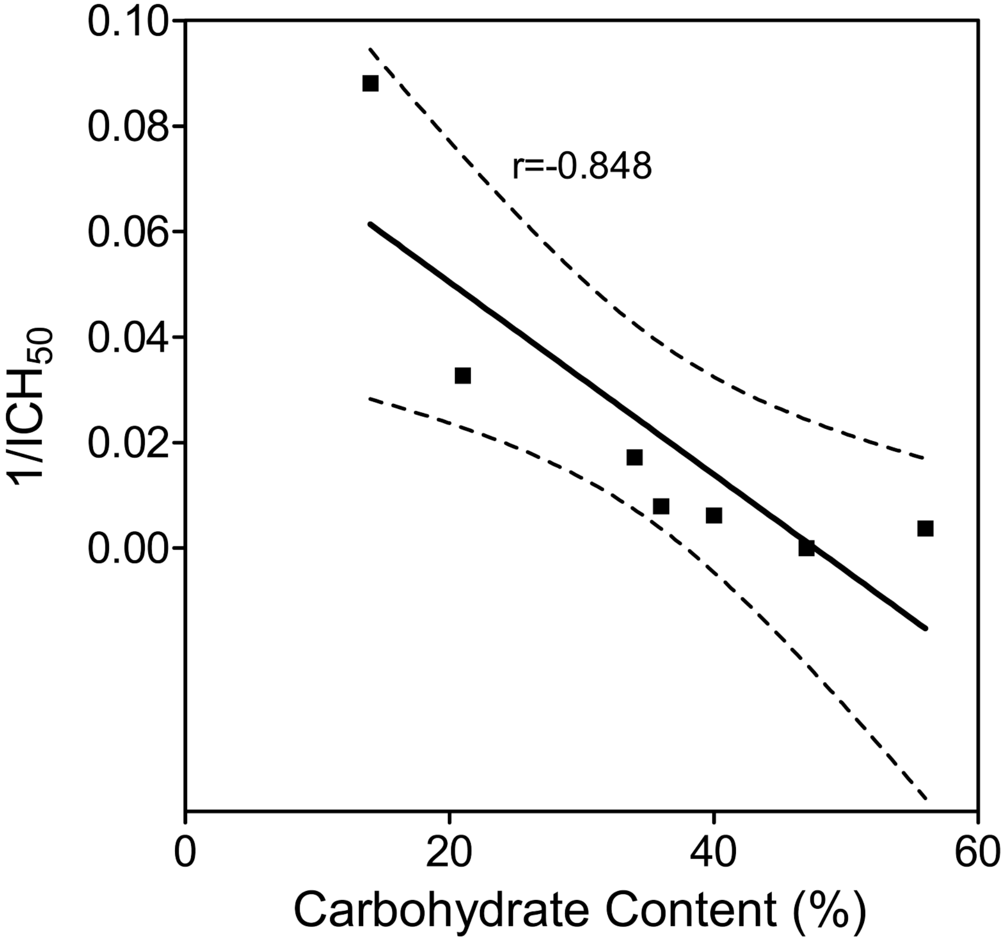


Figure 6.

Plot of complement-fixing activity of Shilajit fractions versus carbohydrate content in the fractions. Complement-fixing activity is represented as inverse ICH_{50} ($1/ICH_{50}$). Dashed lines indicate area of the 95% confidence band.

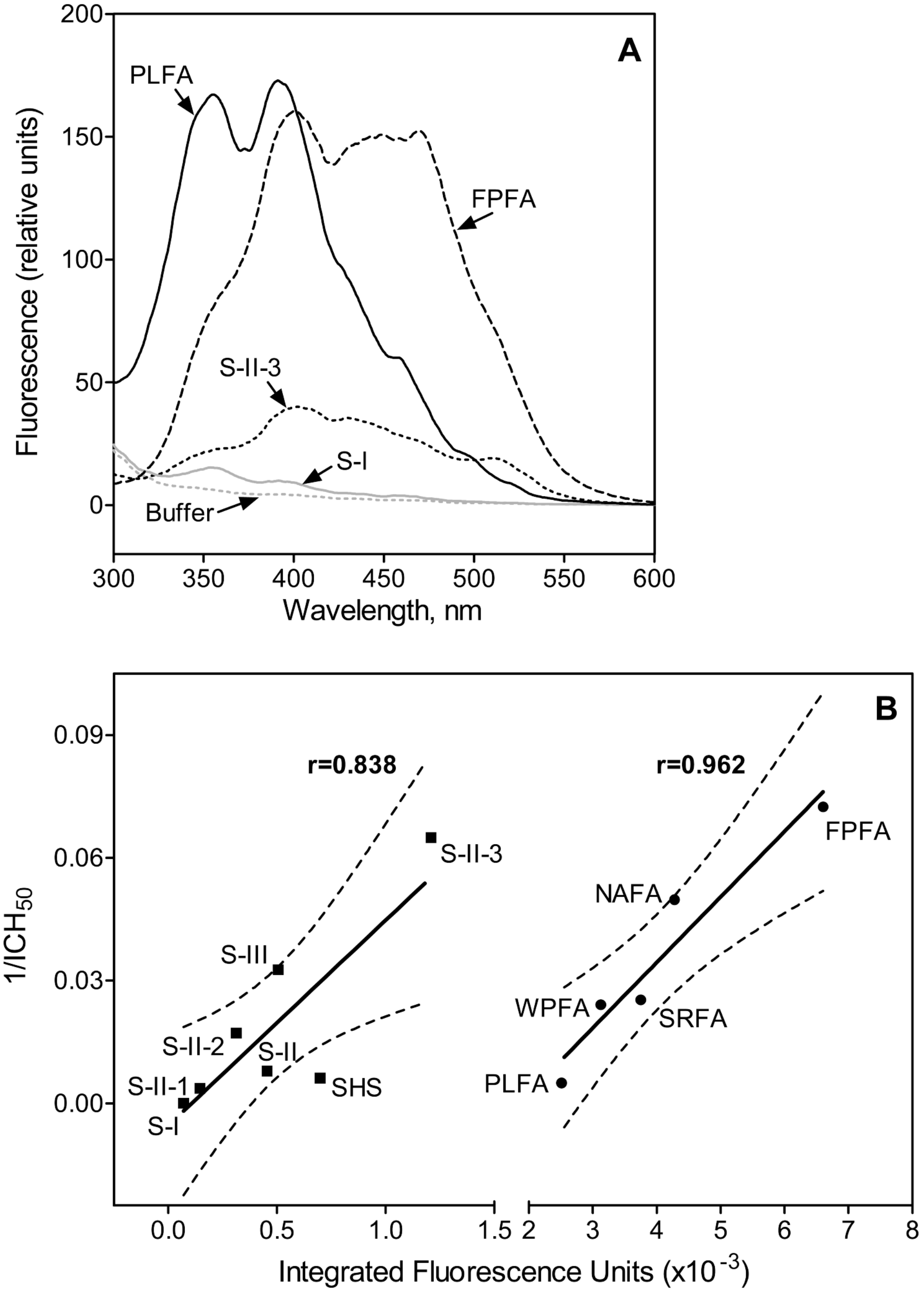


Figure 7.

Fluorescence spectra and plot of complement-fixing activity versus integrated fluorescence for the Shilajit fractions. **Panel A.** Solutions of Shilajit fraction S-I, sub-fraction S-II-3, Pony Lake FA (PLFA), and Florida Peat (FPFA) (20 μg/ml in 25 mM $NaHCO_3$) were analyzed with a scanning fluorometer, and the synchronous spectra ($\Delta\lambda$=20 nm) are shown. **Panel B.** Complement-fixing activity, represented as inverse ICH_{50} ($1/ICH_{50}$) was plotted versus integrated fluorescence of the synchronous spectra from 435-480 nm. Dashed lines indicate area of the 95% confidence band.

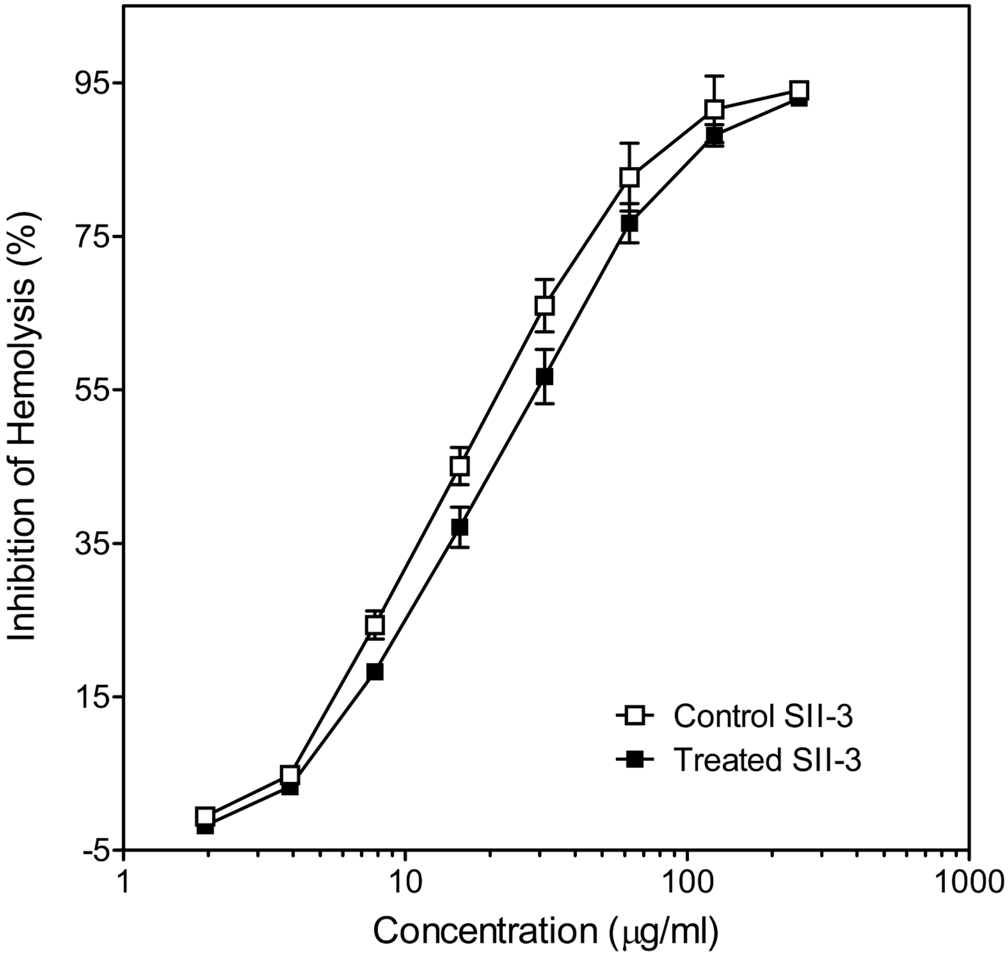


Figure 8.

Evaluation of the role of endotoxin in complement-fixing activity of sub-fraction S-II-3. Complement-fixing activity was determined in control samples of fraction S-II-3 (□) and samples treated with endotoxin-removing gel (■). The data are presented as the mean ± SEM of 3 samples from one experiment that is representative of 2 independent experiments.

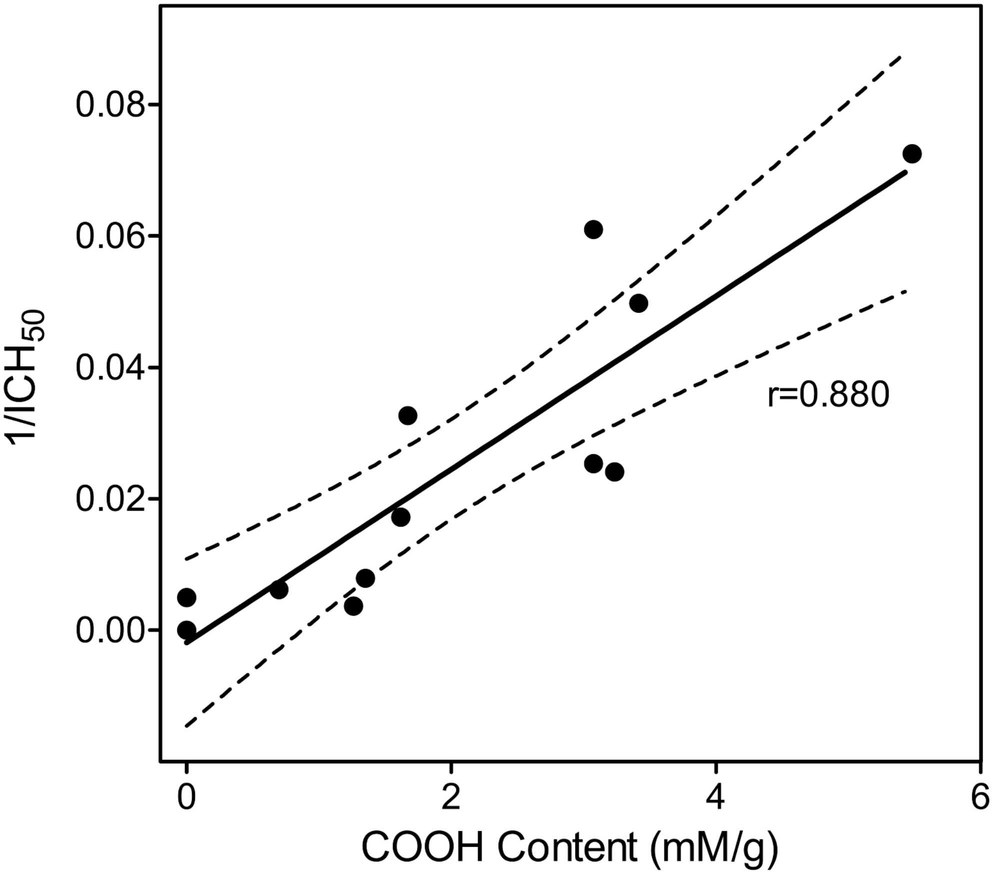


Figure 9.

Plot of complement-fixing activity versus carboxylic group content in the Shilajit fractions and standard FA samples. Complement-fixing activity is represented as inverse ICH_{50} ($1/ICH_{50}$). Dashed lines indicate area of the 95% confidence band.

Table 1

Chemical and physical properties of Shilajit fractions

Fraction	Color (powder)	Chemical features	Carbohydrate content (%)	Yariv test	Protein content (%)
SHS	Dark brown	-	40	Positive	4.5
S-I	White	Neutral	47	Positive	0.2
S-II	Brown	Acidic	36	Positive	2.3
S-III	Black	Acidic	21	Positive	6.8
S-II-1	Brown	Acidic	56	Positive	0.2
S-II-2	Brown	Acidic	34	Positive	2.5
S-II-3	Pale brown	Acidic	14	Negative	6.3

Table 2

Monosaccharide composition (mol %) of the Shilajit fractions

Fraction	Glc	Gal	Xyl	Ara	Rha	GalA	GlcA
SHS	41	32	8	3	7	5	4
S-I	57	26	10	2	5	5	4
S-II	34	35	8	3	10	5	4
S-III	43	24	13	3	13	<2	4
S-II-1	34	38	8	4	10	6	<2
S-II-2	37	31	10	3	9	10	<2
S-II-3	46	7	8	3	17	<2	20

Table 3

Elemental analysis of the Shilajit fractions

	Na	C	H	O	N	S	P	Si	Br	Cl	F	I	Metals	H/C	O/C	C/N
S-I	ND	41.55	6.02	40.16	0.69	0.10	<0.2	ND	ND	ND	ND	ND	ND	1.72	0.73	70.25
S-II	4.05	40.59	5.59	46.86	1.54	0.17	0.11	0.55	<0.1	0.3	<0.1	<0.1	<0.98	1.64	0.87	30.75
S-III	8.78	38.69	4.27	36.32	1.41	0.15	<0.1	0.55	<0.1	8.5	<0.1	<0.1	<1.23	1.31	0.71	30.01

N.D., not detected.

Table 4

Complement-fixing activity and carboxylic group content of the Shilajit fractions and standard FA from IHSS

Sample	IC_{H50} (μg/ml)	COOH (mM/g)
SHS	161.8±14.7	0.70±0.04
S-I	N.A.	N.P. (0)
S-II	126.1±11.3	1.35±0.06
S-III	30.6±4.2	1.67±0.07
S-II-1	272.9±29.4	1.26±0.09
S-II-2	58.2±6.7	1.62±0.11
S-II-3	15.4±3.1	3.07±0.23
Florida Peat FA	13.8±1.6	5.48±0.37
Nordic Aquatic FA	20.1±1.9	3.41±0.25
Suwannee River FA	39.5±4.3	3.07±0.23
Waskish Peat FA	41.5±3.8	3.23±0.24
Pony Lake FA	201.7±18.8	N.P. (0)

N.A., not active; N.P., not precipitated by CTABr. The data are presented as the mean ± SEM of 3 independent experiments.

Table 5

Plant pectin polysaccharides with the highest reported complement-fixing activity

Plant Common Name	**Fraction**	**M_r (kDa)**	**ICH_{50} (μg/ml)**	**Reference**
Plantago major	PMII	46-48	25	(Samuelsen et al., 1996)
Glinus oppositifolius	GOA1	70	34	(Inngjerdingen et al., 2005)
	GOA2	30-39	60	
Avicennia marina	HAM-3-IIb-II	105	23	(Fang et al., 2006)
Vernonia kotschyana	Vk2a	1150	2	(Nergard et al., 2006)
Biophytum petersianum	BP100 III	31	9	(Inngjerdingen et al., 2006)
Trichilia emetica	Te 100 acidic 4	223	<15	(Diallo et al., 2003)

AG-I/II – arabinogalactan type I or type II; RG-I – rhamnogalacturonan type I.