Immunomodulating Polysaccharide Fractions of *Menyanthes trifoliata* L.

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Looking for new plant sources of immunomodulating agents polysaccharide-rich fractions (PS) from *Menyanthes trifoliata* L. (Menyanthaceae) have been isolated. The herb of *Menyanthes trifoliata* L. was sequentially extracted with water, 0.1 M NaOH, 8% CH_3COOH, and 1 M NaOH. After dialysis and resolution on Biogel P-10 four homogenic (B-4, B-5, C-4, D-5) and two nonhomogenic (A-3 and D-4) PS were isolated. About 0.5% of PS over 3500 Da were found in the dry plant material. They were characterized through chemical analysis, NMR and vibrational spectroscopy. Speciation analysis of chosen metal/metaloid elements was performed and an exceptionally high concentration of Se was found in PS of a pure water extract (A-3). The biological tests on the immunomodulating influence with human blood-derived lymphocytes and granulocytes revealed that two fractions, B-4 and B-5, were strong stimulators of immune cells, whereas fractions D-5 and A-3 were found as potent suppressive and anti-inflammatory agents. The applied isolation procedures led to the separation of active compounds into stimulatory and inhibitory fractions.

Key words: Immunomodulating Activity, *Menyanthes trifoliata* L., Speciation of Elements

Introduction

Continuing our search for immunoactive plant polysaccharides (Kuduk-Jaworska *et al.*, 1993; Gąsiorowski *et al.*, 1994) we present the initial studies on *Menyanthes trifoliata* L. (Menyanthaceae) (MT) including isolation, chemical and physical characterization as well as some biological tests of polysaccharide fractions. MT is a plant widely known and distributed in Europe. The extracts of MT were used in traditional medicine as a remedy against scurvy and other diseases (Madaus, 1938; Bisset, 1994). In the UK, *Menyanthidis* folium is present in over 30 anti-inflammatory (antirheumatic) preparations. According to Balitskij and Woroncowa (1982), the herb of MT was one of the components of the mixture which was successfully used together with 5-fluorouracil in the treatment of 25 patients suffering from advanced stomach cancer.

The chemical and biological properties of MT were recently widely studied (Battersby *et al.*, 1981; Junior, 1989; Tunon and Bohlin, 1995; Lindholm *et al.*, 2002; Maksimova *et al.*, 1980) but, to our knowledge, polysaccharides of MT have not been studied so far. We suppose that some of the therapeutic properties announced in literature, especially an antitumor effect of MT, may be associated with the presence of polysaccharides which are known to have immunomodulating and anticancer activity (Kraus and Franz, 1990; Srivastava and Kulshreshtha, 1989; Paulsen, 2001; Fisher and Yang, 2002). We believe, moreover, that the biological and therapeutic properties of plant polysaccharides may be modulated by mineral constituents usually present in herbal material.

In this work we will try to find out if MT may be a new source of immunomodulating polysaccharides and also if selenium and other microelements are bound with these polymers.

This work includes: (1) the isolation of polysaccharide-rich fractions (PS), (2) the characterization of PS by chemical and physical methods including speciation of mineral constituents, NMR and vibrational spectroscopy, (3) a biological study of immunomodulating activity carried out on human blood-derived lymphocytes and granulocytes.

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Results and Discussion

General preparation and characterization

PS were isolated from the herb of MT obtained from commercial pharmaceutical material (Herbalux, Warszawa, Poland). About 0.5% of PS over 3500 Da were found in the dry plant material. The PS, differring in their chemical, physical and biological properties, were obtained using as extractants: water, 0.1 M NaOH, 8% CH_3COOH, and 1 M NaOH, respectively. After purification and chromatographic resolution on Biogel P-10, four homogenic (B-4, B-5, C-4, D-5) and two nonhomogenic (A-3 and D-4) PS (Fig. 1, Table I) were isolated.

Chemical analyses and spectrophotometric evaluation indicate sugar character of all isolated fractions, whereas the chromatographic analysis of hydrolysate products indicates that all fractions are heteropolysaccharide types.

The IR and ^{13}C NMR spectra are typical for polysaccharide structures. The IR absorption at ~ 1260 cm^{-1} together with ~ 1400 cm^{-1} is charactereristic for ν(C-O) and δ(C-O-H) of sugars (Table II) whereas 1630, 1600 and 1700 cm^{-1} indicate the presence of -CONH-, $-COO^-$, -COOR or -COOH functions attached to sugar moieties. The weak bands at 886 cm^{-1} indicate the presence of β-glycoside linkages (Misaki *et al.*, 1986). Absorptions at 1263 and 1073–1076 cm^{-1} are associated with ν(C-OH) vibrations but also ν(S=O) are possible for A-3, B-4, B-5 and C-4 because they contain a small amount of sulfur (by elemental analysis). The clear absorption at 800 cm^{-1} only in B-4 and B-5 may be associated with C-O-S vibrations, which are possible for an anhydrogalactose 2-sulfate moiety (Malfait *et al.*, 1989). Both, D-4 and D-5, do not have the absorption at 1260 cm^{-1}, which may confirm the lack of a sulfate group.

The ^{13}C NMR spectrum of B-4 is not very well resolved and contains main peaks grouped at 20–30, 60–80, 100–110, 180–185 and 214 ppm, characteristic for polysaccharide and polyuronide structures (Table III). The signals grouped at 20–36 ppm are associated with the methyl resonances of acetamido sugar, whereas the methine (C2 to C5) resonances appear in the region 62–85 ppm (Agrawal, 1992; Cartier *et al.*, 1988; Teleman *et al.*, 2002). Signals noticed in the region of the anomeric carbon atoms (100–102 ppm) may be assigned to the β-(1–4)-linked glucopyranosyl and mannopyranosyl residues. Confirmation of this conclusion could be found with the signals of C-4 substituted glucose residues at 79.8 ppm and with the C-6 substituted carbon atoms of glucose as well as mannose at 69.5 and 69.9 ppm, respectively (Cartier *et al.*, 1988). The signal at 110.6 ppm seems to be characteristic for C-1 of a α-L-arabinofuranosyl residue (Joseleau and Ullmann, 1990; Cros *et al.*, 1994). Low field absorption in the region 180–190 ppm reflects the existence of a carboxylate ion and/or the carbonyl groups of acetamido sugars (Agrawal, 1992).

Speciation of trace elements

SEC-ICP MS analyses of A-3, A-3-1, A-3-2, C-4 and D-4 fractions were performed. TotalQuant total analysis mode was used for quick estimation of the content of mineral constituents as a preparatory step for subsequent chromatographic determinations. A set of eight isotopes, ^{82}Se, ^{88}Sr, ^{138}Ba, ^{63}Cu, ^{67}Zn, ^{11}B, ^{208}Pb and ^{140}Ce, was chosen.

Table I. Analyses of polysaccharide fractions.

Fraction	Hexose[a] (%)	Uronic acid[a] (%)	Amino-sugar[a] (%)	Neutral monosaccharide components[b]	C (%)	H (%)	N (%)	S (%)	P (%)	Molecular weight[c] [10^4 Da]
A-3	48.2	11.9	0.89	gal, glc, fru, ara, man, rha, xyl	43.4	6.80	1.78	0.92	trace	6.8; 1.2; 1.0
B-4	31.5	9.3	4.0	gal, glc, fru, ara, man, rha, xyl	41.0	5.82	2.37	1.03	0	1.2
B-5	32.0	9.5	3.8	gal, glc, ara, man, rha, xyl	38.1	5.38	1.95	1.03	0	1.1
C-4	41.0	7.9	1.9	gal, glc, ara, xyl, man, sor	43.5	6.02	2.01	0.97	0	1.2
D-4	40	8.0	1.9	gal, glc, ara, xyl, man, fru	38.88	5.83	2.70	trace	trace	1.2; 1.1
D-5	33.0	7.1	3.5	gal, fru, ara, man, rha, xyl, sor	38.84	5.80	2.62	0	trace	1.2

[a] Estimated spectrophotometrically.
[b] TLC and PC analysis of PS hydrolysates.
[c] Estimated on Sephadex G-50.

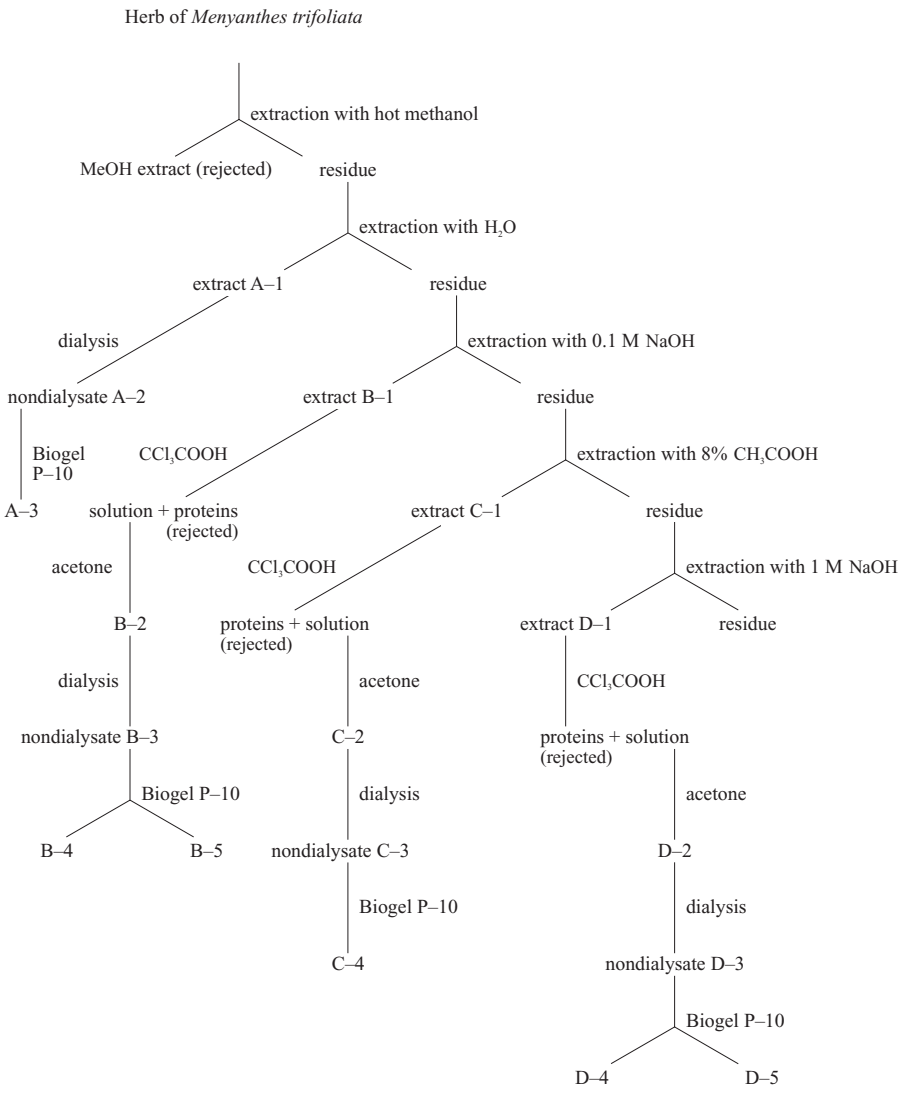

Fig. 1. Isolation of polysaccharides from the herb of *Menyanthes trifoliata.*

Strontium, lead, barium and rare earth elements have been reported to be complexed by the borate ester of rhamnogalacturonan-II from plant cell walls (Szpunar *et al.*, 1998; Matsunaga *et al.*, 1997). These elements were also found to be complexed by high molar mass polysaccharides (> 50 kDa) in the water-soluble fraction of edible plants (Cartier *et al.*, 1988). An extraction procedure with a mixture of pectinolytic enzymes allowed to hydrolyse these species to the borate ester of rhamnogalacturonan-II (Szpunar *et al.*, 1999). Selenium is an element with proved anticancerogenic activity; exceptionally high concentrations of Se (ca. 0.5%) were found in fractions A-3 and A-3-2, whereas Cu and Zn are the essential elements present in plants.

Boron has been known as an essential microelement for plants: it was found that in plant tissue boron existed in the form of borate monoester and diester together with boric acid (Ishi and Matsunaga, 1996). The presence of a boron signal co-eluted with Pb, Sr, Ba and REE can also serve as a proof for the presence of the borate ester of the rhamnogalacturonan-II complex with these metals.

Chromatographic profiles obtained for the analysed fractions are consistent with other data indicating heterogeneity of fractions A-3 and D-4. A multielemental chromatogram of fraction A-3

Table II. Main frequencies in IR spectra of polysaccharide fractions [cm^{-1}].

A-3	B-4	B-5	C-4	D-4	D-5	Assignment
3391 vs, br	3393 s, br	3409 vs, br	3392 vs, br	3394 s, br	3386 vs, br	ν(OH), ν(NH)
2931 m	2932 m	2937 m	2931 m	2937 m	2938 m	ν(CH)
1700 sh			1720 sh			ν(C=O) of (COOH or COOR)
1635 s	1640 sh	1640 s	1635 s	1635 s	1635 s, b	ν(C=O) of (COO^- or -CONH-)
1605 s	1604 s	1606 s		1600 s		
1437 sh	1440 sh	1440 sh	1440 sh	1440 sh	1440 sh	$\delta(CH_2)$
1394 m	1399 m	1405 m	1394 m	1398 m	1399 m	ν(CO), δ(COH)
1262 w	1263 w	1262 w	1260 w			ν(CO), δ(COH), ν(SO)
1074 s	1076 s	1076 s	1073 s, b	1077s	1076 s, b	ν(SO), ν(POC), ν(CC), ν(PO), (CO)
	886 w	886 vvw	886 w	887 w	886 w	C(1)H β-glycoside
	800 sh	800 m				ν(C-O-S)

Table III. ^{13}C NMR of polysaccharide fraction B-4 (ppm).

CH_3	C-2, C-3, C-4, C-5	C-6	C-1	C=O
19.0; 21.2; 25.7; 27.1; 29.8; 30.1; 36.8	62.1; 62.7; 71.1; 71.5; 72.0; 73.2; 73.9; 74.7; 76.1; 77.1; 77.5; 77.9; 79.8; 81.4; 82.6; 83.2; 85.2	69.5; 69.9	100.6; 101.9; 110.6	181.7; 182.7; 183.8; 185.8; 187.8; 196.4

shows the presence of two not completely resolved signals for Se, the first of these signals co-eluted with copper. The signal with the highest molecular weight indicates Pb, Ce, Sr, Ba and B suggesting a polysaccharide aggregate similar to that detected earlier (Szpunar *et al.*, 1999). Further purification of this fraction into a higher molecular polysaccharide A-3-1 and residual A-3-2 led to the differentiation of chromatographic profiles. Fraction A-3-1 (Fig. 2) contains only some residual Se co-eluted with the Pb and B fraction A-3-2 (Fig. 3) keeping the original pattern of A-3. In fraction D-4 two groups of signals were detected by HPLC-ICP MS: Cu, Ce and Pb eluted at ca. 10 kDa and Sr, Zn and Ba eluted in the low molecular weight region. Chromatograms for fraction C-4, which seem to be homogenous with one signal of 18 kDa identified by HPLC-spectrophotometry, give rise to an additional non-identified Sr signal in the low molecular weight region in HPLC-ICP MS.

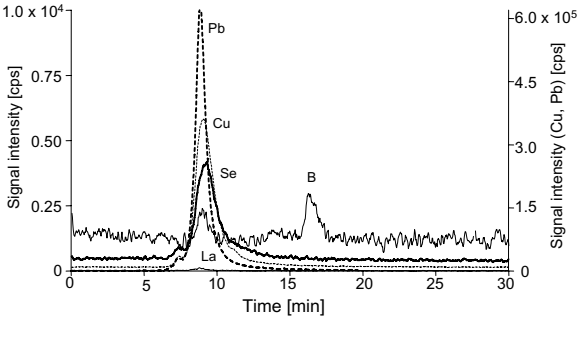

Fig. 2. Chromatographic profile of fraction A-3-1.

Biological tests

MT fractions were not found cytotoxic to human lymphocytes in an 18-h culture as assessed with the standard trypan blue-exclusion test. For direct comparison of immunotropic effects we chose the data obtained in the highest of the tested MT concentrations (100 μg/ml).

All tested fractions decreased the lectin-induced lymphocyte proliferation *in vitro*, mitotic indices were 30–40% lower as compared with the control cultures (without the MT fractions, Table IV). The influence of the MT fractions on the frequency of selected lymphocyte subpopulations of 72-h cultures was different in the case of different frac-

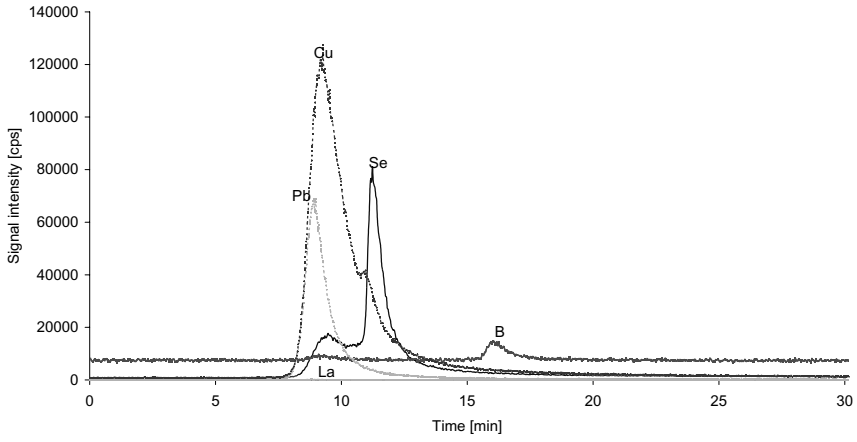

Fig. 3. Chromatographic profile of fraction A-3-2.

Table IV. Immunotropic activity of five chemical fractions isolated from *Menyanthes trifoliata* in human lymphocyte cultures and in human granulocyte samples.

Test*	A-3	B-4	B-5	C-4	D-5
MI	0.59 ± 0.040	0.67 ± 0.124	0.56 ± 0.092	0.73 ± 0.086	0.73 ± 0.092
CD 4	0.73 ± 1.119	0.98 ± 0.213	1.31 ± 0.167	1.10 ± 0.242	0.78 ± 0.090
CD 8	1.05 ± 0.261	1.01 ± 0.185	0.95 ± 0.211	0.91 ± 0.167	0.93 ± 0.193
B	1.10 ± 0.183	1.29 ± 0.245	0.97 ± 0.169	1.23 ± 0.237	1.05 ± 0.258
HLA-DR+	0.62 ± 0.271	1.32 ± 0.195	1.14 ± 0.203	1.08 ± 0.184	0.92 ± 0.193
NBT	0.57 ± 0.187	1.01 ± 0.122	1.48 ± 0.283	1.02 ± 0.124	0.54 ± 0.104

MI, mitotic indices in lymphocyte cultures; CD 4, frequency of T inductory/helper lymphocytes; CD 8, frequency of T supressory lymphocytes; B, frequency of B lymphocytes; HLA-DR+, frequency of lymphocytes expressing HLA-DR antigens; NBT, nitroblue tetrazolium reduction test (NBT reduction by superoxide radicals in human granulocyte samples).
* Tested fractions were added to the cell cultures in a concentration of 100 μg/ml. The results obtained with blood cells isolated from 4 blood donors, healthy volunteers, were expressed in proportion to the relative control samples (without tested fractions) and presented as: $\times \pm$ SD, n = 4.

tions. The CD 4 lymphocyte numbers were markedly elevated in the presence of the B-5 fraction and markedly decreased in the presence of the A-3 fraction. The frequencies of the CD 8 lymphocytes did not differ significantly from those of the control culture with all tested MT fractions. The frequencies of the B cells were elevated (by about 25%) in the presence of the B-4 and the C-4 fractions. The number of HLA-DR-expressing cells was increased by 32% in case of the B-4 fraction in comparison to control cultures, and by 15% in the case of the B-5 fraction, whereas it was decreased by almost 40% by the A-3 fraction.

The generation of free radicals by human granulocyte samples was enhanced by the B-5 and B-4 fractions (by 48% and 15%, respectively) but was lowered by almost 55% by the A-3 and D-5 fractions.

Summing up, we can conclude that the tested MT fractions differ substantially in their immunotropic influence on human lymphocytes and granulocytes *in vitro*, the A-3 and D-5 fraction exhibited potent immunosuppressory action, while B-4 and, especially, B-5 revealed a strong immunostimulatory effect.

It is worth emphasizing that the immunological activity was determined not for pure polysaccharide fractions but for their adducts (or complexes) with some metals/metaloids which should not be considered as a kind of herba contamination. At least in part they are chemically bound with organic compounds. As already observed with *Caltha palustris* L. part of inorganic ions could not be removed during the chemical and chromatographic purification of polysaccharide fractions (Kuduk-Jaworska *et al.*, 1993). A similar situation

seems to be with MT PS preparations. Following the literature (Combs and Gray, 1998; Combs and Clark, 2001; Whanger, 2002; Seregina and Nilovskaya, 2002) on antitumorigenic effects of selenium compounds, we assume that selenium-modified PS might be mainly responsible for immunocorrective action of MT PS studied here. However, the results do not indicate a simple correlation between the content of selenium and activity of tested samples. That is conceivable because the impact of metal-containing polymers on biological processes is very complex. It is difficult to evaluate whether the metal/metaloid components influence the biological targets alone, or rather act as a complex, metal-containing PS polymer. Further investigation should establish the nature of metal/metaloid influence on immunomodulating activity of MT PS.

Experimental

Plant material and polysaccharide fractions

Herb of *Menyanthes trifoliata* L. was purchased from Herbalux (Warszawa, Poland). The dried powdered material (1 kg) was pre-extracted with hot methanol (2.5 l) and left for 24 h, then the solution was discarded. The operation was repeated three times. The residual herb was extracted successively with water, 0.1 M NaOH, 8% CH_3COOH, 1 M NaOH obtaining: water extract (A-1), weak alkali extract (B-1), acidic extract (C-1) and strong alkali extract (D-1), respectively. Extracts A-1, B-1 and C-1 were concentrated to 30, 40 and 40% of volume, respectively, under reduced pressure at a temperature not exceeding 45 °C. Then the solutions were treated with CCl_3COOH (TCA) to precipitate proteins which were centrifuged and rejected. Supernatants were treated with acetone (1:6 v/v) to precipitate polysaccharides. The crude polysaccharide fractions were further processed (as schematically presented in Fig. 1) and analyzed (Table I).

PS fractions of water extract. The crude water extract was obtained by treating the pre-extracted herb with 2 l of hot water and was allowed to stand for 24 h (repeated three times), then the solutions were evaporated. The residue (A-1, 58 g) was dialysed and the nondialysable solution was evaporated, giving 1.2 g of dry substance (A-2) which did not produce any precipitation with CCl_3COOH. A-2 was permeated on Biogel P-10. The resulting, non-homogenic product A-3 (1.17 g) was further separated on Biogel P-10 into A-3-1 and A-3-2 fractions, which were not homogenous, but included constituents of higher and lower molecular weight, respectively.

PS fractions of weak alkali extract. The herb extracted earlier by H_2O was treated 3 times with 2 l of 0.1 M NaOH yielding B-1. After neutralization, evaporation and removal of the precipitated TCA-proteins by centrifugation, the resulting supernatant (0.15 l) was added to 0.9 l acetone. The obtained precipitate was dissolved in water, undissolved material separated by centrifugation and the clear solution was treated with acetone (1:3 v/v). The brown, powdered residue was collected (B-2, 32.2 g) and dialysed. The nondialysable solution was evaporated giving 1.8 g of dry substance B-3 which was resolved on Biogel P-10 yielding fractions B-4 and B-5.

PS fractions of acidic extract. The herb, which remained after 0.1 M NaOH extraction was treated with 8% CH_3COOH, then condensed by evaporation *in vacuo.* The product C-1 was adjusted to 100 ml with water and mixed under stirring with 100 ml of 30% TCA to precipitate the proteins. After centrifugation the supernatant was treated with acetone (2 l). The brown, powdered dry substance was collected (C-2, 15.5 g); 10 g of C-2 were dialysed and the nondialysable solution evaporated giving 0.45 g of a bright black substance C-3 which was purified on Biogel P-10 giving homogenic C-4 (0.42 g).

PS fractions of strong alkali extract. The herb, which remained after the 8%-CH_3COOH extraction was suspended in 1 M NaOH and macerated for 24 h. After filtration, neutralization and centrifugation, 6 volumes of acetone were added. The precipitate recovered by centrifugation, D-1, was dissolved in 75 ml of distilled water and mixed with 75 ml 30% TCA. After centrifugation the resulted supernatant was poured into 0.9 l of acetone. The brown, powdered dry substance was collected (D-2, 28 g) and dialysed. The nondialysable solution was evaporated giving 0.8 g of substance D-3 which was permeated on Biogel P-10 giving D-4 (0.57 g) and D-5 (0.16 g).

General analytical methods

Elemental analyses were performed with a CHN Analyser. IR spectra were recorded on a Specord M 80 and ^{13}C NMR spectra on a Bruker MSL 300 spectrometer at 75.468 MHz in D_2O.

Neutral hexose contents were determined as anhydroglucose after Dubois *et al.* (1956), aminosugars by a modified Elson-Morgan reaction (Rondle and Morgan, 1955), uronic acids by the Blumenkrantz method (Blumenkrantz and Asboe-Hansen, 1973). The sugar analysis was made by TLC and PC after complete acid hydrolysis by treatment with 4 M HCl. TLC was performed on silufol folium sheets (Kavalier, Votice, Czechoslovakia) in methanol/chloroform (3:7 v/v) and sugars were detected by spraying with aniline phthalate.

PC was performed on Whatman No. 1 MM papers, using butanol/pyridine/water (6:4:3 v/v/v) and detection was with alkaline silver nitrate. Dialyses against distilled water were performed for 3 d with dialysing tubes, mol wt. cut off 3500 (Serva, Heidelberg, Germany). The purification of polysaccharides was performed by gel permeation chromatography on columns of Biogel P-10 (Bio-Rad) (80 cm × 4 cm); the columns were eluted with bidistilled water. Molecular weight was estimated on Sephadex G-50, (Pharmacia) (100 cm × 1 cm), using dextrans T-10, T-70, T-500 and T-2000 as calibration substances. The columns were eluted with acetic acid/pyridine/H_2O (1:0.4:100 v/v/v), pH 6.4. The exclusion volume (V_0) of the column was determined by dextran blue T-2000. Polysaccharides were monitored by phenol/H_2SO_4 reagent and absorbances were measured at 490 nm.

HPLC SEC-ICP MS

Chromatographic separations were carried out using a BIO410 HPLC pump (Perkin-Elmer, Palo Alto, CA, USA) as the sample delivery system. Injections were performed using a model 7725 injection valve with a 20 μl injection loop (Rheodyne, Cotati, CA, USA). All the connections were made of PEEK tubing (0.17 mm i.d.). Analyte species were separated on a Progel TSK (30 cm × 7.8 mm × 4 μm) column with an exclusion limit of 80 kDa (TosoHaas, Stuttgart, Germany). A guard column [TSK PW_{XL} (40 mm × 3 mm i.d.) (TosoHaas)] was always used. The columns were calibrated (UV detection was used) with the following standards: rabbit liver metallothionein-Cd complex (M_r 6918), cytochrome C (M_r 12384), bovine albumin (M_r 66000), and thyroglobuline (M_r 660 000).

An ELAN 6000 ICP mass spectrometer (PE-SCIEX, Concord, Canada) was used as an element-specific detector in HPLC. The column eluate was introduced into the ICP *via* a cross-flow nebulizer fitted in a Ryton™ spray chamber. For total analysis the samples were fed by means of a Minipuls 3 peristaltic pump (Gilson, Villiers-le-Bel, France) that also served for draining the spray chamber. Chromatographic data were processed using the Turbochrom4™ software (Perkin-Elmer).

Analytical grade reagents purchased from Sigma-Aldrich (St. Quentin Fallavier, France) were used throughout unless specified otherwise. 18 M Milli-Q water (Millipore, Bedford, MA, USA) was used throughout. The Tris-HCl buffer was prepared by dissolving 30 mM Tris (trihydroxymethylaminomethane) in water and adjusting pH to 7.0 by the addition of hydrochloric acid (1:10, v/v). The buffer solution was degassed in an ultrasonic bath before use.

ICP MS measurement conditions (nebulizer gas flow, RF power and lens voltages) were optimized daily using a standard built-in software procedure. TotalQuant mode was used for quick estimation of the content of mineral constituents as a preparatory step for chromatographic analysis. The chromatographic mobile phase was the 30 mM Tris-HCl buffer, pH 7.2. The flow rate was 0.75 ml min^{-1}. A sample aliquot of 20 μl was injected. The eluate from the column was fed directly into the ICP. The ^{82}Se, ^{88}Sr, ^{138}Ba, ^{63}Cu, ^{67}Zn, ^{11}B, ^{208}Pb and ^{140}Ce isotopes were monitored. The dwell time for each isotope was 100 ms. The number of replicates applied allowed the continuous data acquisition by the peak hopping mode for the duration of the chromatographic run.

Biological tests

The MT fractions were screened for immunomodulating activities in human lymphocyte cultures and in granulocyte samples. Lymphocytes and granulocytes were isolated from heparinized blood of four healthy volunteers. Cells were isolated by centrifugation on the discontinuous density gradient solutions: Histopaque-1077 and Histopaque-1119 (Sigma, St. Louis, MO, USA) following the standard procedure (English and Anderson, 1974). After washing with phosphate buffered saline the cells were counted and cultured in the presence of the MT fractions. Viability of lymphocytes after an 18-h culture in the presence of the MT fractions (CO_2-incubator, 37 °C)

was evaluated with routine trypan blue-exclusion test and examined microscopically.

Lymphocyte proliferation was as follows: mitotic indices were estimated on microscopic slides made from a 72-h culture carried out in the presence of the tested MT fractions (concentration range: 25.0 μg/ml – 100.0 μg/ml) together with the standard lymphocyte mitogenic agent, lectin phytohaemagglutinine (PHA; 10 μg/ml).

Immunocytochemical staining of cell smears was performed after a 72-h culture in the presence of lectin (PHA; 10 μg/ml) and the MT fractions, and was carried out following a protocol given in literature (Wranke and Levy, 1980) with mouse monoclonal antibodies (DAKO, Glostrup, Denmark). The frequencies of stained cell subpopulations were counted under a microscope, compared to the frequency in the control culture (without the MT fractions) and finally expressed as the experimental versus control (E/E_0) ratios.

Free radical generation by human granulocytes during an incubation of cells with the MT fractions (40 min, 37 °C, shaking water-bath) was assessed by quantitative reduction of 0.4% nitroblue tetrazolium (NBT) according to standard procedures (Borregaard *et al.*, 1983). The absorption of NBT reduction-product (formazan) was estimated spectrophotometrically, the results ($A_{515\,nm}$) were subtracted from relative blank samples and were expressed as E/E_0 ratios.

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