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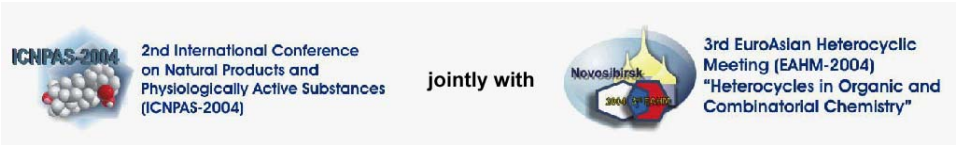

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Poly[3-(3,4-dihydroxyphenyl)glyceric Acid], A New Biologically Active Polymer from *Symphytum Asperum Lepech*. and *S. Caucasicum Bieb*. (Boraginaceae)

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Abstract: Two high-molecular water-soluble preparations with high anticomplementary, antioxidant, antilipoperoxidant and antiinflammatory activities were isolated from the roots of *Symphytum asperum* and *S. caucasicum.* Their main chemical constituent was found to be poly[oxy-1-carboxy-2-(3,4-dihydroxyphenyl)ethylene], according to IR and NMR spectroscopy. The *Symphytum* high-molecular preparations can modulate *in vitro* B-chronic lymphocytic leukaemia (B-CLL) cells apoptosis and cell cycle progression.

Keywords: Caffeic acid, 3-(3,4-dihydroxyphenyl)glyceric acid, *Symphytum asperum, Symphytum asperum caucasicum,* poly[oxy-1-carboxy-2-(3,4-dihydroxyphenyl)ethylene].

Introduction

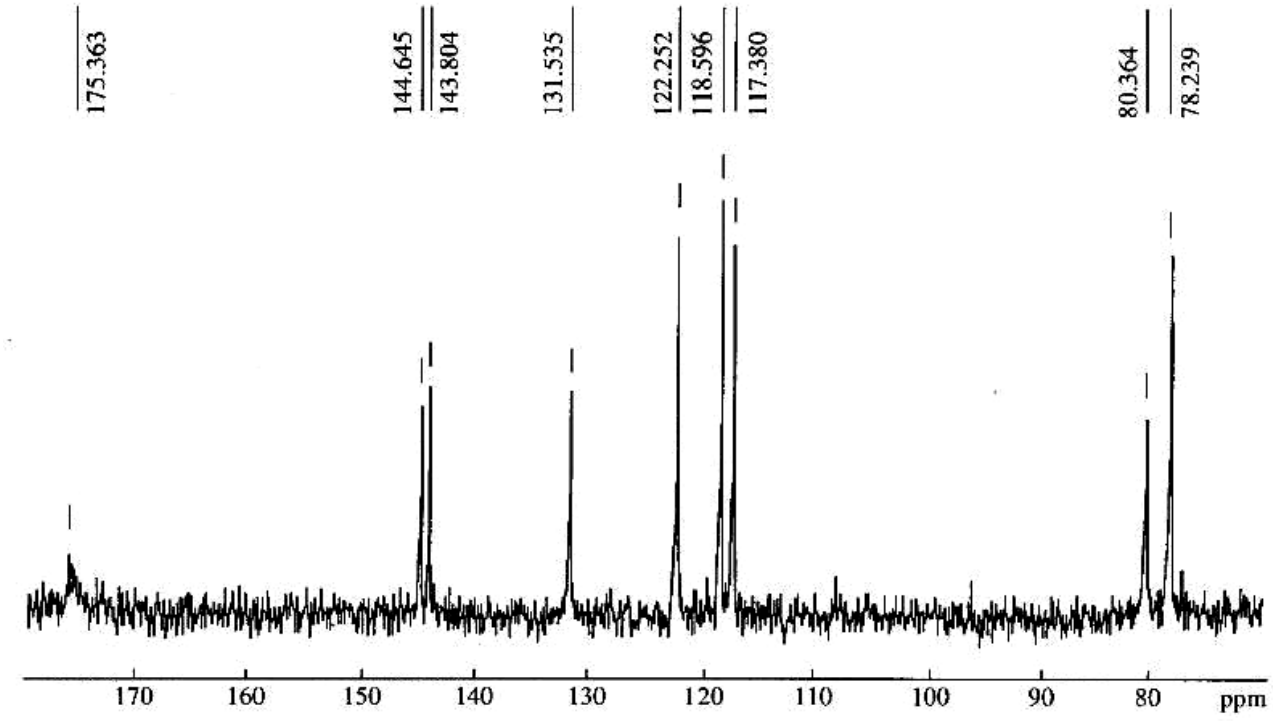
In a previous paper it was shown that the crude polysaccharide preparations from the roots of *Symphytum asperum* and *S. caucasicum* (Boraginaceae) displayed strong anticomplementary activity [1]. In order to determine the chemical nature of the active components both total polysaccharide preparations were fractionated by ultrafiltration on membrane filters with cut-off values of 1000 kDa. This

fractionation procedure allowed us to remove most ballast polysaccharides and to obtain water-soluble high-molecular (>1000 kDa) preparations **1** and **2** from *S. asperum* and *S. caucasicum* roots, respectively [1]. They contained some amounts of carbohydrates (25.7 and 26%, respectively). The monosaccharide composition of the carbohydrate fraction of the preparations differed sharply from those of the starting polysaccharide extracts, in which fructose prevails [1, 2]. Preparations **1** and **2** were found to contain rhamnose, arabinose, mannose, glucose, galactose, and uronic acids, along with only negligible amounts of fructose [1]. Three absorption maxima at 252, 282 (shoulder) and 286 nm in veronal-saline buffer pH 7.35) were observed in the UV spectra of both preparations. The high anticomplementary activity of preparations **1** and **2** decreased sharply upon the treatment of their solutions with a skin powder. On the basis of these data, we hypothesized that the isolated compounds are phenolic polymers [1]. We had shown in our preliminary communications that a regularly substituted polyoxyethylene, namely, poly[oxy-1-carboxy-2-(3,4-dihydroxyphenyl)ethylene], is the main component of preparation **1** [3,4]. We herein report data on the comparative study of preparations **1** and **2** using IR and NMR spectroscopy and their antioxidant activities.

Results and Discussion

The absorption maxima in water at 213, 237 282 (shoulder) and 286 nm were observed in the UV spectra of both preparations. The IR spectra of preparations **1** and **2** are identical and contain absorption bands characteristic of phenol-carboxylic acids [5]: 3400 (OH); 2930 (CH); 1620 (ionized carboxyl); 1600, 1510, and 1450 (aromatic C=C); 1410 and 1220 (phenols); 1270, 1130, 1075 and 1030 (R─O─R'); 880 (C─H in the aromatic ring with one isolated hydrogen atom); and 830 cm^{-1} (C─H in the aromatic ring with two neighboring hydrogen atoms).

Figure 1. The ^{13}C-NMR spectrum of preparation **1**.



The ^{13}C-NMR spectra of **1** and **2** are also completely identical. Interestingly, the signals of the carbohydrate components are practically unobservable in the spectra of these preparations, probably due to their variegated monosaccharide composition; only nine distinct signals corresponding to the carbon atoms of the substituted phenylpropionic acid fragment are observed (Figure 1).

Figure 2. The APT spectrum of preparation **1**.

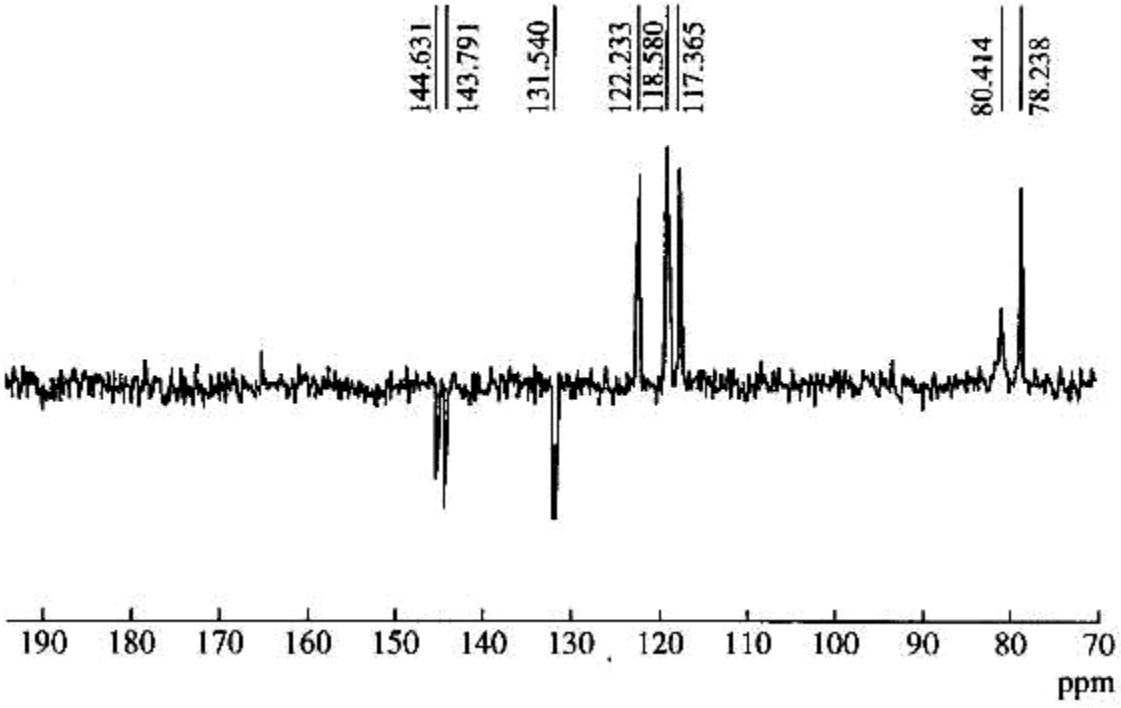
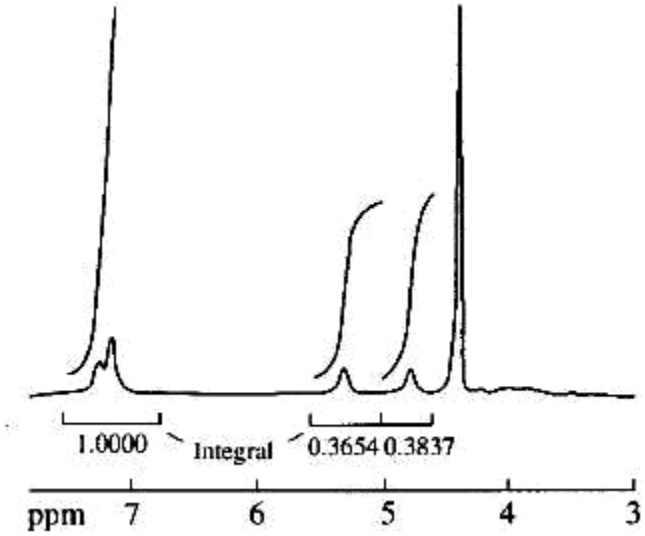


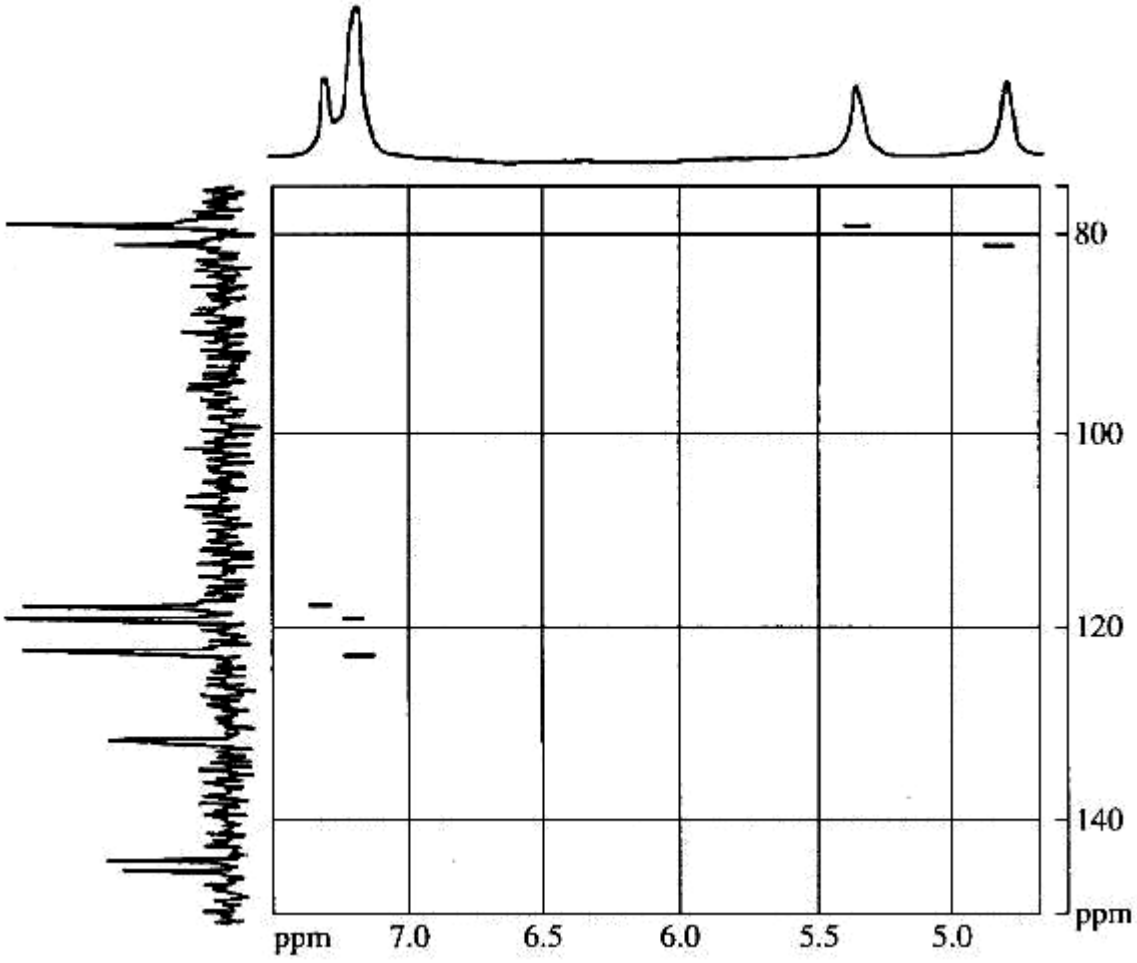
Figure 3. The ^{1}H-NMR spectrum of preparation **1**.



It follows from the spectra obtained using the APT technique [6] (Figure 2) that five signals should be assigned to CH groups and four signals to the nonprotonated carbon atoms. The two signals with chemical shifts of 78.2 and 80.4 ppm obviously belong to oxygen-bound protonated aliphatic carbon atoms. Six signals were assigned to aromatic carbon atoms (protonated atoms at 117.4, 118.6, and 122.3 ppm and nonprotonated atoms at 131.5, 143.8, and 144.6 ppm). The broadened signal at 175.4 ppm was assigned to the carboxyl group in the compound.

The ^{1}H-NMR spectra of both preparations are also practically identical (Figure 3). They contain four signals at 4.88, 5.33, 7.13, and 7.24 ppm, one of them (7.13 ppm) with doubled intensity. Unfortunately, these signals are broadened, and, therefore, the coupling constants cannot be determined. The 2D heteronuclear ^{1}H/^{13}C HSQC spectrum (Figure 4) exhibits the following correlations between protons and carbon atoms: 4.88/80.4, 5.33/78.2, 7.13/118.6, 7.13/122.3, and 7.24/117.4 ppm.

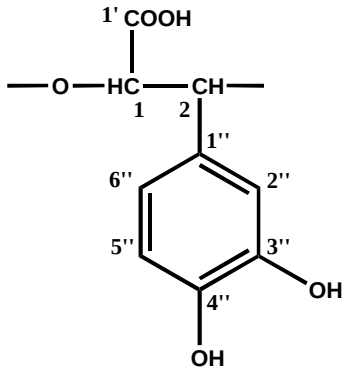
Figure 4. The HSQC spectrum of preparation **1**.



The good resolution and the narrow shape of the ^{13}C-NMR signals indicate that the compounds under study are regular polymers. As shown in our previous communications [3,4], the polyoxyethylene chain is the backbone of the polymer molecule according to the spectral data. Dihydroxyphenyl and carboxyl groups are regular substituents at two carbon atoms in the chain (see Table). The hydroxyl groups in

positions 3 and 4 of the phenyl ring were unambiguously established by a 1D-NOE experiment performed in the difference mode. The pre-irradiation of the proton in position 1 with the chemical shift of 5.33 ppm caused a NOE in the two aromatic protons with the chemical shifts of 7.13 and 7.24 ppm. Hence, these protons occupy positions 2 and 6 in phenyl ring. Therefore, hydroxyl groups cannot occupy *o*-positions. Different values of NOE for these protons, different chemical shifts, and different chemical shifts of resonances of the corresponding carbon atoms in the ^{13}C NMR spectrum exclude the feasibility of symmetric bis-*m*-substitution in the aromatic ring with two hydroxyl groups. Thus, poly[oxy-1-carboxy-2-(3,4-dihydroxyphenyl)ethylene] is the main component of both isolated preparations **1** and **2.** The repeating unit of this polyether contains two asymmetric carbon atoms, but we do not as yet have data on the chirality of these centers.

Table. Signal assignments of the ^{13}C- and ^{1}H-NMR spectra of preparations **1** and **2.**

Repeating unit	Atom no.	^{13}C chemical shift, δ, ppm	^{1}H chemical shift, δ, ppm
	1'	175.4	
	1	78.2	5.33
	2	80.4	4.88
	1"	131.5	
	2"	117.4	7.24
	3"	144.6	
	4"	143.8	
	5"	118.6	7.13
	6"	122.3	7.13

Various phenylpropanoids are well-known fragments of lignans and structural polymers of the plant cell wall. For example, lignols are the main units of lignin [7,8], whereas arylpropionic acids are the main constituents of the aromatic moiety of suberin [9,10]. Both lignin and suberin are practically water-insoluble high-molecular three-dimensional cross-linked polymers with an irregular structure. The information on their structures is mainly based on the data obtained upon their chemical degradation [11, 12]. The ^{13}C- NMR spectra of lignin and suberin obtained for the solid samples give rather poor structural information [10,13,14]. On the other hand, the solubility and behavior upon fractionation make preparations **1** and **2** akin to plant polysaccharide mucilages. One can see that NMR spectroscopy is the most effective method for elucidating their structure. Although the ^{13}C-NMR spectra of solid lignin or suberin samples and solutions of **1** and **2** are not directly comparable, our assignment of the resonances in the ^{13}C-NMR spectrum does not contradict the current spectral data for lignin [13], suberin [14], a number of low-molecular natural compounds containing fragments of 3-(3,4-dihydroxyphenyl)lactic [15], 3-(3,4-dihydroxyphenyl)glyceric acid [16] and model derivatives of caffeic acid [17] reported in literature.

Besides the strong anticomplementary activity of **1** and **2** [1], it was shown that preparation **1** has high antioxidant and antilipoperoxidant activity. It strongly reduced the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical and inhibited the nonenzymatic lipid peroxidation of bovine brain. The ability of preparation **1** to inhibit both degranulation of azurophilic granules and superoxide generation in primed leukocytes indicates that the NADPH oxidase responsible for this process is inhibited, pointing to the preparation **1** as a potent antiinflammatory and vasoprotective agent. In addition, because of its strong antilipoperoxidant activity, it may partly prevent both low-density lipoprotein (LDL) oxidation and formation of byproducts resulting from lipid lipoperoxidation, and therefore, it may have a beneficial effect in the prevention of atherosclerosis and cardiovascular diseases [18].

Aqueous extracts from *Symphytum* species are used in traditional medicine as antiinflammatory and wound-healing agents. Our results demonstrated that the new water-soluble *Symphytum* polymers participate in the global activity of the plant extract. The *Symphytum* polyether could be of significant importance for the treatment of burn wounds. The *Symphytum* caffeic acid-derived polymer mentioned above displayed relevant *in vitro* activities similar to tannic acid (e.g. antioxidant activity) [18], which is used in traditional medicine to treat of burn wounds [19]. However, due to the presence of only ether-linked moieties of caffeic acid, the major advantage of this *Symphytum* new polymer is its low susceptibility to hydrolysis, thus being a much more stable compound than tannic acid which is composed of ester-linked glucose and gallic acid moieties.

Besides generation of superoxide anions by stimulated polymorphonuclear neutrophils (PMNs) these radicals may also arise in chronic wounds where ischemic conditions may convert the enzyme xanthine dehydrogenase into xanthine oxidase which catalyses the conversion of oxygen into superoxide anions causing tissue damage. During this process xanthine oxidase converts hypoxanthine to xanthine and subsequently to uric acid. Consequently, scavenging of superoxide anions either produced by the PMNs or through xanthine oxidase is regarded to be beneficial in the treatment of chronic wounds [20]. Therefore, a superoxide anion scavenging assay of **1** and **2** was carried out in which superoxide anions are generated in a cell-free hypoxanthine/xanthine-oxidase system, and measured as chemiluminescence using lucigenin as light enhancer [21]. Preparations **1** and **2** were found to be strong antioxidants in this assay with IC_{50} values of 2.0 ± 0.8 and 3.2 ± 0.7 μg/mL, respectively [values are depicted as mean IC_{50} values (n = 6) ± standard errors of the mean (SEM)]. Uric acid formation is usually determined to discriminate between actual scavenging of superoxide anions and inactivation of xanthine oxidase by the test sample, but in this case it was not possible to determine uric acid formation spectrophotometrically at 290 nm, due to the strong absorbance of **1** and **2** themselves (with a UV absorption maximum at 286 nm). It should be noticed that inactivation of the enzyme xanthine oxidase by **1** and **2** could not be excluded. Regarding their molecular structures, it is expected that these polyethers easily bind to proteins, thus inactivating enzymes like xanthine oxidase. On the other hand, the molecular structure of **1** and **2** also suggest strong antioxidative properties. The *ortho*-dihydroxyl (catechol) groups of *Symphytum* polymers could act as a donor of hydrogen radicals or electrons what is crucial for enhanced antioxidant efficacy. The strong nonenzymatic DPPH radical-scavenging activity of **1** [18] may be attributed to the catechol moieties contained in this preparation.

Preliminary research regarding the effects of **1** and **2** on the modulation of TNF-α production by human monocytes/macrophages was carried out. The macrophage is a most important cell regulating the inflammatory process by the production of various cytokines, including TNF-α. It was used as marker for the modulation of macrophage functioning. Human adherent mononuclear cells were stimulated with lipopolysaccharide (LPS) from E. coli to produce TNF-α, which was determined using an ELISA [22]. Both preparations inhibited the TNF-α production by human mononuclear cells/macrophages (IC_{50} values 10 - 60 μg/mL). The observed inhibition may be due to binding of these phenolic polyethers to a protein TNF-α, rather than real inhibition of TNF-α production. In order to clarify this theme additional experiments should be carried out.

Conclusions

Thus, our results help establish that one and the same poly[oxy-1-carboxy-2-(3,4-dihydroxyphenyl)ethylene] is the main structural element of both high-molecular water-soluble preparations isolated from the roots of *S. asperum* and *S. caucasicum.* Such a caffeic acid-derived biopolymer is hitherto not known and has been identified for the first time. This compound is a representative of a new class of natural polyethers with a residue of 3-(3,4-dihydroxyphenyl)glyceric acid as the repeating unit. We have no information on the biosynthesis of such a polymer in plants, but from the chemical viewpoint, this process can be conceived as the epoxidation of the double bond in caffeic acid followed by the polymerization of the resulting epoxide.

The similarity in the anticomplementary and antioxidant activities of both preparations can obviously be explained by similarity of their chemical nature. At the same time, preparation **2** is somewhat less soluble than **1,** which could be due to the higher molecular mass of **2** or fine structural differences not reflected in NMR spectra. The disclosure of these differences (including the determination of the structural importance of residual carbohydrates) and further investigation of the biological activity of the polymers isolated will be the subject of future work. Further study should clarify the physiological function of these polyethers in plants and demonstrate whether their biosynthesis is the unique property of the genus *Symphytum* or such compounds are also generated in other plants.

Besides, recently it was shown that the new polymers from the roots of *S. asperum* and *S. caucasicum* can modulate B-chronic lymphocytic leukaemia (B-CLL) cells apoptosis and cell cycle progression [23, 24], therefore these substances can be proposed for further investigations as prospective tumour modulating preparations.

Experimental

General

The total preparations of water-soluble polysaccharides from the roots of *S. asperum* and *S.caucasicum* were obtained as described in [25]. Preparations **1** and **2** were isolated by the procedure described previously [1]. UV spectra were recorded on a Hitachi 150-20 spectrophotometer. IR spectra were registered in KBr pellets on a Perkin-Elmer 571 spectrophotometer. NMR spectra were taken on a Bruker

DRX-500 spectrometer for 1% solutions of the polymers in D_2O at 70-80ºC using acetone (δ_H 2.225 ppm, δ_C 31.45 ppm) as the internal standard (sealed tubes). The preirradiation time for the 1D-NOE experiment was 1 s, and the signal of the preirradiated proton in the differential spectrum was taken as 100%. The 2D HSQC spectrum was obtained using the Bruker standard software. Superoxide anion scavenging assay in a cell-free hypoxanthine/xanthine-oxidase system was carried out as described in [21]. The inhibition of TNF-α production by adherent human mononuclear cells/marophages was measured in accordance with [22]. For spectral data (UV λ_{max} (H_2O) nm, IR ν_{max} (KBr) cm^{-1}; for ^1H and ^{13}C (^2H_2O) δ ppm) see Results and Discussion.

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