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POLY[3-(3,4-DIHYDROXYPHENYL)GLYCERIC ACID] FROM *Symphytum officinale* ROOTS AND ITS BIOLOGICAL ACTIVITY

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Two high-molecular-weight (>1000 kDa) water-soluble biopolymers, the main component of which was poly[3-(3,4-dihydroxyphenyl)glyceric acid] or poly[oxy-1-carboxy-2-(3,4-dihydroxyphenyl)ethylene] according to IR and NMR spectroscopy, were isolated from roots of Symphytum officinale. *They exhibit antioxidant activity as expressed in a decrease of active oxygen species (AOS) by interfering directly in their formation process by polymorphonuclear neutrophils (PMN) and binding directly AOS.*

Key words: *Symphytum officinale*, 3-(3,4-dihydroxyphenyl)glyceric acid, poly[oxy-1-carboxy-2-(3,4-dihydroxyphenyl)ethylene], antioxidant activity.

Comfrey (*Symphytum officinale*, Boraginaceae) has been used since antiquity to treat wounds and fractures. Its high-molecular-weight fraction (>1000 kDa) exhibits immuno-modulating (anticomplementary) activity [1]. It was shown previously that the main component of such high-molecular-weight (>1000 kDa) preparations from roots and stems of Caucasus comfrey species *S. asperum* and *S. caucasicum* is a regularly substituted poly(oxyethylene), namely poly[3-(3,4-dihydroxyphenyl)glyceric acid] or poly[oxy-1-carboxy-2-(3,4-dihydroxyphenyl)ethylene] [2-5], which possesses antioxidant activity [6-9]. This previously unknown in nature phenolic biopolymer was isolated for the first time. This polymer of a caffeic acid derivative is a representative of a new class of natural simple polyethers with 3-(3,4-dihydroxyphenyl)glyceric acid as the repeating unit.

The goal of our research was to isolate water-soluble high-molecular-weight (>1000 kDa) preparations (HP) from total polysaccharides from roots of comfrey and to study the structure of their main component using IR and NMR spectroscopy. Furthermore, we studied their ability to block the formation of active oxygen species (AOS). It is known that AOS formed by activated polymorphonuclear neutrophils (PMN) play an important role in the protection of an organism from foreign microorganisms [9]. However, the surrounding tissue is threatened when PMN begin to produce an excess of AOS [10, 11].

An increase of AOS can also be observed during chronic damage when the enzyme xanthineoxidase (XO) catalyzes conversion of oxygen into superoxide anion, which damages tissue. During this process, XO converts hypoxanthine (HX) into xanthine and then uric acid.

Consequently, binding of superoxide anion formed by activation of PMN and by XO is very important for treating wounds and inflammations [12].

Natural compounds that influence the human complement system and/or the AOS formation process can be used to prevent tissue damage during various pathological conditions.

Total polysaccharides from *S. officinale* roots were isolated by the literature method [13] and precipitated by ethanol to afford TP-et; by acetone from the mother liquor, TP-ac. These were fractionated by ultrafiltration [1, 6] to afford water-soluble high-molecular-weight (>1000 kDa) preparations HP-et and HP-ac, respectively. Table 1 lists the sugar content in these preparations.

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TABLE 1. Monosaccharide Composition of HP-et and HP-ac, %

Preparation	Total sugars, %	Uronic acid	Fru	Rha	Ara	Xyl	Man	Glc	Gal
HP-et	20.3	3.8	2.5	0.8	1.1	0.5	6.0	1.6	4.0
HP-ac	17.1	6.0	2.2	0.4	0.7	0.2	3.3	1.9	2.4

TABLE 2. Assignment of Resonances in ^{13}C NMR and PMR Spectra of HP-et and HP-ac (δ, ppm)

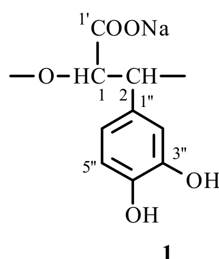
C atom	δ_C	δ_H	C atom	δ_C	δ_H
1′	175.0		3″	144.6	
1	78.3	5.18	4″	143.8	
2	80.2	4.65	5″	118.5	7.03
1″	131.5		6″	122.3	7.03
2″	117.3	7.13			

UV and IR spectra of HP-et and HP-ac were very similar to those of previously isolated simple polyethers from *S. asperum* and *S. caucasicum* [2-5].

HP-et and HP-ac were further characterized using NMR spectroscopy (^{13}C NMR and PMR in addition to 2D heteronuclear ^{1}H/^{13}C HSQC).

Resonances of carbohydrate components in the spectra were practically imperceptible because of the variety of monosaccharides in HP-et and HP-ac (rhamnose, arabinose, xylose, mannose, glucose, galactose, galacturonic acid, fructose).

^{13}C NMR spectra showed only nine distinct resonances for C atoms substituted by phenylpropionic acid, namely 3-(3,4-dihydroxyphenyl)glyceric acid (**1**). Two resonances belonged to protonated aliphatic C atoms bound to oxygen; six, to three protonated and three unprotonated C atoms of an aromatic ring. A broad resonance (175.0 ppm) was assigned to a carboxylic acid.



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PMR spectra contained four resonances, one of which (7.03 ppm) had doubled intensity. Unfortunately, these resonances were broad. Therefore, it was impossible to determine the spin—spin coupling constants.

Table 2 lists all assignments of resonances in ^{13}C NMR and PMR spectra of HP-et and HP-ac and correlations between protons and C atoms that were established using 2D heteronuclear ^{1}H/^{13}C HSQC spectra.

The good resolution and narrow shape of resonances in ^{13}C NMR spectra indicated that the substance was a regular polymer. According to spectral data, the polymer was based on a poly(oxyethylene) chain, two C atoms of which were regularly substituted by 3,4-dihydroxyphenyl and carboxylic acid, respectively.

Thus, ^{13}C NMR, PMR, and 2D heteronuclear ^{1}H/^{13}C HSQC spectra of HP-et and HP-ac were identical to those of the high-molecular-weight fractions from *S. asperum* and *S. caucasicum*, which consisted mainly of poly[oxy-1-carboxy-2-(3,4-dihydroxyphenyl)ethylene] [2-5]. Therefore, we concluded that the main component of HP-et and HP-ac was the same simple polyether with the repeating unit 3-(3,4-dihydroxyphenyl)glyceric acid, which contains two asymmetric C atoms. However, we have no data on the chirality of these centers.

The high-molecular-weight (>1000 kDa) preparation from *S. officinale* is known to exhibit anticomplementary activity [1]. We attempted to establish which constituent of this preparation was the active principle and determine the nature of the relationship between the main component, the phenolic polymer, and other minor polysaccharides. For this, we attempted to fractionate HP-ac by gel chromatography over a column of Sepharose 2B as previously described [6].

TABLE 3. Antioxidant Activity of HP-et and HP-ac

Preparation	IC_{50}, μm/mL			
	CL_{lum} OPZ-stimulated PMN	CL_{luc} OPZ-stimulated PMN	CL_{luc} PMA-stimulated PMN	CL_{luc} in system HX/XO
HP-et	85.4±20	57.4±5.4	97.8±20.0	1.5±0.5
HP-ac	125±19.6	88.4±25.4	70±7.4	0.7±0.3

Inhibition of complement activity by classical (CP) and alternative pathways (AP) was determined in equal volumes of collected fractions. Furthermore, the presence of the phenolic polymer in these fractions was detected by UV absorption at 287 nm (characteristic absorption maximum) and the content of total sugars was determined. A correlation between the elution curves of the phenolic polymer and the anticomplementary activity by the CP and AP was established from the elution profiles. The anticomplementary activity curves by both pathways practically coincided with the elution curve of the phenolic polymer but did not follow the elution curves of two minor polysaccharides. The elution peaks of the polysaccharides were displaced from the peaks of anticomplementary activity and the elution peak of the phenolic polymer. A similar result was obtained earlier from an analogous high-molecular-weight preparation from *S. asperum* [6]. Consequently, the anticomplementary activity of the high-molecular-weight fraction (HP-et and HP-ac) is due to its main component, poly[3-(3,4-dihydroxyphenyl)glyceric acid], which apparently is not covalently bound to the minor polysaccharides.

Fractionation by ultrafiltration removed the majority of ballast polysaccharides and increased the anticomplementary activity [1]. Based on the results, the question about what forces hold the residual polysaccharides together with the phenolic polymer during fractionation of the high-molecular-weight preparations from *S. officinale* (HP-et and HP-ac) by ultrafiltration and gel chromatography is as yet unanswered.

Then, residual carbohydrates were removed from HP-et and HP-ac by acid hydrolysis to cleave glycoside bonds of the polysaccharides while leaving the strong ether bonds of the polyethers untouched. Hydrolysis of HP-et and HP-ac produced a black water-insoluble precipitate. The appearance of this product could be explained by the formation of lignin-like substances via oxidative intermolecular linking of the phenolic polymer and the production of a three-dimensional polymer that was practically insoluble in water [6]. Therefore, the structural significance of these residual carbohydrates will be studied in more detail elsewhere.

Thus, the main component of HP-et and HP-ac, like high-molecular-weight fractions from *S. asperum* and *S. caucasicum*, was poly[3-(3,4-dihydroxyphenyl)glyceric acid] or poly[oxy-1-carboxy-2-(3,4-dihydroxyphenyl)ethylene].

The ability of HP-et and HP-ac to inhibit production of AOS by human PMN due to two different stimulants, receptor-dependent opsonized zymosan (OPZ) [14] or receptor-independent phorbolmyristateacetate (FMA) [15], was determined by measuring luminol- or lucigenin-induced chemiluminescence (CL_{lum} and CL_{luc}, respectively).

Use of luminol produced mainly hypochlorous acid whereas lucigenin was more specific for superoxide anion. It is known that both intra- and intercellular production of AOS can be observed using luminol [16] whereas the lucigenin acts only in intercellular space because PMN are practically impenetrable to lucigenin [17].

A test in which superoxide anion was generated in a cell-free HX/XO system and their CL_{luc} was determined was carried out in order to differentiate inhibition of AOS production from their binding [18].

Table 3 shows the effect of HP-et and HP-ac on CL_{lum} and CL_{luc} generated by OPZ- and FMA-stimulated PMN and on CL_{luc} due to superoxide anion production in the cell-free HX/XO system.

HP-et and HP-ac showed pronounced antioxidant activity as evident in the inhibition of CL_{luc} due to formation of superoxide anion in the cell-free HX/XO system. However, the decrease of CL_{lum} or CL_{luc} due to OPZ- or FMA-stimulated PMN was weaker than would have been expected for the use of a light amplifier, especially one such as lucigenin, which specifically detects superoxide anion. Because all studied fractions had molecular weight >1000 kDa, these large molecules themselves may have activated PMN. Thus, PMN activation also by HP-et and HP-ac would have increased the chemiluminescence and, therefore, decreased the inhibition activity (increased IC_{50}), in addition to the chemiluminescence due to OPZ- or FMA-stimulated PMN.

It may also be that XO was inactivated by HP-et and HP-ac based on the fact that their dominant component was the phenolic polymer that probably bonds readily to proteins, inactivating enzymes like XO and complement convertase. XO is the enzyme responsible for formation of not only AOS but also uric acid, which causes painful inflammation of joints (for example, gout) [19, 20]. Furthermore, XO inhibitors are known to bind superoxide radical [21].

Uric acid formation is usually determined spectrophotometrically (λ_{max} 290 nm) in order to differentiate superoxide anion binding from XO inactivation. In our instance, this was impossible because these fractions themselves had absorption maxima in this same range (λ_{max} 287 nm).

Phenolic polymers HP-et and HP-ac are polycatecholic acids. They can act as H radical or electron donors due to the presence of the *o*-dihydroxyl (catechol) group. This is a decisive factor in increasing the antioxidant activity. Therefore, the observed suppression of chemiluminescence by HP-et and HP-ac (Table 3) may be the result of a decreased AOS concentration.

The similarity of the biological activity of HP-et and HP-ac is easily explained by the similarity of their chemical structures. Their strong antioxidant properties and the observed inactivation of complement convertase and XO are probably related to the phenolic structure.

The principal advantage of this polymer is its resistance to hydrolysis and corresponding high stability owing to the presence of only simple ether bonds.

Thus, the antioxidant and anticomplementary activity of poly[3-(3,4-dihydroxyphenyl)glyceric acid] from roots of *S. officinale* indicates that this polymer is a potential anti-inflammatory, vasoprotective, and wound-healing agent and can be used as an active ingredient for developing therapeutic agents for treating vascular diseases, various types of wounds, and inflammations caused by free radicals or enzymes.

EXPERIMENTAL

Isolation of Total Polysaccharides. Fresh roots (1 kg) of *S. officinale* were supplied by Biohorma (Elburg, Netherlands) in February 2002, cut into small pieces, and dried in air to afford air-dried material (165.8 g, 16.6%) that was ground in a mill. The dry powder (55.6 g) was extracted as before [6]. Total polysaccharides were precipitated by ethanol (5:1) to give TP-et (0.7%). Ethanol was removed from the mother liquor. The bulk of the polysaccharides were precipitated by acetone (4:1) to give TP-ac (11.7%).

Preparations HP-et and HP-ac. TP-et and TP-ac (0.4 g) were fractionated by ultrafiltration on membrane filters (1000 kDa cut-off) as described in the literature [1, 6] to afford water-soluble high-molecular-weight (>1000 kDa) preparations HP-et (19.5%) and HP-ac (15.3%), respectively.

Determination of Carbohydrates of HP-et and HP-ac. HP-et and HP-ac (5 mg) were hydrolyzed by CF_3COOH (2 M) for 1 h at 121°C. Sugars were converted into acetates and determined by GC using myoinosite as an internal standard [16]. GC was performed on a Hewlett—Packard 5890 GC with a flame-ionization detector, Ultra-1 capillary column, and HP 3393A integrator. The carrier gas was N_2. The chromatography conditions were 150°C (1 min) → 290°C (5°/min). Fructose [22] and uronic acid [23] were determined colorimerically.

UV, IR, and NMR Spectra of HP-et and HP-ac. UV spectra were recorded on a Hitachi 150-20 spectrophotometer; IR spectra in KBr disks, on a Jasco FT/IR-410 spectrophotometer; NMR spectra of 1% solutions in D_2O, on a Varian Unity Inova 500 spectrometer (operating frequency 500.13 MHz for ^{1}H; 125.76 MHz, ^{13}C) at 70-80°C with acetone internal standard (δ_H 2.225 ppm, δ_C 31.45 ppm vs. Me_4Si). 2D heteronuclear HSQC spectra were obtained using standard Varian programs.

UV spectrum (H_2O, λ_{max}, nm): 214, 236, 282 (sh), 287. IR spectrum (KBr, ν, cm^{-1}): 3390-3410 (OH); 2928 (CH); 1617 (COO^-); 1510, 1444 (C=C in Ar); 1407, 1217 (ArOH); 1267, 1124, 1075, 1042 (R–O–R′); 870 (C–H in aromatic ring with one isolated H atom); 820 (C–H in aromatic ring with neighboring H atoms).

Table 2 lists the PMR spectrum (500.13 MHz, D_2O, δ, ppm) and ^{13}C NMR spectrum (125.76 MHz, D_2O, δ, ppm).

Determination of Anticomplementary Activity. Anticomplementary activity of HP-et and HP-ac through the CP and AP was determined by the literature methods [24, 25] and expressed in concentrations causing 50% inhibition of hemolysis (IC_{50}, μg/mL). The percent inhibition of complement activity by the CP and AP was calculated as before [25].

Gel Chromatography of HP-ac. Gel chromatography of HP-ac was performed over a column with Sepharose 2B (Pharmacia Biotech, Uppsala, Sweden) by the literature method [6].

Determination of AOS Production and Superoxide Anion Binding/Inhibiting Activity of XO. The activity of HP-et and HP-ac on CL_{lum} and CL_{luc} generated by OPZ- and FMA-stimulated PMN and CL_{luc} due to production of superoxide anion in a cell-free HX/XO system was determined as before [8, 18]. The activity of HP-et and HP-ac was expressed as their concentrations causing 50% decrease of the chemiluminescence signal (IC_{50}, μg/mL).

REFERENCES

1. F. M. van den Dungen, *Symphytum officinale L. - Influence on immune functions and wound-healing processes*, Ph.D. Thesis, University of Utrecht (1993).
2. V. V. Barbakadze, E. P. Kemertelidze, A. S. Shashkov, and A. I. Usov, *Mendeleev Commun.*, **10**, 148 (2000).
3. V. V. Barbakadze, E. P. Kemertelidze, I. L. Targamadze, A. S. Shashkov, and A. I. Usov, *Bioorg. Khim.*, **28**, 362 (2002).
4. V. Barbakadze, E. Kemertelidze, I. Targamadze, K. Mulkijanyan, A. S. Shashkov, and A. I. Usov, *Molecules*, **10**, 1135 (2005).
5. V. Barbakadze, E. Kemertelidze, I. Targamadze, K. Mulkidzhanyan, J. Kemmink, A. J. J. van den Berg, K. J. Beukelman, and A. I. Usov, *Khim. Prir. Soedin.*, 303 (2005).
6. V. V. Barbakadze, E. P. Kemertelidze, A. I. Usov, B. H. Kroes, H. C. Quarles van Ufford, E. van den Worm, C. J. Beukelman, A. J. J. van den Berg, and R. P. Labadie, *Proc. Georgian Acad. Sci. Biol. Ser.*, **25**, 207 (1999).
7. C. M. Barthomeuf, E. Debiton, V. V. Barbakadze, and E. P. Kemertelidze, *J. Agric. Food Chem.*, **49**, 3942 (2001).
8. V. Barbakadze, E. Kemertelidze, K. Mulkidzhanyan, A. J. J. van den Berg, C. J. Beukelman, E. van den Worm, H. C. Quarles van Ufford, and A. I. Usov, *Khim.-farm. Zh.*, **41**, 14 (2007).
9. M. B. Hampton, J. Kettle, and C. Winterbourn, *Blood*, **92**, 3007 (1998).
10. S. J. Weiss, *N. Engl. J. Med.*, **320**, 365 (1989).
11. H. L. Malech and J. I. Gallin, *N. Engl. J. Med.*, **317**, 687 (1987).
12. B. Latha and M. Babu, *Burns*, **27**, 309 (2001).
13. V. V. Barbakadze, R. A. Gakhokidze, Z. S. Shengeliya, and A. I. Usov, *Khim. Prir. Soedin.*, 330 (1989).
14. J. C. Whitin, D. H. Ryan, and H. J. Cohen, *Blood*, **66**, 1182 (1985).
15. N. Schnitzler, K. Schwezer, A. Podbielski, G. Haase, B. Spellerberg, R. Holland, and R. Lutticken, *Adv. Exp. Med. Biol.*, **418**, 897 (1997).
16. C. Dahlgren, P. Follin, A. Jiohansson, R. Lock, and K. Orselius, *J. Biolumin. Chemilumin.*, **4**, 263 (1989).
17. C. Dahlgren, H. Aniansson, and K. E. Magnusson, *Infect. Immun.*, **47**, 326 (1985).
18. E. van den Worm, C. J. Beukelman, A. J. J. van den Berg, B. H. Kroes, R. P. Labadie, and H. van Dijk, *Eur. J. Pharmacol.*, **433**, 225 (2001).
19. J. M. McCord and I. Fridovich, *J. Biol. Chem.*, **243**, 5753 (1968).
20. H. C. Chiang, Y. J. Lo, and F. J. Lu, *J. Enzyme Inhib.*, **8**, 61 (1994).
21. J. E. Chung, M. Kurisawa, Y.-J. Kim, et al., *Biomacromolecules*, **5**, 113 (2004).
22. W. Yaphe and G. P. Arsenault, *Anal. Biochem.*, **13**, 143 (1965).
23. N. Blumenkrantz and G. Asboe-Hansen, *Anal. Biochem.* **54**, 484 (1973).
24. J. P. A. M. Klerx, C. J. Beukelman, H. van Dijk, and H. J. M. N. Willers, *J. Immunol. Methods*, **63**, 215 (1983).
25. J. M. Simons, L. A. 't Hart, H. van Dijk, F. C. Fischer, K. T. D. de Silva, and R. P. Labadie, *J. Ethnopharmacol.*, **26**, 169 (1989).