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Pharmacochemistry

Investigation of Water-Soluble High Molecular Preparation of *Symphytum grandiflorum* DC (Boraginaceae)

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ABSTRACT. According to data of IR, ^{1}H NMR, gCOSY and 2D ^{1}H/^{13}C gHSQCED experiments caffeic acid-derived polymer, namely poly[3-(3,4-dihydroxyphenyl)glyceric acid] (PDPGA) was detected in water-soluble high-molecular preparation of *Symphytum grandiflorum* DC (HMP-SG) by analogy with *S. asperum, S. caucasicum, S.officinale* and *Anchusa italica* high-molecular weight preparations. 2D ^{1}H/^{13}C gHSQCED spectrum of HMP-SG exhibited that in contrast to polymers of other species of *Symphytum*, and likewise some polymer preparations from *A. italica*, most of the carboxylic groups of PDPGA from *S. grandiflorum* are methylated. Besides, the additional signals might have appeared in the gHSQCED spectrum of HMP-SG due to the presence of residual polysaccharide impurities. A 2D DOSY experiment of HMP-SG showed that both sets of signals of methylated in carboxylic group PDPGA and polysaccharides fell in the same horizontal. It means that they have the same diffusion coefficient. This would imply a similar molecular weight for the phenolic polyether and the polysaccharides. This would explain why, unfortunately, it proved not feasible to separate the phenolic polymer from residual polysaccharides by ultrafiltration. However, on the basis of data from IR, ^{1}H NMR, gCOSY, gHSQCED and 2D DOSY experiments, the presence of methylated PDPGA in HMP-SG was sustained by analogy with high molecular preparations of *A. italica.* © 2017 Bull. Georg. Natl. Acad. Sci.

Key words: caffeic acid-derived polymer, poly[3-(3,4-dihydroxyphenyl)glyceric acid], poly[oxy-1-carboxy-2-(3,4-dihydroxyphenyl)ethylene], *Symphytum grandiflorum*

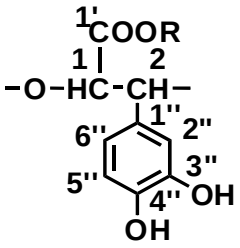

Fig. The repeating unit of **PDPGA**; R=H, CH_3

Symphytum grandiflorum DC (De Candolle) (Creeping comfrey) is a terrestrial perennial herbaceous species with large flowers belonging to *Boraginaceae* family. It is endemic to the Caucasus region, particularly Georgia. *S.grandiflorum* was described for the first time by Swiss botanists Augustin Pyramus de Candolle and published by his son Alphonse De Candolle later in 1846 [1]. Only few papers have been published concerning chemical composition of *S. grandiflorum.* It was documented to synthesis pyrrolizidine alkaloids of retronecine type lycopsamine, echimidine and symphytine. A methanolic alkaloids extract and a hexane extract containing triterpenes and phytosterols were obtained [2,3]. According to some published works [4], the main component of mucilage of *S. grandiflorum* are glucofructans (67 %). Cellulose, uric acid, ketoses, aldoses, saccharose, starch and dextrins in minor extent are also reported [4].

In our previous work high molecular weight preparations [5] from *S. asperum, S. caucasicum, S. officinale* and *Anchusa italica* (Boraginaceae) were isolated. Their main chemical constituent was found to be a novel regular caffeic acid-derived polymer, namely poly[oxy-1-carboxy-2-(3,4-dihydroxyphenyl) ethylene] or poly[3-(3,4-dihydroxyphenyl)glyceric acid] **(PDPGA)**, according to IR and NMR spectroscopy data [6-10]. The repeating unit of this polymer is a 3-(3,4-dihydroxyphenyl)glyceric acid residue (Fig.).

This polymer possesses diverse biological activity, such as anticomplementary, antioxidant, anti-inflammatory [11,12], wound-healing properties [13,14], modulates B-chronic lymphocytic leukaemia cell apoptosis and cell cicle progression [15,16], and completely abrogates the adhesion of murine B16 melanoma cells to tumor-activated hepatic sinusoidal endothelium (HSE) [17]. However, its most important property is anticancer efficacy against prostate cancer cells, both in *in vitro* and *in vivo* experiments [18].

The aim of the present research was to examine high molecular weight preparation of *S. grandiflorum* (HMP-SG) on the presence of PDPGA.

Materials and Methods

Apparatus: The UV spectrum was recorded on a UV/VIS spectrophotometer (Mecasys Optizen Pop, Mecasys Co., Ltd., Daejeon, Korea). The IR spectrum (KBr disc) was obtained on a Varian 660 FT-IR spectrometer (Made in Australia by Varian Australia PTY LTD). The NMR spectra of 1% solutions in D_2O at 80°C and with acetone-d_6 as the internal standard (δ_C 31.42 ppm, δ_H 2.225 ppm vs. Me_4Si) were recorded in a Varian NMR System (Palo Alto, CA, USA), fitted with a CHX $^1H/^{13}C/^{15}N$-^{31}P probehead, gradient module and variable temperature unit. The spectrometer resonance frequency for ^1H was 499.61 MHz. The ^1H 90° hard pulse was optimized for each sample. The spectral width was set to 8012.8 Hz for the monodimensional ^1H experiments. All NMR spectra were processed with the Mestre NOVA software (version 10.0.2, Mestrelab Research, S. L., Santiago de Compostela, Spain). For the 2D DOSY experiment, 15 increments (steps) of 32 scans each were recorded (6009,6 Hz spectral width, 65k spectral size for each scan), and then a Bayesian transformation was employed (128 points in the diffusion dimension).

Plant material: Fresh stems of *S.grandiflorum* were collected from their natural habitat in the Adjara region of Georgia 20.06.2014. Herbarium material of *S. grandiflorum* is available from the I. Kutateladze Institute of Pharmacochemistry (Tbilisi, Georgia).

Extraction and isolation: Stems were cut into small pieces, air-dried and grounded in a mill. Lipids, pigments and low molecular weight compounds were

removed by Soxhlet extractions with chloroform, methanol and acetone. Hot water extraction of 80 g of air-dried, pretreated with organic solvents stems, followed by dialysis [19], afforded 20.69 g of a water-soluble preparation of *S. grandiflorum* (WSP-SG) based on dry biomass (yield, 25.86 %). Further fractionation of 4 g WSP-SG in a stirred ultrafiltration cell, (model 8200, Millipore Corporation, Billerica, MA, USA), fitted with a Biomax-500 ultrafiltration disc (500 000 NMWL), as reported in [5], yielded 0.49 g of water-soluble, high-molecular (>500 kDa) preparation HMP-SG, based on WSP-SG and dry biomass (12.25 % and 3.16%, respectively).

Carbohydrate analysis of WSP-SG: Qualitative monosaccharide composition was analyzed after hydrolysis of samples (5-10 mg) with 2M CF_3COOH at 121°C for 2 h [20]. The acid was removed by multiple evaporations to dryness of methanolic solutions. The monosaccharides were identified by TLC with monosaccharide standards as references. TLCs were performed on 0.25 mm pre-coated silica gel plates (Merck 60, GF-254; Merck, Darmstadt, Germany) treated with *n*-butanol-acetic acid-water (3:1:1). The sugars were visualized by spraying the plates with aniline hydrogen phthalate and heating at 105^{O}C for 10 min. Fructose, a main carbohydrate component of **WSP-SG**, was determined spectrophotometrically [21].

HMP-SG.

UV spectrum (H_2O, λ_{max}, nm): 216, 236 (shoulder), 282 (shoulder), 286.

IR spectrum (KBr, ν, cm^{-1}): 3415.3 (OH); 2928.3 (CH); 1729.6 ($COOCH_3$) 1604.7 (ionized carboxyl); 1511, 1445.3 (aromatic C=C); 1409.8 1218.9 (phenols); 1266, 1122.7, 1076.4, 1047 (R-O-R'); 869.7 (C-H in the aromatic ring with one isolated hydrogen atom); 820.6 (C-H in the aromatic ring with two neighboring hydrogen atoms).

Results and Discussion

The main components of WSP-SG were found to be fructans (66.8 %). On top of that, galacturonic acid, galactose, glucose, arabinose, xylose and rhamnose were also found. According to previous reports on the detection of PDPGA in high molecular weight preparations of *S. asperum, S. caucasicum, S. officinale* and *A. italica* [6-10], WSP-SG was further subjected to ultrafiltration on membrane filter with a cut off value of 500 kDa in order to remove polymers with molecular weights lower than 500 kDa, so that a high molecular (>500 kDa) weight preparation HMP-SG was obtained**.**

The UV spectrum (see materials and methods) of water-soluble HMP-SG showed some absorption maxima that indicated the presence of polymers with phenolic character, and was identical to the UV spectra of polyethers obtained from *S. asperum, S. caucasicum, S. officinale* and *A. italica* [7-10].

The IR spectrum (see materials and methods) of HMP-SG showed absorption bands characteristic of phenol-carboxylic acids. Absorption bands corresponding to the hydroxyl groups attached to the aromatic ring, as well as the carboxyl and ether groups were observed. Again, the IR spectrum of HMP-SG was very similar to that of phenolic polymers from *S. asperum, S. caucasicum, S. officinale* and *A. italica* [7-10].

It was decided to detect PDPGA in HMP-SG using different techniques of NMR spectroscopy.

Two signals in the ^{1}H NMR spectrum (500 MHz, D_2O, 80°C) of HMP-SG with chemical shifts of 5.34 and 4.84 ppm were assigned to H-1 and H-2 (Fig. 1), respectively, linked to oxygen-bound aliphatic carbon atoms C1 and C2 (Fig.). The signal with chemical shift of 7.51 ppm was assigned to the aromatic proton H-2" (Fig.) and the signal with chemical shift 7.40 ppm, which integrated for 2H, was assigned to H-5" and H-6" [6-10]. The gCOSY spectrum showed a cross peak between the signals at 4.84 and 5.34 ppm, which was consistent with the coupling between H-1 and H-2 of PDPGA.

A resonance in the ^{1}H NMR spectrum at 4.19 ppm which correlated with ^{13}C resonance at 55.9 ppm in the $^{1}H/^{13}C$ gHSQCED spectrum suggested the presence of methoxy groups in carboxylic acid methyl

esters (Fig.) [10]. The gHSQCED spectrum also gave a two cross peaks between the ^{13}C resonance at 75 ppm and the ^{1}H peak at 5.34 ppm, and between 81.7 ppm (^{13}C) with 4.84 ppm (^{1}H), consistent, respectively, with CH-1 and CH-2, of PDPGA [6-10].

Other cross peaks were also observed in the $^{1}H/^{13}C$ gHSQCED spectrum, namely correlations between the following proton and carbon atom pairs: δ 4.15/64.2, 4.11/71.4 and 4.35/71.9 ppm. These cross peaks, plus others at 5.51/102.4 and 5.33/103.4 ppm, those clearly anomeric, are most likely due to the presence of polysaccharide impurities in the sample of HMP-SG. Unfortunately, we were unable to detect aromatic C-H correlations in the gHSQCED spectrum due to lack of signal.

A 2D DOSY experiment of high molecular weight preparation of *S. grandiflorum* showed that both sets of signals of methylated in carboxylic group PDPGA (4.2, 4.8, 5.35, 7.4 and 7.5 ppm) and polysaccharides fell in the same horizontal. It means that they have the same diffusion coefficient. This would imply a similar (same order of magnitude) molecular weight for the phenolic polyether and the polysaccharides. This would explain why, unfortunately, it proved not feasible to separate the phenolic polymer from polysaccharides by ultrafiltration. However, on the basis of data from IR, ^{1}H NMR, gCOSY, gHSQCED and 2D DOSY experiments, the presence of methylated PDPGA in HMP-SG was sustained by analogy with high molecular preparations of *A. italica*, which in that case it could be demonstrated by chemical and NMR analysis alone, similarly to high molecular preparations from *S. asperum*, *S. caucasicum*, *S. officinale* and *A. italica* [6-10]. We found that, in this preparation of HMP-SG, these methylated PDPGA were mixed with residual carbohydrates (polysaccharides). The disclosure of the nature, structural importance and quantitative determination of the residual polysaccharides of HMP-SG will be the subject of further studies.

ფარმაკოქიმია

Symphytum grandiflorum-ის DC (Boraginaceae) წყალშიხსნადი მაღალმოლეკულური ფრაქციის შესწავლა

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[*] თბილისის სახელმწიფო სამედიცინო უნივერსიტეტი, იოველ ქუთათელაძის ფარმაკოქიმიის ინსტიტუტი, თბილისი, საქართველო მცენარეული ბიოპოლიმერებისა და ბუნებრივ ნივთიერებათა ქიმიური მოდიფიკაციის დეპარტამენტი,

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***Symphytum grandiflorum* DC-ის წყალში ხსნად მაღალმოლეკულურ პრეპარატში (მმპ-SG), მსგავსად *S. asperum, S. caucasicum, S.officinale* and *Anchusa italica*-ს მაღალმოლეკულური პრეპარატებისა იწ, ^{1}H ბმრ, gCOSY, gHSQCED და 2D DOSY ექსპერიმენტების მონაცემების საფუძველზე დეტექტირებულ იქნა კოფეინის მჟავას წარმოებულის პოლიმერი, კერძოდ, პოლი[3-(3,4-დიჰიდროქსიფენილ)გლიცერინის მჟავა] (პდფგმ). მმპ-SG-ის 2D ^{1}H/^{13}C gHSQCED სპექტრი გვიჩვენებს, რომ *Symphytum*-ის სხვა სახეობების პოლიმერისაგან განსხვავებით და *A.italica*-ს პოლიმერის მსგავსად, *S. grandiflorum*-ის პდფგმ-ის კარბოქსილის ჯგუფების უმეტესი ნაწილი მეთილირებულია. გარდა ამისა, მმპ-SG-ის gHSQCED სპექტრი გვიჩვენებს დამატებით სიგნალებს, რაც შეიძლება განპირობებული იყოს ნარჩენი პოლისაქარიდის მინარევების არსებობით. მმპ-SG-ის 2D DOSY ექსპერიმენტი გვიჩვენებს, რომ კარბოქსილის ჯგუფში მეთილირებული პდფგმ-ის და ნარჩენი პოლისაქარიდების სიგნალების ორივე რიგი მოთავსებულია ერთ ჰორიზონტალში. ეს ნიშნავს, რომ მათ აქვთ მსგავსი დიფუზიის კოეფიციენტები. აღნიშნული ფაქტი მიუთითებს, რომ მეთილირებულ პდფგმ-ს და პოლისაქარიდებს აქვთ მსგავსი მოლეკულური მასები. ამით შეიძლება აიხსნას თუ რატომ არ მოხდა ულტრაფილტრაციით ფენოლური პოლიმერის ნარჩენი პოლისაქარიდებისაგან დაშორება. მაგრამ იწ, ^{1}H NMR, gCOSY, gHSQCED და 2D DOSY ექსპერიმენტების მონაცემების საფუძველზე *A.italica*-ს მაღალმოლეკულური პრეპარატების ანალოგიურად ნათლად დასტურდება მმპ-SG-ში მეთილირებული პდფგმ-ის არსებობა.**

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