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Pharmacochemistry

UHPLC-Q-TOF/MS Characterization of Several Compounds from the Roots and Stems Extracts of *Symphytum Asperum*

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ABSTRACT. Phytochemical study of roots/stems of *Symphytum asperum* Lepech. (prickly or rough comfrey) was carried out in order to define phenolic constituents. Firstly, grinded air-dried *S. asperum* roots (SAR) and stems (SAS) were fore-extracted exhaustively in a Soxlet apparatus with hexane and chloroform in order to remove lipids, pigments and other nonpolar compounds and afterwards these materials were treated with the aqueous mixtures of different organic solvents. Eight analytical samples were obtained. Ultrahigh-pressure liquid chromatography coupled with quadrupole time-of-flight tandem mass spectrometry (UHPLC-Q-TOF/MS) analysis of extracts of *S. asperum* roots/stems was carried out that revealed the presence of low molecular weight compounds such as caffeic, rosmarinic, chlorogenic acids, salvianolic acid, B/lithospermic acid B and several oligomeric compounds. Currently only the brutto-formulas of the oligomeric compounds are known and further investigations are in progress in order to determine exact chemical structures of those. The obtained results revealed that the comfrey roots/stems can be used as a source for the isolation of low molecular weight biologically active compounds.

Key words: *Symphytum asperum* roots/stems, polyphenols, phenolic acids, UHPLC–Q-TOF/MS

Symphytum asperum Lepech. **(**prickly or rough comfrey**)** belongs to the *Boraginaceae* family. It is a weed plant widespread in some regions of Asia, Europe, North America and Caucasus [1,2]. Previously, analgesic, anti-inflammatory, astringent, expectorant, antifungal and decongestant properties of comfrey were reported [3–5]. In folk medicine, the comfrey root has been used externally as a traditional medicinal plant (as ointments, compress, or alcohol extracts) for treating fractures, strains, thrombophlebitis and hematomas, and internally (as tea, tinctures or infusions) in treating gastrointestinal and respiratory tract diseases [6]. Preparations from the leaves and stems were used for treating rheumatism and gout, as well [7]. Previous studies showed that these beneficial properties of comfrey are the result of

the presence of numerous bioactive compounds [3,8]. It is known that comfrey contains allantoin, amino acids, A, B and C vitamins, ellagic acid, auxin, triterpenoids, tannins, rosmarinic acid, steroidal saponins, inulin, pyrrolizidine alkaloids [9-10].

Previously, the isolation of high-molecular (>1000 kDa) water-soluble fractions from the roots and stems of *S. asperum* (SA) and *S. caucasicum* (SC) was reported. Based on the IR and NMR spectroscopy data, poly[3-(3,4 dihydroxyphenyl)glyceric acid] (PDPGA) was confirmed to be the major component of these fractions [11-13]. PDPGA–SA and PDPGA–SC exhibit immunomodulatory (anticomplementary), antioxidant and antiinflammatory activities [14-15] and wound-healing property [16]. PDPGA exerted anti-cancer efficacy *in vitro* and *in vivo* against androgen-dependent and -independent human prostate cancer (PCA) cells via targeting androgen receptor, cell cycle arrest and apoptosis without any toxicity, together with a strong decrease in prostate specific antigen (PSA) level in plasma [17].

However, the biotransformation mechanism of PDPGA is not clear yet. It is obvious that a molecule of such a size and mass as PDPGA could not be passively absorbed from the gastrointestinal tract or interact directly with cell membrane receptors. Therefore, the observed biological effects are possibly caused by much smaller size/mass fragments of the PDPGA. Thus, the aim of the present study was to identify such fragments in stems and roots of aforesaid plants to clarify possible biosynthetic/metabolic pathways for the polymer as well as low molecular weight biologically active compounds.

The solid-liquid extraction technique was chosen as the first step for isolation of the compounds probably containing the fragments of PDPGA followed by the investigation of the composition of the extracts of *S. asperum* roots/stems using UHPLC-Q-TOF/MS method.

Materials and Methods

Initially, the fore-extraction was used that is necessary in order to remove unwanted substances such as waxes, fats, terpenes, and chlorophylls. On the basis of literary data, solid-liquid extraction is the most commonly used procedure prior to the analysis of polyphenolics and simple phenolics in plants. Commonly used extraction solvents are: alcohols (methanol, ethanol), acetone, diethyl ether, and ethyl acetate. However, very polar phenolic acids could not be extracted completely with pure organic solvents and the mixtures of alcohol–water or acetone–water are recommended, therefore aforesaid mixtures of solvents were applied for the extraction procedure [18].

The extracts were further fractionated by TLC and CC and then analyzed using UHPLC–Q-TOF/MS for determination of chemical content of comfrey extracts.

Sample Preparation

Grinded air-dried *S. asperum* roots (SAR) and stems (SAS) were fore-extracted exhaustively in a Soxlet apparatus with hexane and chloroform in order to remove lipids, pigments and other nonpolar compounds. The fraction SAS1 from fore-extracted stems was obtained by processing with 70% acetone. Powdered fore-extracted roots were treated with mixtures of solvents, such as alcohol–water (50% MeOH) or acetone–water (70% acetone) and the extracts SAR 2 and SAR 3 were obtained, respectively. Lastly, SAR 4 fraction was obtained by treatment of preliminary macerated fore-extracted roots with ethanol containing 2% HCl (v/v) with 50% MeOH.

Compounds (even trace amounts) were identified using UV detection at 254 nm. TLC conditions: silicagel GF 254 - stationary phase; BAW (buthanol/acetic acid/water 4:6:2; v/v/v) - mobile phase; $FeCl_3$ (2% in ethanol) or H_2SO_4 (20% in ethanol) - developers. All fractions were further fractionated on Diaion HP 20 using stepwise elution gradient (10-70% MeOH or EtOH) As a result samples SAR 2dw, SAR 2dm, SAR 3dw, SAR 3de, SAR 4dw, SAS 1dw and SAS 1dm were obtained. In addition, fraction SAR 4 was further fractionated on Diaion HP 20 eluted by water and the fraction SAR 3dm was eluted on Sephadex LH-20 column by water and sample SAR 3dsw was

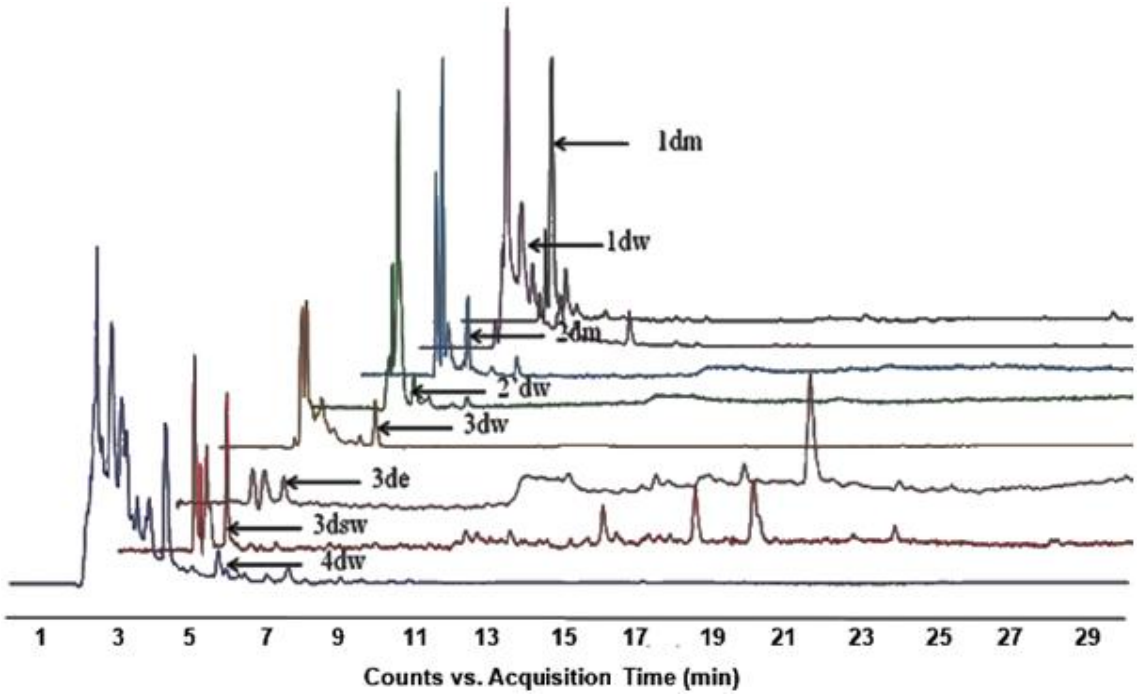

Fig. 1. UHPLC-chromatograms of the 8 samples from roots/stems extracts of *S. asperum* are indicated by arrow marks.

prepared. Finally, all eight water-soluble fractions were freeze-dried.

Apparatus

UV/VIS spectrophotometer (Mecasys Optizen Pop UV/Vis Spectrophotometer) was used for the measurement of absorbance at 286 nm which showed the presence of phenolic compounds; Chromatographic separations were performed using Agilent 1220 Infinity UHPLC System with Agilent Zorbax SB-C18 analytical column (4.6 x 250 mm, 5mm) coupled with diode array detector (DAD).

Chromatographic equipment and UHPLC-Q-TOF conditions. A sensitive, accurate and specific method coupling high-performance liquid chromatography (HPLC) with diode array detector (DAD) and electrospray ionization mass spectrometry (MS) was developed for the separation and identification of extracts of *S.asperum*. The molecular masses of the constituents were assigned by electrospray ionization mass spectrometry. Chromatographic separations were performed on a Agilent Zorbax SB-C18 analytical column (4.6 x 250 mm, 5mm). The samples on the column were eluted with a gradient mixture of acetonitrile (solvent A) and 0.1% formic acid (solvent B). The gradient program was as follows: 0 – 30 min, from 8 to 50% A. The flow rate was at 1 mL/min with column temperature at 30єC. Injection volume was set at 1 µL. Q-TOF/MS method was carried out using nitrogen to assist nebulization ion mode, negative and positive; drying gas (N_2) flow rate, 10.0 L/min; drying gas temperature, 350 єC; nebulizer, 35 psig; sheath gas temperature, 300 єC; sheath gas flow, 11 L/min; capillary, 3500 V; skimmer, 65 V; OCT 1 RF Vpp, 250 V; fragmentor voltage, 120 V. All operations, acquisition and data analysis were controlled by the Agilent Masshunter Workstation Software Qualitative Analysis, version B.01.03

Results and Discussion

All compounds were identified based on retention times, UV–Vis absorption spectrum and mass spectra by matching their molecular ions obtained by ESI-MS/MS methods with theoretical molecular weights from literature data [19-21].

The results obtained show the presence of various phenolic and non-phenolic compounds. The chromatograms of the extracts from *S. asperum* roots/

Table 1. The identified compounds in the extracts of *S.asperum* roots/stems

No.	Name *	R_t(min)	Formula	Exact Mass	ESI-MS m/z					ESI-MS/MS m/z (% base peak)
					$[M-H]^-$	$[M+Cl]^-$	$[2M-H]^-$	$[M+COOH]^-$	$[M-H-H_2O]^-$	
	2	3	4	5	6	7	8	9	10	11
1dm	ChA [19]	8.2	$C_{16}H_{18}O_9$	354.0947	353.0876					191 (100),179 (6), 126 (0.4)
	CA[19]	10.5	$C_9H_8O_4$	180.0412	179.0339					135(100)
	LA B/ SA B [20-21]	15.305	$C_{36}H_{30}O_{16}$	718.1534	717.1455					225(100),109(80)
	RA[19]	17.01	$C_{18}H_{16}O_8$	360.0871	359.0773	395.0538				161(100),197(21), 135(3)
1dw		4.2	$C_7H_{14}O_5$	178.0827	177.0756					
		5.6	$C_9H_{10}O_5$	198.0525	197.0452	233.0219				
		6.9	$C_7H_6O_4$	154.0263	153.019					
		7.4	$C_9H_{10}O_4$	182.0579	181.0505	217.029				
	ChA	8.2	$C_{16}H_{18}O_9$	354.0947	353.0876					191(100),179(6), 126(0.4)
	LA B /SA B	15.305	$C_{36}H_{30}O_{16}$	718.1534	717.1455					225(100), 109(80)
	RA	17.01	$C_{18}H_{16}O_8$	360.0871	359.0773	395.0538				161(100),197(21), 135(3)
2dm		25.753 2.8	$C_{42}H_{70}O_{14}$ C_5H_4NOS	798.4766 125.9998	797.4714 124.9926	833.448		843.4791		473((100),191(30)
		3.5	$C_9H_7N_5O$	201.0653	200.0579					
		4.2	$C_7H_{14}O_5$	178.0827	177.0756					
2dw		4.2	$C_7H_{14}O_5$	178.0827	177.0756					
3dw		4.2	$C_7H_{14}O_5$	178.0827	177.0756					
3de	CA	10.5	$C_9H_8O_4$	180.0412	179.0339					135(100)
	LA B/ SA B	15.252	$C_{36}H_{30}O_{16}$	718.1534	717.1464	753.125				225(100),109(80)
	RA	17.01	$C_{18}H_{16}O_8$	360.0871	359.0773	395.0538	719.1645			161(100),197(21),135(3)
3dw	LA B / SA B	15.305	$C_{36}H_{30}O_{16}$	718.1534	717.1455					225(100),109(80)
	RA	17.01	$C_{18}H_{16}O_8$	360.0871	359.0773	395.0538				161(100),197(21), 135(3)
4dw		25.188 27.577	$C_{22}H_{50}N_4O_8S$ $C_{27}H_{59}N_5O_9S$	530.3353 629.4039	529.3278 628.3964					301(100),260(70), 202 (40) 288(100),242(30)
		29.40	$C_{50}H_{73}N_2O_5S$	829.5189	828.5117					488(100),322(60)
		4.2	$C_7H_{14}O_5$	178.0827	177.0756					
		6.17	$C_6H_{10}O_5$	162.0528	161.0456				143.035	
		6.9	$C_8H_{12}O_7$	220.0583	219.051					
	LA B	15.305	$C_{36}H_{30}O_{16}$	718.1534	717.1455					225(100),109(80)

* RA - Rosmarinic acid; LA-Lithospermic acid B; SA B -Salvianolic acid B; ChA - Chlorogenic acid; CA - Caffeic acid

stems are given in Fig. 1.The identified compounds from these samples are summarized in Table 1.

The UHPLC-Q-TOF/MS analysis of the samples SAS 1dm, SAS 1dw obtained from *S. asperum* stems, showed the first peak at retention time (R_t) of 8.20 min. MS fragmentation of pseudomolecular ion $[M-H]^-$ in the negative mode at 353 m/z showed ion fragments at 191 (100 %), 179 (6%), 126 (0.4%) m/z that indicated the presence of chlorogenic acid [19]. At the same time the samples SAS 1dm, SAR 3de also showed another peak at R_t of 10.50 min., with the presented pseudomolecular ion $[M-H]^-$ in the negative mode at 179 m/z and ion fragment at 135 (100%) m/z after MS fragmentation, proved the existence of caffeic acid [20]. Rosmarinic acid was identified in the samples SAS 1dm, SAR 3de, SAR 3dsw, SAR 4dw as the peak at R_t of 17.01 min. MS fragmentation of pseudomolecular ion $[M–H]^-$ in the negative mode at 359 m/z and that of $[M+Cl]^-$ in the positive mode at 395 m/z showed three ion fragments at 161 (100%), 197 (21%) and 133 (3%) m/z [19]. The peak of the chromatograms of the samples SAS 1dw, SAS 1dm, SAR 3de, SAR 3dsw and SAR 4dw at R_t of 15.31 min identified salvianolic acid B or lithospermic acid B. On the basis of MS fragmentation of the pseudomolecular ion $[M–H]^-$ in the negative mode at 717 m/z which showed fragments at 225 (100%) and 109 (80%) m/z, it can be concluded that salvianolic acid B or lithospermic acid B is presented in the comfrey roots/stems extracts. (Fig. 1.). It is reported that Salvianolic acid B and Lithospermic acid B have identical structures except for the configurational assignments of two stereocenters. Through chemical correlation between a degradation product of salvianolic acid B and synthetic material, the absolute configuration of salvianolic acid B has been corrected to establish that salvianolic acid B and lithospermic acid B are one and the same compound [20-21] (Table 1). The structures of identified compounds are shown in Fig.2.

Conclusion

Thus, caffeic, rosmarinic, chlorogenic, and salvianolic acid B was detected in the comfrey root/stem 1dm, 1dw, 3de, 3dsw and 4dw extracts. On the other hand, the study revealed the presence of several compounds (oligomers and low-molecular weight ones) in the samples 1dw, 1dm, 2dw, 3dw and 4dw. Since currently only the brutto-formulas of the above men-

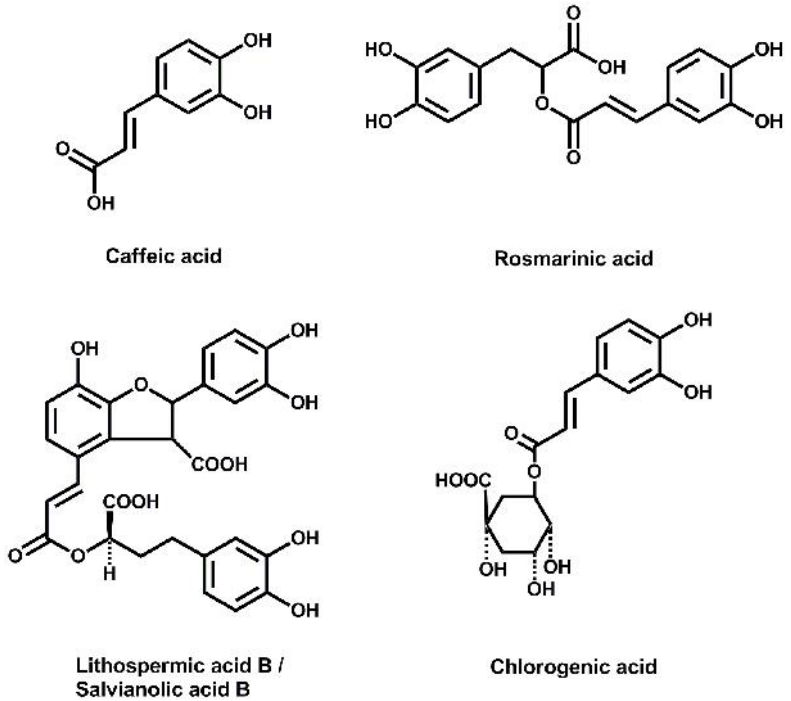

Fig. 2. Structural formulas of the identified compounds in the extracts of *S.asperum* roots/stems

tioned compounds are known, further investigations are in progress in order to determine exact chemical structure of these compounds as well as to identify fragments in stems and roots of aforesaid plants to clarify possible biosynthetic/metabolic pathways for the polymer.

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ფარმაკოქიმია

UHPLC-Q-TOF/MS მეთოდით *Symphytum asperum*-ის ფესვების/ღეროების ექსტრაქტებში შემავალი ზოგიერთი ნაერთის დახასიათება

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ფენოლური ნაერთების შემცველობის განსაზღვრის მიზნით ჩატარებულ იქნა *Symphytum asperum*-ის Lepech. (ბუსუსებიანი ან ხაოიანი ლაშქარა) ფესვების/ღეროების ფიტოქიმიური კვლევა. თავდაპირველად ლიპიდების, პიგმენტების და სხვა არაპოლარული ნაერთების მოსაცილებლად განხორციელდა დაწვრილმანებული ჰაერმშრალი *S. asperum*-ის ფესვებისა (SAR) და ღეროების (SAS) ფორექსტრაქცია ჰექსან/ქლოროფორმით და შემდგომი ექსტრაქცია სხვადასხვა ორგანული გამხსნელის წყლიანი ნარევებით. მიღებულ იქნა რვა საანალიზო ნიმუში. ექსტრაქტების UPLC-Q-TOF/MS ანალიზით დადგინდა მათში დაბალმოლეკულური ნაერთების – კოფეინის, როზმარინის, ქლოროგენის, სალვიანოლის B/ლიტოსპერმის B მჟავების და ცალკეული ოლიგომერული ნაერთების შემცველობა. დადგენილ იქნა ოლიგომერული ნაერთების მოლეკულური ფორმულები, ხოლო მათი ზუსტი ქიმიური სტრუქტურების შესწავლა შემდგომი კვლევის საგანს წარმოადგენს. აგრეთვე ანალიზის შედეგებმა აჩვენა, რომ ლაშქარას ფესვები/ღეროები შეიძლება გამოყენებულ იქნეს როგორც ნედლეული დაბალმოლეკულური ბიოლოგიურად აქტიური ნაერთების მისაღებად.

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