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Pharmacochemistry

Obtaining of Toxic Pyrrolizidine Alkaloid-Free Biologically Active High Molecular Preparations of *Symphytum asperum* and *S.caucasicum*

Lali Gogilashvili, Lela Amiranashvili, Vakhtang Barbakadze, Maia Merlani, Karen Mulkijanyan, Eka Shaburishvili

I. Kutateladze Institute of Pharmacochemistry, Tbilisi

(Presented by Academy Member E. Kemertelidze)

ABSTRACT. The isolation scheme of water-soluble crude polysaccharides and high-molecular (>1000 kDa) preparations from roots, stems and leaves of *Symphytum asperum* and *S. caucasicum* permits to remove completely the toxic pyrrolizidine alkaloids. Due to immunomodulatory, antiinflammatory and antioxidative activity, these pyrrolizidine alkaloid-free preparations may have potential pharmaceutical value and can be recommended for the development of both external and internal medical remedies. *© 2008 Bull. Georg. Natl. Acad. Sci.*

Key words: *Symphytum asperum, S.caucasicum, hepatotoxic, pyrrolizidine alkaloids, ultrafiltration.*

Introduction

Symphytum L. (Comfrey) is a common garden plant that has been used as a herbal medicine for >2000 years [1]. Comfrey is used for the treatment of broken bones, tendon damage, ulcerations in the gastrointestinal tract, and lung congestion. Comfrey promotes wound healing and/or reduces inflammation of joints. Comfrey is rich in many crucial nutrients, such as protein and antioxidant vitamins [1]. Apart from its high protein content, the major components of comfrey implicated in biological activity are allantoin, mucilage, tannin and pyrrolizidine alkaloids (PAs) [2,3] .

Traditionally comfrey has been used both internally and externally, but nowadays its internal usage is not recommended due to the presence of hepatotoxic and carcinogenic PAs. Comfrey has been found to contain as many as nine hepatotoxic PAs, including the most toxic symphytine, echimidine, and lasiocarpine [2-5]. The total alkaloids' content in air-dried plants varies between 0.13-0.18% and 0.31-0.48% in *S.asperum* and *S.caucasicum* respectively. In both species asperumine, echinatine, lasiocarpine, heliosupine, echimidine N-oxide and heliosupine N-oxide were detected [6,7]. Not all PAs are harmful, and it has been shown that PA toxicity is determined by 1,2-unsaturation in the pyrrolizidine ring and an ester function on the side-chain. PAs (1,2-dehydro-pyrrolizidine ester alkaloids) and their N-oxides, which are generally the predominant natural form of PAs, have been found to be not significantly toxic [8]. Besides, medicinal use of comfrey aqueous extracts may be considered less hazardous since the PAs are particularly insoluble in water, and thus should contain only water-soluble N-oxides. Moreover, the external use of comfrey preparations should not be hazardous since the alkaloids are converted to toxic pyrrolic metabolites (dehydroalkaloids) by mammalian liver oxidases only after being ingested. Pyrrolic metabolites cumulate in the liver, damage nuclear DNA and inhibit cytoplasmic protein synthesis. Consequently, PAs are linked with carcinogenic effects on the liver and a range of other deleteri-

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ous effects such as veno-occlusive diseases. Since a small amount of pyrroles may enter the blood and be transported to the lungs, pulmonary lesions have also been reported [3-5, 8-10].

Thus, obtaining of *Symphytum* extracts or preparations free of hazardous PAs as well as the development of rapid and sensitive methods for detection of PAs and their N-oxides even at low concentration that may be present in complex mixtures is of great importance.

One possibility to decrease the concentration of PAs is treating of crude extracts with protonated cation exchangers [11], but due to adsorption processes this procedure may lead to the loss of high molecular constituents.

For PAs determination, a visual spectrophotometrical method was elaborated, based on the reaction of 3,4-dehydro-1,2-unsaturated necine with Ehrlich reagent [12,13]. The same reaction can be applied to detect PAs on thin-layer chromatography (TLC) plates [14]. TLC, high-performance liquid chromatography (HPLC), gas chromatography (GC), and gas chromatography-mass spectrometry (GC-MS) have been applied for separating, characterizing, and quantifying the alkaloids present. Effective use of these procedures requires authentic alkaloids for standards, which are not commercially available [3]. Due to sensitivity, GC and HPLC are mainly used for the analysis of PAs in natural samples. However, in GC analysis the thermal decomposition of labile N-oxides and sometimes also of diesterified PAs is frequently observed. For these reasons and purposes, HPLC is often considered [15].

Previously it was established that glucofructan from *Symphytum asperum* and high molecular phenolic preparations of *S. asperum* and *S. caucasicum* exhibit strong biological activity (immunomodulatory, antiinflammatory and antioxidative) and were considered to be of pharmaceutical importance [16-19].

According to IR and NMR spectral data, the main constituent of high molecular preparations was a new regular caffeic acid-derived polymer, namely, poly[oxy-1-carboxy-2-(3,4-dihydroxyphenyl)ethylene] [20-23].

The isolation of high-molecular (> 1000 kDa) preparations was carried out using hot water extracts dialysis through the semipermeable cellophane membranes followed with ultrafiltration on membrane filters with cut-off value of 1000 kDa. These procedures suggested the removal of PAs with average molecular weight of 340 Da and their absence in the obtained high molecular fractions.

Therefore the goal of this work was to confirm the above-mentioned suggestion. For this purpose the detection of such alkaloids in *S. asperum* and *S. caucasicum* aqueous extracts and their monitoring at different stages of isolation of high molecular (> 1000 kDa) preparations from these organs of plants have been carried out.

The TLC was chosen as a cheap, rapid, simple and sensitive method for fast detection of PAs present in *S.asperum* and *S.caucasicum* extracts and subsequent fractions. After the TLC the alkaloids on the plate are converted to N-oxides by a hydrogen peroxide spray, followed by heating. Treatment with acetic anhydride then converts the N-oxides to pyrroles, which give blue or mauve spots with Ehrlich reagent (4-dimethylaminobenzaldehyde). The use of Ehrlich's reagent greatly improves the sensitivity of detection, bringing the limit down to 0.1 μg [14].

Materials and Methods

Isolation and purification of polysaccharides. Water-soluble crude polysaccharides were extracted from *S.asperum* and *S.caucasicum* roots, stems and leaves as described in [24, 25]. In brief, air-dried roots, stems and leaves ground up and fore-extracted exhaustively in a Soxhlet apparatus using chloroform, methanol and acetone to remove pigments, lipids and low molecular compounds. Then hot water extraction was performed, followed by dialysis and precipitation in 4 volumes of ethanol or acetone, yielding water-soluble crude polysaccharides.

Fractionation of crude polysaccharides by ultrafiltration. *S.asperum* and *S.caucasicum* crude polysaccharides from roots, stems and leaves were fractionated by ultrafiltration in an Amicon chamber (Amicon Corp. Lexington MA, USA) under nitrogen pressure (3 atmosphere) using ultrafiltration membrane with cut-off value of 1000 kDa (NMWL 1 M Filtron omega series).

Extraction of alkaloids. Air-dried ground roots, stems and leaves, crude polysaccharides and high molecular (> 1000 kDa) preparations were extracted exhaustively with methanol as described in [26].

Reagents for detection on TLC plates.

30% Hydrogen peroxide. 100 volumes solution, in which sodium pyrophosphate, 2-4 mg per ml was dissolved [14].

Acetic anhydride. Acetic anhydride, light petroleum (b.p. 80 – 100°C) and benzene, 1:4:5 (v/v/v) [14].

Ehrlich's reagent. Dimethylaminobenzaldehyde (1 g) was dissolved in absolute ethanol (70 ml), carbitol (diethylene glycol monoethyl ether; 30 ml), and hydrochloric acid (1.5 ml) [14].

TLC procedure. The alkaloids, applied as methanol extracts, were placed on silicagel plates (60 F_{254}, Merk or Silufol F_{254}, Czech Republic). The solvent systems used were: a) chloroform-methanol-25% ammonia (85:14:1,

v/v/v) [27,28]; b) chloroform-acetone-ethanol- 25% ammonia (5:3:1:1, v/v/v/v) [14]; c) chloroform–methanol–25% ammonia (100:10:2, v/v/v) [15]; d) dichloromethane–methanol–25% ammonia (85 : 15 : 1, v/v/v) [29]; e) the non-aqueous phase from n-butanol-acetic acid-water (4:1:5, v/v/v) [14]; f) chloroform-methanol (9:1, v/v) [6]; g) chloroform-acetone-methanol (7:2:1, v/v/v) [7]; h) benzene-diethyl ether-methanol (10:5:2, v/v/v) [6,7], i) chloroform-methanol-acetone (10:7.5:2.5, v/v/v) [30], j) chloroform-methanol-acetone (20:5:3, v/v/v) [30]. Runs of 10 cm were usual.

Plates were air-dried, sprayed lightly with 30% aqueous hydrogen peroxide and heated at 90-100°C for about 15 min. The plates were cooled, sprayed with acetic anhydride reagent, and heated as before for 15 min. After this some alkaloids appeared as weak brown spots, fluorescent when viewed in UV light. However, much greater sensitivity was achieved by spraying the plates with Ehrlich's reagent, followed by heating for 5-15 min. The alkaloids then appeared as blue spots against a pale yellow or practically colourless background [14]. Dragendorff's reagent [27] also has been used for detection, but it is non-specific and its sensitivity is limited by the pale colour of its spots.

Ten solvent systems, recommended for alkaloids, were used. The solvent system a) chloroform-methanol-25% ammonia (85:14:1, v/v/v) gave the best separation and distinct spots. The compounds were thus separated into three groups with the following R_f values: (a) 0.06-0.25 (highest retention); (b) 0.25-0.45 (medium retention); and (c) 0.45-0.6 (lowest retention) detectable under UV light and according to positive reaction with Ehrlich's reagent.

Results and Discussion

The detection of PAs was carried out in *S. asperum* and *S.caucasicum* raw and fore-extracted roots (SA-RR, SC-RR and SA-FR, SC-FR), stems (SA-RS, SC-RS and SA-FS, SC-FS) and leaves (SA-RL, SC-RL and SA-FL, SC-FL).

Thereafter we attempted to detect PAs in *S. asperum* and *S.caucasicum* roots, stems and leaves crude polysaccharides (SAR-CP, SAS-CP, SAL-CP and SCR-CP, SCS-CP, SCL-CP, respectively). Finally, the same was done in *S. asperum* and *S.caucasicum* roots, stems and leaves high molecular preparations (SAR-HMP, SAS-HMP, SAL-HMP and SCR-HMP, SCS-HMP, SCL-HMP, respectively). The results are given in the Table.

SA-FR, SC-FR, SA-FS, SC-FS, SA-FL, SC-FL contained only traces of PAs while in SAR-CP, SAS-CP, SAL-CP, SCR-CP, SCS-CP, SCL-CP, SAR-HMP, SAS-

Table

TLC of PAs [solvent system used: a) chloroform-methanol-25% ammonia (85:14:1, v/v/v)]

Samples	Spots*	Compounds R_f
SA-RR	+	0.06
	+++	0.28
	+++	0.52
SA-RS	+	0.10
	++++	0.25
	+	0.40
	+++++	0.45
SA-RL	+++	0.10
	+	0.20
	+	0.25
	+	0.40
	+++++	0.60
SC-RR	+	0.06
	+++++	0.28
	+++	0.52
SC-RS	+++++	0.06
	+++	0.13
	+	0.28
SC-RL	+	0.06
	+	0.13
	+	0.28
	++	0.48
SA-FR, SC-FR, SA-FS, SC-FS, SA-FL, SC-FL	traces	
SAR-CP, SAS-CP, SAL-CP, SCR-CP, SCS-CP, SCL-CP, SAR-HMP, SAS-HMP, SAL-HMP, SCR-HMP, SCS-HMP, SCL-HMP	absent	

* + - relative intensity of distinct spots.

HMP, SAL-HMP, SCR-HMP, SCS-HMP, SCL-HMP PAs were not detected at all.

According to TLC data, the isolation scheme of water-soluble crude polysaccharides and high-molecular (>1000 kDa) preparations from roots, stems and leaves of *S. asperum* and *S. caucasicum* permits to remove completely the toxic PAs. Thus we can conclude, that due to immunomodulatory, antiinflammatory and antioxidative activity [16-19], these pyrrolizidine alkaloid-free preparations may have potential pharmaceutical value and can be recommended for both external and internal medical application.

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ფარმაკოქიმია

ტოქსიკური პიროლიზიდინის ალკალოიდებისაგან თავისუფალი, *Symphytum asperum*-ის და *S.caucasicum*-ის ბიოლოგიურად აქტიური, მაღალმოლეკულური ფრაქციების მიღება

ლ. გოგილაშვილი, ლ. ამირანაშვილი, ვ. ბარბაქაძე, მ. მერლანი, კ. მულკიჯანიანი, ე. შაბურიშვილი

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(წარმოდგენილია აკადემიის წევრის ე. ქემერტელიძის მიერ)

Symphytum asperum **და** ***S. caucasicum*****-ის ფესვებიდან, ღეროებიდან და ფოთლებიდან წყალში ხსნადი პოლისაქარიდების ჯამის და მაღალმოლეკულური (>1000 kDa) ფრაქციების გამოყოფის სქემა საშუალებას იძლევა მთლიანად იქნას მოცილებული ტოქსიკური პიროლიზიდინის ალკალოიდები. ამ პიროლიზიდინის ალკალოიდებისაგან თავისუფალი იმუნომოდულატორული, ანთების საწინააღმდეგო და ანტიოქსიდანტური აქტივობის მქონე *** ***S. asperum*** **და** ***S. caucasicum*****-ის ფრაქციები შეიძლება რეკომენდებული იქნას როგორც გარეგანი, ისე შინაგანი გამოყენების პრეპარატების შესაქმნელად.**

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