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Pharmacochemistry

Allantoin- and Pyrrolizidine Alkaloids-Free Wound Healing Compositions from *Symphytum asperum*

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ABSTRACT. Extracts from the Caucasian species of comfrey – *Symphytum asperum* and *S.caucasicum* have been used in folk medicine in the treatment of some kinds of disorders, mainly fractures and wounds. The aforenamed extracts contain allantoin, claimed to be a cell proliferation-stimulating agent responsible for the wound-healing properties of *Symphytum*, and, on the other hand, hepatotoxic pyrrolizidine alkaloids which strongly restrict internal use of comfrey extracts. In the present investigation, we obtained allantoin- and toxic pyrrolizidine alkaloids-free composition containing crude polysaccharides and novel biopolymer from *S. asperum* roots – poly[3-(3,4-dihydroxyphenyl)glyceric acid] (PDGA), and attempted to appraise its pharmacological properties in *in vitro* (anticomplementary and antioxidant assays) and *in vivo* experiments (mouse excisional wound and skin burn models). PDGA exhibited marked antioxidant and anticomplementary activity in contrast with polysaccharides, which displayed no detectable anticomplementary and antioxidant efficacy. Besides, ointment, containing 2.5% crude polysaccharides and PDGA was found to have pronounced wound-healing properties, by efficacy not yielding to 2.5% allantoin ointment. The obtained results allow assuming with high degree of reliability that wound healing activity of comfrey preparations could be associated not only with allantoin but also with PDGA. *© 2009 Bull. Georg. Natl. Acad. Sci.*

Key words: *Symphytum asperum, wound healing, allantoin, pyrrolizidine alkaloids, excisional wounds, skin burn*

Introduction

Comfrey (*Symphytum* L.), used in ancient times, owes its reputation as a medicine mainly to its external use as a wound-healing agent [1]. In folk medicine, comfrey has been used as an externally applied poultice to promote wound healing and/or reduce joint inflammation. It is used for the treatment of broken bones and tendon damage as well [2]. Through the ages preparations from the Caucasian species of comfrey – *Symphytum asperum* and *S.caucasicum* have also been used in folk medicine in the treatment of some kinds of disorders and wounds [3]. Besides wound-healing, *Symphytum* has been applied to ulcers, and rheumatic and arthritic diseases. Based on these ethnomedical indications, it may be speculated that interference with the immune system may be involved in its activity. On the other hand, comfrey extracts contain hepatotoxic pyrrolizidine alkaloids, which strongly restrict internal use of these extracts in modern medicine. Inflammation as a first stage in the natural wound-healing process usually results from an exogenous injurious stimulus; in these inflammatory reactions, products of activated cells and/or nonspecific humoral systems are involved [4].

The active ingredient in comfrey is thought to be allantoin, which is reported to promote cell division and the growth of connective tissue, bone, cartilage and accelerate the healing of wounds [1,5].

In our previous work crude polysaccharides of *S.asperum* showed significant antiexudative activity in the acute inflammatory process, modeled in mice [6]. To investigate the curative properties of *Symphytum,* test systems dealing with both immunomodulation and wound healing were selected. The immunomodulatory activities of high molecular components of aqueous extract of *Symphytum* roots were assessed by testing their effect on functional parameters of humoral and cellular branches of the innate immune system. For the humoral part human complement and for the cellular part human polymorphonuclear leukocytes (PMNs) were selected as relevant immune parameters [7,8]. Selection of the immunological parameters was based on the claimed therapeutic value of *Symphytum.* The involvement of both immune functions in inflammatory diseases such as rheumatic arthritis and ulcers is well established. Moreover, it is generally accepted that both immune functions participate in the early inflammatory phase of the wound-healing process. Interference of *Symphytum* with these immune functions may basically contribute to its claimed curative effects [4].

The crude polysaccharides of *S. asperum* and *S. caucasicum* exhibited immunomodulatory (anticomplementary) and antioxidant activities [7]. Neutral glucofructan and acidic arabinogalactan were shown to be the principal and minor constituents, respectively, of crude polysaccharides from *S. asperum* and *S.caucasicum* roots [9]. In order to characterize immunomodulatory constituents on the basis of their molecular mass, the *S. asperum* and *S.caucasicum* roots' crude polysaccharides were fractionated by ultrafiltration (UF) using membrane filters with cut-off values of 1000 kDa, 100 kDa and 10 kDa. In the complement assays, the fractions of *S. asperum* and *S.caucasicum* roots' fractions containing molecules with a relative mass (M_r) > 1000 kDa (27 and 26% of crude polysaccharides, respectively) showed higher inhibitory activity than the crude polysaccharides themselves [7]. Fraction with M_r >1000 kDa exhibited also a pronounced antioxidant activity, which is manifested by the suppression of lucigenin-dependent chemiluminescence (CL_{luc}) production related to the superoxide anion formation in the cell-free hypoxanthine/xanthine-oxidase system [8].

The main constituent of *S. asperum* and *S. caucasicum* high-molecular (>1000 kDa) fractions was found to be a new caffeic acid-derived polymer, namely poly[3-(3,4-dihydroxyphenyl)glyceric acid] (PDGA) or poly[oxy-1-carboxy-2-(3,4-dihydroxyphenyl)ethylene] [10-13]. This super-gigantic caffeic acid-derived polymer, but not most polysaccharides removed by ultrafiltration, exhibited anticomplementary, antioxidant and anti-inflammatory activities [7,8,13,14]. Consequently, we supposed that biological activity of crude polysaccharides arises from contaminating new phenolic polymer. Proceeding from the above-mentioned, we suggested that active principle of *Symphytum* as wound healing plant might be not only allantoin but also phenolic polymer – PDGA.

Besides, it is necessary to emphasize that allantoin, claimed to be a cell proliferation-stimulating agent, and responsible for the wound-healing properties of *Symphytum*, exhibited nonsignificant modulatory effects in the complement assays [classical pathway (CP) and alternative pathway (AP): IC_{50}>2.8 mg/ml) and on the generation of chemiluminescence (CL) by zymosan-stimulated human PMNs (IC_{50}>3.8 mg/ml) [4].

The purpose of the present study was to obtain an allantoin- and toxic pyrrolizidine alkaloids-free polysaccharide preparation from *S. asperum* roots and comparative investigation of its wound-healing properties in comparison with allantoin and in combination with allantoin in *in vivo* experiments.

Materials and Methods

Isolation and fractionation of crude polysaccharides. Extraction of water-soluble crude polysaccharides from *S.asperum* roots was carried out as described in [15], and fractionated by ultrafiltration in accordance with [7].

Extraction and detection of allantoin. Allantoin from air-dried raw fore-extracted *S. asperum* roots and crude polysaccharides was obtained with exhaustively methanol treating as described in [16]. Allantoin, applied as methanol extract, was placed on silicagel plates (60 F_{254}, Merk or Silufol F_{254}, Czech Republic). The solvent system used was: methyl ethyl ketone – acetone – formic acid – water (40:2:1:6 v/v). The plates were air-dried in a fume hood. For the detection of allantoin, TLC plates were sprayed with Ehrlich's reagent and heated in an oven at 100°C for 5 minutes. Allantoin appeared as yellow spots on a practically colorless background [17].

Determination of anticomplementary and antioxidant activity. Determination of complement activity by CP, AP and terminal route (TR) and antioxidant activity using the method of luminol-enhanced chemiluminescence (CL_{lum}) by zymosan-stimulated PMNs have been described in [7,8].

Test and reference preparations. Pyrrolizidine alkaloids- and allantoin-free medicinal form has been developed - 2.5 % ointment of water-soluble fraction of

comfrey roots containing polysaccharides and PDGA (**S**). Composition of distilled monoglyceride and glycerine was chosen as an ointment base. Reference preparations - 2.5% allantoin ointment (**A**) and combined ointment **SA** (1.25% allantoin + and 1.25% crude polysaccharides) were prepared using the same ointment base.

Animal pretreatment. White outbred male mice weighting 22-25 g were used in all experiments. Animals were maintained in a 12-h night/day rhythm in groups of 5 animals per cage under constant access to water and food. Wound healing properties were studied using mouse excisional wounds and skin burn models [22, 23]. All procedures adhered to regulations related to animal use and experiments were performed in accordance with the principles of [24]. 24 hours prior to the beginning of each experiment animals were clipped and 4õ2 cm skin area was depilated on back and left flank under light ether anesthesia.

Mouse excisional wound model. Two 1 cm diameter skin rags were cut out on depilated skin area. Operation was carried out under ether anesthesia. Treatment of animals began within 24 h after operation. Five groups of 6 animals were randomized and investigated: groups 1, 2 and 3 were treated with **S**, **A** and **SA** ointments, respectively, group 4 – with ointment base only, while group 5 stayed untreated and served as treated/ untreated control. Wounds were treated with 0.1 ml of ointment per wound once a day. Animals were inspected daily to ensure adequate feeding and mobility and to reapply agents to injured areas. Before applying ointment the wounds were disinfected with 3 % hydrogen peroxide solution.

Mouse skin burn model. Area and depth standardized skin burns were caused on depilated skin area under ether anesthesia using a special device with the temperature controller and contact electroheater (1 sm^2 square copper plate) [8-9]. In our case the temperature of a contact plate was 150^0C, exposition time – 10 sec. In these conditions burn corresponds to IIIA-degree in accordance with clinical classification of burns [22]. Treatment of animals began within 24 h after burn induction. Randomization of experimental animals and treatment scheme were same as the above-mentioned.

Estimation of healing process. Clinical supervision over wound healing process was carried out daily, up to full healing. The general condition of animals was estimated on the basis of behavioural reactions, appetite, body weight, survival rate.

Wound condition (infection, exudation, scab formation) was inspected and wound area was measured using transparent grid template (with the area of a single cell 0.25 cm^2). Once a week wounds were photographed. Wound healing effect was estimated by the reduction of injured area in relation to initial and calculated under the formula:

$$\Delta = (S_{exp} / S_{in}) \times 100\ \%, \text{ where}$$

S_{in} - initial wound area on day 1.

S_{exp} - wound area on day of measurement.

The obtained data were processed statistically using Student's **t**-test [25].

Results and Discussion

S. asperum roots' fraction containing molecules with a relative mass (M_r) < 10 kDa (65 % of crude polysaccharides) contained 96% sugars, which basically consists of glucofructan and practically did not contain uronic acid. Under the conditions of the experiments, these compounds did not exhibit any activity of interest, either in the complement or in the antioxidant (CL_{lum}) assay (IC_{50} > 500 μg/ml). Fractions (1000-100) kDa and (100-10) kDa were found to exhibit some anticomplementary and antioxidant activity (IC_{50} 59.3 and 51.5 μg/ml, respectively), but their yields were negligible – 1.7 and 0.5%, respectively [7].

Fraction <1000 kDa, which is basically a mixture of polysaccharides, displayed no detectable anticomplementary and antioxidant activities (Table) in contrast to fraction >1000 kDa [7,8], the main component of which is PDGA.

Consequently, the active principle of crude polysaccharides of S. *asperum* roots must be its phenolic constituent - PDGA. In conclusion, the pronounced antioxidant and anticomplementary activity of PDGA based fraction isolated from the roots of *S. asperum* suggests that this native polymer is a potential antiinflammatory, vasoprotective, and wound-healing agent. This offer

Table

Inhibitory effects of fraction <1000 kDa on human complement activity and CL_{lum} generated by zymosan-stimulated PMNs

Preparation	IC_{50} (μg/ml) *			
	CP	AP	TR	CL_{lum} by PMNs
<1000 kDa	>333	>667	>286	>500

* Based on 3 independent observations

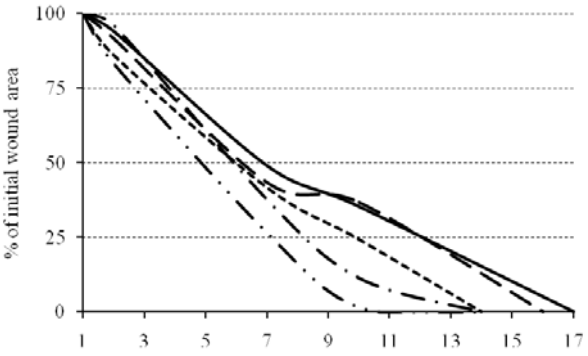

Fig. 1. Wound healing dynamics: — - control; – – - base; — -S; – × - A; – ×× - SA. x-axis – days of experiment

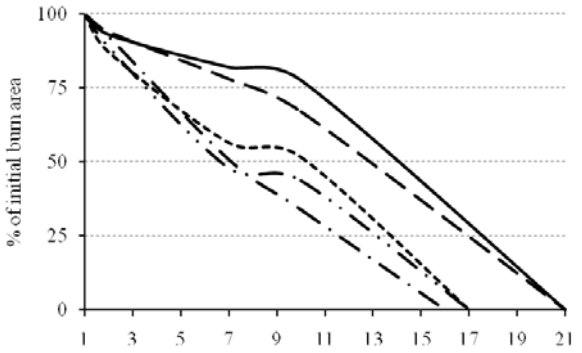

Fig. 2. Burn healing dynamics: — - control; – – - base; — -S; – × - A; – ×× - SA. x-axis – days of experiment

promises for the development of effective therapeutic means for the treatment of vascular disorders, wounds of various etiology, and inflammatory processes caused by free radicals and/or enzymes.

The detection of allantoin was carried out separately in *S. asperum* raw, fore-extracted roots and crude polysaccharides. *S. asperum* raw roots contained allantoin, fore-extracted roots contained only traces of allantoin, while in crude polysaccharides, allantoin was not detected at all. According to TLC data, pretreatment (fore-extraction) of roots using methanol and dialysis of aqueous extracts of crude polysaccharides permits to remove completely low molecular compounds including allantoin and toxic pyrrolizidine alkaloids [21]. Thus, the ointment of crude polysaccharides from *S. asperum* roots does not contain allantoin or toxic pyrrolizidine alkaloids.

Results obtained in mouse excisional wound model are presented in Fig. 1.

From the presented results it is visible that the wound area in animals, treated with ointments "S" "A" and «SA», since day 7 of experiment was significantly less (15, 23 and 45 %, respectively) than in the control group ($p<0.01$). Scab rejection in groups treated with ointments "S" "A" and "SA" began on day 5, whereas in control - on day 7. In mice treated with ointment "SA", full reepithelization was completed 4 days earlier (on day 10) than in animals treated with ointments "S" and "A". Full healing of wounds in control group ended on day 17. No differences in healing dynamics were evidenced between control and ointment base-treated groups.

Results obtained in mouse skin burn model are presented in Fig. 2.

Differences in burn wound area between animals, treated with ointments "S" "A" and «SA» and control group became statistically significant since day 4 of the experiment, and for day 7 burn area in these groups was respectivly, 31, 38 and 41 % less than in the control group ($p<0.01$). Primary eschars in groups "S" "A" and "SA" were torn away on day 8-10, while in control - on day 12-14. Full healing in animals treated with ointment "A" was achieved 1 day earlier (in 16 days) than in "S" and "SA" treated animals, and 5 days earlier than in control group. No differences in healing dynamics were recorded between control and ointment base treated animals.

The results obtained with both excisional and burn wound models revealed the expressed reparative action of all ointments studied.

Besides, it is necessary to mention that after complete healing of wounds animals in control group still had noticeable scar at the place of injury while in trial groups animals the scar was absent.

Conclusions

1. Pharmacological study of wound healing activity of ointment containing allantoin- and pyrrolyzidine alkaloids-free high-molecular polysaccharide fractions from comfrey roots revealed that in efficiency it does not yield to 2.5 % allantoin ointment.

2. The obtained results allow to assume with high degree of reliability that established wound healing activity is associated with poly[3-(3,4-dihydroxyphenyl)glyceric acid].

3. The established pharmacological action of ointment allows recommending it for treatment of wounds and second and third-degree burns.

ფარმაკოქიმია

ალანტოინისა და პიროლიზიდინის ალკალოიდებისაგან თავისუფალი ჭრილობის შემახორცებელი კომპოზიცია *Symphytum asperum*-იდან

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(წარმოდგენილია აკადემიკოს ე. ქემერტელიძის მიერ)

ლაშქარას კავკასიური სახეობების *Symphytum asperum*-ის და *S. caucasicum*-ის ექსტრაქტებს ხალხურ მედიცინაში უძველესი დროიდან იყენებდნენ სხვადასხვა სახის დაავადებების სამკურნალოდ, კერძოდ, მოტეხილობების და ჭრილობების შესახორცებლად. აღნიშნული ექსტრაქტები შეიცავენ უჯრედის პროლიფერაციის მასტიმულირებელ აგენტს – ალანტოინს, რომელთანაც ასოცირდება ზემოთ ხსენებული თვისებები, და, მეორეს მხრივ, ჰეპატოტოქსიკურ პიროლიზიდინის ალკალოიდებს, რომლებიც ძლიერ ზღუდავენ ამ ექსტრაქტების შინაგან გამოყენებას. მოცემული კვლევის ფარგლებში *S. asperum*-ის ფესვებიდან მიღებულია ალანტოინის და პიროლიზიდინის ალკალოიდებისაგან თავისუფალი პოლისაქარიდების ჯამის და ახალი ბიოპოლიმერის პოლი[3-(3,4-დიჰიდროქსიფენილ)გლიცერინის მჟავას] (პდგმ) კომპოზიცია. შეფასებულია ამ კომპოზიციის შემცველი კომპონენტების ფარმაკოლოგიური თვისებები *in vitro* (ანტიკომპლემენტარული და ანტიოქსიდანტური) და *in vivo* (ჭრილობის და კანის დამწვრობის მოდელი თაგვებში) ექსპერიმენტში. დადგენილია, რომ პოლისაქარიდებისაგან განსხვავებით, ცალკე აღებული პდგმ ამჟღავნებს გამოხატულ ანტიოქსიდანტურ და ანტიკომპლემენტარულ თვისებებს. გარდა ამისა, პოლისაქარიდების ჯამის და პდგმ შემცველი 2.5% მალამო ჭრილობის შემახორცებელი ეფექტურობით უტოლდება ალანტოინის 2.5% მალამოს. მიღებული შედეგებიდან გამომდინარე ცხადია, რომ ლაშქარას პრეპარატების ჭრილობის შემახორცებელ უნარს განაპირობებს არა მხოლოდ ალანტოინი, არამედ ასევე პდგმ.

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