PHYTOCHEMICAL STUDY OF SOME *SYMPHYTUM OFFICINALIS* EXTRACTS CONCENTRATED BY MEMBRANOUS PROCEDURES

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Lucrarea prezintă un studiu fitochimic calitativ privind anumite clase de compuşi: taninuri, aminoacizi, terpenoide, steroli, triterpene, flavonoide, aminoacizi, compuşi reducători, saponine, alcaloizi din extracte EtOH 50% şi MeOH 50%, de concentraţie masică 10% de Symphytum officinalis. S-a determinat cantitativ alantoina – compus bioactiv caracteristic pentru tătăneasă.

Extractele au fost ulterior concentrate prin procedee membranare (ultrafiltrare) utilizând o membrană de ultrafiltrare Millipore cu cut-off (masa de excludere moleculară) de 5,000 daltoni (Da). Determinările analitice s-au realizat în extractele iniţiale, în permeate şi în soluţiile concentrate.

This work present a phytochemical qualitative study regarding certain classes of compounds: tannins, amino acids, terpenoides, sterols, triterpenes, flavonoids, reducing agents, saponins, alkaloids in Symphytum officinalis (10% mass concentration) extracts in EtOH 50% and MeOH 50%. The allantoin – a bioactive compound characteristic for the comfrey - was quantitatively determined. The extracts were further concentrated through membranous procedures (ultrafiltration) using Millipore ultrafiltration membranes with 5000 daltoni (Da) cut-off (molecular mass exclusion). The analytical determinations were carried out in the initial extracts, permeates and concentrated solutions.

Keywords: phytochemical, *Symphytum officinalis*, membranous procedures

1. Introduction

Symphytum officinalis (Fam. Boraginaceae) is known from ancient times and was used in folk medicine in externally applied poultices – to promote wound healing, to reduce joint inflammation, in the treatment of broken bones and tendon damages, in rheumatic and arthritic diseases, and it was also internally applied for ulcers [1-2].

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On the other hand, the comfrey extracts contain hepatotoxic pyrrolizidine alkaloids, which strongly restrict the internal use of these extracts in modern medicine [3].

The toxic effects of Comfrey were described early in 1980s and, since then, various pyrrolizidine alkaloids, such as lasiocarpine, lycopsamine, intermedine, symlandine, riddelline and mainly symphytine have been identified [4].

The plants roots contain allantoin (0.6-2%) [5], pyrrolizidine alkaloids (0.02–0.07%), polyphenolic acids, triterpenic saponosides, proteins, caffeic acid, chlorogenic acid, rosmarinic acid, tannins (2.4%), carotene (0.63%), choline, asparagine, coniferin, mucopolysaccharides, starch, gumiresins, phytosterols, carotenoids, A, C, E vitamins, riboflavin and B_{12}. Moreover, they contain an antigonadotropic principle - lithospermic acid and immuno-stimulant polyosides, as well as high amounts of mineral substances (Ca, K, P, Mg, Fe, Mn, Na, Zn) [6,7].

The active compound in comfrey is thought to be the allantoin, which was reported to promote cell division and the growth of the connective tissue, bones, cartilages and to accelerate the wounds' healing [8,9]. The oxidative stress studies have demonstrated that increased levels of allantoin are associated with the oxidative stress [10], like as in diabetes [11-13], inflammatory and autoimmune conditions [14-16] and cardiovascular [17], renal [18], pulmonary [19,20] and Wilson's [21,22] diseases.

The membranous processes have wide application within biotechnologies' domain, being able to efficiently separate and concentrate the heat sensitive compounds – as bioactive compounds from plants. The membranous techniques – microfiltration, ultrafiltration, nanofiltration, inverse osmosis – compared with other classical methods present the advantage of separation, purification or concentration in one single stage, at cold, without the use of any other reagents. Therefore, additional purification operations are eliminated and products with higher quality than those processed by conventional methods were obtained [23].

2. Materials and methods

Reagents: chloroform, H_2SO_4 96%, NaOH 0.5N, Mg, HCl 0.5 N, HCl 2%, acetone, nynhidrin 1%, Fehling reagent (I and II), NH_3, Dragendorff reagent, Hager reagent, 0.1% ferric chloride, phenylhydrazine hydrochloride 0.33%, sodium acetate 15%, potassium ferricyanure 1.67%. All reagents were of analytical purity and were purchased from Sigma Aldrich.

Equipments

The vegetal material was grinded to a fine powder using a GRINDOMIX GM200 mill. The spectrometric determinations were realized using a Jasco V 530 spectrometer.

Extracts' preparation

The finely grinded *Symphytum officinalis* roots were subjected to hydro-alcoholic (methanolic and ethanolic) extraction. The 10% mass concentration extracts were prepared through maceration in 50% ethanol (in water) and 50% methanol (in water), at room temperature, during 7 days, under mild stirring.

Extracts' concentration

The extracts were firstly filtrated, and then concentrated on ultrafiltration using Millipore UF membranes of regenerated cellulose, with 5.000Da cut–off. A KMS Laboratory Cell CF-1 installation, (Koch Membrane – Germany) was used for the ultrafiltration, the concentration ratio being 2:1.

Test for tannins: 2-3 drops of 0.1% ferric chloride were added to 2 mL extract, and a brownish green or blue-black coloration was.

Test for terpenoids (Salkowski test): 2 mL of each extract were mixed in 2 mL chloroform, followed by the careful addition of 1 mL concentrated H_2SO_4 in order to form a distinct layer. If a reddish brown coloration is forming on the interface, then the terpenoids' presence is confirmed [24].

Identification of sterols and triterpenoides: was carried out through the Liebermannn-Bourchard reaction: 3-10 mL hydro-alcoholic extract were evaporated into a porcelain capsule, the residue was dissolved in 0.5 mL chloroform, then 0.5 mL acetic anhydride ware added. The resulted solution was passed into a dry test tube, followed by the addition of 1-2 mL concentrated H_2SO_4 at the test tube bottom; in the case when sterols or triterpenoids are present in solution, a red-brown or violet ring appears after 5-10 min at the contact zone between the two liquids as well as a green–bluish or violet upper layer [25].

Test for flavonoids: Two methods were used to determine the flavonoids [26, 27]: the *lead acetate test* and the *Shinoda reagent test*

- ***Lead acetate test:*** 2 mL hydro-alcoholic extract were treated with few drops of 5% lead acetate solution. The formation of a yellow precipitate indicated the presence of flavonoids [28].
- ***Shinoda reagent test:*** 1-2 fragments of metallic Mg were mixed with 3-4 mL extract, then 0.5 mL concentrated HCl were added; after 5 minutes a red

color appeared– for the *flavonols*, orange – for the *flavons*, red-violaceous - characteristic to *flavanones* or green – in the case of *flavanols* [29].

Identification of amino acids: 5 mL hydro-alcoholic extract were evaporated in a porcelain capsule, the residue being retaken with 1.5 mL water at warm, then they were filtrated in a test tube and 10 drops of 1% acetone solution of nynhidrin were added. The mixture was heated into a boiling bath, 20-25 minutes. The appearance of a violet or blue-violet color indicated the amino acid presence.

Identification of reducing agents: 1 mL hydro-alcoholic extract was diluted with 2 mL distillated water, 1 mL Fehling solution (I +II) was added and it was heated to boiling, 30 minutes. The appearance of a red-brick precipitate (the cuprous oxide) on the test tube bottom indicated the presence of reducing agents.

Identification of saponins - was realized through the foam test: 1 mL hydro-alcoholic extract was diluted with 9 mL water, from which 4 mL were brought into a test tube and stirred 15 sec. The formation of a foam column of 1cm high, persistent minimum 15 min, presumptively showed the presence of saponins.

Identification of alkaloids – 15 mL hydro-alcoholic extract were evaporated into a capsule on water bath; the residue was dissolved in 5-10 mL HCl 2%, by mixing with a glass rod, and by heating (on water bath). The settled down or filtrated acid solution was brought into a separation funnel and concentrated NH_3 (pH=8-10) was added. The alkaline solution was extracted with ether or chloroform. The chloroform extract, washed with distillated water (in a separation funnel), was dehydrated with anhydrous sodium sulfate and evaporated into a capsule on water bath (under hood). The residue was dissolved in 1.5 mL 2% HCl; the decanted solution was separated in 3 test tubes, in equal volumes. To one test tube 2-3 drops of Dragendorff reagent were added, to other one 2-3 drops of Hager reagent, while the third test tube served as control. The occurrence of some white-yellowish precipitates indicated the presence of alkaloids in the analyzed vegetal extract [25].

Quantitative determination of allantoin – 1mL solution was pipetted in to a calibrated flask of 25 mL, 1 mL 0.5 N NaOH solution was added and it was maintained in boiling bath 12 min. (for occurrence of the allantoin hydrolysis to allantoic acid). The samples were maintained in water bath at 20 ^{0}C, then 1 mL 0.5 N HCl was added and it was maintained in boiling bath 2 min. After cooling the samples on ice, 1 mL sol. 0.33% phenylhydrazine hydrochloride and 15%

sodium acetate were added, then the mixture was stirred and the sample was maintained 15 min into a water bath at 30^0 C. After that, it was left into a recipient with ice till freezing (15-30 min). 3 mL 3N HCl and 1 mL of 1.67% potassium ferricyande solution were added, by intermittently stirring 30 min. when a red-brick precipitate (phenylhydrazona glioxilic acid) was formed. The formed compound was dissolved in the distilled water by completing it to the sign, and it resulted a red-brick colored solution which was analyzed by spectrometry (λ = 540 nm).

Determination of the sensitivity parameters of the analytical method used for the allantoin quantitative determination. The *detection limit* and the *quantification limit of the* method were determined.

Limit of Detection - LoD represents the lowest concentration c_L or quantity q_L, obtained for the lowest signal x_L which could be detected with reasonable accuracy for a given analytical method.

The lowest signal x_L is the signal of k times greater than the average value of the blank, σ_{blanc}, where k is a numerical factor chosen according to the requested level of requested confidence. As the k value is higher, the confidence level is higher.

In wide terms, *the detection limit* is the lowest concentration of analyte in the sample which could be surely distinct from zero [30].

In practice, in order to determine *the lowest detectable signal,* 10 blank samples, measured once, each one independently, were used and the signal was calculated according to the formula:

$$X_{LD}=X_{m\ (blanc)}+3\ \sigma_{blanc}$$

where σ_{blanc}=the standard deviation of the blank

Limit of Quantification – LoQ - is the lowest concentration or quantity of analyte which could be quantitatively determined with an acceptable level of repeatability and accuracy.

In order to practically determine *the lowest quantifiable signal* 10 blind samples, measured once, each one independently, were used and the signal was calculated after the formula:

$$X_{LQ}=X_{m\ (blanc)} + 10\ \sigma_{blanc}$$

where σ_{blanc}= the standard deviation of the blank

3. Results and discussions

The results regarding the phytochemical screening of comfrey extracts are summarized in the Table 1.

Table 1

Bioactive compounds identified in *Symphytum officinalis* hydro-alcoholic extracts

No.	Compound	Test	10%extract in 50%EtOH			10% extract in 50%MeOH		
			initial	permeate	concentrate	initial	permeate	concentrate
1.	Tannins	$FeCl_3$	+	-	++	+	±	++
2.	Terpenoides	Chloroform + sulfuric acid	+	-	++	+	±	++
3.	Sterols + triterpenoides	Liebermann-Bourchard reaction	+	±	++	+++	+++	++
4.	Flavonoids	Lead acetate	++	+	+++	++	+	+++
5.	Amino-acids	Nynhidrin	+	++	±	++	+	±
6.	Reducing Agents	I+II Fehling Reagent	++	+	++	++	++	++
7.	Flavones	Schinoda reaction	+	±	++	+	±	+
8.	Saponins	Foam reaction	+	-	++	+	±	++
9.	Alkaloids	Dragendorff reagent / Hager reagent	++	+	++	+++	+	++

Legend: - = absent; ± = low present; + = present; ++ = abundant; +++ = very abundant

It was observed that:

- ✓ Generally, the analyzed bioactive compounds were present in the initial extracts, their presence in the permeates was low or even they missed, while they become abundant in the concentrates – excepting the amino-acids, which were better represented in the permeates comparative to the concentrates;

- ✓ In the case of the reducing agents it was concluded that their presence was almost constant in all types of extracts.

For the *quantitative analysis of allantoin,* a stock water solution of 4mg/ml allantoin was used and the diluted solutions used for the realization of the calibration curve were200, 400, 800, 1200 µg/ mL. The following calibration curve was obtained, having a good correlation factor of R^2=0.9937 (Fig. 1):

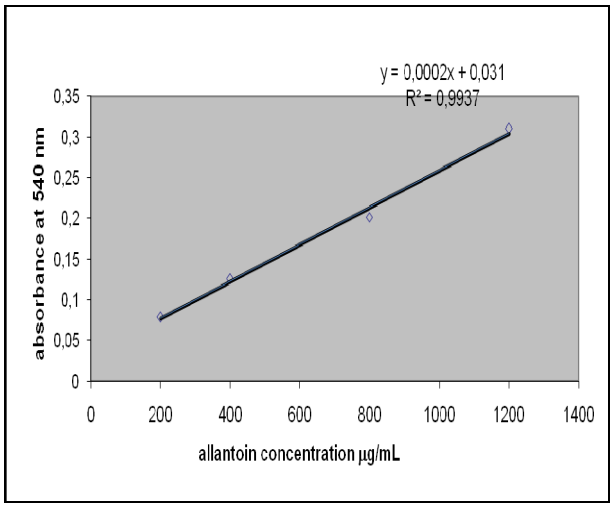

Fig. 1. Calibration curve of allantoin

The results regarding the allantoin determinations in different types of extracts are presented in the Table 2.

Table 2

Allantoin determination in *Symphytum officinalis* extracts

No.	**Extract Type**		**Allantoin conc.** µg/mL
1.	10% -mass 50% EtOH	initial	362.5
		permeate	167
		concentrate	377
2.	10% mass 50% MeOH	initial	373.5
		permeate	198
		concentrate	***436.5***

It was observed that the allantoin concentration was higher in the methanolic extracts than that in ethanolic ones.

The detection limit and the *quantification limit* of the analytical method used for the quantitative allantoin determination were calculated based on the calibration curve and the previously indicated formulas, and they were:

LoD = 173.6 µg/mL

LoQ = 1024.25 µg/mL

The values found by us were in accordance with the theoretical values requested for this analytical method:

LoD < 200 µg/mL and LoQ > 200 µg/mL [28].

4. Conclusions

✓ The quantitative determinations of tannins, amino-acids, terpenoides, sterols, triterpenes, flavonoids, reducing agents, saponins, alkaloids were performed in the obtained extracts. A quantitative determination of allantoin - a compound characteristic for the comfrey - was made, the highest allantoin quantity - *436.5 µg/mL*- being found in the concentrate of 50% methanolic extract;

✓ The obtained hydro-alcoholic *Symphytum officinalis* extracts, 10% mass concentration in 50% EtOH and 50% MeOH were concentrated through membranous procedures (ultrafiltration) on regenerated cellulose membranes with 5.000 Da cut-off ;

✓ The studied bioactive compounds were present in initial extracts, while their presence diminished or even missed in permeates and become abundant in the concentrates.

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R E F E R E N C E S

[1] *D.V. Awang*, The American Herb Association. Quaterly Newsletter, **vol. 6**, no. 4, 1989, pp. 6-7

[2] *D. Rode*, Trends Pharmacol. Sci., **vol. 23**, no. 11, 2002, pp. 497-499

[3] *V. Barbakadze, K. Mulkijanyan, L. Gogilashvili, L. Amiranashvili, M. Merlani, Z. Novikova, M. Sulakvelidze,* Pharmacochemistry, Bull. Georg. Natl. Acad.Sci., **vol.3**, no.1, 2009

[4] *Maria Fernanada Pereira Lavieri Gomes, Cristina de Oliveira Massoco, Jose Guilherme Xavier, Leoni Villano Bonamin* – Comfrey *(Symphytum Officinale L.)* and experimental hepatic carcinogenesis: A short-term carcinogenesis model study, e CAM, 2007, pp. 1-6

[5] *B.H. Havsteen*, Pharmacology &Therapeutics **96**, 2002, pp. 67-202
[6] *A. Saija, M. Scalase, M. Lanza, D. Marzulla, F. Bonina , F. Castelli*, Free Rad. Biol. and Med. **vol. 19**, 1995, pp. 481 -486
[7] *C.S. Fodorea, L. Vlase, S. Suciu, M. Tamas, S. E. Leucuta, L. Bersan*, Ovidius" University Annals of Medical Science – Pharmacy, **vol. II**, no. 2, p. 70, 2004
[8] *D.V.Awang*, The American Herb Association. Quaterly Newsletter, **vol.6**, no. 4, pp. 6-7, 1989
[9] *D. MacKay, A.L. Miller*, Altern Med. Rev., **vol. 8**, no. 4, 2003, pp.359-377
[10] *Adviye A. Tolun a, Haoyue Zhang a, Dora Il'yasova b, Judit Sztáray a, Sarah P. Young a, D.S. Millington*, Allantoin in human urine quantified by ultra-performance liquid chromatography–tandem mass spectrometry, Analytical Biochemistry **402**, 2010, pp. 191–193
[11] *R. Kand'ar, P. Zakova, V. Muzakova*, Monitoring of antioxidant properties of uric acid in humans for a consideration measuring of levels of allantoin in plasma by liquid chromatography, Clin. Chim. Acta **365**, 2006, pp. 249–256
[12] *I.F. Benzie, W. Chung, B. Tomlinson*, Simultaneous measurement of allantoin and urate in plasma: analytical evaluation and potential clinical application in oxidant:antioxidant balance studies, Clin. Chem. **45**, 1999, pp. 901–904
[13] *S.W. Ma, B. Tomlinson, I.F. Benzie*, A study of the effect of oral glucose loading on plasma oxidant:antioxidant balance in normal subjects, Eur. J. Nutr. **44**, 2005, pp. 250–254.
[14] *M. Grootveld, B. Halliwell*, Measurement of allantoin and uric acid in human body fluids: a potential index of free-radical reactions in vivo?, Biochem J. **243**, 1987, pp. 803–808.
[15] *S. Yardim-Akaydin, A. Sepici, Y. Ozkan, B. Simsek, V. Sepici*, Evaluation of allantoin levels as a new marker of oxidative stress in Behcet's disease, Scand. J. Rheumatol. **35**, 2006, pp. 61–64
[16] *S. Yardim-Akaydin, A. Sepici, Y. Ozkan, M. Torun, B. Simsek, V. Sepici*, Oxidation of uric acid in rheumatoid arthritis: is allantoin a marker of oxidative stress?, Free Radic Res. **38**, 2004, pp. 623–628.
[17] *W. Doehner, N. Schoene, M. Rauchhaus, F. Leyva-Leon, D.V. Pavitt, D.A. Reaveley*, *G. Schuler, A.J. Coats, S.D. Anker, R. Hambrecht*, Effects of xanthine oxidase inhibition with allopurinol on endothelial function and peripheral blood flow in hyperuricemic patients with chronic heart failure: results from 2 placebo-controlled studies, Circulation **105**, 2002, pp. 2619–2624.
[18] *R. Kand'ar, P. Zakova, V. Muzakova*, Monitoring of antioxidant properties of uric acid in humans for a consideration measuring of levels of allantoin in plasma by liquid chromatography, Clin. Chim. Acta **365**, 2006, pp. 249–256.
[19] *T. Ogihara, H.S. Kim, K. Hirano, M. Imanishi, H. Ogihara, H. Tamai, R. Okamoto, M. Mino,* Oxidation products of uric acid and ascorbic acid in preterm infants with chronic lung disease, Biol. Neonate **73**, 1998, pp. 24–33
[20] *T. Ogihara, R. Okamoto, H.S. Kim, A. Nagai, T. Morinobu, H. Moji, H. Kamegai, K. Hirano, H. Ogihara, H. Tamai, M. Mino*, New evidence for the involvement of oxygen radicals in triggering neonatal chronic lung disease, Pediatr. Res. **39**, 1996, pp. 117–119
[21] *P. Ferenci,* Pathophysiology and clinical features of Wilson disease, Metab. Brain Dis. **19**, 2004, pp. 229–239
[22] *H. Ogihara, T. Ogihara, M. Miki, H. Yasuda, M. Mino*, Plasma copper and antioxidant status in Wilson's disease, Pediatr. Res. **37**, 1995, pp. 219–226
[23] *Gh. Batranescu, D. Garganciuc, G. Paun Roman, G. Popescu, C. Lehr*, Procese de membrane in biotehnologii, Ars Docendi, Univ. Bucuresti, 2006
[24] *G.A. Ayoola, H.A.B. Coker, S.A. Adesegun, A.A. Adepoju-Bello, K. Obaweya, E.C. Ezennia, T.O. Atangbayila*, Tropical Journal of Pharmaceutical Research, **vol. 7**, no. 3: 1019-1024, 2008

[25] *V. Istudor, I. Ciulei*, Farmacognozie Fitochimie Fitoterapie, **vol II,** 1998
[26] *A. Sofowara*, Medicinal plants and Traditional medicine in Africa. Spectrum Books Ltd, Ibadan, Nigeria. p. 289, 1993
[27] *J.B. Harborne*, Phytochemical methods, London. Chapman and Hall, Ltd. pp. 49-188, 1973
[28] *H. Usman, Fi Abdulrahman and A.Usman*, Qualitative Phytochemical Screening And In Vitro Antimicrobial Effects Of Methanol Stem Bark Extract Of Ficus Thonningii (Moraceae), Afr. J. Trad. Cam **vol. 6,** no**.** 3, 2009, pp. 289 – 295.
[29] *G.E. Trease, W.C. Evans*, Pharmacognosy. 13th edn. Bailliere Tindall, London, p.176-180, 1989
[30] *I.Gh. Tanase*, *A. Pana, G.L. Radu, M. Buleandra*, Validarea metodelor analitice – principii teoretice and studii de caz, Ed. Printech, 2007.