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ORIGINAL PAPER

Concentration of *Symphytum officinale* extracts with cytostatic activity by tangential flow ultrafiltration

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Abstract

Symphytum officinale L (Boraginaceae) species are currently used in the Romanian traditional medicine to treat different human and animal disease, being also active in certain cancer forms.

This work's aim consists in obtaining of Symphytum officinale concentrated extracts by using performance membrane processes, aqueous extracts prepared were concentrated by tangential flow ultrafiltration with a Koch Laboratory Cell CF-1 membrane. The cytostatic activity of the total plant extracts were studied on HeLa cells culture. By comparison to the bystander value of 100%, we noticed that in vitro treatment of HeLa neoplasic cells with these concentrated extracts determined a mitoinhibitory effect with statistical and cytostatical significant amplitude. These values were of almost 57,6% for Symphytum officinale.

Keywords: *Symphytum officinale*, ultrafiltration, cytostatic activity, HeLa cells, potential antitumoral agents

Introduction

Symphytum officinale L – comfrey (Boraginaceae family) are used in Romanian traditional medicine to treat different human and animal disease, being also active in certain cancer forms [1-7]. Comfrey has been used as herbal medicine for more than 2000 years to treat broken bones, tendon damage, ulcerations in the gastrointestinal tract, lung congestion, and joint inflammation, and to promote wound healing [8]. Despite recent scares over the hepatotoxicity of some of its alkaloids, the plant is still widely used by herbalists, especially as external treatment.

Comfrey root contains allantoin, mucopolysaccharides, flavones, steroidal saponins and various amounts of pyrrolizidine alkaloids. It also contains some phenolic acids (rosmarinic, chlorogenic, caffeic and lithospermic acids) with anti-cancer and anti-oxidant action.

Biological active compounds separation and concentration from the liquid media through membrane techniques offer a new approach regarding herb extract processing. Compared with classical methods, membrane techniques bring the advance of the separation and concentration of a certain compound, in a single phase, at environment temperature, without the interference of other chemical reactives [9-11].

A comparative study on hydro alcoholic extracts of *Symphytum officinale*, separated and concentrated by ultrafiltration on specific membranes, are reported and discussed in this

paper. The extracts prepared were concentrated by tangential flow ultrafiltration with a Koch Laboratory Cell CF-1 membrane.
Our study also aimed at investigating the cytostatic effects of the total plant extracts concentrated by ultrafiltration techniques, on HeLa cells. *In vitro* cell cultures were cultivated on Eagle's MEM growing medium, supplemented with different concentration of plant extracts. After 24, 48 and, respectively 72 hours of incubation, all the cyto-physiological parameters of investigated tumor cells were affected. The cytostatic property of the studied biopreparations was appreciated through the standard value imposed for the *in vitro* selection of the potential antitumoral agents (inhibitory impact of minimum 50%) [12].

Materials and Methods

Extract preparation
The extract was prepared by maceration, using ethylic alcohol (70% v/v) as solvent and taking the following factors into account: the root is homogenized into a fine powder by using the GRINDOMIX GM200 mill, the contact duration between the plant and the solvent was of 7 days, sporadic mechanically stirring, working temperature (20^oC). The plant's mass concentration in the solvent was of 10%.
Extract concentration
The extract was processed by filtration, microfiltration (MF) through Millipore membrane with 0.45 μm pores, followed by pre-concentration and concentration by the ultrafiltration (UF) procedure. The concentration ratio was 2:1. Ultrafiltration membranes with cut-off of 50,000 Da (Koch membrane) have been used for the pre-concentration (UF1), while Millipore ultrafiltration membranes with cut-off of 1,000 Da for *Symphytum officinale* have been used for the concentration (UF2).
The concentration ratio (expressed as a volumetric ratio between the permeate and concentrate) was 2:1. The installation on that micro- and ultrafiltration have been used was the KMS Laboratory Cell CF-1 type, furnished by from Koch Membrane – Germany.
Analysis
The polyphenols and flavones from Comfrey characterization have been accomplished by UV-VIS spectroscopy and chromatography HPLC.

Spectrophotometric determinations:
The quantitative analysis of flavonoids and polyphenols was made using the methods described in the Romanian Pharmacopoeia IX^{th} Edition [13].

HPLC analysis:
Apparatus
HPLC separations for method development were carried out on system from Jasco, consisting of Jasco PU – 1580 Intelligent HPLC Pump and Jasco UV 1575 Intelligent UV-Vis detector. Solvents were degassed with Jasco DG-2080-54 4-Line Degasser. Gradient control, data acquisition and analysis were provided by computer running Borwin software from Jasco. The chromatographic column used for the method was Kromasil 250mm x 4.6 mm with 5μm packing.
Sample Preparation
The samples were analyzed on a RP C18 column gradiently eluted with two-phase system consisting of acetic acid, water (pH 2.65) and acetonitrile; detection was made on a diode array detector set between 320-370 nm. Polyphenolic compounds (chlorogenic acid, caffeic

acid, ferulic acid, coumaric acid, rosmarinic acid, rutoside, luteolin and quercetol) were quantified in our samples using RP HPLC method. It was obtained a very good peak-to-peak separation efficiency and a good linearity for all the analytes (R^2>0.998).

Cytostatic activity analysis:

The preliminary tests of cytostatic activity for the Comfrey concentrated extract have been carried out on HeLa cell lines (HeLa ACC 57, DSMZ, Germany).

Mycoplasm-negative negroid human cervix epithelium carcinoma (HeLa ACC 57, DSMZ, Germany) cells were cultured in DMEM medium (Biochrom AG, Germany) supplemented with 10% fetal bovine serum (Sigma, Germany), 100 µg/mL streptomycin (Biochrom AG, Germany), 100 IU/mL penicillin (Biochrom AG, Germany) and 50 µg/mL amphotericin B (Biochrom AG, Germany), at a density of $5x10^5$ cells in 75 cm^2 flasks, in a humidified 5% CO_2 atmosphere at 37°C. When the cells reached confluence they were detached from the flask with 0.25% trypsin + 0.02% EDTA (Biochrom AG, Germany) in the normal medium and then centrifuged at 1800 rpm for 2 minutes. The cells were seeded at a density of $1x10^5$ cells/mL in the experimental tubes containing 2ml of DMEM medium. The medium of the 24 hours cell cultures was changed either with a normal one (control cultures) or with one containing the vegetal extracts (treated cultures), in a variable dose. After 24 and 48 hours of *in vitro* treatment, the total proteins amount [14], protein dynamics, total cell number (cytometry), the dead cells/alive cells ratio (exclusion test with trypan blue) and the cell cultures development were estimated. The cytostatic property of the studied biopreparations was appreciated through the standard value imposed for the *in vitro* selection of the potential antitumoral agents (inhibitory impact of minimum 50%).

Results and Discussions

The etalon curve has been drawn in order to determine the quantitative determination of the polyphenols (as chlorogenic acid) and flavones quantities from the biopreparation from *Geranium robertianum*, by spectrophotometer methods (figs.1 and 2).

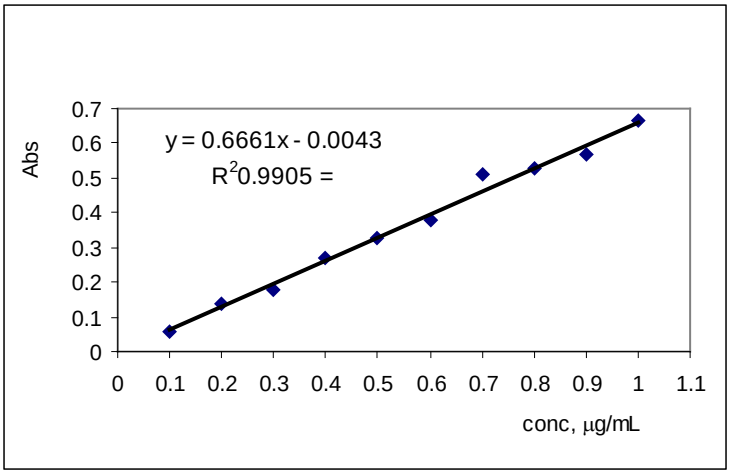

Figure 1. The etalon curve for the polyphenols quantitative determination

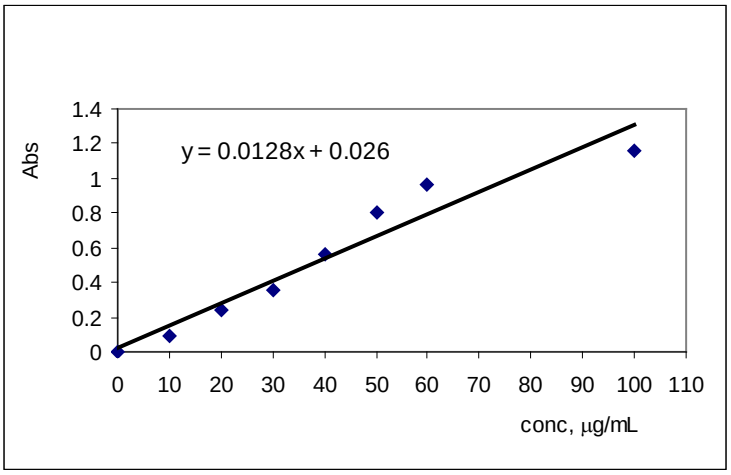

Figure 2. The etalon curve for the flavones quantitative determination

Table no.1 shows the obtained results at the *Symphytum officinale* hydro alcoholic extracts processed by consecutive operations such as microfiltration - ultrafiltration.

Table 1. *The obtained results by the Symphytum officinale hydro alcoholic extract processed by micro- and ultrafiltration*

Process	s.u., %		Polyphenols concentration, mg/L		Permeate flow at 4 bar, L/m^{2}h
	permeate	concentrate	permeate	Concentrate	
Alcoholic extract of *Symphytum officinale*	2,498	-	0,492	-	-
Microfiltration (MF)	1,977	-	0,469	-	119,3
Pre-concentration (UF1)	1,105	2,179	0,473	-	98,3
Concentration (UF2)	1,538	2,742	0,191	0,837	19,3

In *Symphytum officinale* hydro alcoholic extracts the some polyphenolic compounds were identified by HPLC: chlorogenic acid, cafeic acid, ferulic acid, cumaric acid, rutin, rozmaric acid, luteolin and quercetol. The HPLC cromatograms for hydro-alcoholic extract and after ultrafiltration are presented in figures 3 and 4.

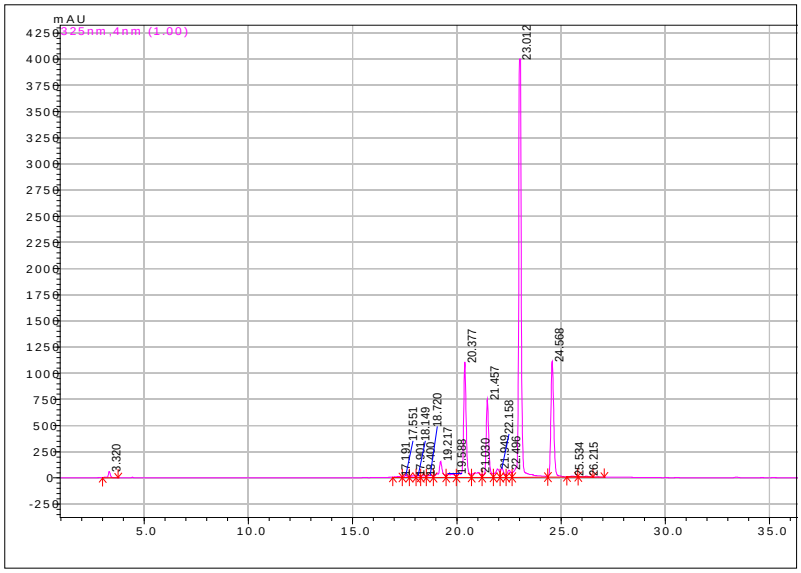

Figure 3. Chromatograms of hydro alcoholic *Symphytum officinale*

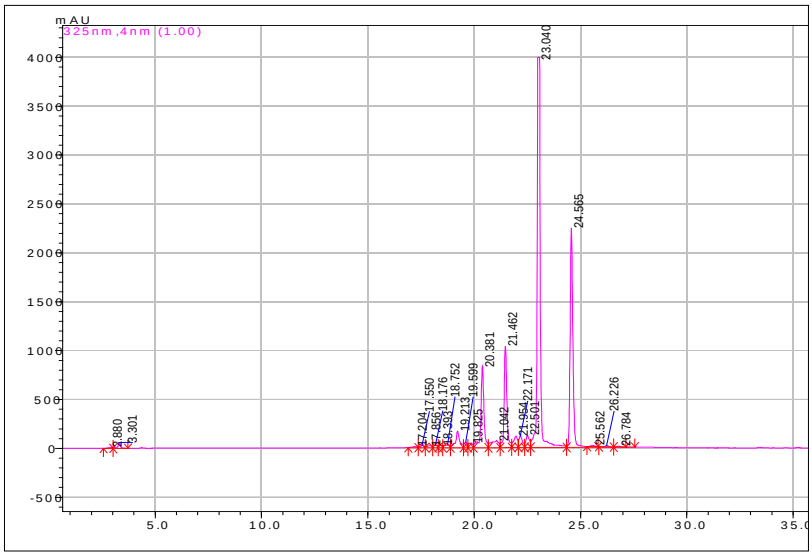

Figure 4. Chromatograms of concentrate hydro alcoholic *Symphytum officinale* extract by UF

In figure no.5 the reactivity of the HeLa cells' cellular mitosis was presented, these cells were treated with concentrate extract from *Symphytum officinale*, such as for the HeLa neoplasic cells the total cellular number registered an important decrease.

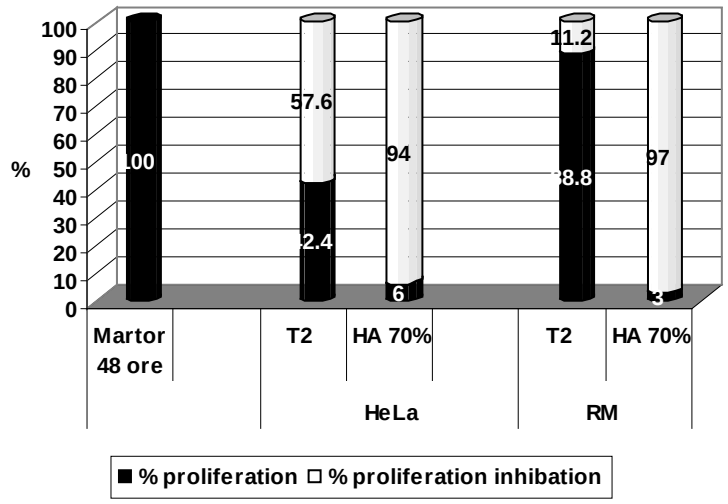

Figure 5. The cellular proliferation process' reactivity amplitude at HeLa neoplasic cell cultures, in case of testing the Comfrey alcoholic concentrate biopreparations' effect, as compared with the quality control cultures

The experimental data were demonstrated the negative impact on the cell cultures development at 48 and at 72 hours overcoming the minimum level of the potential cytostatic agents (of 50%), set up by the pre-screening programs. By comparison to the bystander value of 100%, we noticed that *in vitro* treatment of HeLa neoplasic cells determined a mitoinhibitory effect with a statistical and cytostatical amplitude, these values were 57,6%, while for the normal RM cell cultures the same indicator were only 11,2%.

Conclusion

The processing of vegetal extracts by the MICROFILTRATION – ULTRAFILTRATION line-up leads both to the concentration of biological active compounds from the proteins and polyphenols classes and its separation from smaller molecular weight compounds (free aminoacids, monosaccharides etc.) which pass in the ultrafiltration permeate.
By comparison with the bystander value of 100%, we noticed that *in vitro* treatment of HeLa neoplasic cells determine a mitoinhibitory effect, this proving a statistical and cytostatical significant amplitude, with values of almost 57,6% for *Symphytum officinale*. The *Symphytum officinale* concentrated extract obtained by the membrane processes (micro- and ultrafiltration) might be chosen as potential cytostatic agents.

Acknowledgments

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References

1. C. PARVU, *Universul plantelor,* Editura Enciclopedica, 1991, pp. 476-536
2. D. BOWN., *Encyclopaedia of Herbs and their Uses,* Dorling Kindersley, London, 1995.
3. M. GRIEVE, *A Modern Herbal, Ed*. Penguin, 1984
4. D.R. JR YANCE, S.M. SAGAR, *Targeting Angiogenesis with Integrative Cancer Therapies. Integr. Cancer Ther*., 5(1): 9-29 (2006)
5. A. CHEVALLIER**,** *The Encyclopedia of Medicinal Plants,* Dorling Kindersley, London, 1996
6. A. OLINESCU, G. MANDA, M. NEAGU, S. HRISTESCU, C. DASANU, *Roum Arch Microbiol Immunol* 52 (2):73-80 (1993)
7. W.B. PARK, S.Y. LYU, J.H. KIM, S.H. CHOI, H.K. CHUNG, S.H. AHN, S.Y. HONG, T.J. YOON, M.J. CHOI, *Cancer Biother Radiopharm*., 16(5): 439-447 (2001)
8. D. RODE, *Trends Pharmacol Sci* **23**: 497–499 (2002)
9. M. MULDER, *Basic Principles of Membrane Technology*, 2nd edition, Kluwer Academic Publishers, Dordrecht/Boston/London, 1996, pp.284-297
10. R.E. KESTING, *Synthetic Polymeric Membranes*, Mc Groww –Hill, New York, 1971;
11. GABRIELA PAUN-ROMAN, GHEORGHE BATRINESCU, DANA GARGANCIUC, GEORGETA POPESCU, *Procese membranare la separarea si concentrarea unor compusi bioactivi din medii lichide naturale*, Ed. Cartea Universitara, Bucuresti, 2006, pp.65-80
12. J. LEITER, D.J. ABOTT, S.A.SCHEPARTZ, *Cancer Res.*, 25 : 20-35 (1965)
13. x x x, *Farmacopeea Romana*, Editia a IX-a, Editura Medicala Bucuresti, 1976, pp.260
14. V. I.OYAMA, H. EAGLE, *Proc. Soc. Exper. Biol. & Med*., 91:305-307 (1956)