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Chemical composition and antioxidant activity of *Salvia officinalis* concentrated by ultrafiltration

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Abstract

The aim of this study was to determine the best conditions to obtain the highest total polyphenol content from *Salvia officinalis* hydroalcoholic and aqueous extracts and to study the main bioactive compounds and the antioxidant activity of the concentrate obtained during ultrafiltration. 8%, 10% and 15 % (mass concentration) hydroalcoholic extracts of *Salvia officinalis* in 70% ethanol and an aqueous extract (8%) were prepared. The obtained extracts were further purified and concentrated through membrane procedures (ultrafiltration). The aqueous extracts were successively concentrated through ultrafiltration membranes of regenerated cellulose with 10000 Da, than 5000 Da and 3000 Da, the highest concentration being obtained using the 3000 Da Millipore membranes. For hydroalcoholic extracts 3 types of membranes were used: Millipore ultrafiltration membranes with cut-off of 10.000 Da and 5.000 Da and polysulfone composite membrane (PSF). The contents of total polyphenols, proteins, reducing sugars and flavones were determined. The values ranged between: 321.23 – 404.21 μg/ mL –polyphenols, 1.04 – 1.72 mg/ mL – proteins, 26.01 - 99.48 mg/mL – flavones and 35.05 – 138.16 μg/ mL – sugars. The concentrated extracts showed promising levels of DPPH radical scavenging activity (42.12%–70.82%), the highest values being obtained for the extracts concentrated by Millipore membranes 5000 Da.

Keywords: *Salvia officinalis,* ultrafiltration, flavones, sugars, polyphenols, protein

Introduction

Salvia officinalis L (sage) is an aromatic and ornamental herb, known from Greeks and Romans ancient times and mentioned in the papers of Dioscorides and Galen. It is found in the spontaneous vegetation in Dalmatia, Croatia, Bosnia, Herzegovina, Serbia, Bulgaria, Albania, Macedonia, Greece and the Iberian peninsula / 1 /. Some species have economic importance while others are used as spices or flavors in perfumery and cosmetics /2/.

In Romania, from the approximately 500 species of the genus *Salvia,* 3 species are occurring, from which only 2 species are cultivated (*Salvia officinalis*, *Salvia splendens* – grown as an ornamental), both a spontaneous and a cultivated species (*Salvia sclarea* L. – Serlai) and 10 spontaneous species, many of them common throughout the country (*Salvia Glutionsa* L. Cinstet – shady places, woods, *Salvia pratensis* – on fields, *Salvia nemorosa* L. and *Salvia nutans* L., *Salvia austriaca* Jacq, *Salvia verticillata* L.- in open places). One of these species - *Salvia transilvanica* Schur- is endemic in Romania /1/.

The medicinal *Salvia* has the main importance. The essential oils contained in *Salvia officinalis* are used due to their healing properties in a wide range of diseases of the nervous

system, heart and circulatory system, the respiratory illness, digestive, metabolic and endocrine diseases, while *Salvia officinalis* infusion is used for its haemostatic effect, estrogen, antiperspirant, antiseptic, hypoglycemia and for many other therapeutic properties/ 3 /.

Phytochemical investigation of *Salvia officinalis* revealed a great number of bioactive compounds possesing a variety of biological activities. The main bioactive ingredient of *Salvia officinalis* is its essentials oil /4/. Essential oils of *Salvia stenophyla, Salvia runcinata* and *Salvia repens* exhibit anti-inflammatory and antimalarian properties /5/. Essentials oils from *Salvia officinalis* and *Salvia trilobata* were found to have antibacterial action /2/. *Salvia milthiorrhiza* is one of the most important and popular Chinese medicinal plants and is used for the prevention and tratment of stasis, pains, dysmenorrhea, heart disease, liver and intumescente of the spleen /6/.

Membrane processes, such as microfiltration (MF), ultrafiltration (UF), nanofiltration (NF) and reverse osmosis (RO) represent processes having potentiality for the concentration of medicinal plant extracts. Ultrafiltration processes offer many advantages over the conventional technologies. When compared with other classical methods, the membrane processes have the advantages of separation, purification and concentration of a certain compound, in a single phase, at the ambient temperature, without the interference of any other chemical reactive /7-11/.

The aim of this work was to analyze the potentiality of a membrane-based process in the separation and concentration of polyphenols from *Salvia officinalis* extracts, in order to develop a natural antioxidant. The hydroalcoholic and aqueous extracts of *Salvia officinalis* were then submitted to UF processes in order to evaluate the effect of the nominal molecular weight cutoff (MWCO) on the rejection of the membranes towards proteins, polyphenols, flavones and reducing sugars. Moreover, the polyphenol content of the extracts was determined by High Performance Liquid Chromatography (HPLC), and their antioxidant capacity was determined by the percentage of DPPH inhibition.

Materials and methods

Reagents

Folin-Ciocalteu reagent, phenol, sulfuric acid, ethanol, sodium acetate, aluminum chloride, methanol were provided from Sigma-Aldrich Chemical Co., Germany, while the other reagents were analytical grade.

Equipments

The vegetal material was grounded to a fine powder by means of GRINDOMIX GM200 mill. The spectrophotometric measurements were made using a Jasco V 530 spectrophotometer.

Extract Preparing

The leaves of *Salvia officinalis* were finely grinded using a GRINDOMIX GM200 mill. The extracts of 8%, 10% and 15% mass concentration were obtained by grinding in 70% ethanol at room temperature, during 7 days, under gentle mixing. The 8% aqueous extract was obtained by maceration at room temperature, during 24 h.

Concentration of extracts

Firstly the extracts were filtered and then concentrated on UF ultrafiltration Millipore membranes of regenerated cellulose with 10000 Da, 5000 Da and 3000 Da cut–off and on polysulfone composite membranes, PSF. A KMS Laboratory Cell CF-1 installation was used for ultrafiltration (Koch Membrane – Germany) and the concentration ratio was of 2:1.

Determination of flavones content was done by spectrophotometry using the method described in "Farmacopeea Romana" Xth Edition, using sodium acetate 100 g/L and aluminum chloride 25 g/L /12/.

Determination of polyphenols content was realized by spectrophotometry at 760 nm using the Folin–Ciocalteu reactive/13/. The concentration of polyphenols in sample was calculated based on a calibration curve of cafeic acid in the range 10-100 μg/mL.

Determination of proteins content was realized by spectrophotometry at 660 nm, by Lowry method /14/.

Determination of sugars content was realized by spectrophotometry at 490 nm with phenol – sulfuric acid - Dubois method /15/.

HPLC analysis of the obtained extracts

The chromatographic analyses of *Geranium robertianum* aqueous extracts were performed by HPLC–DAD method. The chromatographic measurements were carried out using a HPLC SHIMADZU system with following components: LC-20AD sp Pump, the Kromasil C18 column for polyphenol-polycarboxilic acids, DGU-20As Degasser, CTO-20AC thermostat column, detector SPD-M20A diode array Shimadzu, LCMS – Shimadzu solution software.

The used standards were: rutin, luteolin, quercetin, kaempferol, gallic acid, cafeic acid, coumaric acid, ferulic acid, myricetin, rosmarinic acid.

Determination of antioxidant activity was made spectrophotometrically, by evaluation of Trolox Equivalent Antioxidant Capacity (TEAC) using the method based on the decrease of the DPPH maximal absorbance, at 519 nm in the antioxidant presence /16,17/ by the determination % DPPH inhibition.

The antioxidant activity (radical scavenging activity) was calculated using the expression:

$$\% \text{ inhibition} = [(A_B - A_A)/A_B] \times 100$$

where: A_B = control absorbance; A_A = sample absorbance.

Results and Discussion

Flavones content of the obtained extracts was determined spectrophotometrically at 430 nm, based on the calibration curve obtained for rutozide in a concentration ranged between 1-12 mg/mL, the correlation factor R = 0.9962 (Tables 1 and 2).

Table 1. Flavones content in *Salvia officinalis* hydroalcoholic (70%) extracts

Type of membrane / Sample	Flavones mg/mL					
	Millipore 10000 Da		Millipore 5000 Da		PSF	
	permeate	concentrate	permeate	concentrate	permeate	concentrate
8% mass extract	27.07	*73.57*	28.01	69.08	38.49	40.22
10% mass extract	42.36	*99.48*	28.69	79.38	50.22	53.88
15% mass extract	32.17	*75.40*	26.01	62.26	45.98	48.27

It was concluded that:

- In the hydroalcoholic extracts of *Salvia officinalis* the content of *flavones* was proportionally with the concentration of plant mass, the highest content was obtained

in the case of the 10% (mass concentration) extract in 70% ethanol, the increase concentration of mass plant above this value resulted in flavones amount decrease.
- For concentration of flavones the Millipore membrane of 10000 Da was more efficient then Millipore of 5000 Da membrane and PSF.

Table 2. Flavones content in *Salvia officinalis* aqueous extract

Type of membrane	Flavones mg/mL					
	Millipore 10000 Da		Millipore 5000 Da		Millipore 3000 Da	
	permeate	concentrate	permeate	concentrate	permeate	concentrate
8% aqueous extract	14.25	17.63	11.47	16.93	3.05	20.27

- In the case of the 8% aqueous extract successively concentrated by Millipore membranes of 10000 Da-5000 Da-3000 Da the content of flavones was smaller than in the case of the hydroalcoholic extracts and the highest concentration was obtained using the Millipore membrane of 3000 Da.

Total polyphenols content. It was spectrophotometrically determined at λ =760 nm, with Folin –Ciocalteu reagent /13/. The polyphenols content was calculated using the calibration curve for caffeic acid within a concentration range of 10-100 μg/mL, the correlation factor being R = 0.9918. The obtained results are presented in Tables 3 and 4.

Table 3. Total polyphenols content in *Salvia officinalis* hydroalcoholic (70%) extracts

Type of membrane	Polyphenols μg/ mL					
	Millipore 10000 Da		Millipore 5000 Da		PSF	
	permeate	concentrate	permeate	concentrate	permeate	concentrate
8%mass extract	341.46	359.26	330.39	391.12	320.56	360.11
10%mass extract	347.92	369.58	349.49	***404.21***	339.66	387.52
15% mass extract	321.23	379.38	347.13	364.66	336.12	360.45

It was concluded that:
- The content of polyphenols found in the hydroalcoholic extracts of *Salvia officinalis* – as to flavones- increased proportionally with the mass plant, the highest concentration being obtained for 10% mass plant extract in 70 % ethanol, the increase in mass plant over this value lead to a decrease in total polyphenolic content.
- Similar results were obtained for all type membranes;
- In the case of the 8% aqueous extract successive concentrated on Millipore membranes of 10000 Da-5000 Da-3000 Da the highest concentration was obtained on Millipore membrane of 3000 Da, but the content of polyphenols is inferior to the content found for the hydroalcoholic extracts.

Table 4. Total polyphenols content in *Salvia officinalis* aqueous extracts

Type of membrane	Polyphenols μg/ mL					
	Millipore 10000 Da		Millipore 5000 Da		Millipore 3000 Da	
	permeate	concentrate	permeate	concentrate	permeate	concentrate
8% aqueous extract	238.09	280.01	225.44	294.33	232.64	336.51

HPLC Analysis of obtained extracts

The plant extracts were analyzed by the interpolation of the areas obtained for each compound. The results were expressed in μg/g dry matter and presented in Table 5. The main polyphenols identified in the analyzed extracts were rosmarinic acid, coumaric acid , caffeic acid, myricetin and quercetin.

Table 5. Content in active substances of interest, expressed in μg/g vegetal mass in *Salvia officinalis* extract

Sample / Compound	70% hydro-alcoholic 15% mass extract			
	initial	Millipore 10000 Da concentrate	Millipore 5000 Da concentrate	PSF concentrate
Gallic acid	0.83	0.78	0.85	0.85
Chlorogenic acid	0.30	0.49	0.50	0.44
Caffeic acid	1.87	1.5	1.91	1.31
Rutin	0.96	0.98	1.3	0.88
Coumaric acid	3.46	4.41	4.5	0.55
Ferrulic acid	1.11	1.11	1.21	1.41
Rosmarinic acid	14.31	12.99	17.25	12.01
Myricetin	1.05	1.06	1.45	1.06
Quercetin	1.33	1.68	2.80	1.70
Kaempferol	-	-	-	-

The selective concentration of some polyphenolic compounds (chlorogenic acid, rutin, coumaric acid, quercetin) in the retentate was observed, whereas other phenolics (caffeic acid, rosmarinic acid) were preferentially found in permeates while others were not separated at all (gallic acid, ferrulic acid and myricetin). The best separation of polyphenols occurred on UF membranes with a nominal MWCO of 5000Da.

Content of proteins in extracts was spectrophotometrically assessed by Lowry method /14/ at λ= 660 nm, using a calibration curve for bovine serum albumin (BSA) on 0-100 μg/mL concentration domain, correlation factor R= 0.9976, the results are shown in Tables 6 and 7.

Table 6. Proteins content in *Salvia officinalis* hydroalcoholic (70%) extracts

Type of membrane	Proteins µg/mL					
	Millipore 10000 Da		Millipore 5000 Da		PSF	
	permeate	concentrate	permeate	concentrate	permeate	concentrate
8% mass extract	1343.42	1526.31	1409.21	1728.95	1305.26	1597.36
10%mass extract	1446.05	***1668.42***	1481.57	***1720.02***	1535.52	***1601.31***
15% mass extract	1332.89	1460.526	1268.42	1469.73	1040.79	1415.78

- The content of *protein* increased proportionally to the increase in mass plant concentration, the highest proteins content being obtained for 10% (mass concentration) extract in 70% ethanol, over this value the content of protein decreased;
- The Millipore membrane with 5000 Da cut-off was more efficient for proteins concentration.

Table 7. Proteins content in *Salvia officinalis* aqueous extract

Type of membrane			Proteins µg/mL			
	Millipore 10000 Da		Millipore 5000 Da		Millipore 3000 Da	
	permeate	concentrate	permeate	concentrate	permeate	concentrate
8% aqueous extract	1432.89	1592.10	1147.37	1692.10	996.05	2080

- In the case of the 8% aqueous extract successively concentrated by Millipore membranes of 10000 Da-5000 Da-3000 Da the highest concentration was obtained on a Millipore membrane of 3000 Da and the polyphenols content is similar to that of hydroalcoholic extracts.

Determination of sugars content was spectrophotometrically done at 490 nm with phenol – sulfuric acid - Dubois method /15/. The results were obtained using a calibration curve realized with 50 mg% dilutions of glucose on a 5- 25 µg/mL concentration domain, correlation factor R= 0.9902. The results for the three variants of hydroalcoholic extracts are shown in Tables 8 and 9.

Table 8. Reducing sugars content in *Salvia officinalis* hydroalcoholic (70%) extracts

Type of membrane	Reducing sugars content µg/mL					
	Millipore 10000 Da		Millipore 5000 Da		PSF	
	permeate	concentrate	permeate	concentrate	permeate	concentrate
8% mass extract	35.05	80.04	36.79	96.25	40.89	48.31
10% mass extract	44.9	118.95	50.52	***138.16***	55.35	59.79
15% mass extract	74.29	96.21	66.59	107.64	74.20	83.78

- The sugar content increased proportionally with solvent content and mass plant. The highest sugar content was obtained for 10% mass extract in 70% ethanol, over this value the sugar content decreased
- The most efficient membrane for sugars concentration was Millipore 5000 Da membrane, then Millipore 10000 Da and the last PSF – similar situation as in the case of proteins and flavones determination.

Table 9. Reducing sugars content in *Salvia officinalis* aqueous extracts

Type membrane	**Reducing sugars content µg/mL**					
	Millipore 10000 Da		**Millipore 5000 Da**		**Millipore 3000 Da**	
	permeate	**concentrate**	**permeate**	**concentrate**	**permeate**	**concentrate**
8% aqueous extract	56.10	61.23	44.49	118.13	34.32	130.33

- In the case of 8% aqueous extract the content of reducing sugar was inferior to that from hydroalcoholic extracts and the highest concentration was obtained with the Millipore membrane of 3000 Da.

Inhibition of DPPH radical

The values obtained for hydroalcoholic extracts of *Salvia officinalis* by the DPPH method were between 42.12% and 70.82%. The highest values obtained for extracts processed through Millipore membrane with 5000 Da (UF2) were: 70.82% - for 10% mass extract and 66.23% - for 8% mass extract, than for the extracts processing by Millipore membrane of 10.000 Da (UF1): 60.75% - for 8% mass extract and 65.53% for 10% mass extract. The existence of correlation between the polyphenols content and antioxidant activity was found (Fig. 1).

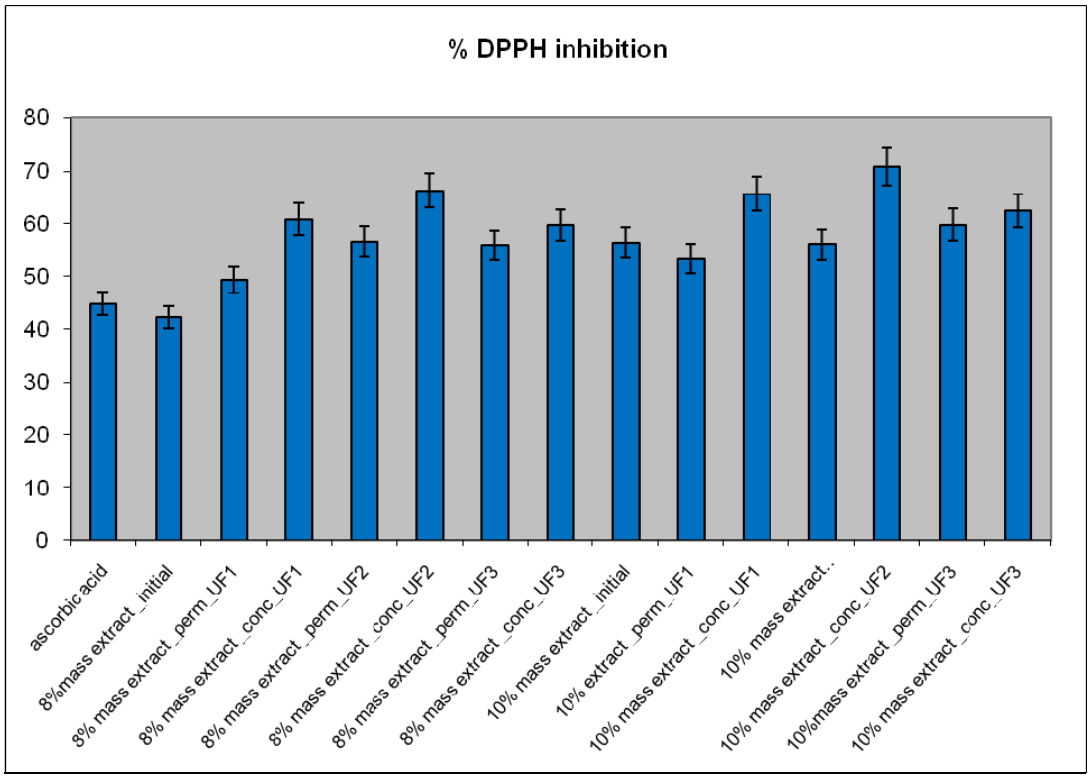

Fig. 1. DPPH inhibition percent

Conclusions

- In this study hydroalcoholic and aqueous extracts of *Salvia officinalis* were obtained, purified and concentrated by membranare procedure – ultrafiltration.
- The used ultrafiltration membranes were: Millipore ultrafiltration membranes with cut-off 10000 Da, 5000 Da and polysulfone composite membrane, PSF for hydroalcoholic extracts and Millipore ultrafiltration membranes with cut-off 10000 Da, 5000 Da and 3000 Da – for 8% aqueous extracts.
- The content of total polyphenols, proteins, reducing sugars and flavones was determined for all types of extracts. The highest concentration of active principles was obtained when a 3000 Da Millipore membrane was used for the aqueous extracts. Generally, the content of active principles in alcoholic extracts was higher than the content of active principles found in the aqueous extracts.
- In the case of hydroalcoholic extracts, the highest content of active principles was found for the 10% hydroalcoholic extracts in 70% ethanol, over this value the active principles content decreased.
- The antioxidant capacity of the tested extracts was determined and the results were expressed as % DPPH inhibition. The obtained values ranged between 42.12% and 70.82%, the highest values being obtained for the extracts processed by Millipore membrane with cut-off 5000 Da (UF2).
- The existence of a correlation between the polyphenols content and antioxidant activity was found. The highest antioxidant activity and the highest DPPH inhibition percent

70.82% – were found in the extracts with 70% ethanol and 10% (mass concentration) concentrated through membranes with cut-off of 5000 Da (UF2).

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