Membrane Processes for the Purification and Concentration of *Helleborus Purpurascens* Extracts and Evaluation of Antioxidant Activity

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This study aim was to prove the efficiency of ultrafiltration process for the concentration of Helleborus purpurascens extracts and to evaluate the extract antioxidant activity. The aqueous and hydro alcoholic extracts of H. purpurascens were concentrated by a multi-stages ultrafiltration cascade, as a novelty in medicinal plant processing. The results revealed that the concentrated extracts have a high antioxidant activity (over 70% DPPH inhibition for hellebore concentrated aqueous extract and over 78% DPPH inhibition for hellebore concentrated hydro alcoholic extract), thus it can be considered a good source for further medicinal applications.

Keywords: ultrafiltration process, Helleborus purpurascens, antioxidant activity

Helleborus purpurascens W. et K. is a perennial plant belonging to the Ranunculaceae family and it is widely spread in Western and Central Europe, from West of Great Britain, Spain and Portugal, eastward across the Mediterranean region and central Europe into Romania and Ukraine [1]. The main active compounds of the *H. purpurascens* are cardiac glycosides, polyphenolic compounds, flavonoids, steroidal saponins and alkaloids [2-5].

Some extracts of *Helleborus* species are used as phytopreparations with immunostimulatory properties in Romanian traditional medicine. An original patent medicine utilizing purified *Helleborus* extracts having antiantalgic and antirheumatic activity has been prepared in Romania with the registered name Boicil (US Patent) [6]. The latest researches proved that the *Helleborus* extract is active against tumors, inhibiting their evolution, but there are few contradictions and debates relative to this issue.

The therapeutic effects of several plants and vegetables, used in traditional medicine, are mainly assigned to their antioxidant compounds. The majority of natural compounds with antioxidant properties contain phenolic groups [7, 8]. Antioxidant substances block the activity of the free radicals, which are involved in pathogenesis of many diseases including atherosclerosis, ischemic heart disease, Alzheimer's disease, Parkinson's disease, cancer and in the aging process [9]. Our main purpose is determination of the *H. purpurascens* extract's antioxidant activities. There are no experimental data reported in the literature on radical scavenging properties of *Helleborus purpurascens*.

This paper is focused on the "non-stressing" process' utilization that, with respect to traditional plants, may occur in milder temperature conditions. A particular attention was provided to investigate the possibility to purify and concentrate hellebore extracts, by means of a microfiltration/ultrafiltration unit, which allows preservation of thermolabile compounds from the extracts. The health concerns have sparked research into methods that would reduce the use of organic solvents in the extraction procedure. Membrane processing is one method that reduces the use of toxic organic solvents and concentrates the final product. Previous studies revealed the ultrafiltration performances could be used for the concentration of medicinal plant extracts [10-12].

Experimental part

The roots of *H. purpurascens* were dried, homogenized and ground to a fine powder, using the GRINDOMIX GM200 mill; the extracts were prepared by maceration in cold distillate water and ethylic alcohol (50%v/v), as solvents. The contact time between the plant and the solvent was maintained of 24 h for aqueous extracts and 7 days for hydro-alcoholic extracts, extracts have been sporadic, mechanically stirring. The herbal mass concentration in the solvent was of 6% (w/v). After filtering the extract through Isolab quantitative filter paper "medium", each filtrates was processed by microfiltration (MF) (Millipore filters with 0.45 μm), followed by a two/three-stages ultrafiltration cascade, using three ultrafiltration plane membrane types from regenerated cellulose (Millipore) with cut-off 30,000 MWCO (UF1), 3.000 MWCO (UF2) and 1.000 MWCO (UF3). The permeate obtained from UF1 was introduced into the cross-flow circuits for UF2 and then the resulted permeate from UF2 was eventually introduced into the cross-flow circuits for UF3 (fig.1). A KMS Laboratory Cell CF-1 installation purchased from Koch Membrane (Germany) was used for both MF and UF (fig.2).

All extracts were sterilized, as passed through a 0.45 μm pore size microfiltration membrane. The concentration ratio in ultrafiltration processes (expressed as permeate and concentrate volume ratio) was of 2:1. All ultrafiltration experiments were carried out at room temperature (cca. 23°C).

All the used membranes in this experiment have an effective area of 0.0028 m^2. The permeate flux for each membrane was calculated with formula:

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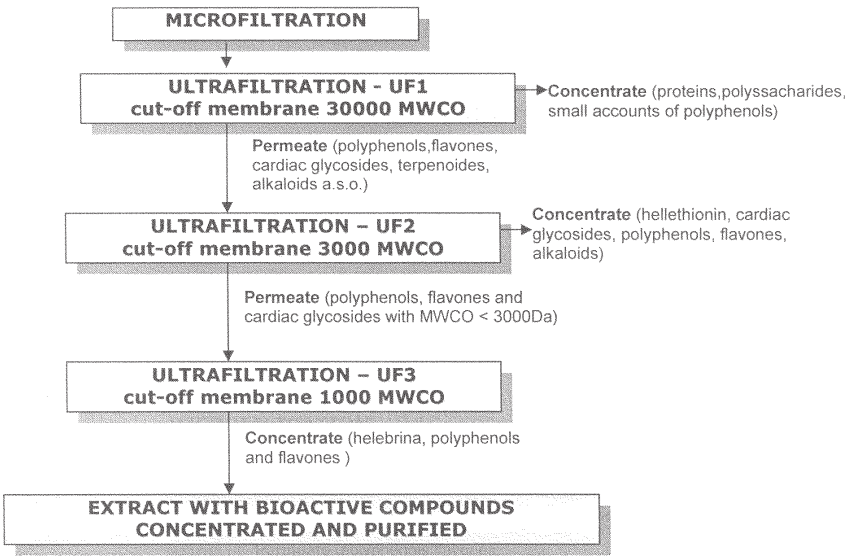

Fig.1 Experimental set-up of cascade ultrafiltration system

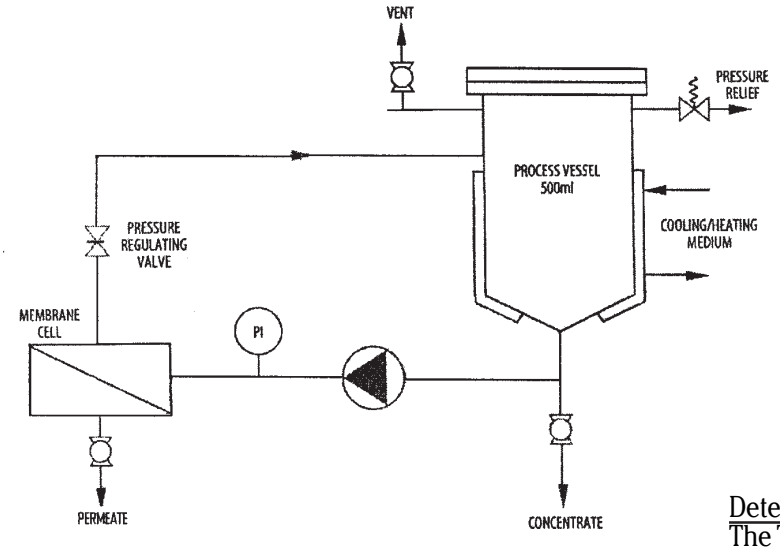

Fig.2 KMS Laboratory Cell CF-1 experimental setup

$$J = \frac{V}{A \cdot t} \quad (Lm^{-2}h^{-1}) \qquad (1)$$

in which:

V is the permeate volume (L);

A - the effective membrane area (m^2);

t - the time (h) necessary for the V liters of permeate to be collected. We counted the requested hours for 100 mL of permeate to be collected and then we calculated the flux.

The polyphenols retention coefficient (R) was calculated with formula:

$$R = (1-C_P/C_R)\cdot 100, \ \% \qquad (2)$$

where C_P and C_R are the polyphenols concentrations in the permeate and retentate.

Phytochemical screening. Preliminary phytochemical screening was performed using the standard procedures [13-15].

Assessment of total polyphenols. The phenolic total content was determined by the Folin–Ciocalteu method [16]. Gallic acid (GAE) was used to calibrate the standard curve; total polyphenols contents were obtained from the regression equation of the calibration curve of gallic acid ($y = 0.0044x + 0.037$, $R^2 = 0.9975$) and expressed as gallic acid equivalent.

Determination of the antioxidant activity

The Trolox Equivalent Antioxidant Capacity (TEAC) was determined by using of two methods.

One method - DPPH (2,2-diphenyl-1-picrylhydrazyl) radical-scavenging - based on the decrease of the DPPH maximum absorbance at 519 nm in the antioxidant presence [17,18].

$DPPH_{\bullet}$ is a stable radical having a maximum absorbance at 519 nm. It can readily undergo reduction by an antioxidant (AH) which runs as the following reaction [19]:

$$DPPH\cdot + AH \rightarrow DPPH\text{-}H + A\cdot$$

The decreasing of the DPPH radical absorption by the action of antioxidants could be use as a measure of antioxidant activity.

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

$$\% \text{ inhibition} = [(A_0 - A_s)/A_0] \times 100 \qquad (3)$$

where:

A_0 = control absorbance;

A_s = sample absorbance.

Another method is ABTS (2,2'-azino-bis 3-ethyl-benzthiazoline -6-sulfonic) radical cation scavenging [20]. Generation of radical cation ($ABTS^{\bullet}$) involves the reaction between ABTS and potassium persulfate and production of the blue/green $ABTS^{\bullet}$ chromophore with a maximum absorption at 731 nm. In presence of antioxidants, the pre-formed radical cation is reduced to ABTS, proportionally to

the antioxidant activity. This assay is used for the determination the total antioxidant activity. The results were obtained using the equation (1), the equivalent of the antioxidant capacity being expressed as Trolox equivalent:

$$TEAC_{sample} = C_{Trolox} \cdot f \cdot \frac{A_{sample} - A_{blank}}{A_{Trolox} - A_{blank}} \quad (4)$$

where:

A_{blank} - control absorbance;
A_{sample} - sample absorbance;
A_{Trolox} - absorbance for Trolox ;
C_{Trolox} - concentration of Trolox;
f - dilution factor.

Statistical Analysis: The measurements were performed in triplicate and for statistical processing was used Excel 2007, standard deviation (STDV) was < 10%.

Results and discussions

Microfiltration (MF) process is meant to perform feed clarification and sterilization, while the ultrafiltration (UF) process was meant for concentration of rejected solutes and fractionation of solutes. Two main issues are focused in this study through sequence of the proposed steps: hellebore extracts purifying (removal of microorganisms and mechanical impurities) by MF and concentrated extract's obtaining (in steroidal glycosides, cardiac glycoside and some polyphenols like caffeic acid) by UF cascade. Finally we obtained a small volume of concentrated extract that is easier to process further. It could used by other fractions, as well, those resulted in the previous UF steps to recover different bioactive compounds.

The phytochemical screening of the extracts studied (initial, after microfiltration and after the last ultrafiltration process) showed the presence of cardiac glycosides, flavonoids, reducing sugars, terpenoids, saponins and aminoacids (table 1). The other compounds like alkaloids and tannins were present in the extracts, as a trace amount.

The data (table 1) showed that all components from microfiltrate will gather as concentrated components in the ultrafiltrate retentate (UF2 /UF3), while in the permeate they will remain in a low concentration.

So far, as plant phenolics represent one of the major groups of compounds acting as primary antioxidants or free radical terminators, it was reasonable to determine their total amount in the selected plant extracts. Total polyphenol content was determined in permeate and retentate after each ultrafiltration process of the extracts (table 2).

The obtained results for hellebore extracts concentration through ultrafiltration ranged between 20% ÷ 64% for the polyphenols retention, depending on the used membrane type.

The reason for the retention of polyphenols by UF1 (membrane with cut-off 30,000 MWCO) is probably due to colloids which are retained together with colloidal matter on membrane. After the UF2 step, the total polyphenols concentration in the retentate was 1.7 times higher than that in permeate and after UF3 process the polyphenols concentration in the retentate was 2.8 times higher than that in permeate (for aqueous extract).

Testing the activity by more than one assay is desirable because different methods measure different characteristics of the antioxidant. In this work were determined the antioxidant activities of hellebore extracts/ fraction by $DPPH_{\bullet}$ and $ABTS^{+}$ radical assay.

The data were sustained by TEAC values obtained for *Helleborus purpurascens* extracts processed by micro- and ultrafiltration (table 3). The scavenging potential of concentrated extracts was compared with known antioxidants, such as Trolox. The higher TEAC value implies a greater antioxidant activity. Comparing the results of two radical scavenging tests, a good correlation between both tests and the determined content of total phenolics can be observed. The obtained results showed that the highest antioxidant activity possess hellebore hydro-alcoholic concentrated extracts, for each ultrafiltration membrane used. A weaker antiradical activity was determined in permeate extracts. For both of the *H. purpurascens* extracts, antiradical activity could be correlated to the polyphenols total content, which is greater in retentate than in permeate.

Extracts are very complex mixtures of many different compounds with antioxidant and pro-oxidant properties, also depending on the chemical structure of each individual compound present in these extracts. This is highlighted by the fact that although concentration of polyphenols in the UF3 retentat is lower than that of microfiltrate, the TEAC value for the UF3 retentat is higher than that of the microfiltrate. Thus, it is revealed that the final extract contains the greatest compounds with antioxidant effect

Table 1
SUMMARY OF PHYTOCHEMICAL SCREENING COMPOUNDS

Constituents	Tests	Hydroalcoholic extract				Aqueous extract			
		initial	MF	UF2		initial	MF	UF3	
				Perm.	Ret.			Perm	Ret.
Alkaloids	a) Dragendorff's	+	+	-	+	+	+	-	+
	b) Hager's	+	+	-	+	+	+	-	+
Cardiac glycoside	Keller-Killiani	++	++	±	++	++	++	-	++
	Kedde's test	++	++	±	++	++	++	±	++
	Liberman-Burchard	++	++	±	++	++	++	±	++
Tannins	Ferric chloride 0,1%	+	+	±	+	+	+	±	+
Flavonoids	a) Alcalin (NaOH 10%)	++	++	+	++	+	+	±	+
	b) Lead acetate	++	++	+	++	++	++	±	++
	c) NH_3 sol.+ H_2SO_4	++	++	±	++	++	++	±	++
Reducing sugars	Fehling	++	++	±	++	++	++	-	++
Terpenoides	Salkowski's test	++	++	+	++	++	++	-	++
Saponins	Froth	++	++	+	++	++	++	+	++
Proteins	Biuret	-	-	-	-	-	-	-	-
Aminoacids	Ninhydrin 0,25%	++	++	+	++	++	++	+	++

++ = Abundant; + = present; ± = poorly present; - = absent
Perm. = permeate; Ret. = retentate.

Table 2
AMOUNT OF POLYPHENOLS ON THE HYDROALCOHOLIC AND WATER EXTRACTS OF HELLEBORUS PURPURASCENS

Sample		Permeate flux, L/m^2h	Total Polyphenols, mg GAE/mL	Retention coefficient, %
Aqueous extract			0.264	
MF aqueous extract		182.6	0.181	
UF1 aqueous extract	retentate		0.195	20
	permeate	129.1	0.156	
UF2 aqueous extract	retentate		0.157	42
	permeate	55.1	0.091	
UF3 aqueous extract	retentate		0.169	64
	permeate	27.9	0.061	
Hydroalcoholic extract			0.189	
MF hydroacoholic extract		191.5	0.129	
UF1 hydroacoholic extract	retentate		0.203	40
	permeate	134.7	0.122	
UF2 hydroacoholic extract	retentate		0.213	52
	permeate	59.2	0.103	

Table 3
ANTIOXIDANT ACTIVITY OF ANALYZED EXTRACTS

Sample		$TEAC_{DPPH}$ (μmol Trolox/L)	$TEAC_{ABTS}$ (μmol Trolox/L)
Aqueous extract		47.81	80.92
MF aqueous extract		31.58	74.26
UF1 aqueous extract	retentate	21.69	64.76
	permeate	31.53	68.65
UF2 aqueous extract	retentate	30.72	68.45
	permeate	21.63	56.12
UF3 aqueous extract	retentate	38.49	75.52
	permeate	22.31	44.43
Hydroalcoholic extract		29.60	80.51
MF hydroacoholic extract		37.10	81.45
UF1 hydroacoholic extract	retentate	38.29	87.39
	permeate	36.51	76.90
UF2 hydroacoholic extract	retentate	41.62	96.06
	permeate	25.07	75.29

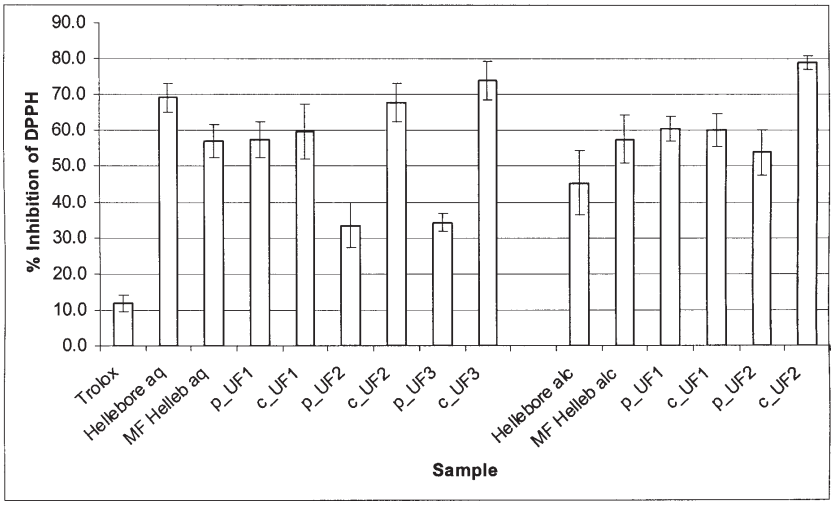

Fig.3 Comparison of DPPH radical scavenging activity of the concentrated Helleborus extracts and those of Trolox p = permeate; c = retentate (e.g. p_UF1 = permeate UF1; c_UF1 = retentate UF1)

by comparing to initially extract and the one obtained by microfiltration.

TEAC different values obtained when using the two methods are due to the ABTS assay to determine total antioxidant activity (associated with all polyphenols types), while the DPPH method is a selective one. DPPH selectivity is expressed through huge reactivity over polyphenols compounds with –OH bonds on condensed rings.

In addition, the inhibition extent of DPPH· radicals was determined, and the obtained results are shown in figure 3.

The values obtained by the DPPH method were over 70% DPPH inhibition for hellebore concentrated extracts. The concentrated extracts obtained after ultrafiltration UF2 (membrane with cut-off 3,000 Da) and UF3 (membrane with cut-off 1.000 Da) had the strongest scavenging activity, in both of the cases, when using aqueous and hydro alcoholic solvents. The retentat from UF2 hydroalcoholic extract showed the highest antioxidant activity with DPPH inhibition value of 79%, while the concentrated aqueous extracts obtained after UF3 step showed less activity (73%

DPPH inhibition) and the initial extract after microfiltration showed an DPPH inhibition value of 69%.

These results proved the ultrafiltration process's efficiency, in case of obtaining the *Helleborus purpurascens* purified and concentrated extracts, with a high antioxidative activity.

Conclusions

Two extracts of *Helleborus purpurascens* were prepared using different solvents and further the extracts were concentrated by a two/three-stages ultrafiltration cascade, as a novelty in medicinal plant processing.

The results revealed that the concentrated extracts have a high antioxidant activity (over 70% DPPH inhibition for hellebore concentrated aqueous extract and over 78% DPPH inhibition for hellebore concentrated hydro alcoholic extract), thus it can be considered a good source for further medicinal applications.

It can be mentioned that until now there are no reports on antioxidant activity of *Helleborus purpurascens*.

It was ascertained that it is possible to obtain concentrated *Helleborus purpurascens* extracts with high antioxidant activity, by means of the membrane processes to concentrate the bioactive compounds.

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