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SELECTIVE FRACTIONS OBTAINED FROM PLANT SPECIES CULTIVATED IN ROMANIA WITH POTENTIAL EFFECT ON COUNTERACTING DISEASES ASSOCIATED WITH AGING PROCESSES

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Abstract

The aim of these studies was to obtain Rosmarini folium selective fractions with beneficial effect in counteracting diseases associated with aging processes.
By four different methods 9 selective fractions were obtained. HPLC analysis and quantitative determination of active principles from selective fraction show that the values obtained from individual assessment by HPLC were well correlated with the values obtained by the spectrophotometrically methods. The selective fractions have a total flavonoid content expressed as rutin from 2.004 to 66.970% and respectively 0.651 to 10.284% polyphenolcarboxylic acids expressed as rosmarinic acid.
Antioxidant activity evaluation showed that polyphenolcarboxylic acids rich fractions such as RIII: 10.284%, RIIC: 10.257%; had 89.44%; 88.12% antioxidant activity in 1% dilution and 87.52%; 89.07% antioxidant activity in 0.1% dilution, similar to rosmarinic acid who has antioxidant activity of 88.99% respectively 89.84% in dilution of 1% respectively 0.1%. The fractions with 2.642% - 3.950% polyphenolcarboxylic acid content exhibited an antioxidant activity of 45.73% - 87.5% in 1% dilution and of 8.75 to 87.500% activity in 0.1% dilution.
Comparing the antioxidant activity of selective fractions and the polyphenolcarboxylic acids content expressed as rosmarinic acid it can be concluded that when the concentration of polyphenolcarboxylic acids increases the antioxidant activity also increases, though not an exact correlation can be made.
A correlation between the flavones content of the selective fractions and antioxidant activity can not be made by this method.

Key words*: Rosmarinus officinalis, selective fractions, antioxidant.*

INTRODUCTION

Some chemical compounds derived from plant species, such as rosmarinic acid, caffeic acid or other compounds from the polyphenolcarboxylic acids class; diosmin, diosmetin or other flavonoidic compounds are known for their antioxidant properties.
Since free radicals are considered the main responsible agents of premature aging and also of diseases associated to aging status, compounds with antioxidant activity are regarded as basic elements of the anti-aging strategy (Ashok et al., 1999).
Among the plant species with antioxidant properties is *Rosmarinus officinalis* L (Lamiaceae family) (Yeset-Celiktas et al., 2007; Babovic et al., 2010).
Rosmarinus officinalis L. has been used since ancient times in traditional medicine. Due to its special therapeutic properties, this species was widely cultivated.
The most important active components of this species are: polyphenolcarboxylic acids, flavornoids, di- and triterpenoids and volatile oil (Begum et al., 2013).
Recent studies have confirmed the pharmacological activity of *Rosmarinus officinalis* leaf extract, based on active substances such as acids polyphenolcarboxylic including rosmarinic and caffeic acid or flavones including diosmin (Begum et al., 2013; Hernadez-Hernadez et al., 2009; Cosio et al., 2006).
For example, *Rosmarinus officinalis* L. leaves (Rosmarini folium) stimulates cerebral circulation and microcirculation due to rosmarinic acid composition (Aruoma et al., 1994) and exhibits an anti- stress action based on antioxidant properties against free radicals and peroxides (Frankel et al., 2000). Due to

polyphenolcarboxylic acids content, this species show hypocholesterolemic and choleretic-cholagogue action and due to flavones (including diosmin) exhibit antiseptic and healing action (Istudor et al., 2001).

MATERIALS AND METHODS

The vegetal material consisting of leaves of *Rosmarinus officinalis* L. (Rosmarini folium) was obtained from culture, dried and ground as a fine powder (sieve VII).

Selective fractions obtainment:

Method I consisted of repeated extraction - two times of the active substances from 200 g Rosmarini folium, with 50% ethyl alcohol v/v (vegetal material / solvent ratio = 1/10 m/v for the first extraction and 1/5 m/v for the second extraction) at boiling temperature of the solvent for 1 hour per extraction with continuous mechanical stirring, followed by cooling and filtration of the extracts.

The reunited solutions were rota-evaporated for alcohol removal. The resulting aqueous solutions were spray-dried and selective fractions RI were obtained.

Method II consisted of active principles extraction from 300 g Rosmarini folium with 50% alcohol (plant / solvent = 1 /10 m/v ratio) at boiling temperature for 1 hour with continuous mechanical stirring, followed by cooling and filtration extracts. Hydroalcoholic extract solution was evaporated to a volume of 1/1 m/v plant/solvent mixture and centrifuged. A precipitate (which was labeled as RII0 after drying) and an aqueous solution were obtained. In order to obtain selective fractions, aqueous solution was further processed by:

- Three successive liquid-liquid extractions with ethyl ether, followed by solvent removal from the reunited etheric extracts resulting RIIA;
- Three successive liquid-liquid extractions with chloroform, followed by solvent removal from the reunited chloroformic extracts resulting RIIB;
- Three successive liquid-liquid extractions with ethyl acetate, followed by solvent removal from the reunited ethyl acetate extracts resulting RIIC;
- Three successive liquid-liquid extractions with 1-butanol followed by solvent removal from the reunited butanolic extracts resulting RIID;
- Adding acetone in a 2/1 v/v acetone/ aqueous extract ratio resting for 24 hours at 4-6^{0}C, filtration, and drying the precipitate resulting RIIE (Figure 1).

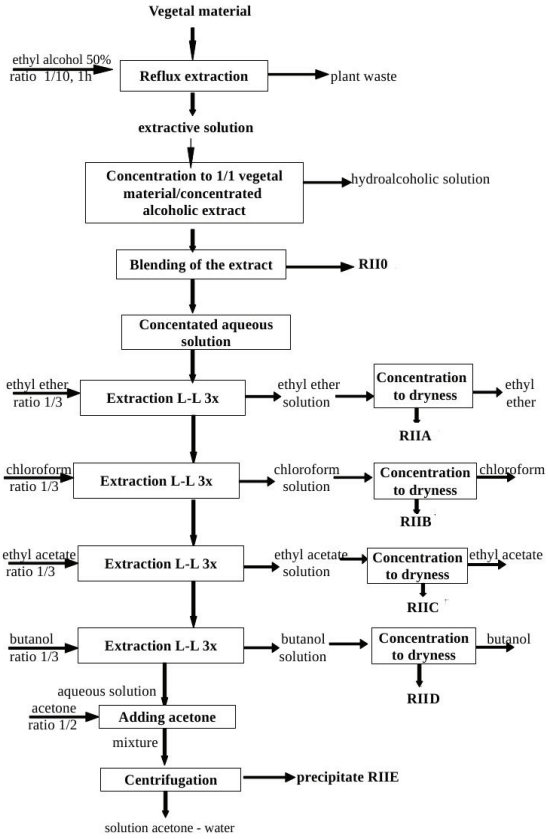

Figure 1. Selective fractions obtainment - method II

Method III consisted of repeated extractions times of the active substances from 200 g Rosmarini folium with methylic alcohol (plant / solvent ratio = 1 / 10 m / v for the first extraction and 1/5 m/v for the second extraction) at boiling temperature of the solvent for one hour per extraction with continuous mechanical stirring, followed by cooling and filtration of the extracts. Methanolic solutions were reunited, the solvent were removed by rotaevaporation resulting RIII selective fractions.

Method IV consisted of macerating 200g Rosmarini folium in acetone (plant/solvent ratio = 1/7 m/v), removing the solvent from acetone solution and re-extracting the residue

in methanol. The active substances were extracted from moist plant material with 20% ethanol (plant/solvent ratio = 1/10 m/v) at boiling temperature of the mixture for 2 hours, followed by hydroalcoholic solution evaporation to an aqueous extract. Methanolic and aqueous extract were reunited and filtered. The resulting precipitate was dried and selective fractions RIV were obtained.

HPTLC analysis of selective fractions was performed using Silica Gel $60F_{254}$ as stationary phase and a mixture of ethyl acetate - acetic acid - formic acid - water (100:11:11:27v/v/v/v) for chromatographic elution. The plates were scanned under 360 nm after the derivatization with NP/PEG. The reference compounds for HPTLC analysis were from Sigma-Aldrich: caffeic acid, rosmarinic acid, chlorogenic acid, rutin, hyperoside and diosmin. (Wagner et al., 1996; Reikh et al., 2008)
HPLC analysis of selective fraction consisted in chromatographic separation on a Purosher ODS column (250x4,6 mm, 5µ) at 40^0C, using a gradient elution (both mobile phase and flow). The mobile phase was a binary gradient: water with orthophosphoric acid (pH=2,5) and methanol. The eluent absorbance was monitored at 330 nm. The reference substances were from Sigma-Aldrich: caffeic acid, rosmarinic acid, rutin, diosmin and luteolin.
Quantitative determination of active principles from selective fractions consisted of determination of flavones by a colorimetric method based on their property to form intensely yellow complex with Al_3^+ and of determination on polyphenolcarboxylic acids by a colorimetric method based on the property of phenols to form nitrocompouds or nitro oxime with nitrous acid which give red stain when dissolve in alkaline solutions due to their weak acid character. For the quantification of flavones, rutin was used as reference substance and for polyphenolcarboxylic acids quantification rosmarinic acid was used as reference substance. (Roumanian Pharmacopoeia the X^{th} Edition, 1993)

Analysis of antioxidant action
DPPH assay: In each reaction tube 100 µL vegetal extract of different concentrations was mixed with 3900 µL of 0.0025 g/L DPPH at room temperature for 30 min. 50% methanol solution was used as control.
The reduction of the DPPH free radical was measured by reading the absorbance at the wavelength 515 nm. Rosmarinic acid (from Sigma-Aldrich) was used as reference substance. Inhibition ratio (percent) was calculated from the following equation:

% inhibition = [(absorbance of control – absorbance of sample)/ absorbance of control] x 100

DPPH radicals react with suitable reducing is measured spectrophotometrically at 515 nm. (Sanchez Moreno, 1998).
For determination of antioxidant activity, the selective fractions were chosen according to the yield obtained from 100 g plant and depending on the flavones and polyphenolcarboxylic acids content.

RESULTS AND DISCUSSIONS

Nine Rosmarini folium selective fractions selective fractions were obtained by experimental methods mentioned above. The quantities of product obtained from 100g plant are shown in table 1.
Flavonoids (rutin, hyperoside, diosmin) and polyphenolcarboxylic acids (rosmarinic acid, caffeic acid, chlorogenic acid) were identified by HPTLC in most of selective fractions .
The content of caffeic acid, rosmarinic acid, diosmine, rutin and luteolin in each fraction was determined by HPLC method. The values obtained from individual assessment by HPLC were well correlated with the values obtained by the spectrophotometrically methods mentioned above. For example, rosmarinic acid content from the selective fractions, determined by HPLC, correlates with the polyphenolic acids content expressed in rosmarinic acid, determined by the headline method.
The flavonoid content expressed as rutin and polyphenolcarboxylic acids expressed as rosmarinic acid of Rosmarini folium selective fraction selective fraction are shown in table 1.
The selective fractions obtained by processing the leaf of *Rosmarinus officinalis* contain 0651-10.284% polyphenolcarboxylic acids expressed as rosmarinic acid and 2.004 – 66.970% flavonoids expressed as rutin.

Table 1. The content of active principles of Rosmarini folium selective fractions

Active principles/ Bioactive product	Quantity of product from 100g plant	Polyphenolcarboxylic acids expressed as rosmarinic acid % g/g	Flavonoids expressed as rutin % g/g
RI	16.45g	3.950	7.856
RII0	4.43g	2.642	2.078
RIIA	0.31g	3.792	13.634
RIIB	0.13g	0.651	4.800
RIIC	0.63g	10.257	66.970
RIID	1.06g	3.041	16.653
RIIE	3.10g	2.380	2.004
RIII	18.73g	10.284	2.929
RIV	5.94g	3.016	2.745

The most affluent fractions in polyphenolcarboxylic acids expressed as rosmarinic acid are RIII (10.284%) and RIIC (10,257%) followed by RI (3,950%), RIIA (3,792%), RIID (3,041%) and RIV(3,016%), RII0 respectively RIIE contains the 2,642% respectively 2,380% and the fraction with most low content in polyphenolcarboxylic acids expressed as rosmarinic acid are RIIB (0,651%). The most affluent fractions in flavonoids expressed as rutin it is RIIC (66,970%) followed by RIID (16,653%), RIIA (13,634%), RI (7,856%) and RIIB (4,800%). The other factions contain 2,929% (RIII), 2.745 % (RIV), 2,078% (RII0) and 2,004 % (RIIE).

Antioxidant activity of selective fractions is shown in figure 2.

Using the method for the analysis of the antioxidant activity described, it can be noted that rosmarinic acid in a percentage of 1; 0,1; 0,01 and 0,001 exhibits an antioxidant activity of 88,99% ; 89,84; 66,15% and 9,14% .

The selective fractions RIII respectively RIIC containing 10.284%, respectively 10.257% polyphenolcarboxylic acids in dilution of 1% and 0.1% had a similar antioxidant activity with the reference substance - rosmarinic acid in the same dilution. Thereby RIII shows an antioxidant activity of 89,44% and 87,52% respectively RIIC an antioxidant activity of 88,12% and 89,07%.

Some selective fractions with a lower polyphenolcarboxylic acids content such as RI (3.950%), RII0 (2.642%) exhibit over 80% antioxidant activity in dilutions of 1% and 0.1%. Thereby RI shows an antioxidant activity of 87,50% and 80,00% and RII0 manifest an antioxidant activity of 84,32% and 87,88%.

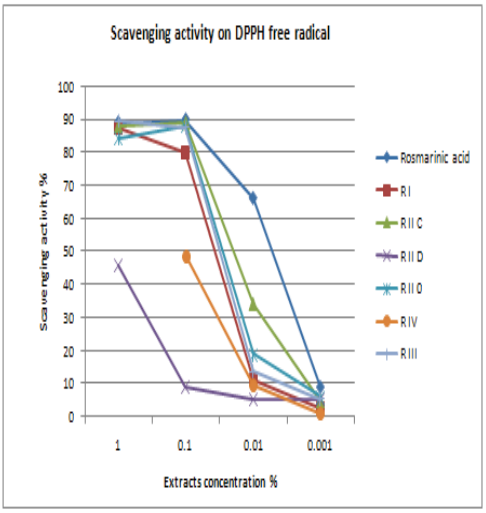

Figure 2. The antioxidant activity of the selective fractions from *Rosmarinus officinalis*

Even the polyphenolcarboxylic acids content of the selective fraction RIID (3,041%) and RIV (3,016%) is similar to the content of RI (which exhibits a good inhibitory potential), these fractions show a weaker antioxidant activity. Thereby RII0 in a dilution of 1% and 0,1% exhibits an antioxidant activity of 45,73% and 8,75% and RIV in a dilution of 0,1 % exhibits an antioxidant activity of 48,69%.

Comparing the antioxidant activity of selective fractions and the polyphenolcarboxylic acids expressed as rosmarinic acid and flavones expressed as rutin content it can be concluded that when the concentration of polyphenolcarboxylic acids increases, the antioxidant activity also increases, though not an exact correlation can be made (Colceru-Mihul S., et al.,2016).

A correlation between the flavones content of the selective fractions and antioxidant activity can not be made by this method. Thus, the selective fraction RIIC with 66.970% flavones content expressed as rutin shows antioxidant activity similar to RIII with 2.292% flavones content expressed as rutin.

CONCLUSIONS

From *Rosmarinus officinalis* L. leaves (Rosmarini folium) 9 selective fractions enriched in flavones and polyphenolic acids were obtained by different methods.

Six selective fractions were tested for antioxidant activity resulting that the two selective fractions exhibited an antioxidant activity comparable with the rosmarinic acid.

It can be concluded that a high content of polyphenolcarboxylic acids expressed as rosmarinic acid lead to a higher antioxidant activity but an exact correlation can not be made.

The antioxidant activity of the flavones was not highlighted by the method used in this study for the antioxidant activity evaluation.

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