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SELECTIVE FRACTIONS WITH ANTIOXIDANT ACTIVITY FROM ROMANIAN CULTIVATED *CYNARA SCOLYMUS* L.

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Abstract
It is known that oxidative stress and inflammation play an important role in the onset of arterial disorders, very common in the elderly. Cynara scolymus L. *is one of the best recommended species for prevention and control of diseases associated with aging processes, mostly due to its high polyphenol content - luteolin, luteolin-7-glucoside, caffeic acid, chlorogenic acid.*
The aim of the study was to obtain some selective fractions from Cynara scolymus L. *leaves with various contents of caffeic, chlorogenic and rosmarinic acids, cynarin, luteolin-7-glucoside, apigenin-7-glucoside and rutin determined by HPLC and to establish the relationship between concentration and antioxidant activity.*
Eight selective fractions obtained by two distinct methods containing 0-0122% caffeic acid, 0-0.443% rosmarinic acid, 0.007-1.504% chlorogenic acid, 0-0.097% cynarin, 0.054-1.6662% luteolin-7-glucoside, 0.009-1.366% apigenin-7-glucoside and 0-0.396% rutin exhibited antioxidant activity at 0.001, 0.01, 0.1 and 1% dilution, varying from 0.27 to 87.77%. More precisely, selective fraction C_6 containing 16.662% luteolin-7-glucoside and C_8 selective fraction containing 5.568% luteolin-7-glucoside and 1.504% chlorogenic acid exhibited 87.77%, respectively 84.44% antioxidant activity at 1% dilution and 85.27%, respectively 69.44%. antioxidant activity at 0,1% dilution. The reference substance luteolin-7-glucoside showed 87.16% antioxidant activity at 1% concentration and 85.56% at 0.1% concentration. All selective fractions exhibited antioxidant activity and the action was correlated with their active substances concentration.

Key words*: selective fractions, Cynara scolymus, aging, antioxidant activity.*

INTRODUCTION

Cynara scolymus L. (Asteraceae) is one of the best recommended species for prevention and control of diseases associated with aging processes.
It is known that oxidative stress and inflammation play an important role in the onset of arterial disorders. It was demonstrated that extracts from the leaves of *Cynara scolymus* show antioxidant effect against oxidative stress-inducing factors and exhibit a cytoprotective effect both *in vitro* on rat hepatocytes (Gebhardt, 1997, 1998; Miccadei et al., 2008) and erythrocytes (Jimenez-Escrig et al., 2003), on human cells: neutrophil leukocytes (Perez-Garcia et al., 2000), endothelial cells and monocytes (Miccadei et al., 2008; Zapolska-Downar et al., 2002; Wang et al., 2003) but also *in vivo* (Jimenez-Escrig et al., 2003). The antioxidant effect is due to the polyphenolic content of this species including luteolin, luteolin-7-glucoside, caffeic acid, chlorogenic acid. (Gebhardt, 1997, 1998; Perez-Garcia et al., 2000; Wang et al., 2003).
Hypercholesterolemia is associated with an increased risk of coronary heart disorders and other sequelae of atherosclerosis. Extracts or some vegetal active substances such as luteolin, luteolin-7-glucoside from the *Cynara scolymus* L. leaves show hypocholesterolemic properties on rat hepatocytes cultures (Gebhardt, 1997) or on human hepatocytes cultures (Gebhardt, 2002).
Moreover, intraperitoneally administration of these extracts on rats decreases cholesterol and triglycerides levels (Saenz Rodriguez et al., 2002).
Other positive effects of extracts from *Cynara scolymus* extracts consist in: increasing the bile secretion demonstrated both *in vitro* on heaptocytes cultures (Gebhardt and Fausel, 1997)

and *in vivo*; hepatoprotective effects demonstrated *in vitro* on rat hepatocytes due to caffeic acid and less to cynarin (Gebhardt, 2002) and also *in vivo* by oral administration in rats (Adzet et al., 1987); spasmolytic effect demonstrated on guinea-pig ileum (Emendorfer et al., 2005).
The therapeutic properties of total extracts from the leaves of *Cynara scolymus* have been demonstrated also by clinical testing. These extracts are capable to decrease cholesterol and triglycerides levels (Petrowicz et al., 1997; Wider et al., 2007), to improve LDL / HDL ratio (Schmiedel, 2002; Fintelmann and Petrowicz, 1998), to exhibit choleretic (Kirchhof et al., 1994) and antidispeptic effects by improving symptoms like vomiting, abdominal pain, nausea, flatulence (Fintelmann and Petrowicz, 1998) and generally improving quality of life (Bundy et al., 2004) and also to have benefficial effects in the treatment of irritable colon syndrome (Holtmann et al., 2003).
The aim of this work was to study the antioxidant activity of fractions obtained by processing *Cynara scolymus* containing caffeic, chlorogenic and rosmarinic acids, cynarin, luteolin-7-glucoside apigenin-7-glucoside and rutin, secondary plant metabolites selectively extracted and distributed in fractions.

MATERIALS AND METHODS

The vegetal material consisted of *Cynara scolymus* L. leaves (Cynarae folium) obtained from cultivated crops, dried and ground as powder with a IV sieve, containing 1.38 polyphenolcarboxylic acids expressed as chlorogenic acid and 1.75 total flavones expressed as luteolin-7-glucoside .
Chemicals: 2,2-diphenyl-1-picrylhydrazyl (DPPH), cynarin, chlorogenic, rosmarinic and caffeic acids, luteolin-7-glucoside apigenin-7-glucoside and rutin were purchased from Sigma Aldrich-Fluka. All other chemicals were analytical reagent grade.

Extraction of selective fractions

Method I consisted in repeated extraction - two times of the active substances from 100g Cynarae folium with methylic alcohol (selective fraction C_1), ethylic alcohol (selective fraction C_2) 50% ethylic alcohol v/v (selective fraction C_3) (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 mixture for 1 hour per extraction, cooling and filtering, gathering all extractive solutions, solvent removal at reduced pressure (72-74 mmHg) and drying of remaining residue at 40^0C (Figure 1).

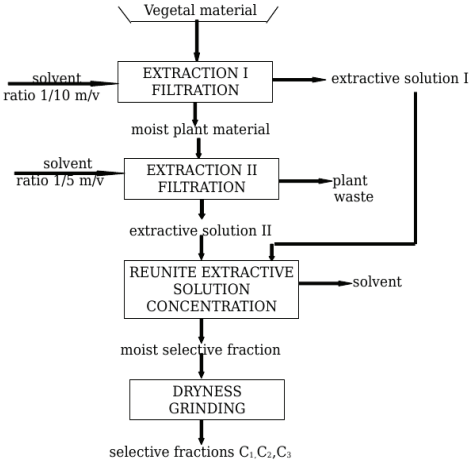

Figure 1 - Extraction scheme, method I

Method II consisted in repeated extraction of the active substances from 100g plant material with 50% ethanol (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 mixture for 1 hour per extraction, cooling and filtering, gathering all extractive solutions, hydroalcoholic solution concentration at reduced pressure (72-74 mmHg) to a volume of 1/1 V/m from plant material. The aqueous solution obtained was centrifuged at 4000 rot/min, the insoluble substances were dried at a temperature of 40^0C and grinding as fine powder with a IV sieve to obtain selective fraction C_4. The selective fractions C_5, C_6, C_7 from clear aqueous solution were obtained by successive liquid-liquid extraction three times with methylene chloride, four times with ethyl acetate and six times with n-butyl alcohol, followed by solvent removal, drying and grinding.. In remaining aqueous solution, a small quantity of acetone was added to obtain a precipitate which was further filtrated, dried and grinded resulting in selective fraction C_8 (Figure 2).

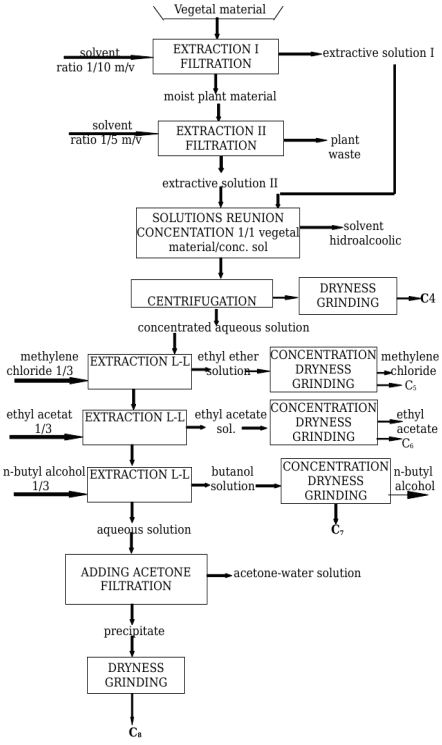

Figure 2 - Extraction scheme, method 2

Selective fractions analysis by HPLC

Chromatographic separation was achieved on a Kromasil ODS column (250 x 4.6 mm, 5µm) at 40ºC, using a gradient elution. The mobile phase was a binary gradient: water with ortho-phosphoric acid (pH = 2.0) and acetonitrile. The first step, the linear gradient started at 10% to 25% acetonitrile in 25 minutes, followed by isocratic elution with 25% acetonitrile over 8 minutes. The second step, the gradient elution was from 25% to 90% acetonitrile in 7 minutes, followed by isocratic elution with 90% acetonitrile for 5 minutes. The eluent absorbance was monitored at 330 nm.

Determination of antioxidant activity

DPPH assay: In each reaction tube 100 µL vegetal extract of different concentrations was mixed with 3900µL of 0.0025g/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 515 nm. Luteolin-7-glucoside (PHYTOPLAN Diehm & Neuberger GmbH) was used as positive control. 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 agents losing color stoichometrically with the number of electrons consumed which is measured spectrophotometrically at 515 nm (Sanchez Moreno, 1998).

RESULTS AND DISCUSSIONS

Using the experimental methods mentioned above, eight selective fractions from *Cynarae folium* were obtained and further analyzed by HPLC for polyphenolcarboxylic acids and flavones content in order to establish the relationship between the content in active substances and antioxidant activity.

The chosen methods allowed an excellent separation of reference substances (Figure 3) and also of the specific phytochemical compounds (Figure 4).

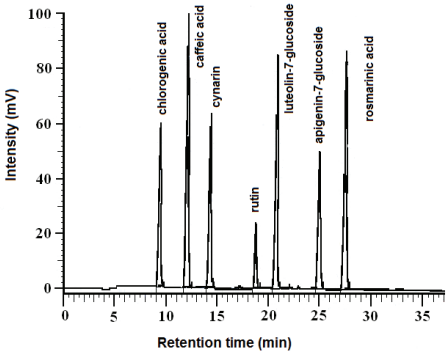

Figure 3 Standard solution chromatogram obtained under the selected chromatographic conditions (HPLC)

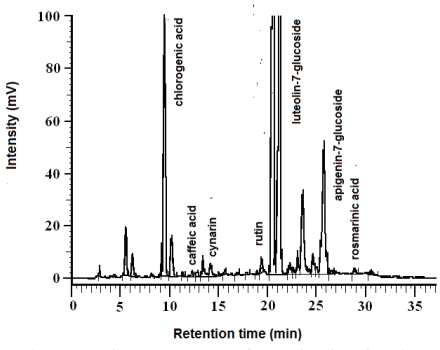

Figure 4 Chromatogram of C_8 selective fraction obtained from *Cynara scolymus*

Table 1. The content of polyphenolcarboxylic acids of selective fraction

Active substance/ Selective fraction	Quantity (g)	Polyphenolcarboxylic acids			
		Chlorogenic %	Rosmarinic %	Caffeic %	Cynarin %
1	12.08	0.112	0.014	0	0.018
2	8.02	0.052	0.008	0	0.013
3	18.773	0.365	0.011	0.018	0.088
4	8.330	0.120	0.356	0.004	0.012
5	1.670	0.306	0.443	0.014	0.013
6	0.534	0.113	0.240	0.122	0
7	5.770	0.007	0	0	0.004
8	2.370	1.504	0.022	0.019	0.097

The results regarding the amount of selective fraction and their content of active substance are presented in Tables 1 and 2.

Table 2. The content of flavones of selective fraction

Active substance/ Selective fraction	Quantity (g)	Flavones		
		Luteolin-7-glucoside %	Apigenin-7-glucoside %	Rutin %
1	12.08	1.672	0.110	0.056
2	8.02	1.909	0.111	0
3	18.773	1.846	0.254	0.218
4	8.330	1.625	0.106	0.043
5	1.670	1.599	0.043	0.117
6	0.534	16.662	1.366	0.034
7	5.770	0.054	0.009	0.009
8	2.370	5.576	1.253	0.396

The antioxidant activity of the pure active substances (reference substances) is presented in Table 3.

Table 3. Antioxidant activity of reference substances (%)

Dilution/ active substances	1%	0.1%	0.01%	0.001%
cynarin	89.44%	88.61%	27.22%	9.50%
chlorogenic acid	90.83%	87.77%	51.38%	7.50%
rosmarinic acid	90.27%	90.55%	61.66%	7.77%
caffeic acid	90.27%	90.55%	85.27%	17.50%
luteolin-7-glucoside	87.16%	85.56%	43.31%	3.74%
rutin	88.23%	86.55%	29.97%	4.76%

The experiments performed confirmed that all polyphenolcarboxylic acids, their derivatives and flavonosides, in pure form or as constituents of *Cynarae folium* selective fractions, exhibit a significant antioxidant activity as determined by DPPH method.

The antioxidant activity of the selective fractions are presented in Table 4.

The results obtained confirm the findings presented in the literature (Gebhardt, 1997, 1998; Perez-Garcia et al., 2000; Wang et al., 2003) concerning the antioxidant activity of extracts obtained from *Cynara scolymus* L. leaves and also the fact that the antioxidant effect is due to the polyphenols content (luteolin-7-glucoside, caffeic acid and chlorogenic acid).

The correlation between the chemical composition of flavonosides and polyphenolcarboxylic acids content and the antioxidant activity of each fraction is showed in Tables 1, 2 and 4.

Table 4. Antioxidant activity of selective fractions (%)

Dilution/ selective fraction	1%	0.1%	0.01%	0.001%
C1	78.05%	21.38%	3.88%	0.27%
C2	71.38%	20.55%	2.77%	2.50%
C3	70.83%	40.83%	8.05%	4.44%
C4	62.22%	33.33%	7.22%	4.72%
C5	60.83%	28,61%	8.05%	4.16%
C6	87.77%	85.27%	17.50%	5.27%
C7	37.22%	11.94%	4.72%	3.33%
C8	84.44%	69.44%	11.66%	4.65%

Comparing the antioxidant activity of selective fractions with the activity of reference substances, it can be said that antioxidant activity depends on the active substances concentration, namely it increase with the increase of concentration.

Selective fraction C_6 especially, which is rich in luteolin-7-glucoside (16.662%) exhibits an antioxidant activity higher than other fractions (87.77% at 1% dilution and 85.27% at 0,1% dilution), similar to the reference substance luteolin-7-glucoside (87.16% at 1% dilution and 85.56% at 0.1% dilution) which shows a significant antioxidant activity.

A dose-effect correlation is obvious, certifying that both flavones and polyphenolcarboxylic acids are responsible for antioxidant activity. In this respect, we showed that C_8 selective fraction containing only 5.576% luteolin-7-glucoside but a higher amount of chlorogenic acid (1.504%) than the other selective fractions

exhibits a good antioxidant activity (84.44% at 1% dilution and 69.44% at 0.1% dilution), close to selective fraction C_6 .
Still, some selective fractions exhibit antioxidant activity even though they have low concentrations in active substances (for example C_7) which might show that these compounds act synergistically.

CONCLUSIONS

Eight selective fractions from *Cynara scolymus* leaves were obtained; their concentration of active substances varies with the chosen extraction method from 0-0.122% caffeic acid, 0.007-1.504% chlorogenic acid, 0-0.443% rosmarinic acid, 0-0.097% cynarin, 0.054-16.662 % luteolin-7-glucoside, 0.009 - 1.253% apigenin-7-glucoside and 0-0.396% rutin.
All selective fractions exhibited antioxidant activity and the action is correlated with their active substances concentration.

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