In vitro study of antioxidant activity and phenolic content of *Chrysanthemum balsamita* varieties

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Abstract: The purpose of our study was to identify the phenolic substances of two varieties of *Chrysanthemum balsamita* (*balsamita* and *tanacetoides*) and to measure the overall antioxidant activity. The phenolic compounds were determined by HPLC. The evaluation of the polyphenolic content was performed by colorimetric analysis. The antioxidant activity was measured by three *in vitro* assay models: the DPPH, the silver nanoparticles antioxidant capacity (SNPAC) and EPR radical detection. Using HPLC-MS analysis, phenolic acids, flavonoids and flavonoid aglycone were detected. The highest antioxidant activity was showed by *Chrysanthemum balsamita* var. *balsamita*, while the lowest for the *Chrysanthemum balsamita var. tanacetoides* extract, in accord with the polyphenolic content. The results show that *Chrysanthemum balsamita* var. *balsamita* might be a source of antioxidant flavonoids, especially rutin and isoquercitrin.

Keywords: *Chrysanthemum*, variety, SNPs, antioxidant, phenolic compound.

INTRODUCTION

The genus *Tanacetum* is an *Asteraceae* family member (Ciocarlan, 2009; Kumar and Tyagi, 2013). The *Tanacetum* species contain sesquiterpenoids, flavonoids and essential oils with several properties: antimicrobial, antihelmintic, anti-inflammatory, antiulcer, antioxidant, anticancer, insecticidal (Hassanpouraghdam *et al*., 2008; Hassanpouraghdam *et al.*, 2009; Kumar and Tyagi, 2013). The Romanian flora comprises around 12 species and a few subspecies and varieties of *Tanacetum* (Ciocarlan, 2009). An important representative of the genus is the *Chrysanthemum balsamita* species. Two Romanian varieties of this species, *Chrysanthemum balsamita* varietas *balsamita* and *Chrysanthemum balsamita* var. *tanacetoides* are the subject of this research study compared.

Chrysanthemum balsamita (L.) Baillon *syn. Tanacetum balsamita* L. (costmary) has Eurasian origin and it has been naturalized and cultivated in different parts of the world such as the Balkan and South American countries (Hassanpouraghdam *et al*., 2008). Costmary is an important medicinal and aromatic plant of Romanian cultivated flora, with a long traditional usage in folk medicine (Marculescu, 2013). In Romanian flora, *C. balsamita* presents two infraspecific taxa with well defined morphological and chemical characteristics: *Chrysanthemum balsamita* var. *balsamita* with the both white ray florets and yellow disk florets on capitulum and camphora as the main component of the essential oil and *Chrysanthemum balsamita* var. *tanacetoides* with only yellow disk florets, but no ray florets and carvone as the main component of the essential oil (Ciocarlan, 2009; Kumar and Tyagi, 2013; Marculescu, 2013). The most important active principles of costmary are essential oil, phenylpropane derivatives, flavonoids, tannins (Hassanpouraghdam *et al.*, 2009; Kumar and Tyagi, 2013; Marculescu *et al*., 2001; Marculescu, 2013). The essential oil composition varies based on genetic, geographic and climatologic factors. For therapeutic purposes this plant has been used as a hepatoprotective, antialergic, tonic, sedative, insecticidal, antioxidant agent (Derakhshani *et al*., 2012; Kumar and Tyagi, 2013; Pukalskas *et al*., 2010; Rusu *et al*., 1994; Yousefzadi *et al*., 2009). There are only a few studies available on the antioxidant activity of *Chrysanthemum balsamita* (from Iran and Lithuania), showing that the extract has antioxidant action, by using DPPH and FRAP methods (Derakhshani *et al*., 2012; Pukalskas *et al*., 2010). Researching information on both local varieties (*balsamita* and *tanacetoides*) of *Chrysanthemum* are poor.

The purpose of our study has been to make a screening of the major polyphenolic compounds of the alcoholic extracts from the aerial parts of the two taxa of *Chrysanthemum*, and to measure antioxidant capacity.

MATERIAL AND METHODS

Plant material and preparation of plant extracts

The aerial parts of *Chrysanthemum balsamita* (L.) Baillon with two varietas: *Chrysanthemum balsamita* L. var. *balsamita* (Voucher No. 26) and *Chrysanthemum balsamita* L. var. *tanacetoides* (Voucher No. 27) from

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Cluj (Romania) were collected at flowering stage in July (2013) and were authenticated by Dr. D. Hanganu from the Department of Pharmacognosy of the Faculty of Pharmacy, "Iuliu Hatieganu" University of Medicine and Pharmacy, Cluj-Napoca, Romania**.** Vouchers are preserved in our herbarium. Fresh aerial plant parts were dried for 24 hours in the Excalibur dehydrator at 35^0C. The plant material was reduced to a proper degree of fineness. Five grams of powder was extracted with 50 mL of 70% ethanol (Merck), for 30 min on a water bath, at 60°C (Benedec *et al*., 2013, Romanian Pharmacopoeia, 1993).

HPLC-MS analysis

HPLC was used to analyse the phenolic derivatives. HPLC-MS analysis has been made on an Agilent system using the conditions described by other authors (Anton *et al*., 2013; Benedec *et al*., 2013; Fodorea *et al*., 2005; Moldovan *et al*., 2014). Quantification has been done by external standard method; retention times were determined with a standard deviation ranging from 0.04 min to 0.19 min. For all compounds, the accuracy was between 94.13% and 105.3%. In sample the compounds were identified by comparison of their retention times and recorded electro spray mass spectra with those of standards in the same chromatographic conditions.

Determination of phenolic content

The spectrophotometric assay based on aluminum chloride complex formation has been used for analysis of flavonoids and the result was expressed as g rutin equivalent (RE) using a calibration curve based on rutin (R^2=0.999) (Benedec *et al*., 2013; Romanian Pharmacopoeia, 1993). The total polyphenolic content (TPC) of extract has been measured using a previously described protocol and the result was expressed as g gallic acid equivalent (GAE) (Benedec *et al*., 2013; *Ph. Eur*., 2005; Slinkard and Singleton, 1977). The phenolic acids have been determined using an Arnows' spectrometric method (Benedec *et al*., 2013; Romanian Pharmacopoeia, 1993). The percentage of phenolic acids has been determined using the calibration curve caffeic acid. All values were calculated as g per 100g of dried plant material (g RE %, g GAE % and g CAE %, respectively).

Determination of antioxidant activity

In this regard, the ethanolic extracts have been screened for its antioxidant capacities by the DPPH, the silver nanoparticles antioxidant capacity (SNPAC), and an EPR radical's detection (Benedec *et al*., 2013; Espin *et al*., 2000; Espinoza *et al*., 2009; Nikolova and Dzhurmanski, 2009; Liu *et al*., 2012; Ozyurek *et al*., 2012; Szydlowska Czerniak *et al*., 2012).

The antioxidant capacity of hydrophilic compounds from studied herb extracts has been measured by silver nanoparticles-based assay (SNPAC), a method described by Ozyurek with a slight modification (Ozyurek *et al*., 2012). Thus, a sensitive spectrophotometric method has been used in this research by reducing of Ag (+) ions to spherical nanoparticles (SNPs) by phenolic compounds in the presence of trisodium citrate and silver seeds produced a very intense surface plasmon resonance (SPR) absorption band of SNPs at 423 nm. Antioxidant activity has been expressed as µmol of gallic acid equivalents/g (Liu *et al*., 2012; Ozyurek *et al*., 2012; Szydlowska Czerniak *et al*., 2012).

EPR measurements have been performed on a Bruker Elexsys E500 spectrometer operating in X band (~ 9.4 GHz) with 100 kHz modulation frequency, at room temperature using the conditions described by other authors (Espinoza *et al*., 2009; Kalita *et al*., 2011; Mocan *et al*., 2014).

RESULTS

Chromatographic analysis

The polyphenols in the extracts of *C. balsamita* have been studied by the reference HPLC method. In this study, 19 standard phenolic compounds were employed: 8 phenolic acids and 11 flavonoids. The simultaneous analysis of different classes of polyphenols has been performed by a single column pass and the separation of all examined compounds was carried out in 35 min (Anton*et al*., 2013; Benedec *et al*., 2013; Fodorea *et al*., 2005; Moldovan *et al*., 2014). The values of identified compounds in these two varietas have been tabulated (table 1) and the HPLC chromatograms of the two ethanolic extracts (*Chrysanthemum balsamita* var*. balsamita* and *Chrysanthemum balsamita* var*. tanacetoides*) were shown in figs. 1-2. The hydroxycinnamic and caffeic acid derivatives (*p*-coumaric acid, ferulic acid and chlorogenic acid) have been found in all extracts. 2,5-dihydroxybenzoic acid (gentisic acid) and 3,4-dihydroxy-cinnamic acid (caffeic acid) have been present only for *Chrysanthemum balsamita* var*. tanacetoides*. Their concentrations are lower than 10µg/g. Also, in the same sample, were quantified several flavonoids. Three glycosides derived from the flavonoid quercetin (rutin, quercitrin, isoquercitrin) and two aglycones (kaempferol/ quercetin) have been found in these extracts. Isoquercitrin and rutin are most abundant constituents in *Chrysanthemum balsamita* var*. balsamita* and are found in high concentrations, in the range of hundreds and thousands of micrograms per gram (780.81 and 8042.62µg/g, respectively). Isoquercitrin was determined just in the *Chrysanthemum balsamita* var*. balsamita* extract*. Chrysanthemum balsamita* var*. balsamita* was distinctly the richer species in quercitrin, quercetin and kaempferol than *tanacetoides* variety (table 1).

Extract yield, polyphenolic content and the antioxidant activity

The extract yield prepared by refluxing using 70% ethanol was 6% for *Chrysanthemum balsamita* var*. balsamita* and

Table 1: Polyphenolic compounds in *C. balsamita* varieties (µg/g dried plant material)

Polyphenolic compounds	Rt ± SD (min)	*m/z*	*Chrysanthemum balsamita* var. *balsamita*	*Chrysanthemum balsamita* var. *tanacetoides*
Gentisic acid	2.15±0.07	179	-	<0.2
Caffeic acid	5.60±0.04	179	-	<0.2
Chlorogenic acid	5.62±0.05	353	<0.2	<0.2
p-Coumaric acid	8.70±0.08	163	9.83 ± 0.17	8.02±0.07
Ferulic acid	12.20±0.10	193	<0.2	6.58±0.42
Isoquercitrin	19.60±0.10	463	780.81 ± 8.20	-
Rutin	20.20±0.15	609	8042.62 ± 11.38	112.48±1.52
Quercitrin	23.64±0.13	447	42.91 ± 0.09	20.48±0.53
Quercetin	26.80±0.15	301	26.51 ± 0.50	3.39±0.21
Kaempferol	32.48±0.17	285	30.69 ± 1.52	13.45±0.55

Note: NF - not found, below limit of detection. Values are the mean ± SD (n = 3).

Table 2: The content of polyphenols in the extracts of *C. balsamita* varieties

Samples	Flavonoids (g RE %)	TPC (g GAE %)	Caffeic acid derivatives (g CAE %)
Chrysanthemum balsamita var. *balsamita*	2.23±0.32	4.40±0.61	1.60±0.40
Chrysanthemum balsamita var. *tanacetoides*	1.19±0.51	2.92±0.23	1.02±0.31

Each value is the mean ± SD of three independent measurements. RE: rutin equivalents; GAE: Gallic acid equivalents; CAE: caffeic acid equivalents

Table 3: Results of the antioxidant activity of *C. balsamita* varieties

Samples	IC_{50} (µg/mL)	SNPAC (µmoli GAE/g)	EPR Integral intensity
Chrysanthemum balsamita var. *balsamita* (32)	59.70±2.30	71.44±3.56	228.04±15.95
Chrysanthemum balsamita var. *tanacetoides* (31)	121.13±5.21	34.25±4.75	361.50±8.49
Quercetin	5.47±0.03	-	-
DPPH	-	-	797.01±43.64

5.2% for *Chrysanthemum balsamita* var. *tanacetoides.* The ethanolic extract of *Chrysanthemum balsamita* var. *balsamita* contained a higher amount of flavonoids (2.23 g RE %), total polyphenols (4.40 g GAE %) and caffeic acid derivatives (1.60 g CAE %), than the extract of *Chrysanthemum balsamita* var. *tanacetoides* (1.19 g RE %, 2.92 g GAE %, and 1.02 g CAE %, respectively) as can see in table 2.

The antioxidant capacity of *C. balsamita* has been measured using the DPPH, the silver nanoparticles based, and EPR methods (table 2).

The antioxidant capacity of the *C. balsamita* extract using DPPH· bleaching assay is shown in table 3. Quercetin has been used a positive blank. IC_{50} value is the concentration where the inhibition reached 50%. As a result of this study, the extract of *Chrysanthemum balsamita* var. *balsamita* was more powerful antioxidant activity with IC_{50}=59.70µg/mL, than the *Chrysanthemum balsamita* var. *tanacetoides* extract withIC_{50} value of 121.13µg/mL.

Silver nanoparticles (SNPs) have attracted many uses in various fields (biotechnology, nanomedicine etc.) (Ozyurek *et al*., 2012). The nanomaterial-based methods were rarely used for assessing antioxidant activities of some compounds like polyphenols (Liu *et al*., 2012; Ozyurek *et al*., 2012; Szydlowska Czerniak *et al*., 2012).

The silver nanoparticles absorption spectrum (fig. 3) has revealed that SNPs prepared by citrate reduction shows a surface plasmon absorption band with a maximum of 423 nm, thus showing the presence of approximately spherical silver nanoparticles. The spherical shape was further confirmed by Transmission electron microscopy (TEM) (fig. 4). The mean size of the nanoparticles without antioxidant compounds was 30 nm (Ozyurek *et al*., 2012). The extract of *Chrysanthemum balsamita* var. *balsamita* showed a higher antioxidant capacity; the results were expressed in µmol gallic acid/ gram (table 3).

A method often used to determine the antioxidant capacity is Electron paramagnetic resonance (EPR) or electron spin resonance (ESR) spectroscopy that use the DPPH as a free radical (Espinoza et al., 2009; Kalita et al., 2014). The reaction rate between polyphenols and DPPH has been monitored by normalized double integrated residual EPR signal (Integral intensity), which was correlated with the paramagnetic species number (fig 5). The results of the two varieties sintegral intensity: *Chrysanthemum balsamita* var. *balsamita* (sample No. 32) and *Chrysanthemum balsamita* var. *tanacetoides* (sample No. 31) are given in table 3. These EPR results are compared with the DPPH value.

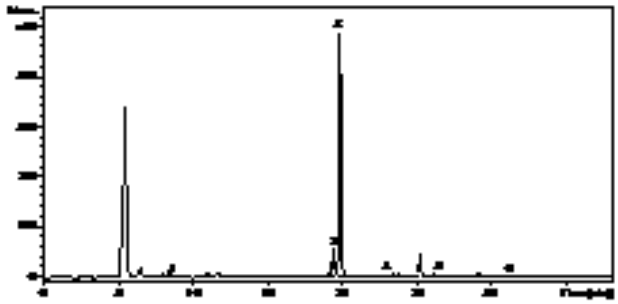


Fig. 1: HPLC chromatogram of *C. balsamita*

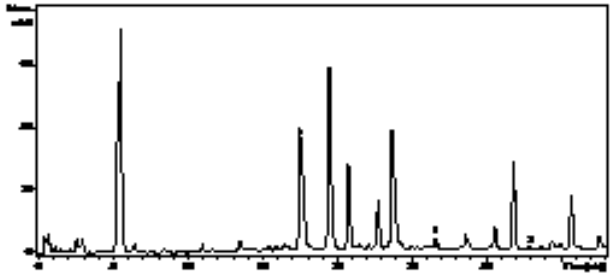


Fig. 2: HPLC chromatogram of *C. tanacetoides*

DISCUSSIONS

The simultaneous analysis of polyphenols was carried out using a highly-accurate HPLC method coupled to MS. The aerial parts of *Chrysanthemum balsamita* have been shown to be rich especially in quercetin and its glycosides, isoquercitrin, rutin and quercitrin, the *balsamita* variety being richer in these flavonoids than *tanacetoides* variety. Rutin was found as dominant flavonoid in both varieties, but the best source of rutin was found to be *Chrysanthemum balsamita* var. *balsamita* (8042.62µg/g). Isoquercitrin identified and quantified only in *balsamita* variety and it can be used as a marker to identify of varieties. It can be considered that *Chrysanthemum balsamita* aerial part could be a good source of natural isoquercitrin and rutin, which is significant for the development of potential pharmaceuticals. Regarding the phenolic acids, just chlorogenic acid was determined in both samples. In summary, this study revealed differences, mainly quantitative, between these two varieties of *Chrysanthemum balsamita*, the richest variety in flavonoidic compounds being *Chrysanthemum balsamita* var. *balsamita.*

In terms of total polyphenolic content, the spectrophotometric results are in good agreement with those obtained by HPLC analysis; the richest variety in these compounds being *Chrysanthemum balsamita* var. *balsamita* and could be an important source of phenolic compounds.

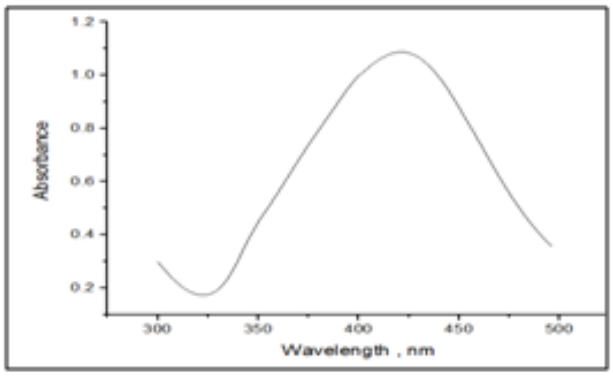


Fig. 3: UV-VIS spectrum of SNPs synthesized

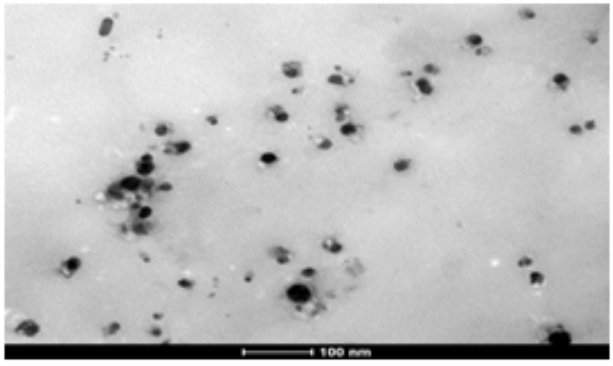


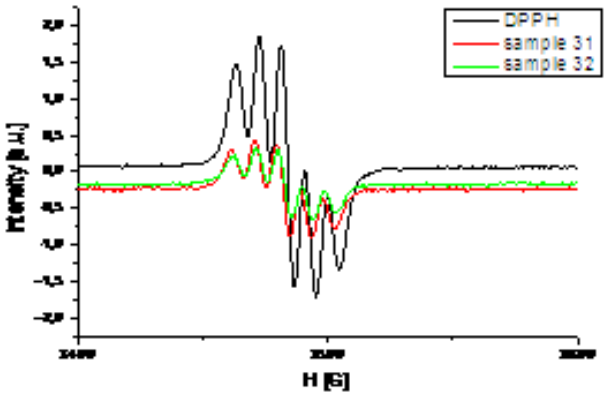
Fig. 4: TEM image of SNPs synthesized

The antioxidant capacity of *C. balsamita* has been evaluated using the DPPH test, SNPAC and EPR spectroscopy methods. The findings obtained by the three methods (table 3) indicated that *Chrysanthemum balsamita* var. *balsamita* was more powerful antioxidant than *Chrysanthemum balsamita* var. *tanacetoides* in line with the phenolic compounds values (table 2).

Until the present, scientific data on the antioxidant activity of *C. balsamita* using the silver nanoparticle-based the electron paramagnetic and resonance (EPR) spectroscopy methods are not available.

In this study, for the first time, the antioxidant property of the *Chrysanthemum* extracts was assessed by the silver nanoparticle (SNP)-based method of O□zyu□rek

(Ozyurek *et al*., 2012). This is a colorimetric method based on the reduction of Ag ions (Ag+) by polyphenols of medicinal plant in the presence of citrate-stabilized silver seeds was used. The principle is that the polyphenolic compounds of the two extracts of *C. balsamita* make monovalent silver ions reduced to form SNPs and the optical properties of nanoparticles produced are in good concordance with the antioxidant capacity of these samples. Alsoin this case, the extract of *Chrysanthemum balsamita* var. *balsamita* showed a higher antioxidant capacity than *Chrysanthemum balsamita* var. *tanacedoites*, related with the polyphenolic total content (table 2) and with the DPPH radical-scavenging assays results (table 3).



Note: sample 31 - *Chrysanthemum balsamita* var. *tanacetoides*; sample 32 - *Chrysanthemum balsamita* var. *balsamita*

Fig. 5: The rate of reaction between antioxidant compounds and DPPH radical

Regarding the EPR spectroscopy method, the DPPH integral intensity in combination with the two samples (for sample no. 31: 361.50 and for sample no. 32: 228.04, respectively) has decreased compared to with DPPH single (797.01). The EPR spectra (fig. 5) showed that it was registered a smaller intensity of the signal function of the extract. It represents the oxidation-reduction rate of the DPPH radical. By comparing the computed rates of both extracts, it can be seen that the extract of *Chrysanthemum balsamita* var. *balsamita* (sample 32) exhibited a higher antioxidant capacity than *Chrysanthemum balsamita* var. *tanacetoides* (sample. 31). The EPR results are consistent with the results of previous methods. With respect to the antioxidant potential measured by three methods, one can conclude that the extract of *Chrysanthemum balsamita* var. *balsamita* showed a higher antioxidant capacity than *Chrysanthemum balsamita* var. *tanacedoites.*

CONCLUSIONS

The phenolic content and antioxidant activities of two varieties of *Chrysanthemum balsamita* were determined. The obtained results provide new useful data concerning the polyphenolic composition and biological activities, which give a wide range of possibilities to use these medicinal plants as the source of phenolic compounds. The highest total amounts of caffeic acid derivatives, flavonoids and polyphenols compounds were determined in *Chrysanthemum balsamita* var. *balsamita*, this variety can be employed as a rich potential source of rutin and isoquercitrin. Nevertheless, the chemical analysis of *Chrysanthemum balsamita* var. *tanacetoides* reveals a lower polyphenolic content. Regarding the antioxidant activity, the best results were found for the ethanolic extract of *Chrysanthemum balsamita* var. *balsamita* in accord with the content of active principles. The results reported in this study showed a great potential of *Chrysanthemum balsamita* var. *balsamita* taxon for the development of the pharmaceuticals rich in compounds with antioxidant properties. Additionally, the differences between these two varieties of *Chrysanthemum balsamita* could be used as chemotaxonomic markers to distinguish the two local varieties.

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