Fingerprint and Quantification of Phenolic Dervatives in *Melissa off.* and *Calendula off.* extracts in Relation to Their Antioxidant Potential

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Abstract. Our experiments aimed the extraction of *Melissa off.* and *Calendula off.* dried plants in two types of solvents (ethanol and glycerine mixed with water, named extract A and B, respectively) and the evaluation of phenolic derivatives either by spectrometry (Folin-Ciocâlteu method) and by HPLC method. The antioxidant activity was determined then and correlated with the phenolic (qualitative and quantitative) composition. Ethanolic extract A was more rich in phenolics than the glycerin-based extract B. We noticed that *Calendula off* is around two times more concentrated in phenolics and contains 3 times more rosmarinic acid than *Melissa off.* Comparing these data with the total phenolics determined by Folic-Ciocâlteu method, we consider the HPLC gives higher concentration values and more accurate. The antioxidant capacity was proportional to the quantity of phenolics and is related to rosmarinic, sinapic and o-coumaric acids for *Calendulla off.*. *Melissa off.* has a also complex composition, but with lower phenolic concentration, its antioxidant activity being mainly due to rosmarinic acid, as in *Calendula off.* Therefore, we recommend a mixture of *Calendula* and *Melissa* extracts as excellent candidates for a food supplement dedicated to the prevention of viral and bacterial infections, due to their powerful antioxidant capacity.

Introduction

Medicinal plants are rich sources of antioxidants belonging to hydrophilic or lipopphilic classes of phytochemicals, such as phenolic derivatives (phenolic acids, catechins, anthocyanins) or terpenoids, sterols, tocopherols , carotenoids, respectively (Socaciu et al., , 2002, 2008; Zavoi et al., 2011).

*Melissa officinalis (*Labiatae fam.) is well known described for her rich composition in phytochemicals, especially terpenoids and phenolic derivatives to mention citronellal, geranial, neral, rosmarinic acid and catechins (Duke, 1995 at http://www.ars-grin.gov/cgi-bin/duke ; Duda et al., 2007; Patora J., et al., 2002). The hydroalcoholic leaf extracts have a spasmolytic, sedative, wound healing, analgesic action and especially anti-inflamatory and antiviral effects against *Herpes simplex* (Hohmann *et al.*, 1999; Sengul M. et al., 2009), due to rosmarinic acid, as main phenolic molecule responsible for these effects. It contains also small quantities of other phenolics such as protocatecuic, caffeic acid and their methyl esters, as well flavonoids like quercetine and kaempherol. The oil of *Melissa off.* is used in aromatherapy against depressions and thyroid hyper-excitability.

Calendula officinalis L. (Asteraceae fam.) is a medicinal plant with a very complex chemical composition (Isaac, 1992; Pintea, 2001). The essential oil content in fresh flowers ranges between 0,1-0,2% and contains both terpene hydrocarbons (α-

pinene, α-thujene, β-cariophyllene, α-humulene, germacrene D, cadinenes) and oxygenated derivatives (cadinols, terpineol, etc). Terpenes of Calendula extracts exhibit antibacterial and fungicide activity. The phytosterols, such as stigmasterol, sitosterol, campesterol, are found in free form, but mainly esterified and glicosylated. *Calendula off.* L. contains also important amounts of triterpenic saponins, estimated at 3-6 %. The common characteristic of Calendula saponosides is the presence of oleanolic acid. Triterpenic alcohols are well represented in Calendula flowers. β-amyrin, taraxasterol, faradiol, calenduladiol and heliantriol are the main triterpenic alcohols. They can exist in free form but also esterified with fatty acids (Zitter-Eglseer et al., 1996,), its anti-inflammatory and antimicrobial properties being related to these derivatives (Della Radioza, 2007; Zitter-Eglseer et al., 1997). Carotenoids are concentrated especially in flowers at concentrations of 0,1-0,2 %, the yellow varieties being rich in epoxides (flavoxanthin, luteoxanthin, violaxanthin) while orange varieties contain mainly hydrocarbons (β-carotene, γ-carotene, lycopene) and monoxantophylls (Isaac, 1992; Bako et al, 2002, Pintea et al., 2003). Carotenoids are related to wound healing properties of pot marygold extracts. In all plant organs were identified also α, γ and δ-tocopherol, estimated at 1980 μg/10 g dry weight (Janiszowska and Rygier, 1985).

Among phenolic derivatives, flavonoids such as quercetin and isorhamnetin glycosides are found especially in *Calendula* flowers. (Vidal-Ollivier et al., 1989) and represents 0,6 - 1% , beside other minor derivatives , e.g. umbelipherone, scopoletine and esculetine which were also identified (Isaac, 1992). Phenolic acids content in dry material is reported to be around 100 mg/100 g and are responsible for the choleretic and cholagogue activities of *Calendula off.* Main compounds of this class are: salicylic, para-hydroxy-benzoic, gentisic, protocatechic, vanillic, syringic, o- and p-coumaric, caffeic, ferulic, sinapic, cinnamic, chlorogenic acids (Isaac, 1992). Alantoine can be also found and has a contribution to the healing, antiinflamatory (Preethi K. C. et al.,2009) and antimicrobian properties (Korakhashvili A., et al.,2007, Radioza S. A.. et al., 2007). Calendula is also rich in fatty acids, besides palmitic, palmitoleic acids, contain an unusual conjugated fatty acid, the calendic acid: 18:3 (8t,10t,12c), inhibitor of cicloxygenase (Isaac, 1992; Pintea et al., 1995, 2000, 2001).

Our experiments aimed the extraction of *Melissa off.* and *Calendula off.* plants in two types of solvents (ethanol and glycerine mixed with water) and the evaluation of phenolic derivatives either by spectrometry and by HPLC method. The antioxidant activity was determined then and correlated with the phenolic (qualitative and quantitative) composition.

Materials and methods

There were used dried plants of *Melissa officinalis* (wild flora - from Timişoara region), and *Calendula oficinallis* (cultivated). Two different extracts were obtained from each plant, as follows: 10g dried plant was mixed with 100 ml solvent A (mixture of ethanol and water, 45:65) or solvent B (mixture of glycerin and water, 50:50). The extraction lasted for 7 days, in dark, at 25 ºC.

The extracts A and B were analyzed by **UV-Vis spectrometry**, identifying the absorption maxima in the region 200-800 nm. **Total phenolics** were determined by Folin Ciocâlteu method adapted by Huang et al., (2005), as follows: to 23 µl extract it was added 115 µl Reactiv Folin Ciocalteu, 345 µl Na_2CO_3 (7,5%) and 1.817 ml bidistilled water. The calibration curve was obtained using gallic acid as pure standard (concentration range 0,03 –1 mg/ml). The regression equation was y =-0,0934x + 0,6867 and the coefficient of correlation R^2 = 0,9833. The results were expressed in mg/ml equivalents gallic acid /g plant.

The antioxidant activity was determined by spectrometry method DPPH (1,1,-difenil-2-picrilhidrazil) to evaluate the quenching of free radicals (Miliauskas et al., 2004). The working method (Brand-Williams si colab., (1995) included the mixing of 200 µl extract with 1.4 ml DPPH 80 µM. Trolox at concentrations ranging from 7,81 to 1000 µM was used as positive control and the absorption was registered at 515 nm. The inhibition of DPPH was calculated as mM trolox equivalents/ ml extract.

To evidentiate the main **individual phenolic derivatives, an HPLC-PDA** (High Performance Liquid Chromatography coupled with Photodiode Array) separation was applied, according to a protocol established in our laboratories (Zavoi, 2011) . A calibration curve was firstly obtained with a complex mixture of phenolic acids: galic, protocatecuic, acid clorogenic, caffeic, p-cumaric, ferulic, sinapic, o-cumaric acids, rosmarinic acid, rutin, miricetin, quercetine, kaempherol. The phenolic derivatives from extracts A and B were separated and then, considering the regression equation for each phenolic compound, it was calculated its concentration, expressed per g of dried plant.

All determinations were done in duplicate and the results represent the mean values.

Results and discussion

1. **Phenolic derivatives quantification in relation to the antioxidant activity, as determined by UV-Vis Spectrometry**

Extracts A and B showed specific UV-Vis spectrometric fingerprint with maxima at 280 nm, indicating the presence of phenolics. The hydroalcoholic extract A had a higher absorption comparing with the glycerine extract B. Table 1 includes the data obtained from the determination of total phenolics by method Folin Cicâlteu, in both extracts A and B. It was observed that extract A contains more phenolics than extract B and that Calendula off. is more rich in phenolics than Melissa. The antioxidant capacity was proportional to the concentration of phenolics, being higher in *Calendula off.*

1. **Fingerprint and quantification of phenolic derivatives using HPLC - PDA method**

The calibration curve was firstly obtained, based on the HPLC-PDA separation (Fig.1) of a complex mixture of phenolic acids: galic, protocatecuic, acid clorogenic, caffeic, p-cumaric, ferulic, sinapic, o-coumaric acids, rosmarinic acid, rutin, miricetin, quercetine, kaempherol.

Table 1

Mean values obtained for total phenolics (mg/100ml extract) and antioxidant activity (mM Trolox/ml extract) of *Melissa off.* and *Calendula off.*

Plant	Total phenolics mg/100ml extract A	Antioxidant activity mM Trolox/ml extract A	Total phenolics mg/ml extract B	Antioxidant activity mM Trolox/ml extract B
Melissa off.	89	8.37	74	7.07
Calendula off.	198	9.52	141	8.22

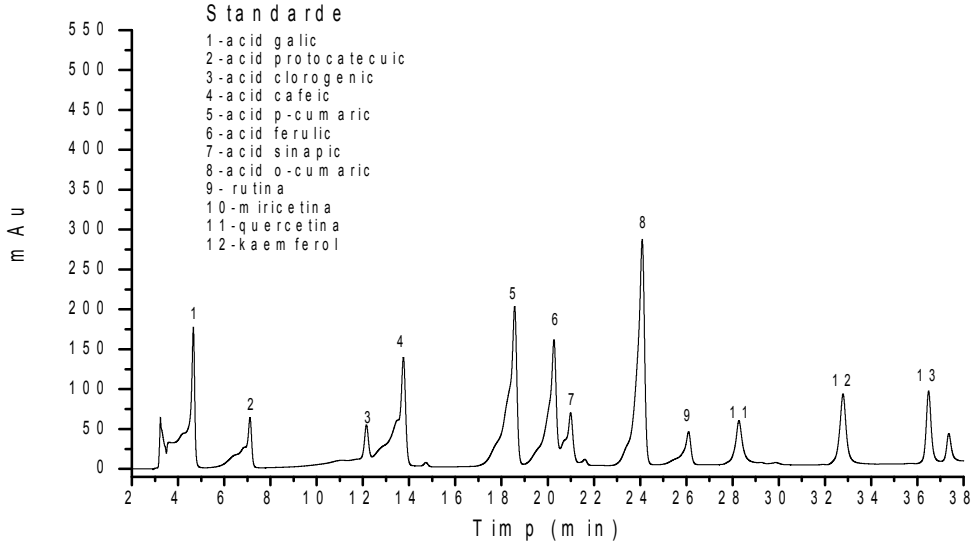


Fig.1. HPLC-PDA chromatogram of pure phenolic acid mixture which are ussually found in medicinal plants. Numbers correspond to the legend description.

Table 2 presents the retention times corresponding to and regression curves determined for each of the pure phenolic acid derivatives separated by HPLC. These data were used to calculate the individual concentrations of major phenolics found in *Melissa off.* and *Calendula off.* One can notice a very good separation and significant regression coefficients which demonstrates a very good resolution.

The extract A of *Melissa* contained rosmarinic acid (peak 10) as a major compoenent together with other non-identified peaks (n.i.) while the extract A of *Calendula off.* contained also rosmarinic acid (peak 10) as major peak together with sinapic acid (peak 7), p-coumaric acid (peak 5) gallic acid (peak 1) and other minor components.

Considering the calibration data, we calculated for each identified polyphenol, the concentration being expressed in mg/ 100 g plant (Table 3).

We noticed that *Calendula off* is around two times more concentrated in phenolics and contains 3 times more rosmarinic acid than *Melissa off.* Comparing these data

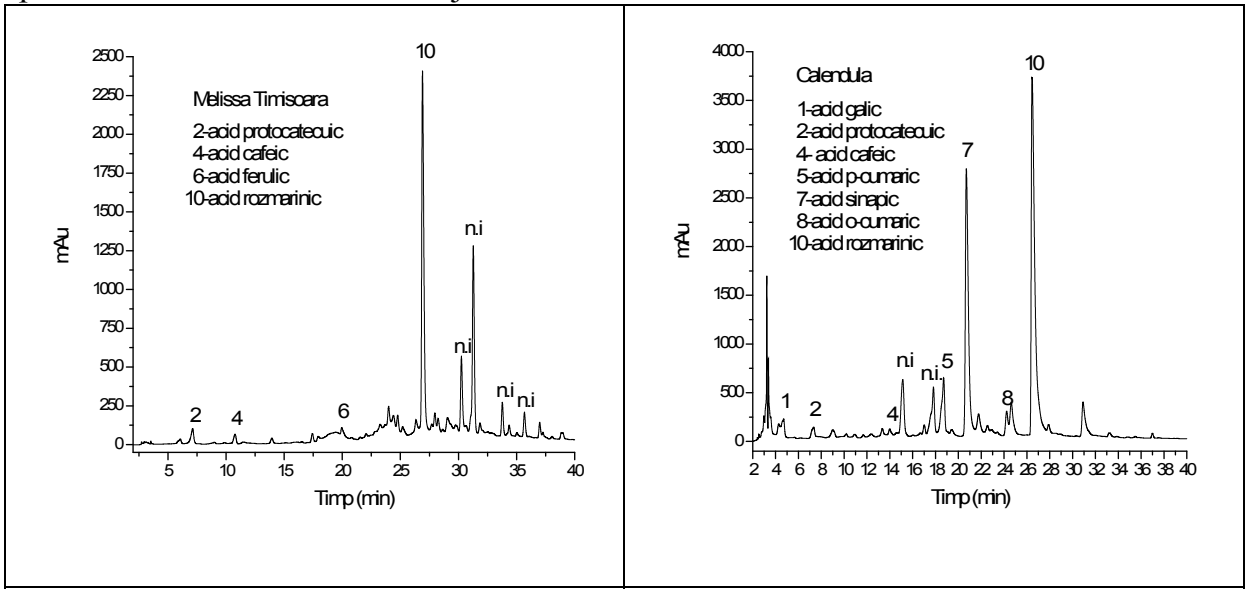
with the total phenolics determined by Folic-Ciocâlteu method, we consider tha HPLC gives higher concentration values and more accurate. The antioxidant cacapicity was proportional to the quantity of phenolics and is related to rosmarinic, sinapic and o-coumaric acids for *Calendulla off.*. *Melissa off.* has a also complex composition, but with lower phenolic concentration, its antioxidant activity being mainly due to rosmarinic acid, as in *Calendula off.*

Table 2.

Retention times and regression curves determined for each of the pure phenolic acid derivatives separated by HPLC-PDA.

Nr.	Pure standard	t_R(min.)	Regression equation	Regression coefficient (R^2)
1.	Galic Acid	4.66	y = 52633x + 70171	0.9972
2.	Protocatecuic Acid	7.11	y = 28805x + 42240	0.9994
3.	Chlorogenic Acid	12.15	y = 68765x –367340	0.9929
4.	Caffeic Acid	13.75	y = 44428x +511659	0.9812
5.	p-cumaric Acid	18.56	y = 54487x +621684	0.9899
6.	Ferulic Acid	20.27	y = 67828x -416401	0,9999
7.	Sinapic Acid	20.99	y = 26533x + 31301	0.9981
8.	o-cumaric Acid	24.09	y = 56842x + 23458	0.9972
9.	Rutin	26.09	y = 38664x –26884	0.9994
10.	Rosmarinic Acid	26.90	y = 48542x –26562	0.9994
11.	Miricetin	28.27	y = 47695x +28830	0.9812
12.	Quercetin	32.78	y = 67597x +432454	0.9899
13.	Kaempherol	36.48	y = 77695x +23471	0.9999

As represented in Fig. 3, for the extracts A we obtained a good separation of phenolics and identified the major molecules.



A. B.

Fig. 3. The HPLC-PDA fingerprint of the extracts A obtained for each plant (A- *Melissa off.* and B-*Calendula off.*)

Table 3.

Concentration of individual phenolics as determined by HPLC-PDA and the calculation of total phenolic derivatives (mg/ 100 g plant) in *Melissa off.* and *Calendula off.* extracts A.

Plant	Acid galic	Acid protocatecuic	Acid cafeic	Acid p-cumaric	Acid ferulic	Acid sinapic	Acid o-cumaric	Acid rosmarinic	**TOTAL (mg/100 g plant)**
Melissa off.	0	54.05	4,09	0	**15.38**	0	0	23.92	**97.44**
Calendula off.	**27.77**	26.5	**14,83**	5,72	0	**52.45**	**40**	**80**	**219.50**

Conclusion

Considering phenolic derivatives as main responsible molecules for the antioxidant, antiviral and antibacterial affects of *Calendula off.* and *Melissa off.,* we can conclude that our studies demonstrated that rosmarinic acid is the main component which confer their antioxidant and antiviral effects. Also, the total phenolic composition is proportional to the antioxidant capacity. Ethanolic extract A was more rich in phenolics than the glycerin-based extract B, for each plant.

Therefore, we recommend a mixture of *Calendula* and *Melissa* extracts as excellent candidates for a food supplement dedicated to the prevention of viral and bacterial infections, due to their powerful antioxidant capacity.

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