Evaluation of Antioxidant Activity and Phenolic Content of 13 Selected Herbs from Romania

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13 herbs alcoholic extracts from 11 botanical families grown in Romania were investigated in order to evaluate their antioxidant capacity (DPPH, ABTS, FRAP assays) and phenolic contents (Folin–Ciocalteu assay). Total antioxidant activity expressed as mM Trolox/L plant extract ranged from 0.928 to 31.425 (DPPH), 0.402 to 34.856 (ABTS) and 1.111 to 31.869 (FRAP) and the total phenolics from 1023.694 to 5853.650 mg GAE/L plant extract, the highest value being obtained for S. alba. Between the antioxidant activity and the phenolic content exists a good correlation, the phenolics being responsible for these species antioxidant activity.

Keywords: plant alcoholic extracts; antioxidant capacity; total phenolics

Polyphenolic compounds, commonly found in both edible and inedible plants have been reported to possess various biological effects, including antioxidant activity [1-5]. As antioxidants, polyphenols can protect cell constituents against oxidative degradations, thus limiting the risk of various degenerative diseases associated with oxidative stress [6, 7]. Their antioxidant capacity is thought to be predominantly responsible for the protection against cardiovascular diseases and cancer [8]. Such plants are used in domains like nutrition, flavoring, beverages, dyeing, cosmetics, fragrances and some of them are known to have beneficial influence on health due to their biological effects [9-11].

The food industry is interested in plant extracts rich in polyphenols because these retard oxidative degradation of lipids and improve the nutritional value and quality of food. Some researchers have studied the antioxidant capacities of freshly and dried culinary herbs and suggested that polyphenolic compounds may be the major bioactive compounds in culinary herbs responsible for the antioxidant effect. These studies also revealed that the levels of polyphenols in the studied herbs are similar to those of conventional antioxidant sources: vegetables, fruits, red wine [12, 13].

The main goals of this research were to obtain some alcoholic herbal extracts of 13 Romanian species, to establish and compare the antioxidant capacity of the extracts using three chemical methods, to evaluate their phenolic content and to determine the relationships between the antioxidant activity and the phenolic compounds in order to determine if the phenolic constituents are responsible for antioxidant activity of the plants.

Experimental part

Plant materials

Plant materials (wild or cultivated) were collected in 2013, from the Cluj County (Province of Transylvania, Romania) during the blooming period (May-August), except the *Salix alba* (March-April), *Lycopodium clavatum* (August) and *Viscum album* (November-December) (table 1). The samples were identified, authenticated and voucher specimens were deposited in the Herbarium of the Quality Control Laboratory, PlantExtrakt Laboratories, Radaia, Romania.

Chemicals

Ethanol (pharmaceutical grade), methanol (HPLC grade), sodium carbonate, sodium acetate, potassium persulfate, Folin-Ciocalteu reagent, 1,1-diphenyl-dipicrylhydrazyl (DPPH), gallic acid and 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid 97% (Trolox) were purchased from Sigma-Aldrich, Germany. Iron (III) chloride, 2,4,6-tris(2-pyridyl)-1,3,5-triazine (TPTZ) and 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) were purchased from Sigma, Germany. Hydrochloric acid 37 % was obtained from Merck, Germany.

Extracts

Fresh or dried material was grinded with a laboratory cutter and extracted with 90% (V/V) ethanol. The plant-solvent ratio (table 1) was established according to European Pharmacopoeia (EP) 8.5 (Homeopathic Preparations – Methods 1.1.3, 1.1.5, 1.1.8) [14c]. The extraction is carried out at room temperature (not exceeding 20°C) by 10 days maceration with repeated shaking, followed by pressing, five days standing in a closed container and then filtration.

The extracts were characterized by the aspect, relative density, dry residue and ethanol content, determined according to EP. Aspect is determined by observation [14a]. The relative density was determined using an Anton Paar DMA 35 digital densitometer [14a]. The dry residue was determined by evaporation of 3.000 g of extracts in oven, at 105-110°C, for 2 h [14b]. The ethanol content was

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Table 1
INFORMATION ON SELECTED HERBS

No.	Binomial name (Family) / Common name	Part of plant / status	Wild flora or cultivated	Harvesting period	Processing method
1	*Aristolochia clematitis* L. (Aristolochiaceae) / European Birthwort	Herba / fresh	Wild flora	August	1.1.3
2	*Arnica montana* L. (Asteraceae) / Arnica	Root / dry	Wild flora	August	1.1.8
3	*Chelidonium majus* L. (Papaveraceae) / Greater Celandine	Entire flowering plant / fresh	Wild flora	May-June	1.1.5
4	*Hypericum perforatum* L. (Hypericaceae) / St. John's wort	Herba / fresh	Wild flora	July	1.1.5
5	*Lycopodium clavatum* L. (Lycopodiaceae) / Stag's-horn Clubmoss	Spora / dry	Wild flora	August	1.1.8
6	*Melissa officinalis* L. (Lamiaceae) / Lemon Balm	Herba/ fresh	Cultivated Organic culture	June-July	1.1.5
7	*Salix alba* L. (Salicaceae) / White Willow	Bark/ fresh	Wild flora	March-April	1.1.5
8	*Salvia officinalis* L. (Lamiaceae) / Sage	Leaf / fresh	Cultivated Organic culture	June-July	1.1.5
9	*Thymus vulgaris* L. (Lamiaceae) / Thyme	Herba / fresh	Wild flora	July	1.1.5
10	*Tilia tomentosa* Moench (Malvaceae) / Silver Lime	Flowers/ fresh	Wild flora	June	1.1.3
11	*Vaccinium myrtillus* L. (Ericaceae) / Bilberry	Fruits / fresh	Wild flora	August	1.1.5
12	*Viola tricolor* L. (Violaceae) / Heartsease	Herba / fresh	Wild flora	May	1.1.3
13	*Viscum album* L. (Santalaceae / Viscaceae) / European Mistletoe	Herba with fruits / fresh	Wild flora (from apples and pears)	November-December	1.1.3

determined by distillation and by correlation of the distillate density with the data from alcoholmetric table [14d].

DPPH radical-scavenging activity.

The free radical scavenging activity of the extracts was performed using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay according to the procedure described in [15] with some modifications. In order to evaluate antioxidant activities, each sample has been diluted appropriately with methanol. Antioxidant solution in methanol (0.1 mL) was added to 2.9 mL of a solution ~$9 \cdot 10^{-5}$ mol/L DPPH in methanol. The inhibition of DPPH was followed by monitoring the decrease of absorbance at 515 nm during 2 h, using a Jasco V 530 UV-Vis spectrophotometer. Trolox was used as antioxidant reference compound. The calibration curve was obtained using standard solutions in the range 0.2-1.0 mmol/L Trolox (y = 78.217916x + 2.884240 (R^2 = 0.997570)). Each sample was analyzed in triplicate.

$ABTS^{\cdot+}$ radical-scavenging activity

The ABTS (7 mmol/L (2,2-azinobis-(3-ethyl benzthiazoline-6-sulfonic acid)) in 20 mmol/L sodium acetate buffer, *p*H=4.5) reacts with potassium persulfate (2.45 mmol/L in the same solution) [16]. The resulted dark blue-green stable radical solution is incubated 16-18 h, at room temperature, in the dark. The solution was then diluted to an absorbance 1.0±0.02 at 734 nm. The reaction between 0.1 mL sample (diluted appropriately with methanol) and 2.9 mL ABTS reactive, was followed at 734 during 2 h, against dd water, using a Jasco V 530 UV-Vis spectrophotometer. Trolox was used as the antioxidant reference compound. The calibration curve was obtained using standard solutions in the range 0.2-1.0 mmol/L Trolox (y = 82.14529x + 4.93265 (R^2 = 0.99669)). Each sample was analyzed in triplicate.

Ferric reducing/antioxidant power (FRAP) assay

The fresh FRAP solution was prepared by mixing 300 mmol/L sodium acetate buffer *p*H=3.6 with 10 mmol/L TPTZ (2,4,6-tripyridyl-triazine) in 40 mmol/L HCl and 20 mmol/L $FeCl_3 \cdot 6H_2O$ in dd water in vol. 10:1:1 ratio [17]. The resulting solution was diluted with 2 volumes of dd water and was incubated at 37°C for 30 min. 2.9 mL of working FRAP solution were mixed with 0.1 mL of extract (diluted appropriately with methanol) and were kept in dark for 2 h, at room temperature. An intense blue colour is formed when the ferric-tripyridyl-triazine complex is reduced to ferrous form. The absorbance of the samples and a blank was measured at 593 nm against dd water using a Jasco V 530 UV-Vis spectrophotometer. Trolox was used as the antioxidant reference compound. The calibration curve was obtained using standard solutions in the range 0.2-1.0 mmol/L Trolox (y = 1.35430x – 0.03477 (R^2 = 0.99974)). Each sample was analyzed in triplicate.

Total phenolics

The content of total phenolics was determined according to the Folin-Ciocalteu method [18] using gallic acid as standard. This assay is based on chemical reduction of the Folin-Ciocalteu reagent, a phosphowolframate-phosphomolybdate complex, to blue coloured products by phenolic compounds. The intensity of blue colour is proportional to the concentration of phenolic compounds.

Briefly, 200µL of each extract (previously diluted 1:10 with double distiled water) or standard solution, 15 mL dd water and 1 mL Folin-Ciocalteu reagent were added to a 20 mL volumetric flask. The contents were mixed and incubated for 5 min at room temperature. Then, 3 mL of 20% (w/v) sodium carbonate solution was added, followed by the addition of dd water to volume and mixing. After incubation for 2 h at room temperature, the absorbance at 765 nm using a Jasco V 530 UV-Vis spectrophotometer was determined against a blank reagent prepared with dd water.

The calibration curve of gallic acid (GA) was obtained using 10 standard solutions in the range 50-550 mg/L. Total phenolics content of the extracts was calculated from the calibration curve (the absorbance at 765 nm vs. gallic acid solution) using the following equation determined by linear regression: A = 0.0012335·C – 0.0505227 (R^2 = 0.9971552).

Total phenolics content was expressed as mg gallic acid equivalents per liter of plant extract (mg GAE/L). All samples were analyzed in triplicate.

Table 2
CHARACTERISTICS, ANTIOXIDANT CAPACITY AND TOTAL PHENOLIC CONTENT OF THE ALCOHOLIC EXTRACTS

No.	Binomial name	Aspect	Relative density	Dry residue [%]	Ethanol content [% vol.]	Antioxidant activity [mM Eq. Trolox/L]			Total phenolics [mg GAE/L]
						DPPH	ABTS	FRAP	
1	*Aristolochia clematitis* L.	dark brown liquid	0.949	2.97	48	3.106	3.140	4.721	1863.380
2	*Arnica montana* L.	dark yellow liquid	0.845	1.52	87	0.950	1.274	1.651	1188.402
3	*Chelidonium majus* L.	brownish-green liquid	0.935	1.22	49	2.339	1.555	2.555	1282.916
4	*Hypericum perforatum* L.	reddish-brown liquid	0.905	2.74	64	10.317	11.068	11.648	2825.880
5	*Lycopodium clavatum* L.	yellow liquid	0.834	1.54	89	0.928	0.402	1.111	1023.694
6	*Melissa officinalis* L.	greenish-brown liquid	0.905	1.55	69	12.534	14.552	15.974	2774.810
7	*Salix alba* L.	reddish-brown liquid	0.897	3.10	69	31.425	34.856	31.869	5853.650
8	*Salvia officinalis* L.	greenish-brown liquid	0.904	2.05	65	10.387	11.925	12.600	2899.860
9	*Thymus vulgaris* L.	brown liquid	0.904	1.57	64	22.751	25.262	24.729	4770.485
10	*Tilia tomentosa* Moench	orange-brown liquid	0.918	1.81	56	9.981	11.017	11.396	2745.083
11	*Vaccinium myrtillus* L.	brownish-violet liquid	0.922	4.17	60	11.255	13.791	13.738	2965.459
12	*Viola tricolor* L.	yellowish-brown liquid	0.952	3.50	45	4.378	5.382	7.215	2268.260
13	*Viscum album* L.	yellowish-brown liquid	0.969	9.29	38	11.760	15.210	17.211	4239.746

Results and discussions

The quality parameters of the extracts are detailed in the table 2. DPPH radical, ABTS radical cation and FRAP assays were used for evaluation of free radical-scavenging properties of 13 Romanian plants. The experimental results are also presented in table 2.

The plant species evaluated as their Trolox equivalent antioxidant capacity (TEAC) values indicated large variation in antioxidant activity. Total antioxidant activity, measured by the DPPH method, ranged from 0.928 to 31.425 mM Trolox equivalents per 1 L plant extract (mM Trolox/L plant extract); total antioxidant activity, using ABTS method, ranged from 0.402 to 34.856 mM Trolox/L plant extract; the antioxidant capacity, determined with the FRAP method, ranged from 1.111 to 31.869 mM Trolox/L plant extract. *S. alba* exhibited the highest antioxidant activity (DPPH: 31.425 mM Trolox/L plant extract; ABTS: 34.856 mM Trolox/L plant extract; FRAP: 31.869 mM Trolox/L plant extract), followed by *T. vulgaris* (DPPH: 22.751 mM Trolox/L plant extract; ABTS: 25.262 mM Trolox/L plant extract; FRAP: 24.729 mM Trolox/L plant extract). It was found that 7 of the 13 species exhibited an antioxidant activity greater than 10 mM Trolox/L plant extract, only 3 species being lower than 2 mM Trolox/L plant extract.

The amount of total phenolics, measured by Folin–Ciocalteu assay, varied widely in plant materials ranging from 1023.694 to 5853.650 mg GAE/L plant extract (table 2). The highest content of phenolics was found in *Salix alba*, while the lowest was in *Lycopodium clavatum*. *Thymus vulgaris* (4770.485 mg GAE/L plant extract) and *Viscum album* (4239.746 mg GAE/L plant extract) also exhibited very high levels of phenolics. Other herbs with high levels of phenolics were *Vaccinium myrtillus* (2965.459 mg GAE/L plant extract), *Salvia officinalis* (2899.860 mg GAE/L plant extract), *Hypericum perforatum* (2825.880 mg GAE/L plant extract), *Melissa officinalis* (2774.810 mg GAE/L plant extract) and *Tilia tomentosa* (2745.083 mg GAE/L plant extract). *Viola tricolor* (2268.260 mg GAE/L plant extract) and *Aristolochia clematitis* (1863.380 mg GAE/L plant extract) had relatively low levels of phenolics, whereas in *Chelidonium majus* (1282.916 mg GAE/L plant extract), *Arnica montana* (1188.402 mg GAE/L plant extract) and *Lycopodium clavatum* (1023.694 mg GAE/L plant extract) total phenolics was the lowest.

In Lamiaceae family, with 3 representatives in this study, the highest content in phenolics was obtained in case of *Thymus vulgaris*, the other two *Salvia officinalis* and *Melissa officinalis* being also among the plants with high levels of phenolics.

Regarding the relationship between the antioxidant activity and the phenolic compounds was found that, generally, exists a good correlation among the two of them (DPPH: $R^2=0.9170$; ABTS: $R^2=0.9436$; FRAP: $R^2=0.9626$), which proves that the phenolic constituents are responsible for antioxidant activity of the plants. Our results are in agreement with those reported by Djeridane et al. [9], Katalinic et al. [19] and Vicas et al. [20] concerning this linearity. The single exception is in case of Melissa officinalis, when the phenolic content is lower compared to its antioxidant activity. This can be explained by the fact that the most powerful scavenging compounds found in Melissa officinalis were monoterpene aldehydes and ketons (neral/geranial, citronellal, isomenthone, and menthone) and mono- and sesquiterpene hydrocarbons (E-caryophyllene) [21], which, besides phenolic compounds, contribute to the antioxidant capacity of the plant.

Antioxidant activity showed the same relationships by all three methods and TEAC values were almost similar (ABTS-DPPH: $R^2=0.9933$; FRAP-ABTS: $R^2=0.9904$; FRAP-DPPH: $R^2=0.9739$). Wojdy³o et al. studied the antioxidant capacity and total phenolics of some Polish herbs [1], five of them being studied also by us. They claimed great differences in antioxidant capacity measured by the FRAP method compared with those obtained with DPPH and ABTS assays and good correlation between the content of total phenolic compounds and their antioxidant capacity just within one family. These variances may be due to the different plant material characteristics, extraction parameters and way of expressing the antioxidant activity.

Conclusions

The experimental results showed that the studied Romanian species are rich in phenolic compounds and demonstrated good antioxidant activity measured by different chemical methods.

More than half of the analyzed species exhibited an antioxidant activity greater than 10 mM Trolox/L plant extract, and among them *Salix alba* demonstrated the highest antioxidant activity, being followed by *Thymus vulgaris*. These two species also proved to be the richest in phenolics.

The three representatives of the Lamiaceae family were situated among the plants with high levels of phenolics, the highest content in phenolics was obtained in case of *Thymus vulgaris*, followed by *Salvia officinalis* and *Melissa officinalis*.

These plants, rich in phenolic constituents, could be a good source of natural antioxidants.

The obtained data proved a linear correlation between the content of total phenolic compounds and their antioxidant capacity established by the three methods: DPPH, ABTS, FRAP assays. Also, there is a good correlation between the antioxidant activity data obtained by these three methods. The results confirm the importance of phenolic compounds in the antioxidant behavior of herbal extracts and also their significant contribution to the total antioxidant capacity.

Alcoholic extracts of *Salix alba* and *Thymus vulgaris* were exceptional free-radical-scavengers and a potential natural phenolic antioxidants for commercial consideration.

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