

POLYPHENOL COMPOSITION AND ANTIOXIDANT ACTIVITY OF SELECTED MEDICINAL HERBS

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The aim of this study was to characterize aqueous and alcoholic extracts [30%, 50% and 70% (w/v)] obtained from medicinal herbs (*Calendula officinalis*, *Hypericum perforatum*, *Galium verum*, and *Origanum vulgare*) used in traditional medicine from our country. Samples were examined for total and individual content of phenolics and antioxidant activities. The highest content of total polyphenols (9.9 ± 0.02 mg gallic acid equivalents (GAE) L^{-1} extract) and antioxidant activities expressed as Trolox Equivalent Antioxidant Capacity [307.51 TEAC $mmol\ g^{-1}DW$ by the ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) method and 20.90 TEAC $mmol\ g^{-1}DW$ by the DPPH (1,1-diphenyl-2-picrylhydrazyl) method] was found in *Origanum vulgare* (50%) extract. Polyphenolic compounds were quantified using RP-HPLC.

Keywords: medicinal herbs, antioxidant activity (TEAC), phenolics, HPLC.

Many medicinal herbs from our country are found in spontaneous flora and represent about 60% of the European medicinal herbs species. Four herbs frequently used in our folk medicine for their remedial qualities are: *Calendula officinalis*, *Hypericum perforatum*, *Galium verum*, and *Origanum vulgare*.

The main constituents of *C. officinalis* include flavonoids, such as flavonols (isorhamnetin, quercetin), flavonol glycosides (isoquercitrin, narcissin, neoliesperoside, and rutin), coumarins (scopoletin and aesculetin), volatile oils (α -cadinol and δ -cadinol), triterpenoids (saponins with oleanolic acid as an aglycone), sterols (present as free alcohols, esters, and glycoside), carotenoids present as carotene- and xanthophyll-derivatives and polysaccharides [1–3].

Generally, one of the most important herb studied was *Hypericum perforatum* (St. John's wort), belonging to the Guttiferae family [2]. *H. perforatum* extracts contain flavonoids, phenolic acids, naphthodianthrone (hypericin, pseudohypericin, protohypericin, protopseudohypericin), and phloroglucinols (hyperforin and adhyperforin) [4–7].

Origanum vulgare is an aromatic herb used as a food ingredient and medicinal herb. The major constituents are thymol, carvacrol, mono-sabinene, *cis*-sabinene hydrate, β -ocimene, terpinen-4-ol and sesquiterpenoids (β -caryophyllene, caryophyllene oxide). The essential oil of the aromatic herb is used in pharmaceuticals for its flavoring, antimicrobial, and antifungal effects [8–10]. The properties of *Origanum vulgare* seem to be related to the content of phenolic compounds present in this herb (protocatechuic acid, caffeic acid, coumaric acid, rosmarinic acid, quercetin).

Another herb with important effects in health is *Galium verum* (Lady's Bedstraw or Yellow Bedstraw). Lady's Bedstraw is an herbaceous perennial plant belonging to the Rubiaceae family. Extracts of this herb contain iridoids (asperuloside, asperulosidic acid, deacetylasperulosidic acid, monotropein, loganin, secologanin), two flavonol glycosides, astragaloside and rutin, tannins (catechin, epigallocatechin), and enzymes. The main objectives of this paper were to determine the content of bioactive compounds and antioxidant activities of different extracts from *Calendula officinalis*, *Hypericum perforatum*, *Galium verum*, and *Origanum vulgare*.

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TABLE 1. Some Chromatographic Parameters of Phenolic Acids and Flavonoids

Analyte	$T_R \pm SD$, min	SD, %	RSD, %, n = 3	R^2	LOD	LOQ
Chlorogenic acid (1)	17.52 \pm 0.10	1.81	4.1	0.9999	0.22	0.73
Caffeic acid (2)	19.18 \pm 0.10	0.82	3.7	0.9993	0.08	0.26
Ferulic acid (3)	20.11 \pm 0.12	0.12	1.9	0.9999	0.21	0.70
Coumaric acid (4)	21.13 \pm 0.02	0.26	2.4	0.9998	0.068	0.22
Rutin (5)	22.13 \pm 0.02	0.30	2.6	0.9998	0.058	0.19
Rosmarinic acid (6)	23.10 \pm 0.07	0.08	1.3	0.9985	0.25	0.85
Luteolin (7)	27.45 \pm 0.07	0.07	1.8	0.9992	0.10	0.35
Quercetol (8)	27.71 \pm 0.07	0.08	2.1	0.9990	0.23	0.77
Apigenin (9)	31.87 \pm 0.03	0.05	1.2	0.9992	0.15	0.53
Kaempherol (10)	33.46 \pm 0.06	0.06	1.4	0.9992	0.23	0.79

Linearity: 2.5–60 $\mu\text{g mL}^{-1}$.

T_R – average migration time of three consecutive analysis of standard solution; RSD – repeatability of migration in %; RSD, n = 3 – intra-assay repeatability in %; R^2 – correlation coefficient; LOD – limit of detection ($\text{g} \times 10^{-9}$ L); LOQ – limit of quantification ($\text{g} \times 10^{-9}$ L).

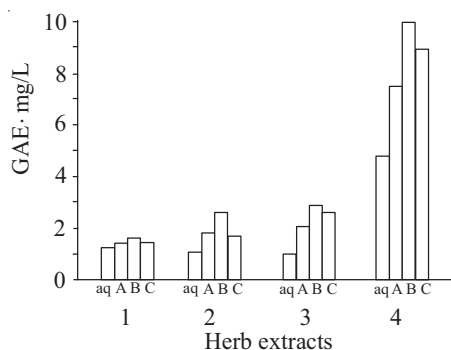


Fig. 1. Total phenolic content of the extracts (aqueous and alcoholic): *Calendula officinalis* (1), *Hypericum perforatum* (2), *Galium verum* (3), *Origanum vulgare* (4). A – 30%, B – 50%, C – 70%. Each value is expressed as mean (n = 3).

The amount of total phenolics, measured by the Folin–Ciocalteu method, varied widely in herb samples and ranged from 0.90 to 9.89 mg GAE L^{-1} (Fig. 1). The highest level of phenolics for aqueous extracts was found in *O. vulgare* (4.71 mg GAE L^{-1}), while the lowest was in aqueous extract of *G. verum* (0.9 mg GAE L^{-1}). For alcoholic extracts the highest content of phenolics was found in 50% alcoholic extract of *O. vulgare* (9.89 mg GAE L^{-1}), and the lowest content was found in 30% alcoholic extract of *C. officinalis* (1.34 mg GAE L^{-1}). It was observed that the amount of polyphenols was dependent on the medium of extraction. The lowest antioxidant activity for all the samples was found in aqueous extracts.

The antioxidant activities for all the extracts are shown in Figs. 2 and 3. Total antioxidant activity measured by the ABTS⁺ method ranged from 18.46 to 307.51 TEAC mmol g^{-1} DW, and the highest antioxidant concentration was found in 50% alcoholic extracts of *O. vulgare*.

Antioxidant activity values measured by the DPPH method are lower than those obtained by ABTS⁺ method. While DPPH does not react with flavonoids without substituted OH in the B-ring or with aromatic acids with a single OH group, ABTS does not discriminate between OH phenolics, providing a response related to total groups able to quench a radical reaction. Consequently, it could be said that DPPH scavenging highly depends on the degree of electron delocalization. Moreover, in the DPPH approach (protocol), the radical scavenging/antioxidant capacity increases if the partial phenolic ionization occurs in the system of analysis [11]. Total antioxidant activity, measured by the DPPH method, ranged from 0.43 to 20.90 TEAC mmol g^{-1} DW, and the highest values were measured in the 50% alcoholic extract of *O. vulgare*.

TABLE 2. Content of the Phenolic Acids and Flavonoids Present in Samples, mg g⁻¹ DW

Herb	Sample	1	2	3	4	5	6	7	8	9	10
<i>Calendula officinalis</i>	Aq. extr.	0.38	0.49	0.31	0.07	0.06	0.013	0.012	0.015	0.013	0.026
	30% alc.extr.	0.82	0.68	0.27	0.17	0.13	0.038	0.0162	0.058	0.061	0.043
	50% alc.extr.	3.23	0.34	0.39	0.97	0.82	0.036	0.035	0.054	0.072	0.04
	70% alc.extr.	2.48	0.36	0.48	1.72	1.32	0.048	0.044	0.069	0.14	0.057
<i>Hypericum perforatum</i>	Aq. extr.	1.78	0.71	0.0154	0.33	0.29	0.063	0	0	0	0
	30% alc.extr.	2.21	1.25	0.61	0.66	0.54	0.28	0.085	0.87	0.068	0.12
	50% alc.extr.	2.96	0.64	3.52	0.67	0.57	0.51	0.14	10.03	0.2	0.38
	70% alc.extr.	0.470	0.092	3.04	0.42	0.074	0.041	0.02	0.85	0.018	0.036
<i>Galium verum</i>	Aq. extr.	2.77	1.97	1.44	0.88	0.84	0	0.053	0.068	0	0
	30% alc.extr.	9.31	2.15	4.56	1.37	1.18	0.29	0.102	0.405	0.062	0.087
	50% alc.extr.	1.35	1.23	8.54	1.85	1.74	0.68	0.16	0.52	0.079	0.089
	70% alc.extr.	1.33	0.592	4.025	2.33	2.25	0.862	0.204	0.471	0.0653	0.245
<i>Origanum vulgare</i>	Aq. extr.	0.522	0.410	0.501	1.401	1.327	1.767	0.230	0.056	0.026	0.024
	30% alc.extr.	0.605	1.314	3.010	1.586	1.605	4.07	0.347	0.035	0.101	0.037
	50% alc.extr.	3.69	0.842	1.82	2.503	2.362	8.57	0.393	0.238	0.142	0.216
	70% alc.extr.	0.292	0.881	0.372	1.677	1.596	7.41	0.368	0.425	0.297	0.635

DW – dry herb weight.

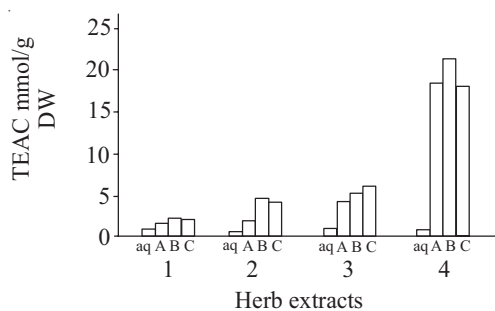


Fig. 2

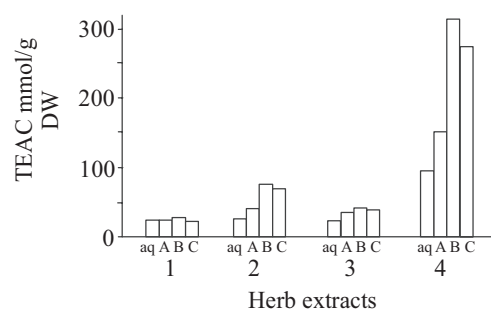


Fig. 3

Fig. 2. DPPH radical-scavenging activity of the various herb extracts (aqueous and alcoholic): *Calendula officinalis* (1), *Hypericum perforatum* (2), *Galium verum* (3), *Origanum vulgare* (4). A – 30%, B – 50%, C – 70%. Each value is expressed as mean (n = 3).

Fig. 3. ABTS scavenging activity of the various herb extracts (aqueous and alcoholic): *Calendula officinalis* (1), *Hypericum perforatum* (2), *Galium verum* (3), *Origanum vulgare* (4). A – 30%, B – 50%, C – 70%. Each value is expressed as mean (n = 3).

For most categories of phenolics in plants, specific HPLC methods have been developed [12]. Phenolic compounds identified in the present study include the following constituents: phenolic acids (chlorogenic acid, caffeic acid, ferulic acid, coumaric acid, rosmarinic acid) and flavonoids (rutin, luteolin, quercetol, apigenin, and kaempferol). The whole HPLC profiles of all the identified phenolics were obtained within 50 min. Phenolic compounds were identified by comparing the retention time (T_R) of the standard mixture with the retention time of the natural extracts. Each calibration curve contains eight points, and each point was performed in triplicate. A good peak-to-peak separation efficiency was obtained, and a good linearity for all analytes. The limit of detection (LOD) was calculated by $3S/N$, and the limit of quantification (LOQ) was calculated by $10S/N$ (Table 1).

Repeatability was investigated by injecting the same standard solution of $5 \text{ g} \times 10^{-9} \text{ L}$ six times. The values obtained ranged from 0.086 for apigenin to 3.774% for chlorogenic acid. To evaluate the intermediate precision, three standard solutions ($5 \text{ g} \times 10^{-9} \text{ L}$; $10 \text{ g} \times 10^{-9} \text{ L}$; $15 \text{ g} \times 10^{-9} \text{ L}$) were analyzed on three different days. Results were reported in terms of relative standard deviation, and the values were between 0.476 for luteolin to 2.821% for caffeic acid. Accuracy was determined by

calculating recoveries of standards by the method of standard addition. Known amounts of reference substance were added to a diluted sample ($2.5 \text{ g} \times 10^{-9} \text{ L}$; $7.5 \text{ g} \times 10^{-9} \text{ L}$; $12.5 \text{ g} \times 10^{-9} \text{ L}$). Each level was injected in triplicate. A good degree of accuracy was achieved for all the compounds, and the average of recovery was higher than 97.6%.

The results showed that the alcoholic extracts had a higher content of phenolic acids and flavonoids than aqueous extracts. The content of chlorogenic acid was significantly different in extracts, and the highest content was found in 30% *G. verum* ($9.31 \text{ mg g}^{-1} \text{ DW}$). The contents of caffeic and coumaric acids were rather low, except for the 30% extract of *G. verum*. The highest concentrations of ferulic acid and rutin were found in aqueous and ethanolic extracts of *O. vulgare*. Rosmarinic acid was the dominant compound quantified in *O. vulgare* extracts, and the highest content was found in the 50% ethanolic extract ($8.57 \text{ mg g}^{-1} \text{ DW}$).

We found lower concentrations of flavonoids (rutin, luteolin, quercetol, apigenin, and kaempferol) in all samples. In aqueous extract of *H. perforatum*, luteolin, quercetol, apigenin, and kaempferol were not detected. Also, apigenin and kaempferol were not found in aqueous extracts of *G. verum* (Table 2).

The results showed that the medicinal herb extracts studied are rich in phenolic constituents and have good antioxidant activity. These herbs, *C. officinalis*, *H. perforatum*, *G. verum*, and *O. vulgare*, rich in flavonoids and phenolic acids, are a good source of natural antioxidants. The HPLC method proposed for analysis of the polyphenols enables reproducible and accurate determination of five phenolic acids and five flavonoids. This method can be recommended as simple standard procedure for analysis of herb extracts.

The highest values for total and individual phenolic content and antioxidant activity were obtained for the 50% alcoholic extracts. This approach highlighted the differences among phenolic compound concentrations as a function of extraction medium.

Obviously the results of this study suggest that *C. officinalis*, *H. perforatum*, *G. verum*, and *O. vulgare* from our spontaneous flora may be used as medicinal herbs for improvement of health.

EXPERIMENTAL

Plant Material and Total Reagents. 5 mg of dried plant material was added to 50 mL water and 30%, 50%, and 70% ethanol, with shaking daily during seven days at 4°C in the dark. Samples were filtered and brought to 50 mL constant volume. Samples were stored at 4°C until analyzed. Each analysis was performed in triplicate. Chlorogenic acid (1), caffeic acid (2), ferulic acid (3), coumaric acid (4), rutin (5), rosmarinic acid (6), luteolin (7), quercetol (8), apigenin (9), kaempferol (10), and 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 1,1-diphenyl-2-picrylhydrazyl (DPPH), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), potassium persulfate, sodium acetate, and sodium carbonate were purchased from Fluka (Germany). Ultrapure water was Mili Q grade (Millipore). Acetonitrile, acetic acid, Folin-Ciocalteu reagent, methanol, and ethanol, grade HPLC purity, were purchased from Sigma-Aldrich (Germany).

Total Phenolic Assay. The content of total phenolic compounds in samples was determined by using the Folin-Ciocalteu method [13]. Samples were mixed with Folin-Ciocalteu reagent and sodium carbonate (7.5%). After 1 hour at 50°C , the absorbance at 765 nm was measured. Total phenolic content in samples was expressed as gallic acid equivalents (GAE) L^{-1} .

Free-Radical-Scavenging Activity on DPPH. The reaction mixture contained methanol, $80 \mu\text{mol}$ DPPH, and different concentrations of test samples at a final volume of 3 mL [14]. After 5 min of incubation [15] at room temperature, the absorbance was recorded at 517 nm. The control solution contained only methanol and DPPH. The absorbance was recorded at 517 nm and was measured against a Trolox standard and expressed as TEAC (Trolox equivalent antioxidant capacity). The DPPH radical-scavenging activity was calculated according to the following equation:

$$\text{TEAC}_{\text{sample}} = C_{\text{Trolox}} \times f \times (A_{\text{sample}} - A_{\text{blank}} / A_{\text{Trolox}} - A_{\text{blank}}) \times 1/m,$$

where A_{blank} represents the maximum absorbance of the blank solution, A_{Trolox} represents the maximum absorbance of Trolox stock solution, A_{sample} represents the maximum absorbance of sample, f is the dilution factor of the sample, C_{Trolox} is the concentration of Trolox in mmol L^{-1} , and m is dry herb weight in g.

Scavenging Activity of ABTS Radical Cation. The ABTS radical cation (ABTS^+) scavenging activity was measured according to the method described by Van den Berg et al. [16] and Re et al. [17] with some modification. ABTS cations are produced by reacting ABTS stock solution (7 mmol) and potassium persulfate (2.45 mmol). The mixture was incubated at room temperature in the dark for 12–16 h to allow completion of radical generation. Different concentrations of the samples were added to the ABTS working solution to give a final volume of 3 mL. The absorbance was recorded at 734 nm and was

measured against a Trolox standard and expressed as TEAC (Trolox equivalent antioxidant capacity), see equation [18, 19]. Absorbance measurements for antioxidant activities and total phenolic content were made on a UV/vis spectrophotometer (Jasco, V-530, Japan).

Chromatographic Analysis. For the polyphenol separation, we used a Kromasil 100-5C18 column (250 × 4.6 mm). The temperature was kept constant at 25°C by the column oven. Injection volume was 20 µL. The mobile phase consisted of acetonitrile (solvent A) and 1% acetic acid (solvent B). The gradient was 30–90% B in 50 min at flow rate 0.8 mL min⁻¹. For analysis by PDA detection, UV spectra were recorded between 320–370 nm at a rate of 0.8 spectrum s⁻¹ and a resolution of 4.0 nm. Stock standard solutions (10⁻³g L⁻¹) were prepared in methanol.

To identify and determine phenolic compounds in the samples, we used a Shimadzu LC-DAD (Shimadzu, Japan) chromatographic system consisting of pumps (LC-10ADvp), a diode array detector (SPD-M20A), a degasser (DGU-20A5), a column oven (CTO-10ASvp), and a system controller (SCL-10Avp).

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