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Cent. Eur. J. Biol. • 6(3) • 2011 • 388-396
DOI: 10.2478/s11535-011-0028-6

Central European Journal of **Biology**

Total phenolic content and antioxidant activity of plants used in traditional Romanian herbal medicine

Research Article

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Received 07 December 2010; Accepted 24 February 2011

Abstract: A number of herbal plants from Romania widely used as natural food additives or for health promotion in traditional medicine were investigated for their antioxidant activity. Methanol extracts were obtained from plants belonging to the *Lamiaceae* family (lavender *Lavandula angustifolia* L.; lemon balm *Melissa officinalis*; sage *Salvia officinalis*; oregano *Origanum vulgare* L.; rosemary *Rosmarinus officinalis* L.; thyme *Thymus vulgaris* L.; mullein *Verbascum phlomoides*; mint *Mentha longifolia*), *Clusiaceae* family (St John's wort *Hypericum perforatum* L.), and *Compositae* family (elecampane *Inula helenium*). Total phenolic concentration was determined using the Folin-Ciocalteu phenol reagent method, while total flavonoids were measured using the aluminium chloride colorimetric method. Relationships between total antioxidant activity and composition of plant extracts were evaluated. *Origanum vulgare* extract showed the highest antioxidant activity and total phenolic content compared to the other plants extracts. A positive correlation was observed between total antioxidant activity and total phenolic content of the analyzed extracts.

Keywords: *Lamiaceae* • *Clusiaceae* • *Compositae* • *Flavonoids* • *DPPH assay*

1. Introduction

The potency of different medicinal plants is related to their individual mechanisms of action in different disorders. Humans consume and use a variety of vegetable materials in the form of leaves, roots, seeds and fruits. Although medicinal plants are widely considered to be of lower risk compared with synthetic drugs, they are not completely free from the possibility of toxicity or other adverse effects [1]. However, there is considerable interest in identifying natural antioxidants from plants that protect against free radical damage as an alternative to synthetic medicines. The literature provides a wealth of information that correlates a diet enriched in fruits and vegetables with the maintenance of health and disease prevention [2,3].

Phenolic compounds from plants belong to a class of bioactive components with antioxidant activities [4-7]. The antioxidant activity of plants has been demonstrated in many recent studies [8,9]. Flavonoids represent one of the most studied classes of phenolic compounds containing carbohydrate units important for their biological activities [10]. Flavonoids exhibit a wide range of biological effects (antibacterial, antiviral, anti-inflammatory and anti-allergic) by reducing low-density lipoproteins in plasma, inhibiting platelet aggregation, scavenging free radicals, and preventing cell proliferation [11].

Typical phenolic compounds that possess antioxidant activity are predominantly phenolic acids and flavonoids. Phenolic acids, including caffeic acid, ferulic acid, and vanillic acid, are widely distributed in the plant kingdom and have been repeatedly implicated as natural antioxidants. The most widespread and diverse phenolics are the flavonoids which have the same C15 (C6-C3-C6) skeleton and possess antioxidant capacity toward a variety of easily oxidizable compounds. In many herbs, the main flavonoid constituents are flavonol aglycones such as quercetin, myricetin, kaempferol, and their glycosides. In general, flavonoids containing multiple hydroxyl groups have higher antioxidant activities against peroxyl radicals than do phenolic acids. The

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presence of different antioxidant components in the plant tissues confounds quantification of individual antioxidant components. Therefore, in many studies, several intermediate extractions are used to ensure a maximum extraction of the available antioxidants. The antioxidant activity of phenolic compounds is mainly due to their redox properties which enable them to act as reducing agents, hydrogen donors, and singlet oxygen quenchers. Phenolics also may have metal chelating properties.

Oregano (*Origanum vulgare*) is a *Lamiaceae* herbaceous plant native to the Mediterranean regions. Lemon balm (*Melissa officinalis*) is also a representative of the *Lamiaceae* family, used in traditional medicine. These plants were previously reported to have antioxidant activities [12,13]. Recent research suggested anti-inflammatory, sedative and antibacterial properties of *Lavandula* species [14]. *Inula helenium*, a perennial plant widely occurring in Europe and East Asia, belongs to the *Compositae* family [15,16]. Mullein (*Verbascum*) flowers are highly valued in herbal medicine, their phenolic constituents being considered to be responsible for the anti-inflammatory and antimicrobial activity of the herb [17].

Common sage (*Salvia officinalis* L., *Lamiaceae*) is an aromatic and medicinal plant well known for its antioxidant properties and anti-inflammatory effects [18]. *Rosmarinus officinalis* L. (*Lamiaceae*) is an edible evergreen shrub native to the Mediterranean area, producing an essential oil with an antimicrobial effect. Ursolic, oleanolic, and micromeric acids were identified as the compounds responsible for its anti-inflammatory effects [19]. *Thymus vulgaris* L. (*Lamiaceae*), commonly known as thyme, is a perennial aromatic and medicinal plant from the Mediterranean region. Studies have indicated that thyme possesses antibacterial, and antioxidant activities [20,21].

St. John's wort (*Hypericum perforatum* L., *Clusiaceae* family) is currently one of the most commonly used herbal remedies in Europe. Flower extracts are used as an antidepressant in the treatment of mild to moderate depression [22], hypericin being the main active ingredient. *Mentha longifolia* is an aromatic perennial herb, being valued especially for its antiseptic properties and its beneficial effects on the digestion. The leaves of *M. longifolia* contain about 0.75% essential oils, exhibiting strong antibacterial and antioxidant activities [23].

The objective of this work was to estimate the total phenolic and flavonoid content and to evaluate the total antioxidant activity of herbal extracts from plants commonly used in Romanian traditional medicine.

2. Experimental Procedures

2.1 Plant samples and extraction

The herbs were selected based on information regarding their traditional uses in Romanian folk medicine (Table 1).

Dried plants were obtained from native crop sources in Iasi, Romania. Samples were ground and extracted with 40% methanol at room temperature for 4 days with a magnetic stirrer. The plant extracts were filtered and concentrated under vacuum at 45ºC. The lyophilized extracts were further extracted with ethyl acetate and

Common name	Botanical name	Part used	Therapeutic uses	References
oregano	*Origanum vulgare* L.	aerial plant	antitussive, expectorant, sedative, choleretic, cholagogue	[12]
lemon balm	*Melissa officinalis*	aerial plant	nerves, insomnia, loss of memory, sedative, digestive, analgesic, intestinal anti-inflammatory, hepatic protector, for sea-sickness	[13]
lavender	*Lavandula angustifolia*	aerial plant	anxiety, insomnia, anorexia, bronchitis, cough, nerves, rheumatism, heart disturbance	[14]
elecampane	*Inula helenium*	root	cholagogue, diuretic, antihelminthic, expectorant, on wounds healing	[15,16]
mullein	*Verbascum phlomoides*	aerial plant	diuretic, analgesic, expectorant and antiseptic properties	[17]
sage	*Salvia officinalis*	aerial plant	condiment, anorexia, flatulence, menopause, nerves, anti-impotence	[18]
rosemary	*Rosmarinus officinalis* L.	aerial plant	analgetic and antiphlogistic effects rheumatic disorder	[19]
thyme	*Thymus vulgaris* L.	aerial plant	polyarthritis and gout	[20,21]
St John's wort	*Hypericum perforatum* L.	aerial plant	wound healing, anti rheumatic, diuretic and antidepressant activities	[22]
mint	*Mentha longifolia*	aerial plant	spasmolytic and digestive disorders	[23]

Table 1. Plant species used in the present study.

dried. Working (standard) solutions with different concentrations were prepared by dilution with methanol. Analysis of total phenolic and flavonoid contents was conducted using standard methods.

We can state here that in such studies, an extraction procedure must remove non-phenolic substances such as sugars, proteins and pigments which may interfere during the total phenolic content evaluation.

2.2 Reagents and Instrumentation

All chemicals used were purchased from Sigma-Aldrich Ldt (Germany).

All absorbance measurements for determination of total phenolic and flavonoid contents were conducted using a JENWAY 6405 UV–VIS spectrophotometer. A 1.0 cm optical path length glass cell was used in all measurements.

2.3 Determination of total phenolic content

Total phenolic content was estimated as gallic acid (GA) equivalents per gram of dried plant extract, according to the Folin-Ciocalteu phenol reagent method [24]. First, a standard curve was generated using gallic acid as a standard. Different concentrations of gallic acid were prepared in 80% methanol, and their absorbance values were measured at 765 nm. For sample measurement, 0.5 mL (1/10 dilution) of Folin-Ciocalteu phenol reagent and 1000 mL of distilled water were added to 100 µL of plant extract. The solutions were mixed and incubated at room temperature for 1 min. After 1 min, samples were combined with 1500 mL of 20% sodium carbonate (Na_2CO_3) solution, mixed, and incubated for an additional 120 minutes. Absorbance at 765 nm was measured. Data presented are average values of four measurements for each sample.

2.4 Determination of total flavonoid content

Total flavonoid content was estimated following the aluminium chloride colorimetric method [25]. Briefly, aliquots of 2 mL (200 µg/mL) of the extracts were added to 2 mL of a 3% $AlCl_3$ solution in methanol, incubated for 10 min at room temperature, and absorbance measured at 430 nm. Total flavonoid content was calculated from a calibration curve of rutin analyzed under the same conditions. The flavonoid content was expressed in rutin equivalents per gram of dried plant extract. Measurements were conducted in triplicate and values are expressed in mean ± SD.

2.5 Determination of DPPH radical scavenging capacity

Free radical scavenging potentials of the extracts were measured using a methanol solution of 1,1-diphenyl-2-picryl hydrazyl (DPPH) [26,27]. 80 to 800 µg/mL of extract, 160 to 800 µg/mL of ascorbic acid, as standard in 500 µL methanol, were added to 5 mL of 100 µM DPPH in methanol. The control was prepared as above without extract. Absorbance at 517 nm was measured using methanol as the blank. The change in absorbance of the samples was measured over 20 minutes. Scavenging activity is expressed as the inhibition percentage calculated using the following equation:

Anti-radical activity (%) = {(control absorbance – sample absorbance) / control absorbance} x 100 (Eq. 1)

Each determination was carried out in triplicate.

The IC_{50} values for the concentration required for 50% scavenging activity were calculated from the above equation.

2.6 Determination of total antioxidant capacity

For the total antioxidant capacity assay [28], 2 mL of each plant extract (100 µg/mL) dissolved in methanol was combined with 2 mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate) and incubated at 95ºC for 90 min. After cooling to room temperature, the absorbance of each solution was measured at 695 nm. Ascorbic acid was used as standard and the total antioxidant capacity is expressed as equivalents of ascorbic acid. The experiment was performed in triplicate and values are expressed in mean ± SD.

2.7 Statistical analysis

Results are presented as the means ± SD. Correlation between analysis of antioxidant activity and the total phenolic and flavonoid contents were carried out using the correlation and regression applications in the Microsoft Excel.

3. Results and Discussion

The functional role of herbs and spices and their constituents is of recent interest in food-related plant research. Various plant species known for their uses in Romanian traditional medicine were studied. Benefits of topical application of herbal compounds are influenced by biologically active components, such as antioxidants. Due to great structural diversity, the antioxidant profiles differ greatly from one plant to another. Activity of natural extracts depends on the plant compounds as well as type and polarity of the extraction solvent and the isolation procedure [29].

There is a wide range of total phenolic content in the extracts of plants under study. The standard curve

generated with gallic acid for total phenolic content determination is presented in Figure 1.

As seen in Figure 2, the total phenolic content ranges from 40.8 mg gallic acid/g extract (*Inula helenium*) to 67.8 mg gallic acid/g extract (*Origanum vulgare*). It is evident from our analyses that all plants studied are rich in flavonoids. The total flavonoid content varies from 17.5 mg rutin/g extract (*Inula helenium*) to 43.6 mg rutin/g extract (*Origanum vulgare*).

The antioxidant capacity of a compound can be measured by the ability of the compound to intercept free radicals by scavenging or trapping methods [30]. Total flavonoid content has been shown to be correlated with antioxidant activity [2]. All plant extracts in the present study demonstrate antioxidant activity based on the reduction of Mo(VI) to Mo(V) and the subsequent formation of a green phosphate/Mo(V) complex at an acidic pH. The total antioxidant activity measurements of methanol extracts are presented in Figure 3.

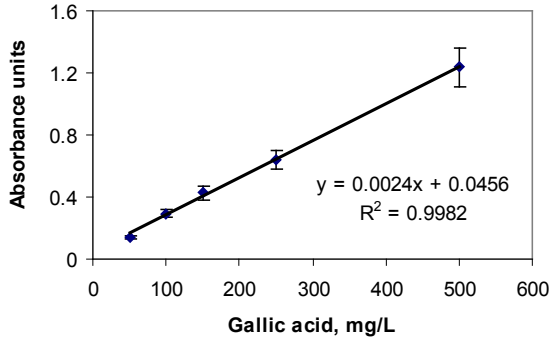


Figure 1. The standard curve obtained using gallic acid for total phenolic content determination.

Species within the Lamiaceae family, including *Origanum vulgare*, *Melissa officinalis,* and *Lavandula angustifolia*, showed the highest antioxidant activities, expressed as ascorbic acid equivalent antioxidant capacity.

A linear correlation (R^2=0.9041) between total phenolic content and antioxidant activity was observed in the present study (Figure 4). The correlation coefficient between the total flavonoid content and antioxidant activity was determined to be R^2=0.7941 (Figure 5).

These results suggest that 90% of the antioxidant activity of the plant extracts under study is due to the contribution of the phenolic and flavonoid compounds. The antioxidant activity of plant extracts may also be influenced by some particularly active individual phenolic compounds. The unclear relationship between the antioxidant activity and the total phenolic content may be explained in numerous ways; in fact, the total phenolic content does not incorporate all the antioxidants.

In addition, the synergism between the antioxidants in the mixture makes the antioxidant activity not only dependant on the concentration, but also on the structure and the interaction between the antioxidants. This can explain why samples such as *Lavandula angustifolia*, *Rosmarinus officinalis* and *Salvia officinalis* with similar values of total phenolic content vary in their antioxidant activities. The results suggest that the phenolic compounds contribute significantly to the antioxidant capacity of the studied plants. However, due to the diversity and complexity of the natural mixtures of phenolic compounds in these plant extracts, it is not easy to characterize every compound and assess their antioxidant activities. Each herb contains a different

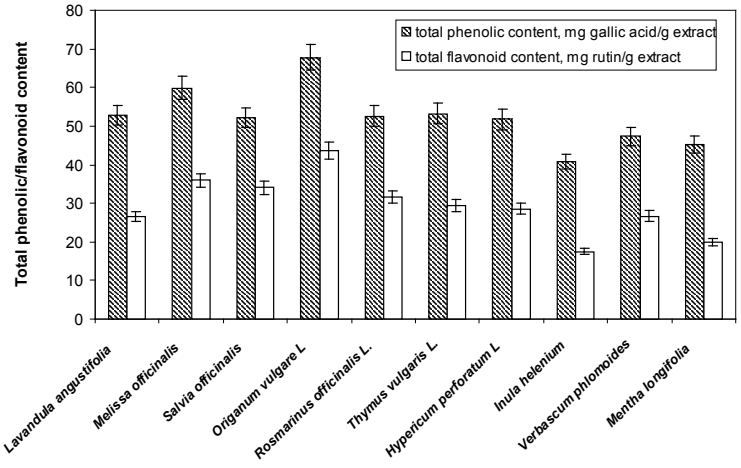


Figure 2. Total phenolic and flavonoid content of herbal extracts.

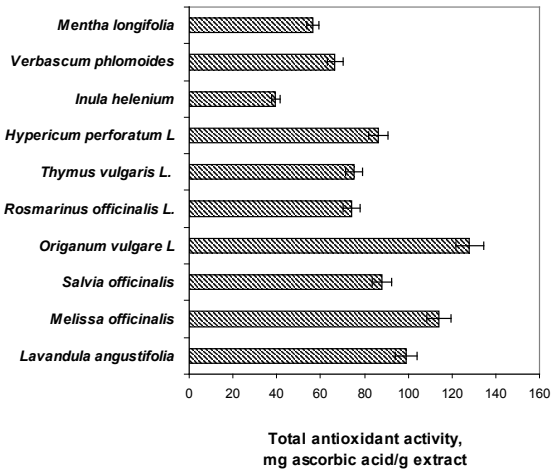


Figure 3. Total antioxidant activity of methanol herbal extracts.

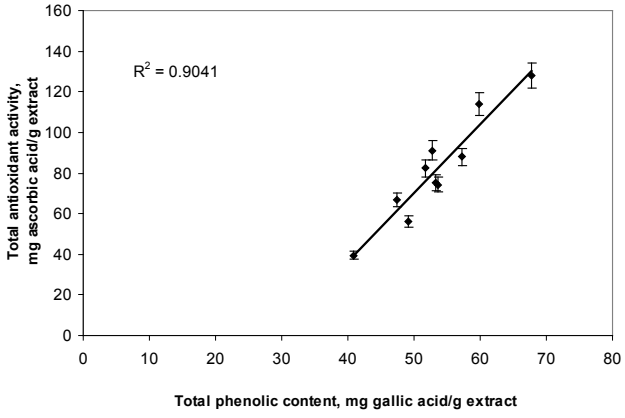


Figure 4. Correlation between total phenolic content and total antioxidant capacity of plant extracts.

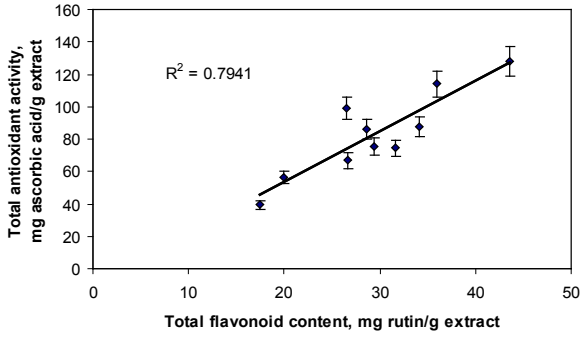


Figure 5. Correlation between total flavonoid content and total antioxidant capacity of plant extracts.

population of phenolic compounds resulting in varying levels of total antioxidant activity.

Because the antioxidant/antiradical activity of plant extracts is usually correlated with the presence of flavonoids and phenolics, a verification of this hypothesis was carried out by recording the UV-VIS spectra. UV-VIS spectra have long been used for structural analysis of flavonoids. The typical flavonoid spectrum consists of two maxima in the range 240–285 nm (Band II) determined by the A ring, and 300–550 nm (Band I), which is more specific and useful for obtaining information regarding identification [31]. The position and relative intensities of these maxima yield information on the nature of the flavonoid and its oxygenation pattern; variation within these ranges will depend on the hydroxylation pattern and on the degree of substitution of the hydroxyls [32]. For example, quercetin 7-glycoside has λmax values of 254 and 370 nm, while quercetin 3-glycoside has λmax values of 254 and 354 nm. The latter shows a hypsochromic shift of 16 nm due to the glycosidation at the C3 position whereas the former does not show this effect, having the same spectrum as the aglycone.

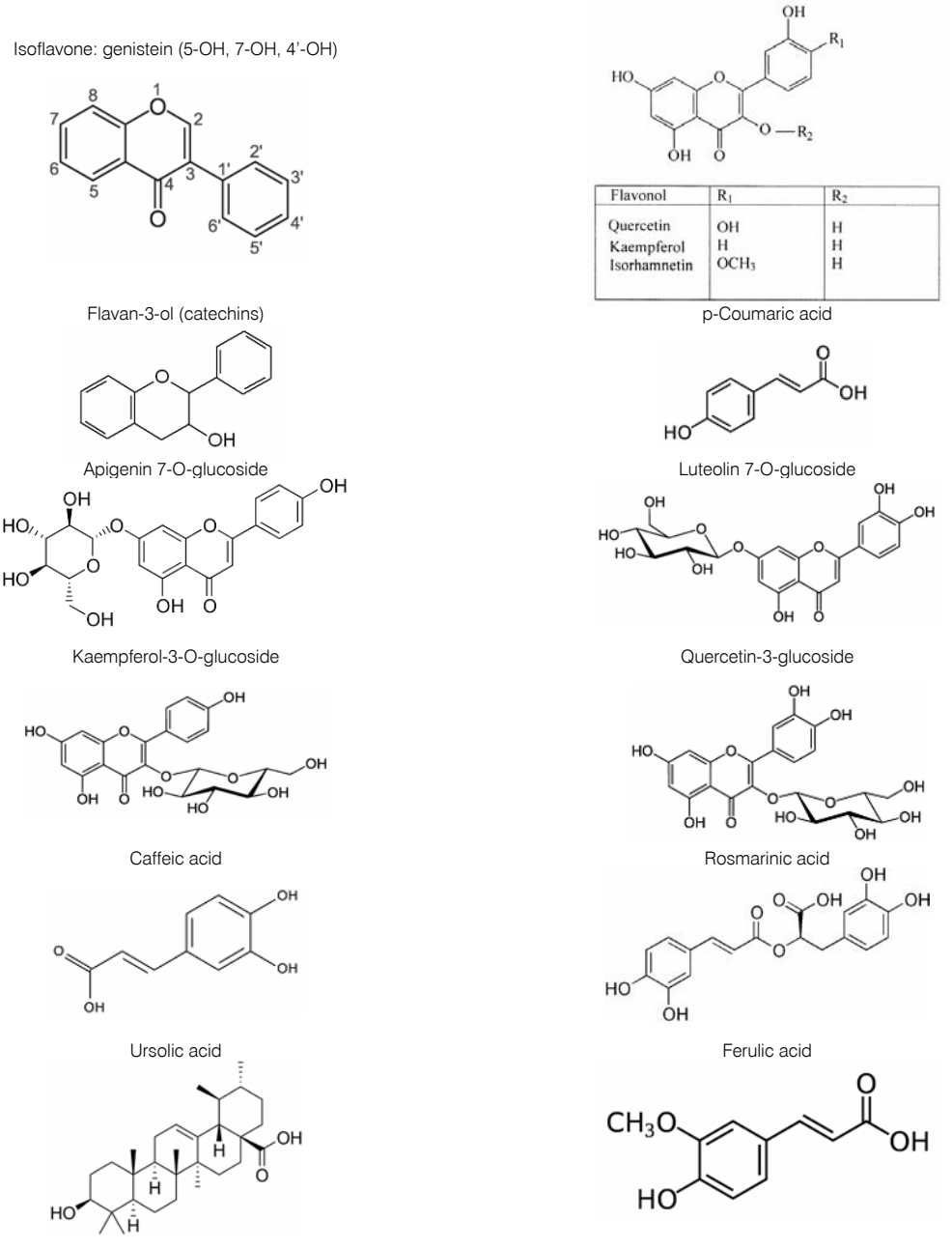
It is known that the introduction of a glycoside on the hydroxyls at positions 7, 3 or 4 has no effect on the wavelength maximum or the spectrum shape. Kaempferol 3-glycoside and luteolin 7-glycoside could be differentiated on the basis of the UV-VIS absorption due to the B rings, where the former has a Band II peak at 264 nm while luteolin 7-glycoside has one at 254 nm. This fact could be explained by the difference in the positions of OH groups between these flavonoids.

The UV spectrum of apigenin 7-glycoside is characterized by the presence of two maxima at 262 and 333 nm, while the spectrum of genistein consists of a prominent band at 286 nm with a shoulder in the 350 nm (Band II) region. Because there is little or no conjugation between the A- and B-rings, UV spectra of flavanones and isoflavones usually have an intense Band II peak but a small Band I peak. Flavanones and their glycosides exhibit a very strong maximum at 285 nm (Band II) and a small peak or shoulder at 320–330 nm (Band I). Isoflavones are generally detected at 236 nm, 260 nm, 262 nm, and 280 nm. This lack of conjugation also results in small Band I peaks for the catechins, usually quantified at 210 nm, 278 nm, and 280 nm. UV spectra of flavones, flavonols, and flavonol glycosides have a Band II peak at around 240-280 nm and a Band I peak around 300-380 nm.

Most of the flavonoids detected in this study were glycosides of apigenin (235 nm, 240 nm, 292 nm, 337 nm), luteolin (265 nm, 330 nm), kaempferol (256 nm, 265 nm, 272 nm, 354 nm), and quercetin (256 nm, 354 nm). Besides flavonoids, phenolic acids such as caffeic acid (240 nm), ursolic acid (210 nm), rosmarinic acid (218 nm, 330 nm), ferulic acid (214 nm, 325 nm), chlorogenic acid (218 nm, 325 nm) and p-coumaric acid (224 nm, 309 nm) were found in the plant extracts under study.

The structural features of some flavonoids and phenolic acids are presented in Figure 6.

The UV-VIS characterization of the studied plant extracts was analyzed between 200–900 nm. Table 2 shows the absorption maxima of the herbal extracts compared to the rutin and gallic acid standards. The 200–400 nm bands, characteristic to flavonoid-type structures can be attributed as follows: 200 nm–250 nm for un-saturated (linear or cyclic) structures; 250 nm–295 nm for un-saturated aromatic structures; 300 nm–400 nm: chromophores bounded to structures with extended conjugation (flavonoids). Thus, the presence of flavonoids in the extracts might explain their antioxidant properties. In addition to the bands that are common with standards, characteristics of other functional groups include absorbance values of 405–419 nm for hydroxyl and carbonyl groups attached to structures with extended conjugation, 510-541 for



Flavonol	R_1	R_2
Quercetin	OH	H
Kaempferol	H	H
Isorhamnetin	OCH_3	H

Figure 6. Structural features of some flavonoids and phenolic acids.

conjugated quinones, and 600–700 nm for structures with extended conjugation and other functional groups from tannins, flavones, and polyphenols.

Some of the observed values may be due to the involvement of sulphur and nitrogen functional groups. Comparison of absorbance spectra at 500–700 nm of the herbal extracts with those of chlorophyll reveals a close resemblance between them. Consequently, the 500–700 nm absorbances may be due to porphyrin-type structures which are photo-sensitizers that promote oxidation. Therefore, removal of chlorophyll during extraction may be necessary in order to obtain accurate measurements of antioxidants. However, it has been reported that the phenolic content is not the only factor influencing the antioxidant capacity, which may also be affected by other bioactive compounds such as minerals and vitamins which could induce synergistic effects [33].

The DPPH radical is a stable organic free radical with an adsorption band at 515-528 nm. When a solution containing DPPH radical absorbs hydrogen from an antioxidant, the colour turns from purple to yellow followed by the formation of DPPH. Addition of an aqueous-methanol DPPH solution to a sample facilitates the extraction of antioxidant compounds from the sample, thereby increasing the measured antioxidant activity. The antioxidant effect is proportional to the disappearance of DPPH• in test samples. Therefore, the antioxidant effect can be easily evaluated by measuring the decrease in UV absorption at 517 nm. Results have been reported as IC_{50}, which is the amount of antioxidant necessary to decrease the initial DPPH• concentration by 50%. The correlation coefficient between DPPH scavenging ability and the total phenolic content is R^2=0.85 (Figure 7), whereas the correlation between the total flavonoid content and IC_{50} is determined to be R^2=0.69 (Figure 8).

Plant extract	λ_{max}
Lavandula angustifolia	210 nm; 218 nm; 235 nm; 240 nm; 325 nm; 330 nm; 338 nm; 354 nm
Melissa officinalis	223 nm; 240 nm; 256 nm; 265 nm; 309 nm; 330 nm; 354 nm; 675 nm
Salvia officinalis	202 nm; 214 nm; 230 nm; 284 nm; 290 nm; 328 nm
Origanum vulgare L	210 nm; 218 nm; 223 nm; 309 nm; 325 nm; 330 nm; 354 nm; 407 nm; 542 nm; 615 nm; 670 nm
Rosmarinus officinalis L.	224 nm; 249 nm, 292 nm; 313 nm; 363 nm; 410 nm; 497 nm ;540 nm; 614 nm; 662 nm
Thymus vulgaris L.	225 nm ; 284 nm; 337 nm; 417 nm; 472 nm; 541 nm; 615 nm; 670 nm
Hypericum perforatum L	201 nm; 214 nm; 226 nm; 235 nm; 272 nm; 330 nm; 652 nm
Inula helenium L.	204 nm; 216 nm; 240 nm; 272 nm; 291 nm; 466 nm; 670 nm
Verbascum phlomoides	204 nm; 226 nm; 234 nm; 254 nm; 265 nm; 272 nm; 320 nm; 480 nm; 497 nm; 652 nm
Mentha longifolia	203 nm; 219 nm; 241 nm; 252 nm; 265 nm; 292 nm; 359 nm; 505 nm; 633 nm; 652 nm; 661 nm; 679 nm; 685 nm; 694 nm
Rutin	203 nm; 207 nm; 210 nm; 249 nm; 362 nm
Gallic acid	220 nm; 271 nm

Table 2. UV-VIS characterization of the methanol herbal extracts.

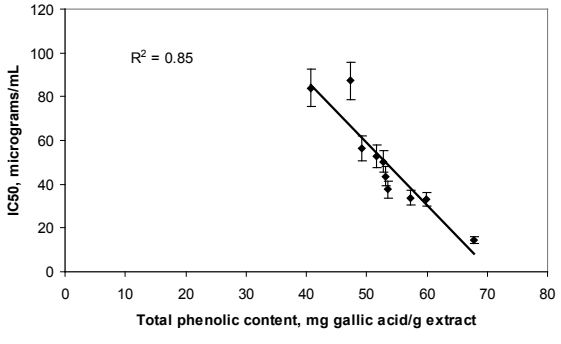


Figure 7. Correlation between total phenolic content and scavenging capacity of plant extracts from regression analysis.

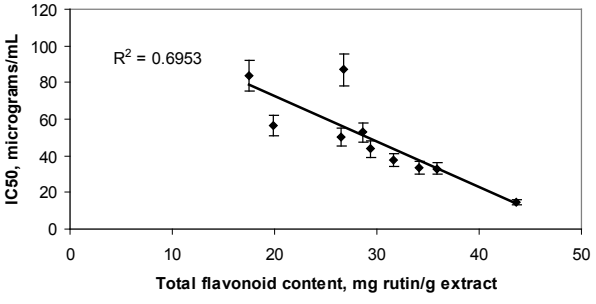


Figure 8. Correlation between total flavonoid content and scavenging capacity of plant extracts from regression analysis.

4. Conclusions

We have demonstrated that flavonoid and phenolic compounds are present in high quantities in the herbal extracts examined, and showed significant antioxidant activities. The plant species belonging to Lamiaceae family, including *Origanum vulgare*, *Melissa officinalis,* and *Lavandula angustifolia*, showed the highest antioxidant activity of all the plants evaluated.

Lavandula angustifolia, *Rosmarinus officinalis* and *Salvia officinalis* have similar total phenolic levels but vary in their antioxidant activities. The results suggest that the phenolic compounds contribute significantly to the antioxidant capacity of the studied plants. The methanol extract of *Origanum vulgare* L showed significant antioxidant activity and presented the highest phenolic content of all the plants studied.

A positive correlation was observed between total antioxidant activity and total phenolic content, revealing that these plants may have considerable benefits when used as food ingredients and for human nutrition.

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