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In vitro evaluation of antioxidant activity of some plant methanol extracts

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The aim of our study was to evaluate in vitro antioxidant potential of methanolic extracts (ME) of 14 medicinal plants, 8 of which are endemic species of Anatolia. Scavenging activity was tested by 1,1-diphenyl-2-picrylhydrazyl (DPPH) method and the inhibitory effect on lipid peroxidation was examined by the ferric thiocyanate (FTC) and thiobarbituric acid (TBA) methods. The obtained results showed significant differences in the antioxidant potential amongst the tested methanolic plant extracts. Among the plant samples, Crataegus microphylla C. Koch, Salvia hypargia Fisch. & Mey., Cotinus coggygria Scop., Origanum sypyleum L. and Rosa damascena Miller exhibited the highest DPPH scavenging activity. Five extracts (Centaurea nerimaniae Š. Kühr, C. coggygria, Scorzonera tomentosa L., R. damascena and Colchicum sanguicole K.M. Perss) showed strong antioxidant activity in the FTC and TBA tests, with per cent inhibition ranges of 72%–84% and 84%–92%, respectively. The ME of C. coggygria and R. damascena exhibited potent antioxidant activity by the DPPH, FTC and TBA methods.

Keywords: antioxidant activity; methanolic extract; free-radical-scavenging activity; DPPH; FTC; TBA

Introduction

Herbal medicine has gained popularity in health care; according to the World Health Organization, about 65%–80% of the world’s population which lives in developing countries depends essentially on plants for primary health care.[1] Since the middle of the nineteenth century, different classes of bioactive compounds have been isolated and characterized. Many of these are used as the active ingredients of modern medicines, or as the lead compounds for new drugs discovery. Several plant-derived medicines are rich in phenolic compounds, flavonoids, alkaloids, tannins etc., used in the treatment of various degenerative ailments.[2,3]

Overproduction of free radicals or reactive oxygen species (ROS) contributes to oxidative stress, which leads to damage of proteins, DNA and lipids that is associated with chronic degenerative diseases, including cancer, coronary artery diseases, hypertension and diabetes etc.[4,5] The human body has an elaborate antioxidant defence system. Antioxidants are manufactured within the body and can also be extracted from the food humans eat such as fruits, vegetables, seeds, nuts, meats and oil. For review, see [6]. There are two lines of antioxidant defence within the cell. The first line, found in the fat soluble cellular membrane consists of vitamin E, β-carotene and co-enzyme Q; of these, vitamin E is considered the most potent chain-breaking antioxidant within the membrane of the cell. Inside the cell, water-soluble antioxidant scavengers are present.[6,7] Antioxidants occurring naturally in leafy vegetables and seeds, such as ascorbic acid, vitamin E and phenolic compounds possess the ability to reduce the oxidative damage associated with many diseases. That is why many researchers have focused on natural antioxidants and, in the plant kingdom, numerous crude extracts and pure natural compounds have been reported to have antioxidant properties.[4] A large number of medicinal plants has been investigated for their antioxidant properties. Natural antioxidants either in the form of raw extracts or their chemical constituents are very effective to prevent the destructive processes caused by oxidative stress.[8,9]

Our aim was to evaluate in vitro antioxidant activity of the crude methanolic extracts (ME) of 14 medicinal plants, 8 of which are endemic species to Anatolia, by commonly used methods [1,1-diphenyl-2-picrylhydrazyl (DPPH), ferric thiocyanate (FTC) and thiobarbituric acid (TBA) methods].

Materials and methods

Plant selection and collection

Fourteen medicinal plants were chosen for in vitro antioxidant activity testing based on literature reviews and ethnobotanical information. The selected plants, some of

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which are endemic, belong to different family groups and were collected from different districts of Turkey (Table 1). Information about the plant chemical constituents with antioxidant activity is shown in Table 2.

### Preparation of extracts

The dried plant material (Table 1) was percolated with methanol (95%) at room temperature. The ME were evaporated to dryness under pressure and controlled temperature (40 °C–50 °C) in a rotary evaporator. The dried cormus of *Colchicum sanguicolle* were extracted with methanol (95%) in a Soxhlet apparatus. All the extracts were kept at −20 °C and then were lyophilized. The crude ME, thus, obtained were dissolved in distilled water.

### In vitro antioxidant activity tests

#### DPPH radical method

In evaluating the free-radical-scavenging activity of crude extracts and single compounds, the agent of choice is often the DPPH radical.[16,17] Here, the radical scavenging activities of the crude ME of 14 different plants were assayed by measuring the decrease in absorbance of DPPH in the presence or absence of the extract in the assay mixture. The method used was adapted from Ko et al. [16], Hossain et al. [18] and Lakshmi et al. [3]. Equal volumes of 10⁻⁴ mol/L DPPH (in methanol) and crude ME (E₁-E₁₄) or reference solution [10 mg/mL and 100 μg/mL vitamin E and ascorbic acid as natural antioxidants and 3-tert-butyl-4-hydroxyanisol (BHA) as a synthetic antioxidant] were mixed and incubated at room temperature.

### Table 1. List of plants used in the study.

| Botanical name                                      | Family       | Code | Specimen number (ISTE) | Plant part used | Districts of collection |
|-----------------------------------------------------|--------------|------|------------------------|----------------|------------------------|
| Crataegus microphylla C. Koch                       | Rosaceae     | E₁   | 76223                  | Leaves         | Gölçükl-Bolu           |
| Teucrium sandrasicum O. Schwarz (endemic)           | Lamiaceae    | E₂   | 87526                  | Aerial parts   | Köyçeğiz-Muğla        |
| Centaurea nerimaniae Ş. Kültür (endemic)            | Asteraceae   | E₃   | 98163                  | Aerial parts   | Mersin                 |
| Olea europaea L.                                    | Oleaceae     | E₄   | 106286                 | Leaves         | Şarköy-Tekirdağ        |
| Salvia hypargeia Fisch.& Mey. (endemic)              | Lamiaceae    | E₅   | 98205                  | Aerial parts   | Mersin                 |
| Cotinus coggyria Scop.                              | Anacardiaceae| E₆   | 80926                  | Leaves         | Kırklarelı             |
| Hypericum kotschyanum Boiss. (endemic)              | Hypericaceae | E₇   | 98173                  | Aerial parts   | Mersin                 |
| Nepeta italica L.                                   | Lamiaceae    | E₈   | 98192                  | Aerial parts   | Mersin                 |
| Stachys cretica L. subsp. vacillans Rech. fil       | Lamiaceae    | E₉   | 98166                  | Aerial parts   | Mersin                 |
| Scorzonera tomentosa L. (endemic)                   | Asteraceae   | E₁₀  | 98954                  | Aerial parts   | Malata                 |
| Origanum sipyleum L. (endemic)                      | Lamiaceae    | E₁₁  | 86060                  | Aerial parts   | Mersin                 |
| Rosa damascena Miller                               | Lamiaceae    | E₁₂  | 106285                 | Flowers        | Isparta                |
| Colchicum sanguicolle K.M. Perss (endemic)          | Colchicaceae | E₁₃  | 48868                  | Cormus         | Antalya                |
| Centaurea antiochia Boiss. var. praevalta (Boiss. & Bal) Wagenitz (endemic) | Asteraceae | E₁₄  | 98247                  | Aerial parts   | Mersin                 |

#### Table 2. Plants and their chemical constituents with known antioxidant activity.

| Plants                                         | Chemical constituents                                                                 | References          |
|------------------------------------------------|----------------------------------------------------------------------------------------|---------------------|
| *Crataegus microphylla* C. Koch                 | Flavonoids                                                                            | Melikoglu et al. [10]|
| *Centaurea nerimaniae* Ş. Kültür (endemic)      | Phytochemical research has not been done yet                                           | Topcu et al. [11]   |
| *Salvia hypargeia* Fisch.& Mey. (endemic)        | Diterpenes                                                                            | Marcetić et al. [12]|
| *Cotinus coggyria* Scop.                        | Monoterpenes, hydrolysable tannins, gallic acid, methyl gallate, pentagalloyl glucose, flavonoids, aurones, glycosides of myricetin, quercetin and kaempherol | Sari et al. [13]    |
| *Scorzonera tomentosa* L. (endemic)              | Stigmasteryl 3β-glucoside, β-sitosterol, lupeol, lupeol acetate, α-amyrin, hydrangenol, hydramacrophyllol | Ozkan et al. [14]   |
| *Origanum sipyleum* L. (endemic)                 | Apigenin, naringenin, rosmarinic acid, rutin, vitexin, carvacrol (phenolic monoterpene) | Basim and Basim [15]|
| *Rosa damascena* Miller                         | Monoterpene alcohols, hydrocarbons                                                   |                     |
| *Colchicum sanguicolle* K.M. Perss (endemic)     | Phytochemical research has not been done yet                                           |                     |
temperature for 15 min in darkness. The control was prepared as above without any extract and methanol was used for the baseline correction. The absorbance of samples was measured at 517 nm. Decrease in absorbance indicated the antioxidant activity. Radical scavenging activity was expressed as percentage inhibition of DPPH and was calculated as follows:

\[
\text{DPPH radicals scavenged (\%) = } \left[ \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \right] \times 100,
\]

where \(A_{\text{control}}\) is the absorbance of the control reaction and \(A_{\text{sample}}\) is the absorbance of the tested extract samples.

**Ferric thiocyanate (FTC) method**

In this assay, hydroperoxide produced by linoleic acid added to the reaction mixture, which has been oxidized by air during the experimental period, is indirectly measured. [19-22] An assay mixture of 2 mL sample [or methanol (as blank) or BHA/vitamin E (as reference)], 2.05 mL of 2.51% linoleic acid in 99.8% ethanol, 4 mL of 0.05 mol/L phosphate buffer (pH 7.0) and 1.95 mL of distilled water was placed in an Erlenmeyer flask in a rotary incubator (150 r/min, 40°C) in a dark place. To measure the antioxidant activity, 0.1 mL of the reaction mixture was transferred into a test tube. Then, 9.7 mL of 75% ethanol was added to it, followed by 0.1 mL of 30% ammonium thiocyanate, and 0.1 mL of 2 \( \times 10^{-2} \) mol/L ferrous chloride in 3.5% hydrochloric acid. Three minutes after the addition of ferrous chloride to the reaction mixture, the absorbance was measured at 500 nm (μ Quant Universal Microplate Spectrophotometer, Bio-Tek). Measurements were taken every 24 h until the absorbance of the control reached its maximum value. This mixture was also prepared without linoleic acid as a negative control. Vitamin E and BHA were used as positive controls. Antioxidant activity was calculated using the following equation:

\[
\text{% inhibition} = \left[ \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \right] \times 100,
\]

where \(A_{\text{control}}\) is the absorbance of the control reaction and \(A_{\text{sample}}\) is the absorbance of the tested extract samples.

**Thiobarbituric acid (TBA) method**

The TBA method was used to represent the inhibition of the production of carbonyl compounds degraded from the peroxides at a later stage. This test is used to measure the second product of peroxide oxidation such as aldehyde and ketone. [22] Briefly, 2 mL of 20% trichloroacetic acid and 1 mL of 0.67% TBA were added to 2 mL of the assay mixture, which was prepared and incubated as described for the FTC method. [19–22] This mixture was placed in a boiling water bath for 10 min and after cooling was centrifuged at 3000 r/min for 10 min (Thermo Scientific Heraeus Megafuge® 40/40R). The absorbance of the supernatant was recorded at 532 nm. Antioxidant activity was calculated using the following equation:

\[
\text{% inhibition} = \left[ \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \right] \times 100,
\]

where \(A_{\text{control}}\) is the absorbance of the control reaction and \(A_{\text{sample}}\) is the absorbance of the tested extract samples.

**Data analysis**

All experiments were performed in triplicate. Values presented in tables and figures are means with standard deviation. Statistical analysis was performed using GraphPad Prism version 5.0 (San Diego, California, USA) and KCJunior software.

**Results and discussion**

Substantial evidence has accumulated and indicated key roles for ROS and other oxidants in causing numerous disorders and diseases. [25] As previously highlighted, [9] the evidence has brought the attention of scientists to an appreciation of antioxidants for prevention and treatment of diseases and maintenance of human health. [25]

There are several techniques that are commonly used to determine the antioxidant activity in vitro for the purpose of rapid screening of substances, since substances that have low antioxidants activity in vitro will probably show little activity in vivo. [9,17] In this work, three different methods based on various aspects of ROS action and scavenging were used to evaluate and compare the antioxidant potential of the studied plant extracts.

It is known that the presence of three aromatic rings in DPPH makes its molecule very stable. [26] This radical has an absorbance maximum at 517 nm. Every substance that can scavenge DPPH, decreases the absorbance in this wavelength, therefore, the DPPH assay could be considered an appropriate method for evaluation of the potential of samples to scavenge free radicals. [26] The radical scavenging activity of the standards (BHA, vitamin E and ascorbic acid) and ME tested is summarized in Table 3. As shown in Table 3, five ME (Crataegus microphylla (E1), Salvia hypargeia (E5), Cotinus coggygria (E6), Origanum sipyleum (E11) and Rosa damascena (E12)) demonstrated potent free-radical-scavenging activity with 86.64%-95.69% DPPH radicals scavenged. Interestingly, the radical scavenging capacity of these five extracts was found to be higher even than that of the reference natural antioxidant vitamin E.

The FTC method was used to evaluate the level of lipid peroxidation by measuring the absorbance of hydroperoxide of linoleic acid. In this method, the concentration of peroxide decreases as the antioxidant activity increases.
Figure 1 illustrates the absorbance versus incubation time, showing the activities of five active ME compared to standards at different time intervals. It was found that hydroperoxide formation started at day 3 and reached the maximum value at day 6. The highest percentage inhibition was shown by the \textit{R. damascena} (E12) extract (84.27%) followed by the extract of \textit{Centaurea nerima-niae} (E3), an endemic species, (80.68%).

The third (TBA) method was used to evaluate the inhibition of the production of carbonyl compounds degraded from the peroxides at a later stage. The results showed that the same five active extracts caused a decrease in the production of carbonyl compounds (Figure 2). Three out of these five active extracts were most effective and showed antioxidant potential comparable to that of the synthetic antioxidant BHA. Comparative analysis of the results obtained by the FTC and TBA methods (Figure 3) showed that the antioxidant activity of the five extracts was found to be slightly higher by the TBA method than by the FTC one. The ME that exhibited potent antioxidant activity in all the three assays used, DPPH, FTC and TBA assay, were those of \textit{C. coggygria} (E6) and \textit{R. damascena} (E12).

A review of the known chemical constituents of the eight plants, whose extracts demonstrated most pronounced antioxidant activity by DPPH and/or FTC and/or TBA methods, revealed that most of them contain flavonoids (Table 2). Flavonoids are known to be related to antioxidant activity.\cite{19,21,29} Additionally, ME have been reported to contain a significantly higher concentration of flavonoids than extracts prepared in other solvents.

![Figure 1](image1.png)

**FTC method**

![Figure 2](image2.png)

**TBA method**

![Figure 3](image3.png)

**Comparison between FTC and TBA**

| Extracts | Concentration* (µg/mL) | Absorbance (517 nm) | DPPH radicals scavenged (%) |
|----------|------------------------|---------------------|-----------------------------|
| E1       | 500                    | 0.111 ± 0.0015      | 92.82 ± 0.79                |
| E2       | 50                     | 0.783 ± 0.001      | 15.53 ± 4.2                 |
| E3       | 500                    | 0.718 ± 0.008      | 23.81 ± 2.38                |
| E4       | 500                    | 0.323 ± 0.018      | 71.84 ± 9.02                |
| E5       | 500                    | 0.306 ± 0.015      | 86.64 ± 9.27                |
| E6       | 500                    | 0.082 ± 0.002      | 95.69 ± 0.17                |
| E7       | 50                     | 0.809 ± 0.003      | 4.52 ± 0.47                 |
| E8       | 250                    | 0.756 ± 0.005      | 13.5 ± 1.90                 |
| E9       | 50                     | 0.792 ± 0.024      | 13.77 ± 4.28                |
| E10      | 50                     | 0.756 ± 0.016      | 10.8 ± 0.01                 |
| E11      | 500                    | 0.139 ± 0.083      | 93.19 ± 3.80                |
| E12      | 500                    | 0.085 ± 0.002      | 95.67 ± 0.68                |
| E13      | 250                    | 0.779 ± 0.015      | 15.5 ± 3.40                 |
| E14      | 500                    | 0.693 ± 0.007      | 26.7 ± 3.88                 |
| BHA      | 100                    | 0.122 ± 0.0075     | 89.49 ± 4.63                |
| Vitamin E| 100                    | 0.223 ± 0.011      | 72.01 ± 5.85                |
| Ascorbic acid | 100 | 0.075 ± 0.001 | 96.78 ± 0.24 |

*In vitro noncytotoxic concentration for the methanolic extracts against the Vero cell line [27].

\cite{22,26,28} Figure 1 illustrates the absorbance versus incubation time, showing the activities of five active ME compared to standards at different time intervals. It was found that hydroperoxide formation started at day 3 and reached the maximum value at day 6. The highest per cent inhibition was shown by the \textit{R. damascena} (E12) extract (84.27%) followed by the extract of \textit{Centaurea nerima-niae} (E3), an endemic species, (80.68%).

The third (TBA) method was used to evaluate the inhibition of the production of carbonyl compounds degraded from the peroxides at a later stage. The results showed that the same five active extracts caused a decrease in the production of carbonyl compounds (Figure 2). Three out of these five active extracts were most effective and showed antioxidant potential comparable to that of the synthetic antioxidant BHA. Comparative analysis of the results obtained by the FTC and TBA methods (Figure 3) showed that the antioxidant activity of the five extracts was found to be slightly higher by the TBA method than by the FTC one. The ME that exhibited potent antioxidant activity in all the three assays used, DPPH, FTC and TBA assay, were those of \textit{C. coggygria} (E6) and \textit{R. damascena} (E12).

A review of the known chemical constituents of the eight plants, whose extracts demonstrated most pronounced antioxidant activity by DPPH and/or FTC and/or TBA methods, revealed that most of them contain flavonoids (Table 2). Flavonoids are known to be related to antioxidant activity.\cite{19,21,29} Additionally, ME have been reported to contain a significantly higher concentration of flavonoids than extracts prepared in other solvents.
For two endemic species, *C. nerimaniae* and *C. sanguicoculle*, no data related to their chemical constituents and biological activities were found in the available literature. The antioxidant activity of these two endemic species was, to the best of our knowledge, determined for the first time in this study. Further studies should be directed at identifying the active antioxidant constituents in these extracts, as it is well known that there may be different interactions (additive, synergistic or antagonistic) among components of a given extract.[30,31]

In recent years, considerable effort has been directed towards identifying naturally occurring substances that can protect against oxidative stress.[3] Antioxidants stabilize or deactivate free radicals, often before they attack targets in living cells.[17,32] Free radical reactions are well known to play a role in disease pathology, e.g. in many acute and chronic disorders in humans, such as diabetes, atherosclerosis, aging, immunosuppression and neurodegeneration.[33] Antioxidants, such as phenolics, flavonoids, tannins and proanthocyanidins, have been reported to be present in many medicinal plants. It is generally considered that the antioxidant contents of medicinal plants may contribute to the protection against diseases. For instance, the ingestion of natural antioxidants has been inversely associated with morbidity and mortality from degenerative disorders.[9,34] Today, there is a growing interest in antioxidant-based drugs/formulations for the prevention and treatment of various complex diseases. Hence, the increased use of natural sources of antioxidants. Natural resources have been reported to be the main sources of medicinal plants which are used as antioxidants.[18] Antioxidants, however, when applied at high dosages, may begin to function as mutagens and tumour promoters. This effect appears to be expressed to a higher extent for synthetic antioxidants than for natural ones. Thus, natural antioxidants could be considered to give a greater degree of safety, even at higher dosages. Natural antioxidants can protect the human body against free radicals, and have also been shown to delay the progress of a variety of chronic diseases such as cancer, heart disease and diabetes.[16,35]

The results from our study indicated that the following plants can be considered potent antioxidant sources: *C. microphylla*, *S. hypargeia* (endemic species), *C. coggyrigia*, *O. sipyleum* (endemic species) and *R. damascena*, according to the DPPH method; and *C. nerimaniae* (endemic species), *C. coggyrigia*, *Scorzonera tomentosa* (endemic species), *R. damascena* and *C. sanguicoculle* (endemic species), according to the FTC and TBA methods. These plant ME may be used for discovery of new drugs in future.

**Conclusions**

Five ME (*C. microphylla* C. Koch, *S. hypargeia* Fisch. & Mey. (endemic species), *C. coggyrigia* Scop., *O. sipyleum* L. (endemic species) and *R. damascena* Miller) have potent free-radical-scavenging activity. The plant ME of *C. nerimaniae* Ş. Kılıtür (endemic species), *C. coggyrigia*, *S. tomentosa* L. (endemic species), *R. damascena* and *C. sanguicoculle* K.M. Perss (endemic species) showed strong antioxidant activity in the FTC and TBA methods, whereas the ME of *C. coggyrigia* and *R. damascena* exhibited potent antioxidant activity in the DPPH, FTC and TBA methods. The results indicated that the eight plants in this study could be considered promising antioxidant sources. These plants extracts could be used for isolating the active compound(s), which in turn could be used for discovery of new drugs in future.

**Disclosure statement**

No potential conflict of interest was reported by the authors.

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