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Nepeta humilis Bentham: First evaluation of phenolic profile and radical scavenging potential

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ABSTRACT: In traditional medicine, Lamiaceae plants are widely used as a cure for various health problems. *Nepeta humilis* is one of the Lamiaceae plants of Iranian-Turkish district growing naturally in the South East Anatolia. The present study was designed to investigate the radical scavenging activity and total phenolic content of *Nepeta humilis* as well as to determine the phenolic constituents of the extracts of flowers, leaves and roots using a previously validated RP-HPLC-DAD method for the first time. The total phenolic contents of flowers, leaves and roots were found as 123.18 ± 1.01, 66.20 ± 0.49 and 54.77 ± 1.23 mg GAE/g extract, respectively. In accordance with the higher total phenolic content, flower extract of the plant displayed more radical scavenging activity with both assays. HPLC results revealed that rosmarinic acid was detected as one of the main compounds in all the investigated parts of the plant, especially in the flowers (0.397 ± 0.01 g/100g dry weight). Also, chlorogenic acid, luteolin and apigenin were found in particular amount in the flower extract. *Nepeta humilis* is rich in phenolic compounds and can be evaluated as a promising antioxidant source.

KEYWORDS: *Nepeta*; Lamiaceae; radical scavenging; HPLC; phenolic compounds.

1. INTRODUCTION

The genus *Nepeta* is represented by approximately 250-300 species that are widespread in Asia, Europe and Africa, and especially in the Mediterranean region [1, 2]. In Turkish flora, *Nepeta* genus has 40 taxa, 16 of which are endemic with an endemism ratio of approximately 40% [3]. *Nepeta* species are widely used in traditional medicine for their expectorant, diuretic, antispasmodic and antiasthmatic activities, so that the local people mostly benefit from the *Nepeta* species as spasmolytic, diuretic and bronchodilator agents. Terpenes, flavonoids, phenolic acids, iridoids and essential oil have been reported, and especially terpenoids and flavonoids are the dominant constituents within the genus *Nepeta* [1, 3-5].

Nepeta humilis Bentham is an annual plant with erect stem usually branched from base, and having violet corolla. It's an Iranian-Turkish element that grows naturally in South East Anatolia, near Hakkari [2, 3]. Although there are many studies on *Nepeta* species, very few studies have been conducted on *N. humilis*, and especially there is no record on its phytochemistry. So that, we aimed to perform the qualitative and quantitative analysis of the phenolics using a previously validated RP-HPLC-DAD (Reverse Phase-High Performance Liquid Chromatography-Diode Array Detector) method as well as to evaluate the total phenolic content and radical scavenging activity of *N. humilis* flowers, leaves and roots for the first time [6, 7].

In brief, standardization of drug means confirmation of its identity, quality and purity throughout all phases of its production process. After true identification of the plant, physical, chemical and biological techniques are frequently used for this purpose. The most important of all these parameters is the determination of the chemical content of the plant and the qualitative and quantitative analysis of its active components [8]. Herein, we established and used an HPLC-DAD protocol to present the phenolic profile of *N. humilis* which is possibly responsible for the examined radical scavenging activity of the plant. We know that for medicinal purposes different parts of the plants are used for different problems, and it is very common that different parts of the plants do not have the same chemical profile. Therefore, the flowers, leaves and roots were analyzed separately to see the distinction of phenolics in the different parts of the plant, and to take advantage of efficiently.

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2. RESULTS

According to the total phenolic content assay results, the total phenolic contents of flowers, leaves and roots were estimated as 123.18 ± 1.01, 66.20 ± 0.49 and 54.77 ± 1.23 mg GAE/g extract, respectively (Table 1). Radical scavenging activity results indicated that the flower extract was able to scavenge DPPH radical with an IC_{50} of 1.29 ± 0.02 mg/mL, and ABTS with an IC_{50} of 0.35 ± 0.01 mg/mL, which the results represent more radical scavenging power compared to the leaf and root extracts. The flower extract having more total phenolic content exhibited more radical scavenging activity with lower IC_{50} data (Table 1). This finding is very important for the consistency between total phenolic content and antioxidant potential.

Table 1. Total phenol contents and radical scavenging activities of the *N. humilis* (NH) extracts.

	Total Phenol Content (mg GAE/g extract)	**DPPH (IC_{50})** mg/mL	**ABTS (IC_{50})** mg/mL
NH Flower	123.18 ± 1.01	1.29 ± 0.02	0.35 ± 0.01
NH Leaf	66.20 ± 0.49	5.02 ± 0.35	0.93 ± 0.01
NH Root	54.77 ± 1.23	13.13 ± 0.2	1.73 ± 0.01

For DPPH assay; Trolox (IC_{50}: 0.043 ± 0.0001 mg/mL) and caffeic acid (IC_{50}: 0.052 ± 0.0001 mg/mL). For ABTS assay; Trolox (IC_{50}: 0.042 ± 0.0001 mg/mL).

For the qualitative and quantitative analyses of secondary metabolites, chromatographic techniques such as HPLC, HPTLC and GC equipped with different detectors are frequently used. Herein, to the best of our knowledge, we performed an RP-HPLC-DAD method to evaluate the phenolic compound characterization of *N. humilis* with our previously validated method for the first time. The experimental conditions and the validation parameters are same, and the results were given in our previous studies [6, 7]. HPLC analyses indicated that among the investigated phenolics only chlorogenic acid, caffeic acid, rosmarinic acid, luteolin and apigenin were detected and quantified in the flower, leaf and root methanol extracts of *N. humilis*. (Figure 1-4). It was also indicated that rosmarinic acid was detected as one of the main compounds in all the investigated parts of the plant, especially in the flowers (0.397 ± 0.01 g/100g dry weight). Rosmarinic acid is a key component in most of the Lamiaceae plants displaying various biological activities. It's common to find many publications on the medicinal benefits of this tannin compound which is contributing too much to the medicinal value of the taxa of the Lamiaceae [9]. While chlorogenic acid, luteolin and apigenin were found in significant amount in the flower extract, any of the investigated flavonoids were not detected in the root extract (Table 2). The high antioxidant potential of the flowers of the plant could be attributed to the substantial amount of rosmarinic acid supported by phenolic acids and flavonoids. In the root extract, rosmarinic acid is the major compound, and any of the other investigated phenolics were not found.

Table 2. The content of phenolic compounds in *N. humilis* (NH).

	Chlorogenic acid (g/100g dw)	**Caffeic acid (g/100g dw)**	**Rosmarinic acid (g/100g dw)**	**Luteolin (g/100g dw)**	**Apigenin (g/100g dw)**
NH Flower	0.203 ± 0.005	0.013 ± 0.001	0.397 ± 0.01	0.012 ± 0.001	0.008 ± 0.001
NH Leaf	0.005 ± 0.0001	-	0.019 ± 0.001	-	-
NH Root	-	-	0.016 ± 0.001	-	-

-: Not Detected; dw: dry weight of the plant part

3. DISCUSSION

According to our comprehensive literature survey, a few studies on radical scavenging activity and phenolic compound profile of *Nepeta* species were found.

In a recent study, the essential oils of *Nepeta heliotropifolia* (NHE) and *N. congesta* subsp. *cryptantha* (NCC) were determined by gas chromatography equipped with different detectors (GC-MS and GC-FID). Liquid chromatography-tandem mass spectrometry (LC-MS/MS) was used to present the phenolic constituents. Moreover, antioxidant and some other biological activities of essential oils gained from aerial parts and ethanol extracts of the plants were studied. Results revealed that the flower extracts were found to contain higher amount of phenolic compounds and the major compound was indicated as rosmarinic acid (8,909.91 and 4,317.20 mu g/g for NHE and NCC, respectively). Similar to our results, flower extracts of the *Nepeta* species exhibited strong antioxidant activity in DPPH and ABTS radical scavenging assays [4].

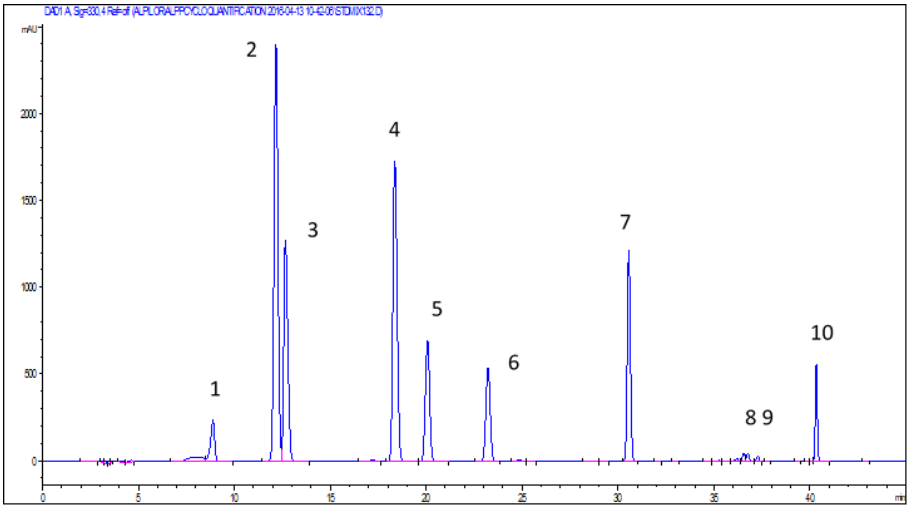

Figure 1. HPLC chromatogram of the standard mixture: 1. Chlorogenic acid, 2. Caffeic acid, 3. Caffeic acid derivative 4.*p*.coumaric acid, 5. Ferulic acid, 6. Rutin, 7. Rosmarinic acid, 8. Quercetin, 9. Luteolin, 10. Apigenin.

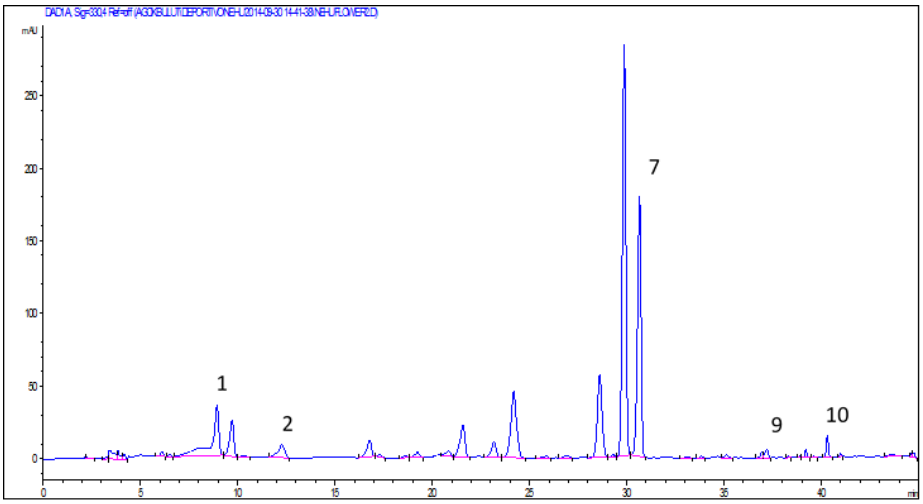

Figure 2. HPLC chromatogram of the flower extract of *N. humilis*.

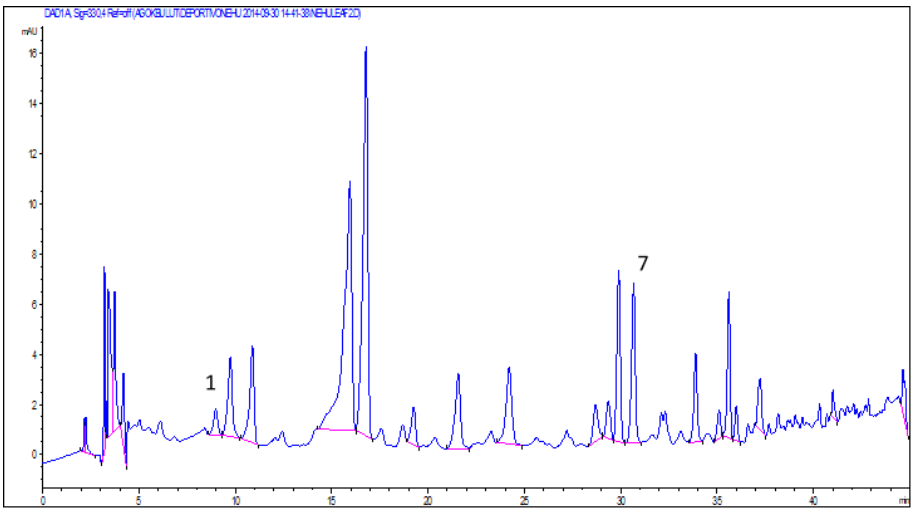

Figure 3. HPLC chromatogram of the leaf extract of *N. humilis*.

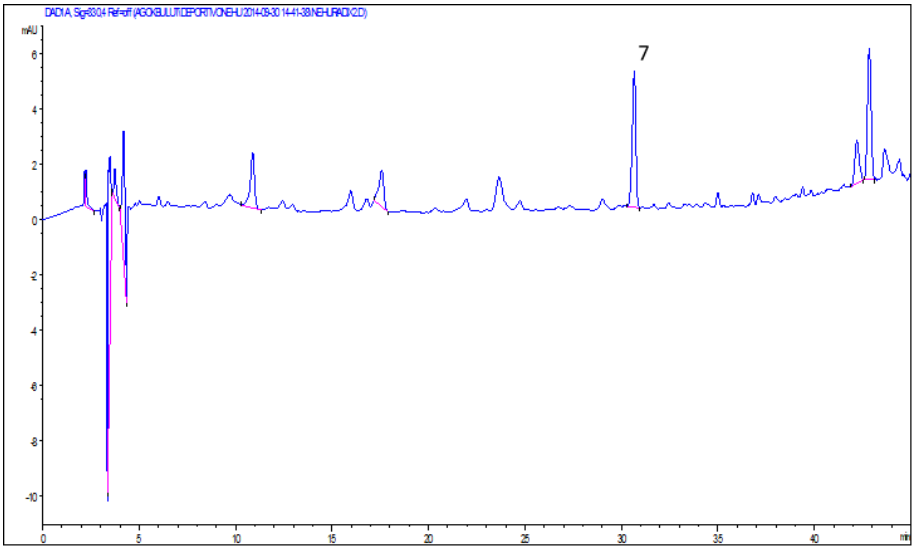

Figure 4. HPLC chromatogram of the root extract of *N. humilis*.

Nasirkandi et al. (2019) studied the antioxidant activity and the phenolic compound characterization of *Nepeta fissa*. The total phenolic content (43.07 mg GAE/g dw), flavonoid content (3.77 mg quercetin/g dw), DPPH assay (IC_{50} value; 197.85 µg/mL) and FRAP assay (1.15 µmol Fe++/g dw) values were estimated. To the HPLC/MS/MS analysis results; rutin, ferulic acid, and chrysophanol were the significant compounds in *N. fissa* extracts [10]. Rutin and ferulic acid were not detected in our sample.

Köksal et al. (2017) performed the HPLC-MS/MS analysis of *N. trachonitica*, and the results showed that more than 10 major phenolic compounds exist, and the main compounds were indicated as rosmarinic, chlorogenic and quinic acids. Also, the radical scavenging activity was evaluated and the plant was suggested as a promising antioxidant source [3].

Phenolic components in the hydroethanolic extracts of *N. italica* subsp. *cadmea* were studied by using HPLC, and quercetin, epicatechin, 2.5-dihydroxybenzoic acid and gallic acid were detected as the most abundant compounds, respectively. Also, the antioxidant property of *Nepeta italica* subsp. *cadmea* was determined using the scavenging methods (DPPH and ABTS), and better radical scavenging activity was observed with both hydroethanolic and hydromethanolic extracts with lower IC_{50} values than our results (ranging from 169.21 to 190.46 µg/mL) [5].

In a comparative study, *Nepeta* species (*Nepeta nuda* L. subsp. *glandulifera* and *N. cadmea*) from Turkey were investigated for their phenolic composition and antioxidant activity. *N. nuda* subsp. *glandulifera* was rich in phenolics than *N. cadmea*. Chlorogenic and ferulic acids in *N. nuda* subsp. *glandulifera* were determined as 63.52 and 14.65 mg/g extract, respectively. It was concluded that higher antioxidant activity should be due to the significant amount of phenolics [11]. The amount of chlorogenic acid was quite high, but it should be better not to compare with our results when we consider the sample source difference as extract and dried plant.

Nepeta cadmea Boiss., an endemic species of Turkey, was studied for its antioxidant potential and phenolic compound characterization. Among the investigated samples of *N. cadmea*, the water extract showed the highest amount of radical scavenging potential (IC_{50} of 25.54 g/mL for DPPH and 14.51 g/mL for ABTS). Also, the phenolic content of the ethanol extract was examined by using HPLC, and epicatechin, chlorogenic, and caffeic acids were determined [12]. In our study, chlorogenic and caffeic acids were detected and quantified for the first time in *N. humilis*.

Sharma and Cannoo (2016) performed the evaluation of antioxidant potential of different extracts obtained from the leaves and flowers of *Nepeta leucophylla*. Among the polyphenolic standards studied, only seven were identified in the methanol extract as chlorogenic, caffeic, syringic, vanillic acids, rutin trihydrate, myricetine and catechin hydrate by using RP-HPLC-DAD method [13].

Aforementioned studies were all about the antioxidant potential and phenolic compound analysis of various *Nepeta* species. However, to the best of our knowledge, in the literature no report demonstrating the antioxidant potential and the phenolic characterization of different parts of *N. humilis* is found. Therefore, the aim of the present study is to analyze the antioxidant potential and the phytochemical composition in terms of phenolics of *N. humilis* using RP-HPLC-DAD method for the first time. The findings of this study shed light to the chemical composition and radical scavenging activity of *N. humilis*. Also, the HPLC method should be used for standardization purposes of any *Nepeta* species and their potential pharmaceutical preparations.

4. CONCLUSION

This study revealed that *N. humilis* is rich in phenolic compounds that are supposed to be responsible for the determined radical scavenging potential. It was also demonstrated that different parts of the plant contain particular amounts of rosmarinic acid together with other phenolics which makes this Lamiaceae plant a valuable medicinal source in the fight against chronic diseases.

5. MATERIALS AND METHODS

5.1. Chemicals

Double-distilled water, methyl alcohol (HPLC purity), acetonitrile (HPLC purity) and analytical grade trifluoroacetic acid were preferred for HPLC analysis. Folin Ciocalteu reagent and the phenolics such as rosmarinic acid (536954), chlorogenic acid (C3878), caffeic acid (C0625), luteolin (L9283), apigenin (A10798), *p*-coumaric acid (C9008), ferulic acid (128708), quercetin (Q4951) and rutin (R5143) were provided from Sigma (Germany). Also, DPPH (D9132) and ABTS (A1888) were supplied from Sigma (Germany). All other chemical staff were of analytical grade.

5.2. Plant material

Nepeta humilis Bentham was collected in its flowering stage from Hakkari, South East Anatolia. The Herbarium sample was placed in Ankara University Faculty of Pharmacy (AEF 19888).

5.3. Preparation of the extracts

Dried and grounded flowers, leaves and roots of the plant (200 mg each part) were extracted with methanol using magnetic stirrer (300 rpm) for 6 h (50°C). Extracts were then filtered and dried.

5.4. Antioxidant activity

5.4.1. DPPH radical scavenging activity

The potential to scavenge the stable free radical DPPH was evaluated according to the modified method of Barros et al. [14]. 0.25 mL aliquot of each extract was mixed with 2.75 mL of the methanolic solution of DPPH radical. The sample was gargled and relaxed for 10 min in a dark place. The measurement of the reduction of the DPPH radical was estimated at 517 nm. The radical scavenging activity (RSA) was calculated as a percentage of DPPH discoloration;

$$RSA\ (\%) = \{(Ab - As)/Ab\}100 \qquad [Eq.\ 1]$$

where As is the absorbance of the solution when the sample extract was added at a significant level, and Ab is the absorbance value of the DPPH solution. IC_{50} was estimated using the graph of inhibition percentage against the extract concentration. Trolox (Sigma, Germany) and caffeic acid were preferred as the standards.

5.4.2. 2,2'-Azino-bis(3-ethylbenzthiazoline-6- sulphonic acid) (ABTS) assay

ABTS radical scavenging activity was monitored using a modification of the method of Re et al. [15]. ABTS was dissolved in methanol and the concentration was arranged to 7 mM. ABTS radical cation was produced by the interaction of ABTS stock solution with 2.45 mM $K_2S_2O_8$ (as an oxidant for changing of ABTS into a radical cation). The blue-green color was obtained for the resulting solution. This radical solution was incubated in a dark place at room temperature for 12 - 16 h. 96% ethanol was used to dilute the ABTS radical cation solution obtaining an absorbance of 0.70 ± 0.02 at 734 nm. 0.25 mL aliquot of each extract was mixed with 2.75 mL of diluted ABTS solution. After 6 min, the reduction in absorbance at 734 nm was recorded. The radical scavenging activity (RSA) was calculated as a percentage of ABTS inhibition as;

$$RSA\ (\%) = \{(Ab - As)/Ab\}100 \qquad [Eq.\ 2]$$

where As is the absorbance of the solution when the sample extract was added at a significant level, and Ab is the absorbance value of the ABTS solution. IC_{50} was calculated from the graph of inhibition percentage against extract concentration. Trolox (Sigma, Germany) was preferred as the standard.

5.5. Phytochemical content

5.5.1. Estimation of total phenol content

A modified Folin Ciocalteu method was used to present the total phenolic content of the extracts [16]. A blue colour was formed after the reduction of the reagent, and the data was recorded at 765 nm. Methanol extract of each plant part was treated with distilled water. Folin reagent was added and the contents of the flask were mixed. 8 min later, 20 % Na_2CO_3 was added. After incubation at room temperature for 2 h, the absorbance was recorded at 765 nm. Gallic acid was used as the standard. The mean values of the triplicate measurements were used to express the mg of gallic acid equivalents (GAE)/g dry extract.

5.5.2. RP-HPLC-DAD experiments

The HPLC experiments to present the phenolic compounds in the extracts were done according to the validated following procedure [6, 7]. LC system consisting of a HP Agilent 1260 series quaternary pump, degasser and photo-diode array detector was used. ACE column (5µ, 250mm × 4.6 mm) was preferred. Gradient elution was used with a flow rate of 0.8 mL/min and temperature was arranged to 30 °C. The mobile phase was a mixture consisting of trifluoroacetic acid 0.1 % in water (solution A), trifluoroacetic acid 0.1 % in methanol (solution B), and trifluoroacetic acid 0.1 % in acetonitrile (solution C). The lay out of the gradient was (A:B:C), 80:12:8 at 0 min, 75:15:10 at 8 min, 70:18:12 at 16 min, 65:20:15 at 24 min, 50:35:15 at 32 min, 25:60:15 at 40 min and 80:12:8 at 45 min. The post run time was 2 min. 10 µL injections were performed for the samples. Chlorogenic, caffeic, rosmarinic acids and apigenin were analyzed at 330 nm, while luteolin was at 360 nm.

Different concentrations of chlorogenic acid, caffeic acid, rosmarinic acid, luteolin and apigenin were prepared in methanol ranging between 0.37-500 µg/mL, 0.07-520 µg/mL, and 0.24-1030 µg/mL, 0.03-75 µg/mL, 0.07-200 µg/mL concentrations, respectively. 10 µL of triplicate was injected into the system The peak areas and the standard concentrations were used to construct the calibration graph.

5.6. Statistical analysis

All analyses were executed in triplicates and the mean values were recorded. All the data are presented as the mean ± standard deviation (S.D.), linear regression analyses and calculations were performed using Microsoft Excel.

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Conflict of interest statement: The authors declared no conflict of interest.

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