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Phenolic Composition and Antioxidant Capacities of *Helichrysum plicatum*

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Abstract

Helichrysum species (Asteracea) are widely found in Anatolia. *Helichrysum* species has used ethnomedicine for centuries. We report here the total phenolic contents and antioxidant activities of the methanolic extracts of *Helichrysum plicatum DC.* subsp. *plicatum* species, together with their HPLC analysis results of individual some phenolic acids and flavonoids. 17 different phenolic constituents were measured by reverse phase-high performance liquid chromatograpy (RP-HPLC) in the three parts of the plant. Total phenolic compound and ferric reducing antioxidant power (FRAP) were used as antioxidant capacity determinants. All parts of the plants showed high antioxidant activity containing large amounts of antioxidant compounds. Chlorogenic acid, quercetin and rutin found in the three parts of the samples as main phenolic components and absisic, ferulic, epicathecin and cinnamic acids have minor concentration or haven't been detected at all. The methanolic extracts of the plants proved to be a good source of phenolic compounds and antioxidants agents that might serve to protect health and fight against several diseases.

INTRODUCTION

The treatment methods with plants is called phytotherapy have been approved and have spread gradually in the last years, and Turkey is a paradise of phytotherapic plants. Many species of plants have been traditionally used for the remedy of a many of diseases. *Helichrysum plicatum DC.* subsp. *plicatum,* a mountain flower, locally named, ölmez çiçek, altınotu or maranda, is a summer flower highly characteristic of Black sea region and known for its unique smell and ethno-pharmacological uses including its diuretic, antidiabetic, alleviate several pains [1-4]. *Helichrysum* plicatum also was used as lithagogue and stomachache [5].

Medically important plants have strong antioxidant activities and their usability have spread gradually in recent years. Phenolic compounds are the largest class of plant secondary metabolites, which, in many cases, serve in plant defense mechanisms to counteract reactive oxygen species (ROS) in order to survive and prevent molecular damage and damage by microorganisms, insects, and herbivores [6-8]. Plants are capable of limitless synthesizing aromatic compounds. They are primarily synthesized by pentose phosphate, shikimate, and phenylpropanoid pathways [9]. The existence of phenolic compounds and other antioxidant components in various forms as soluble or attached

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to membranes or cell walls renders their extraction a problematic issue. The antioxidant activity of phenolic substance is related to a number of different mechanisms such as free radical scavenging, hydrogen donation, singlet oxygen quenching, metal ion chelating, and acting as a substrate for radicals such as superoxide and hydroxide [7]. Antioxidants can inhibit or delay the oxidation of an oxidizable substrate in a chain reaction, therefore, appear to be very important in the prevention of many diseases [10]. A direct relationship has been found between the phenolic content and antioxidant capacity of many fruits and vegetables. Several methods have been developed in recent years to evaluate the total antioxidant capacity of biological samples. The basis of most of these methods relies on a substrate that is oxidized in the procedures, and oxygen consumption, oxidation products, or substrate loss is monitored in different manners by various methods.

Previously, phenolic composition and antioxidant properties of *Helichrysum* species have been studied [11] but our samples of the *Helichrysum plicatum* was collected a different area of Black Sea region, from Zigana plateu. Therefore, the aim of the study was designed to assess the phenolic composition including phenolic acids and flavonoids, and *in vitro* biological activities, in terms of antioxidant, of a medicinally potential plant of *Helichrysum plicatum* species, well as to evaluate their medicinal properties, and whether is the phenolic composition related to grown areas or not.

MATERIALS AND METHODS

Chemicals and Instrumentation

Standards (purity> 99.0%) phenolic compounds for HPLC analysis as follows: gallic acid, protocathechuic acid, *p*-hydroxy benzoic acid, vanillic acid, caffeic acid, chlorogenic acid, syringic acid, epicatechin, *p*-coumaric acid, ferulic acid, benzoic acid, *o*-coumaric acid, *trans*-cinnamic acid, absisic acid, catechin, rutin, quercetin, propylparaben as internal standard (IS) were supplied from Sigma-Aldrich (St. Louis, MO, USA) and Merck (Darmstadt, Germany). The used solvents of methanol, acetic acid, acetonitrile were obtained from and Merck (Darmstadt, Germany). Trolox (6- hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid), TPTZ (2, 4, 6-tripyridyl-s -triazine), Folin-Ciocalteu's phenol reagent, and were from Fluka Chemie GmbH (Switzerland). Polytetrafluoro-ethylene membranes (porosity 0.2 µm) for the filtration of the extracts were obtained from Sartorius (Goettingen, Germany).

HPLC (Agilent 1000) analysis of phenolic compounds was performed on a reverse phase Zorbax Eclipse XDB-C18 column (4.6 x 150 mm, 5 µm), using a gradient program with two solvents system (A: 0.5 % acetic acid in acetonitrile: water (1:1); B: 2 % acetic acid in water) at a constant solvent flow rate of 1.2 mL/min [12]. Injection volume was 20 µL. The signals were detected at 280 nm by UV-VIS detection.

An ATI-Unicam UV-2 UV-Vis spectrophotometer (Cambridge, U.K.) was used in all absorbance measurements. All solutions were prepared with deionized water purified in an Elgacan C104 (Elga, England) filtration system.

Samples and preparation of extracts

Samples of the species were collected from Zigana plateu (1200-1300 m) Trabzon - Turkey July, 2008. Voucher species were identified by Dr. Mustafa Karaköse and have been deposited at the Herbarium of the Department of Faculty of Forestry, Karadeniz Technical University, Trabzon, Turkey. The specimens were dried at room temperature for later analyses and dried materials were taken to biochemistry laboratory for antioxidant activity and

phenolic substance analyses. The plant was divided of three parts, flower, leaves and trunk. About 5-10 g of dried powder of the samples was extracted with 100 mL methanol in a Soxhlet apparatus until the extraction medium appeared. Then the extracts were evaporated until dryness with rotary evaporator at 50ºC. The crude methanol extract was hydrolyzed with 2 N HCI at constant temperature 90ºC at 2 h (IKA, Werke, USA). The acidic mixture was first extracted with 50 mL ethyl acetate and then with 50 mL diethyl ether. The organic solvent was evaporated to dryness under reduced pressure in a rotary evaporator at 40°C. The residue weighed and dissolved in methanol for HPLC analysis or test for antioxidant activities.

Determination of antioxidant capacity

Total phenolic contents were determined by the Folin-Ciocalteau procedure [13] and using gallic acid as standard. Briefly, 0.1 mL of various concentrations of gallic acid and methanolic samples (1 mg/mL) were diluted with 5.0 mL distilled water. 0.5 mL of 0.2 N Folin-Ciocalteu reagents was added, and the contents were vortexed. After 3 min incubation, 1.5 mL of Na_2CO_3 (2%) solution was added, and, after vortexing, the mixture was incubated for 2 h at 20ºC with intermittent shaking. The absorbance was measured at 760 nm at the end of the incubation period. The concentration of total phenolic compounds was calculated as mg of gallic acid equivalents per g of 100 g FW, by using a standard graph.

The antioxidant activities of the samples were determined by FRAP assays. The antioxidant method is based on the measurement of the iron reducing capacities of the propolis. Working FRAP reagent was prepared as required by mixing 25 mL of 0.3 M acetate buffer at pH 3.6 with 2.5 mL of 10 mmol/L 2,4,6-tripyridyl-S-triazine (TPTZ) solution in 40 mmol/L HCl and 2.5 mL of 20 mmol/L $FeCl_3$. 6 H_2O solution [14]. 100 ml of the sample were mixed with 3 mL of freshly prepared FRAP reagent. Then, the reaction mixture was incubated at 37°C for 4 min. After that, the absorbance was determined at 593 nm against a blank that was prepared using distilled water and incubated for 1 h instead of 4 min. A calibration curve was used, using an aqueous solution of ferrous sulphate $FeSO_4.7H_2O$ concentrations in the range of 100-1000 mM, R^2 = 0.96). In order to make comparison, trolox® was also tested under the same conditions as a standard antioxidant compound FRAP values were expressed as a wet weight of the propolis samples basis as millimoles of ferrous equivalent Fe(II) per 100 g dry weight (DW) of the methanolic sample.

Determination of phenolic compounds by HPLC

HPLC analysis of phenolic compounds were performed on a reverse phase Zorbax Eclipse XDB-C18 column (4.6 x 150 mm, 5 µm), using a gradient program with two solvent systems (A: 0.5 % acetic acid in 50:50 acetonitrile: water; B: 2 % acetic acid in water) at a constant solvent flow rate of 1.2 mL/min. Injection volume was 20 µL. The signals were detected at 280 nm. Internal standard (IS) technique was applied to the analysis to increase the repeatability. Propylparaben was a suitable IS for this system.

Statistical analyses

The results were presented as the mean values and the standard deviations (mean ± SD). The data were tested using SPSS (version 9.0 for Windows 98, SPSS Inc.). Statistical analysis of the results was based on Kruskal–Wallis test and Pearson correlation analyses. The significant differences were statistically considered at the level of $p < 0.05$ otherwise given.

Table 1. Phenolic constituents of *Helichrysum plicatum* determined by reverse phase-high-performance liquid chromatography (mg phenolic compound / 100 g).

Phenolic compounds	Flowers	Leaves	Stem - barks
Gallic acid	0.06	0.22	0.04
Proto catechuic acid.	0.19	1.23	0.16
p-OH benzoic acid	0.76	10.65	1.40
Cathecin	0.45	5.64	-
Chlorogenic acid	11.05	10.85	2.38
Vanillic acid	0.35	3.63	1.10
Caffeic acid	0.79	0.81	0.16
Syringic acid	0.08	0.55	0.09
Epicatechin	-	1.63	1.15
p-Coumaric acid	0.25	0.62	0.44
Ferulic acid	-	1.35	-
Benzoic acid	1.35	8.29	0.76
Rutin	29.10	9.68	3.38
o-Coumaric acid	0.63	3.41	0.40
cis,trans-Abscisic acid	-	-	-
trans-Cinnamic acid	0.08	-	0.08
Quercetin	16.23	8.08	0.54
Total phenolic compounds	34.09	45.08	12.08

RESULT AND DISCUSSION

Analysis of phenolic compounds in the *Helichrysum plicatum* species has been carried out by high-performance liquid chromatography. Most of the phenolic acids have absorption maxima in the UV absorption spectra at wavelength of 280 nm and 315 nm and they were identified by comparison of retention times (Peak normalization, PN) and UV spectra of samples with those of authentic standards. Seventeen phenolic acids were analyzed and fourteen compounds were determined: Gallic acid, *proto*-catechuic acid, *p*-OH benzoic acid, chlorogenic acid, vanillic acid, syringic acid, caffeic acid, *p*-coumaric acid, ferulic acid, benzoic acid, *o*-coumaric acid, abscisic acid, trans-cinnamic acid, quercetin, catechin, epicatechin and rutin, either high or low or not in methanolic extracts of the flower. HPLC chromatograms of seventeen standards of phenolic compounds include thirteen phenolic acids and four flavonoids are given in Figure 1. The amounts of the phenolic acids as mg/100 g DW were presented in Table 1. The phenolic chromatograms of the three parts of *Helichrysum plicatum* species, flowers (Figure 2), leaves (Figure 3) and stem-barks (Figure 4) are different from each other. Seventeen phenolic compounds were analysed and about 1-2 individual phenolic compounds were not detected of them. Chlorogenic acid, rutin and quercetin are major phenolic components in flowers of the species (Figure 5). Benzoic, chlorogenic acid **(2)**, rutin **(1)** and quercetin **(3)** were found highly level in leaves. Gallic acid, ferulic acid, catechuic acid, and cinnamic acids were found in very small concentrations. Ferulic acid was found only in the leaves of and in highly concentration of rutin was detected in the flowers of the species (29.10 mg/100 g DW). The phenolic

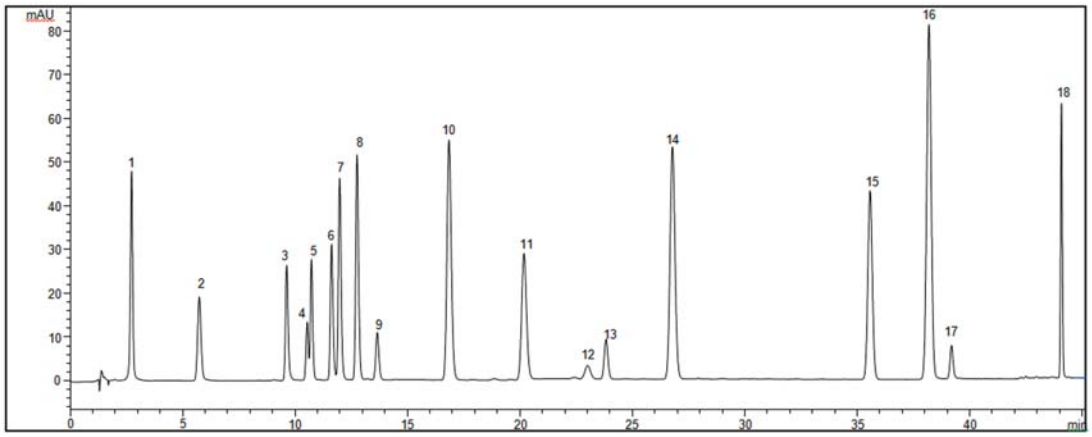

Figure 1. A high-performance liquid chromatography-UV-VIS detection procedure for separating 17 standard phenolic compounds. All peaks were identified by comparison of retention time and UV spectra with commercial standards as follows. Zorbax Eclipse XDB-C18 column (4.6 x 150 mm, 5 µm), gradient eluent acetic acid / acetonitrile/ water/, flow rate 1.2 mL/min. Peak identification: (1) gallic acid, (2) protocatechuic acid, (3) *p*-OH benzoic acid, (4) catechin, (5) chlorogenic acid, (6) vanillic acid, (7) caffeic acid, (8) syringic acid, (9) epicatechin, (10) *p*-coumaric acid, (11) ferulic acid, (12) benzoic acid, (13) rutin, (14) *o*-coumaric acid, (15) *cis, trans*- abscisic acid, (16) *trans*-cinnamic acid, (17) quercetin, and (18) propil paraben (IS).

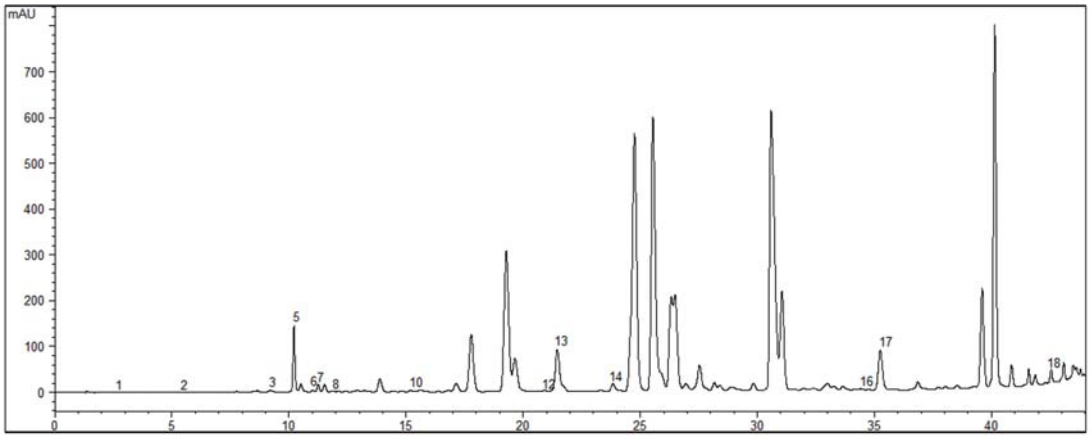

Figure 2. A high-performance liquid chromatography-UV-VIS detection of the methanolic extracts of *Helichrysum plicatum* flowers. (1) gallic acid, (2) protocatechuic acid, (3) p-OH benzoic acid, (4) catechin, (5) chlorogenic acid, (6) vanillic acid, (7) caffeic acid, (8) syringic acid, (9) epicatechin, (10) *p*-coumaric acid, (11) ferulic acid, (12) benzoic acid, (13) rutin, (14) *o*-coumaric acid, (15) *cis, trans*- absisic acid, (16) *trans*-cinnamic acid, (17) quercetin and, (18) propil paraben (IS).

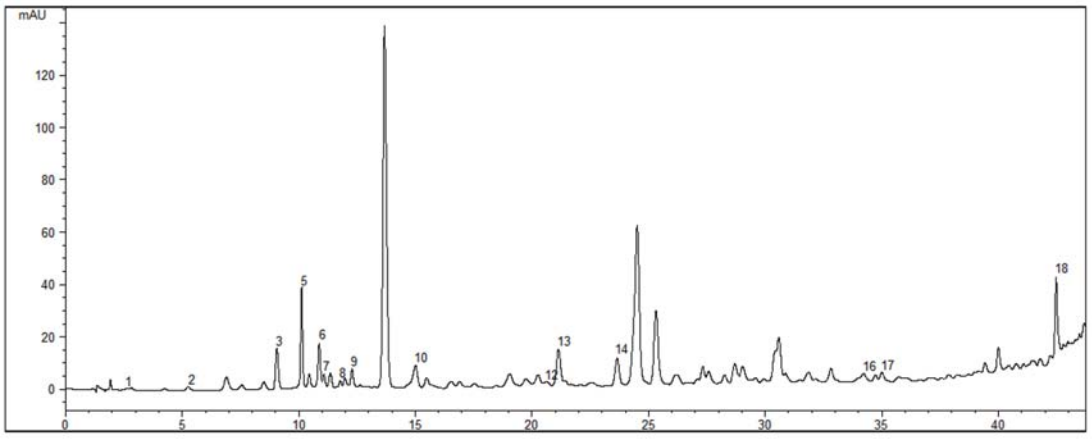

Figure 3. A high-performance liquid chromatography-UV-VIS detection of the methanolic extracts of *Helichrysum plicatum* stem-bark (1) gallic acid, (2) protocatechuic acid, (3) *p*-OH benzoic acid, (4) catechin, (5) chlorogenic acid, (6) vanillic acid, (7) caffeic acid, (8) syringic acid, (9) epicatechin, (10) *p*-coumaric acid, (11) ferulic acid, (12) benzoic acid, (13) rutin, (14) *o*-coumaric acid, (15) *cis, trans*- absisic acid, (16) *trans*-cinnamic acid, (17) quercetin and, (18) propil paraben (IS).

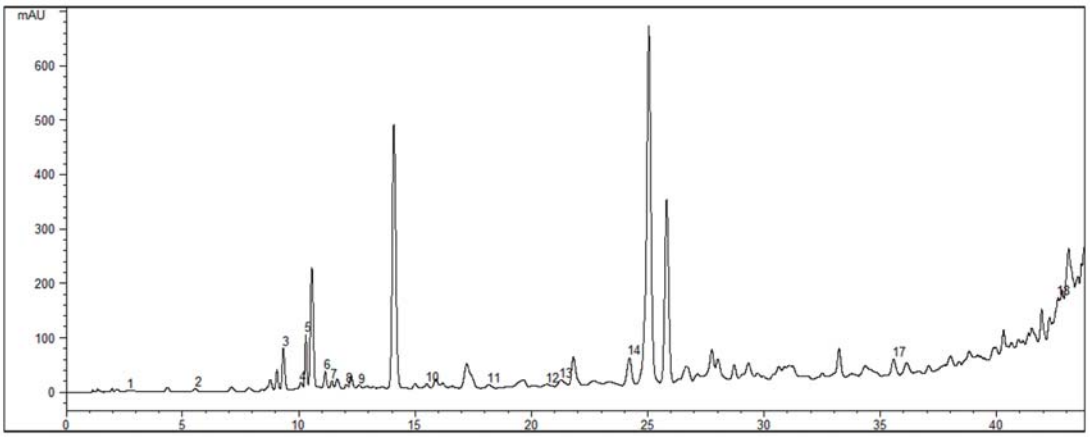

Figure 4. A high-performance liquid chromatography-UV-VIS detection of the methanolic extracts of *Helichrysum plicatum* leaves. (1) gallic acid, (2) protocatechuic acid, (3) *p*-OH benzoic acid, (4) catechin, (5) chlorogenic acid, (6) vanillic acid, (7) caffeic acid, (8) syringic acid, (9) epicatechin, (10) *p*-coumaric acid, (11) ferulic acid, (12) benzoic acid, (13) rutin, (14) *o*-coumaric acid, (15) *cis, trans*- absisic acid, (16) *trans*-cinnamic acid, (17) quercetin and, (18) propil paraben (IS).

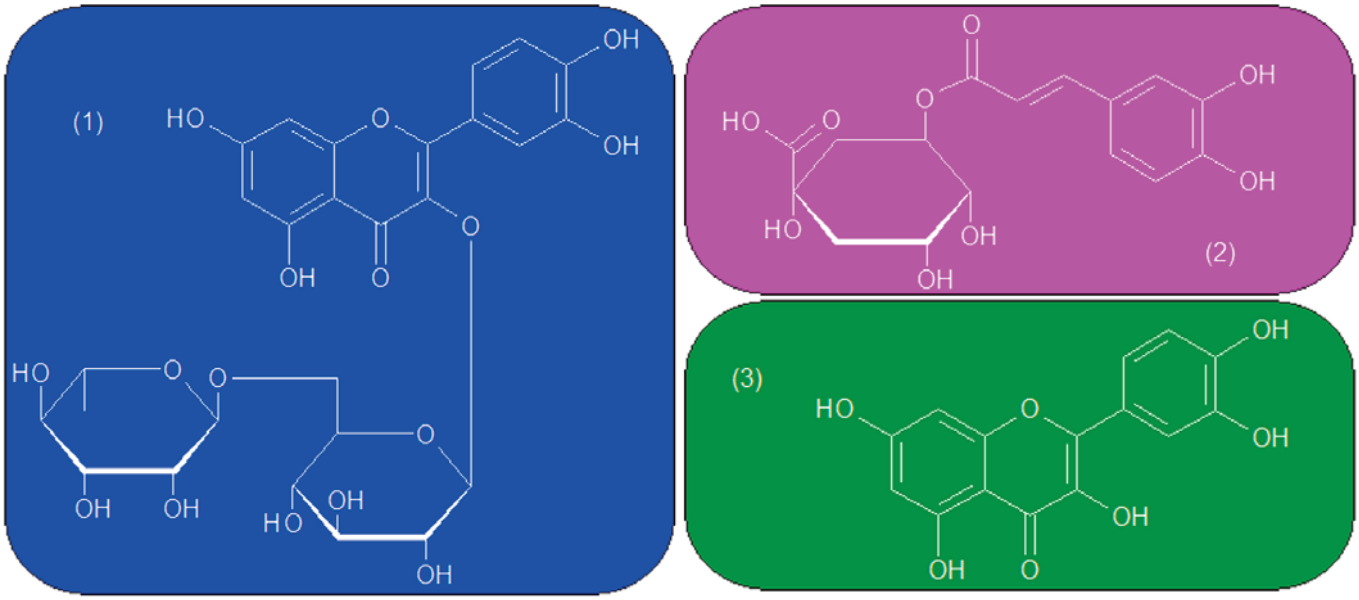

Figure 5. Rutin (1), Chlorogenic acid (2), Quercetin (3). The phenolic compounds were found the most abundant of *Helichrysum plicatum.*

composition of *H. plicatum* subsp. plicatum has been studied previously [11,15]. The researches showed that chlorogenic acid and apigenin were found main phenolic component in *H. plicatum* species. Albayrak et al. [11] have found that a species of flavonoid quercetin was not detected in *H. plicatum.* However, we have already found that quercetin in highly amount in parts of the flowers and leaves of the species. Beside, gallic acid, cathecin, and benzoic acids were found in the species of *H. plicatum.* Therefore, the differences in the quality and quantity of phenolic compounds may be due to a variety of reasons such as growing areas, climate, a many of geography differences and extraction procedures used [11].

In this study, we investigated in vitro antioxidant activity of methanolic samples collected from Black Sea region of Turkey. Total phenolic content and FRAP methods were used as antioxidant capacity determinants with gallic acid and trolox〉 used as reference. The total phenolics and the antioxidant activities of the honey samples were given in Table 2. The amount of the total phenolic content of the three parts of the methanolic extracts of the *H. plicatum* ranged from 5.56-43.12 mg gallic acid /g

Table 2. The antioxidant capacity of *Helichrysum plicatum.*

	Total polyphenol mg gallic acid/ g DW	FRAP 1000 µM Trolox/ 100 g DW
Leaves	40.78 ± 0.67	336.25 ± 0.05
Flowers	43.12 ± 0.08	272.41 ± 0.07
Stem-barks	5.56 ± 0.05	200.18 ± 0.06

DW by using Folin-Ciocalteu method in the three parts of the plant. As seen from Table 2, total phenolic content of the three parts of the *H. plicatum* decreases in order to flowers > leaves > stem-barks. Although total phenolic contents was found the highest in the flower samples, the differences were not found statistical important ($p > 0.05$). Total phenolic contents and flavonoids had studied in the whole *H. plicatum* species collected from Erzurum area [4] and the results were 113.5 ± 8.6 mg gallic acid equivalent/g ethanolic extract and 50.5 ± 1.9 mg quercetin equivalent/g ethanolic extract, respectively. In the other study, total phenolic content and antioxidant activity were studied the same plant species and total phenolic content was found 87.36 ± 0.6 mg gallic acid/g methanolic extract that the samples were collected from Yozgat areas, Turkey [11]. Consequently, when we compared the results the two studies, total phenolic content belongs to *H. plicatum* were the highest in Zigana species. It show that phenolic content and variety founded in the species or may another plants, named secondary metabolism agents, depend on a many of environmental factors such as postharvest processing, soil type, climate, and genetic properties.

We have measured total antioxidant capacity by using FRAP test. The FRAP test is considered to be a good indicator for total antioxidant power because of total reducing power is the sum of the reducing powers of individual compounds present in a sample**.** The increased absorbance is an indication of higher reducing power in this method. The highest FRAP value was obtained for leaves, flowers and stem-bark in the decreasing order of the species. The reducing power measured for all honey samples showed a concentration dependent pattern.

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