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PHENOLIC COMPOUNDS DETERMINATION AND ANTIOXIDANT ACTIVITY OF *TEUCRIUM CAVERNARUM*

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ABSTRACT

The current study was aimed to determine phenolic compounds profile and antioxidant activity of 70% MeOH extract of *Teucrium cavernarum* P.H. Davis which is an endemic plant of Turkey.

According to LC-MS/MS analysis of the extract, forsythoside A, forsythoside B, luteolin-7-gucoside, cirsimaritin and cirsiliol were determined as the major compounds. The total phenolic and radical scavenging activities of the extract were also determined. According to antioxidant activity results, IC_{50} value of 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging effect was determined as 0.12 ±0.01 mg/mL. 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) radical scavenging activity was also determined as 1.2 mM trolox equivalent (TEAC).

Keywords: *Teucrium cavernarum* , Antioxidant activity, LC-MS/MS, Phenolic

1. INTRODUCTION

The genus *Teucrium* with 260 species (370 taxa) is a widespread member of the Lamiaceae family. Most of this genus occurs within the Mediterranean region (96%) besides Asia and America.

In Turkish flora, the genus is represented by 34 species within 46 taxa. *Teucrium* species is a well known folk remedy for Anatolian people and consumed as a tea [1]. Some species of this genus are used as antidiabetic, antispasmodic, anthelmintic and they are also used for treatment of asthma and bronchitis. Antioxidant activity and cytotoxic effects of the genus were also reported [2,3].

The aim of this work was to evaluate antioxidant capacity and phenolic compounds of *Teucrium cavernarum* P. H. Davis for the first time. Radical scavenging activities of the extract were evaluated using 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) scavenging activity test and Trolox equivalent antioxidant capacity (TEAC) assay. Total phenol content was determined using the Folin–Ciocalteu reagent. Phenolic compound analysis was performed via liquid chromatography coupled to Qtrap mass spectrometer (LC-MS/MS) with an electrospray ionization (ESI) interface.

2. MATERIALS AND METHODS

2.1. Chemicals

All chemicals were purchased from Sigma-Aldrich and analytical grade solvents were used.

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2.2. Plant Material

Teucrium cavernarum was collected from three different locations in Turkey.
Sample 1: Gülnar, Ermenek-Gülnar Road 30 km, rocky surface, 1300 m, 05.07.2012.
Sample 2: Gülnar, Gülnar-Mut Road 6 km, rocky surface, 1100 m, 05.07.2012.
Sample 3: Ermenek, Oyuklupınar Village, rocky surface, 1300 m, 05.07.2012.)

2.3. Extraction Procedure

Air-dried herbal parts of *Teucrium cavernarum* samples were powdered, and sonicated with 70% MeOH for 1 h, then macerated with the same solvent at room temperature for 24 h.
Methanol was evaporated from the extract than the aqueous part was lyophilized and used for LC-MS/MS analysis.

2.4. Antioxidant Activity

Total phenols were estimated as Gallic acid equivalents (GAE), expressed as mg Gallic acid/g extract [4].

2.5. Trolox Equivalent Antioxidant Capacity (TEAC) Assay

The assay was performed according to previous study [5].

2.6. DPPH radical scavenging Activity

The equal amount of DPPH was added to diluted solutions of the extracts. After 30 min, UV absorbance was recorded at 517 nm. [6].

2.7. Phenolic Compound Determination

Phenolic compounds were determined with a Shimadzu 20A HPLC system attached to an Applied Biosystems 3200 Q-Trap LC- MS/MS system. Mass spectrum analysis was performed in the negative ionization mode at mass range of 150-800 amu. 150x4.6 mm, 3 µm, ODS analytical column was used at 40ºC for the chromatographic analysis. UV Chromatograms were taken at 280 and 320 nm. CH_3OH : H_2O: HCO_2H (10:89:1, v/v/v) (solvent A) and CH_3OH : H_2O : HCO_2H (89:10:1, v/v/v) (solvent B) were used for the gradient analysis at flow rate of 1 mL/min. The composition of B was increased from 15% to 100% in 40 min.

3. RESULTS AND DISCUSION

According to the LC-MS/MS analysis, eighteen compounds were determined “Table 1”. Some of the major compound mass spectra were given in Figure 1. All chromatograms and major compounds formulas were given in Figure 2 and Figure 3 respectively..

Table 1 LC-MS/MS analysis of *Teucrium cavernarum*

	RT	$[M-H]^-$	Fragments	Identified as
1.	5.8	353	191, 179, 135	3-Caffeoylquinic acid
2.	8.9	353	191, 173, 179	5-Caffeoylquinic acid
3.	15.5	755	623, 593, 461, 179, 161, 135	Forsytoside B
4.	16.3	623	461, 315, 297, 179, 161, 135	Verbascoside
5.	18.6	623	461, 444, 401, 315, 179, 161	Forsythoside A
6.	19.2	447	285, 199,151,133	Luteolin-7-glucoside
7.	19.7	593	447, 285, 327, 175, 151, 133	Luteolin-7-rutinoside
8.	11.9	593	503, 473, 395, 383, 353, 325, 297	Vicenin-2
9.	17.5	755	623, 593, 461, 439, 179, 161, 135	Teucroside
10.	20.5	477	315, 299, 285, 271, 255, 199	Nepetin glucoside
11.	22.6	431	268, 159, 117	Apigenin glucoside
12.	27.6	315	300, 271, 243, 227, 201, 137	Nepetin
13.	28.9	521	475, 445, 313, 298, 283, 269	Cirsimaritim acetyl glucoside
14.	23,8	461	299, 283 , 269, 255	Diosmetin glucoside
15.	35.5	285	199, 175, 151, 133	Luteolin
16.	36.4	313	298, 283, 255,183,163	Cirsimaritin
17.	37.7	299	284, 256	Diosmetin
18.	37.9	329	314, 299, 285, 271, 199, 133	Cirsiliol

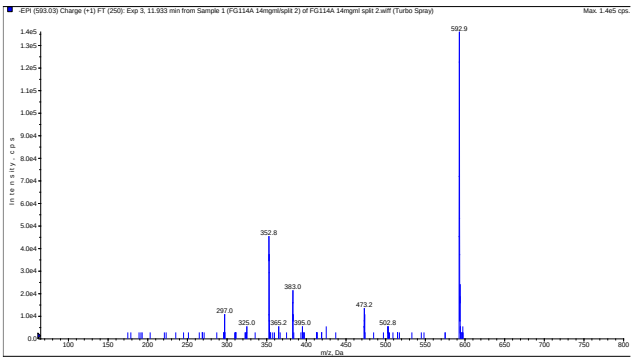
Compound **1** and compound **2** showed the same pseudo molecular ion at *m/z* 353 which was yielded at 191 as a base peak for both. Differences were only observed at ion at *m/z* 179. Compound **1** was showing at 179 as approximately 50% of intensity of the base peak whereas compound **2** showed the same ion in low abundance. The loss of -162 amu between the molecular ion peak and the base peak ion denotes a caffeoyl or hexose unit. Other fragment ions related to caffeic acid such as 135 indicate that the losing part is caffeic acid moiety. According to the previously published data, compound **1** was identified as 3-caffeoylquinic acid and compound **2** was identified as 5-Caffeoylquinic acid, respectively [7].

Compound **3** and compound **9** presented 755 molecular ions $[M-H]^-$, associated with a pentose loss (-132 amu) and caffeic acid loss (-162 amu) and were observed at *m/z* 623 and 593, respectively. The combined loss of these units produced the ion at *m/z* 461. Two molecules determined for *Teucrium* with the same molecular weight and the same fragmentation pattern which were teucrioside and forsythoside B. After comparing their elution times among the different compounds, compound **3** was identified as forsythoside B and compound **9** was identified as teucroside.

Compound **4** and compound **5** gave the same molecular ion peak at *m/z* 623 $[M-H]^-$ and were identified as verbascoside and forsythoside A, respectively. Both of the compounds gave the same ion at *m/z* 461 due to the loss of a caffeoyl moiety. The *m/z* 315 ion was also observed for both compounds owing to the loss of rhamnoglucosyl moiety (162 amu + 146 amu). Distinction of compound 4 and 5 was done accourding to the previous *Teucrium* study in similar conditions which verbascoside eluate earlier than forsythoside A[8].

Compounds **6-13** were determined as flavonoid glycosides. Compound **6** and compound **7** showed characteristic MS fragmentation of luteolin glucoside and luteolin rutinoside with molecular ion peaks at *m/z* 447 and *m/z* 593, respectively. Due to the loss of a glucose and a rutinose moiety, both compounds gave the same base peak ion at *m/z* 285 (luteolin) which allowed to us to identify the compounds as luteolin glucoside and luteolin rutinoside respectively. Confirmation of these compounds was also made by using authentic standards.

a



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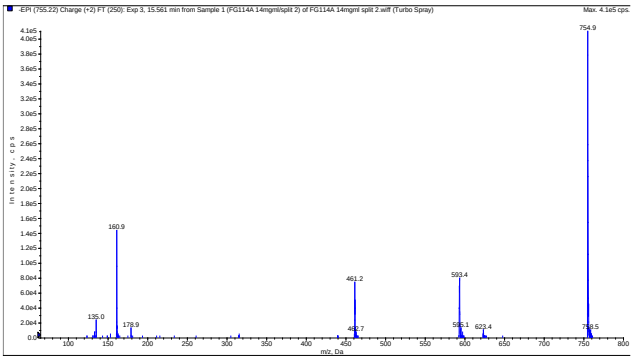

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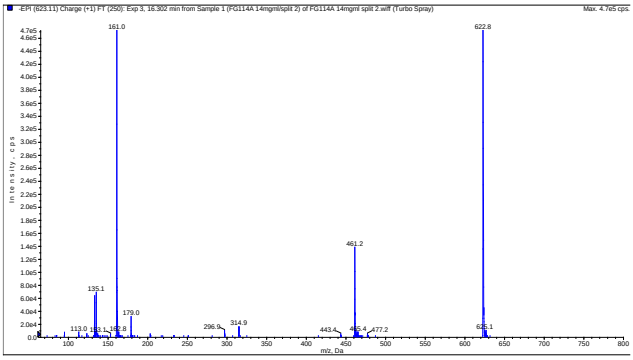


Figure. 1. Mass spectum of vicenin 2 (a), forsythoside B (b), forsythoside A (c)

Compound 8 presented a $[M-H]^-$ molecular ion at *m/z* 593 and product ions at *m/z* 473 ($[M-H]^-$-120) and *m/z* 353 ($[M-H]^-$ -120-120). On the other hand losing of 90 amu from *m/z* 383 ($[M-H]^-$-120-90) indicated that compound 24 is a C-dihexosyl flavone. According to 269 amu between deprotonated molecular ion and two hexose units, apigenin was determined as aglycon, hence compound 8 was identified as apigenin-6,8-di-C-glycopyranoside (vicenin-2) [9]

Compound **10** showed a molecular ion peak at *m/z* 477 $[M-H]^-$ and also showed *m/z* 315 as a main product ion due to the loss of a glucose moiety. Other fragments of ions were observed at *m/z* 299, 285, 255, and 199. The fragmentation pattern of aglycon is not unique for only one compound, according to the compound molecular formula and sterochemistry, whereby 6-methoxy luteolin (nepetin), Quercetin-4'-methylether (tamarixetin), Rhamnetin (7-O-Methylquercetin) gave similar fragmentation patterns. Considering that the biosynthetic pathway of aglycon is possibly related with luteolin, then maybe the ions at *m/z* 285, 151, and 133 are evidence for the theory. Hence, aglycon was determined as 6-methoxy luteolin (nepetin) and compound **10** was tentatively identified as nepetin glucoside. The aglycon part was also determined as nepetin because it eluted at28 min and was numbered as compound **12**.

Compound **11** showed a molecular ion at *m/z* 431 $[M-H]^-$ and a base peak ion at m/z 269 due to the loss of a glucose moiety. fragmentation of aglycon gave ions at *m/z* 159 and 117. Comparing these data with the data in the literature data meant that compound **11** was identified as apigenin glucoside [10].

Compound **13** and compound **16** showed similar fragmentation patterns. Compound **13** showed a precursor ion at *m/z* 521 and product ion at *m/z* 475 due to the loss of an acetyl moiety (-46 amu). Other fragments at *m/z* 313, *m/z* 298, and *m/z* 283, associated with glucose (-162 amu) and methyl loss (-15 amu) were observed. Cirsimatin is previously identified in *Teucrium* [8] according to previously reported work. Compound **16** was identified as cirsimaritin which was previously determined in Teucrium and compound **13** was tentatively identified as cirsimaritin acetylglucoside.

Compound **17** was identified as diosmetin with its molecular ion at *m/z* 299 and base peak ion at *m/z* 284, owing to the loss of a methyl unit. Compound **14** showed a molecular ion at *m/z* 461 and product ions which were same as diosmetin (*m/z* 299) due to the loss of glucose unit, therefore compounds **14** and **17** were identified as diosmetin and diosmetin glucoside respectively using the previously reported data in the literature and fragmentation behaviors [8]. Compound **15** was identified as luteolin after comparison of its retention time and MS/MS spectrum with the standard luteolin compound.

Compound **18** showed a deprotonated molecular ion peak at *m/z* 329 and yielded ions at *m/z* 314, 299, and 285 which were similar with cirsimaritin. Hydroxy cirsimaritin, compound **17**, was identified as cirsiliol which was previously reported for *Teucrium* [8].

3.2. Antioxidant Activity Results

For all samples, total phenol contents were found to be similar to Sample 1: 150±3 mgGAE/g ext, Sample 2: 142±2 mgGAE/g.ext, Sample 3: 156±2 mgGAE/g ext.

There were no significant differences between DPPH scavenging activity results and collecting locality of plant material. Sample **1** IC_{50}: 0.12 ±0.01 mg/mL, Sample **2** IC_{50}: 0.18 ±0.02 mg/mL, Sample **3** IC_{50}: 0.12 ± 0.01 mg/mL. None of the extracts were determined to be as effective as the standard BHT: IC_{50}: 0.01 ± 0.00 mg/ml (figure 4)

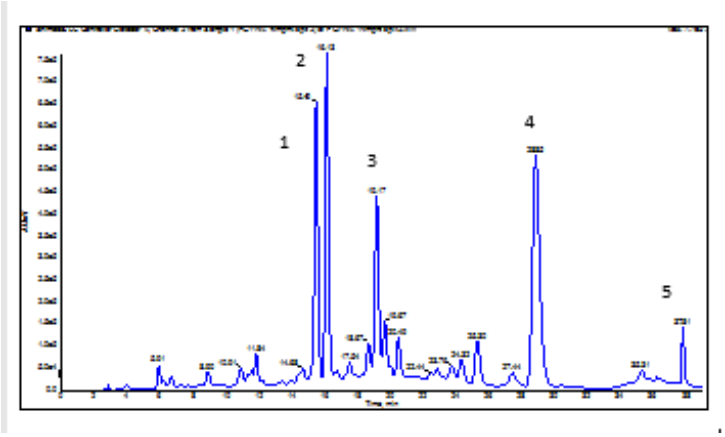
TEAC assay results were found to be parallel to DPPH radical scavenging activity results. Similar to the DPPH activity, the TEAC activity results were also showing no significant differences based on the locality of the plant material. All of the extracts were showing similar results at concentration 1%, and none of them were determined to be as good as the positive control BHT. 1.9 mM TEAC, and sample **1**. was shown as 1.2 mM TEAC, sample **2** and sample **3** were shown as 1.1 mM TEAC and 1.4 mM TEAC, respectively (figure5)

Phenolic acids and flavonoids are antioxidative molecules which reduce the risk of some diseases such as carcinoma and atherosclerosis [11]. According to the results that we obtained from antioxidant activity, the indications are that it is not possible to evaluate *T. cavernarum* polar extract as an alternative synthetic antioxidant compound, such as BHT and BHA. Finding antioxidant active extract as effective as a standard compounds is unfortunately very rare. When comparing other *Teucrium* species from Anatolia: *T. cavarnarum* the total phenol content was found to be much higher for *T. montbret* and *T polium* [11, 12]. We determined antioxidant activity levels that are more active than the previously reported data noted in the above reporting. LC-MS/MS results were highly matched with the previously reported data of Mitreski and colleagues [8].

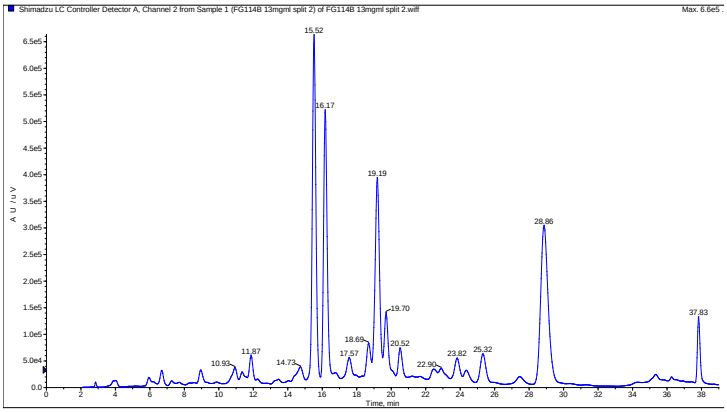
Two hydroxycinnamic acids, four phenylethanoid glycosides, and seven flavonoid O-glycosides, one of them is C glycoside (vicenin 2) and five flavonoid aglycons were identified. It was not possible to find any different phenolic compounds in different locations.

To the best of our knowledge, this is the first identification of this species for all of these compounds, but not for this genus. Nepetin and nepetin glucoside were first reported for this genus.

Sample 1.



Sample 2.



Sample 3.

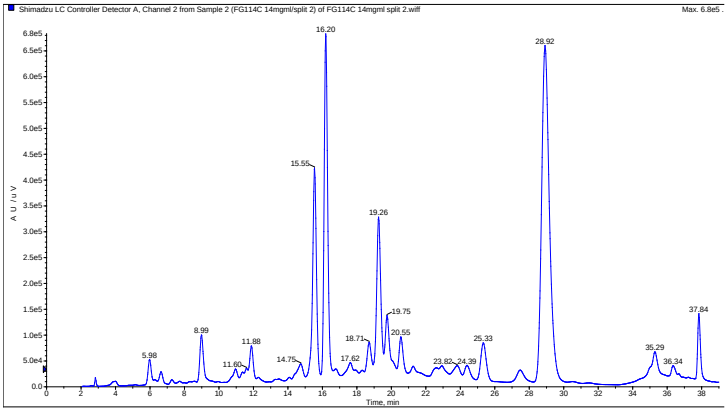


Figure 2. LC chromatograms of the samples. Sample 1: C4 Mersin: Gülnar, Ermenek-Gülnar, Rock surface, 1300m, 05.07.2012. Sample 2: C4 Mersin: Gülnar, Gülnar-Mut rock surface, 1100m, and 05.07.2012. Sample 3:C4 Karaman: Ermenek, Oyuklupınar, rock surface, 1300m, 05.07.2012. Peak 1: Forsytoside B, Peak 2:verbascoside, Peak 3:Luteolin 7 glucoside, Peak 4: cirsimaritim acetyl-glucoside, Peak 5: cirsiliol

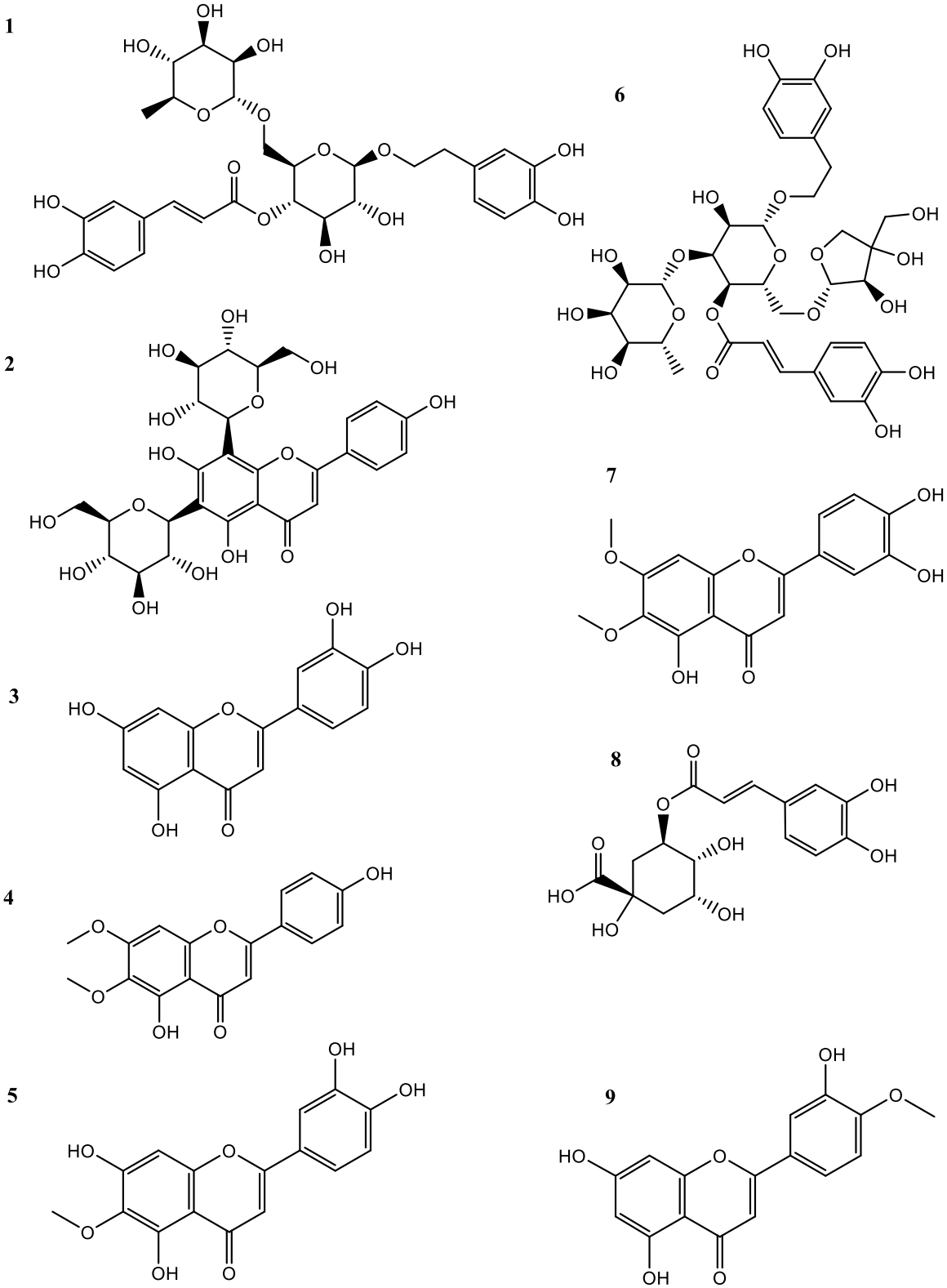


Figure. 3. Molecular formulas of main compounds. 1: Forsythoside A, 2: Vicenin 2, 3: Luteolin, 4:Cirsimaritim, 5: Nepetin, 6: Forsythoside B, 7: Cirsiliol, 8: 3-caffeoylquinic acid, 9: Diosmetin

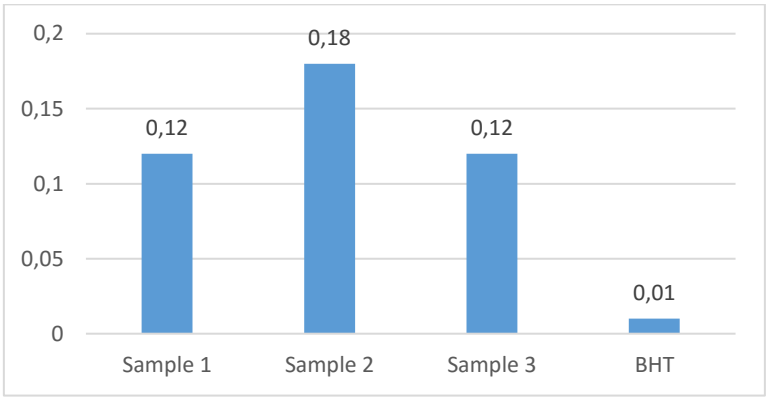


Figure. 4. DPPH radical scavenging effect of the extracts (IC_{50}.mg/mL)

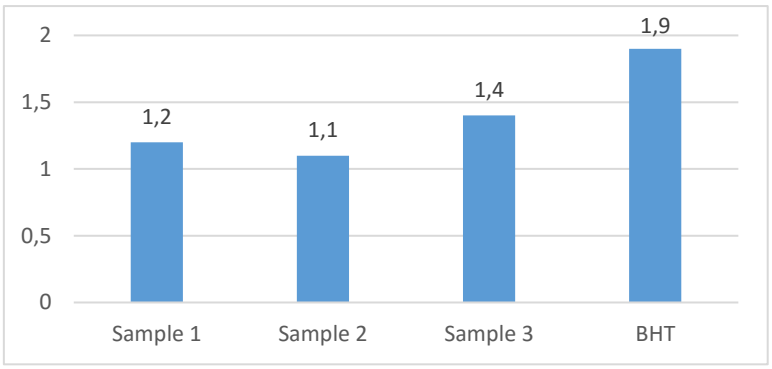


Figure. 5. mM TEAC of the extracts

4. CONCLUSION

There was only one report with the chemical profile of *Teucrium cavernarum* concerning essential oil composition [13]. Determining polyphenolic compounds of this species was as important as volatile determination of this species because the genus *Teucrium* is used for folk remedies in tea form in Anatolia, Turkey.
LC-MS/MS analysis is the fast method for tentative identification of polar compounds. The present study was aimed at determining the phenolic profile of *Teucrium cavernarum* .
WHO notes that “bacon, sausages and ham are among the most carcinogenic substances along with cigarettes, alcohol, asbestos and arsenic”. Even though these substances are very dangerous, manufacturers are trying to preserve such kinds of foods as long as they can achieve the addition of all kinds of synthetic antioxidants, food preservatives. That is why these are listed above as dangerous as cigarettes and alcohol.

We believe that phytochemical studies will be a key to finding new alternative methods of fighting against diseases and natural antioxidant substances in the form of alternative food preservatives.

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