ORIGINAL ARTICLE
DOI: 10.4274/tjps.galenos.2020.44538

# Phytochemical study and antioxidant activities of the water soluble aerial parts and isolated compounds of *Thymus munbyanus* subsp. *ciliatus* (Desf.) Greuter & Burdet

# *Thymus munbyanus* subsp. *ciliatus* (Desf.) Greuter & Burdet bitkisinin suda çözünen topraküstü kısımları ve izole edilen bileşenler üzerine fitokimyasal çalışmalar ve antioksidan aktiviteleri

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25.01.2020
11.10.2020

**ABSTRACT**

**Objectives:** The aim of this study was to determine the phenolic compounds from the water soluble extract of Thymus munbyanus subsp. ciliatus using HPLC-TOF/MS, isolation and characterization of the isolated compounds and their antioxidant activities.
**Methods:** Air-dried aerial parts of *Thymus munbyanus* subsp. *ciliatus* were powdered and extracted with water:methanol three times. The concentrated hydromethanolic extract was dissolved in $H_2O$, then filtered and successively extracted with ethyl acetate, chloroform and *n*-butanol. *T. munbyanus* extracts were purified in column chromatography and studied i*n vitro* antioxidant assays.
**Results:** Two compounds previously undescribed named methyl 2,3,5,6-tetrahydroxybenzoate and 4-hydroxy- 5-methoxy-2-oxo-2*H*-pyran-3-carboxylic acid, along with 14 known compounds as three flavonoids: 3',5,5',7- tetrahydroxyflavanone, luteolin and isorhamnetin-3-*O*-*β*-glucoside, a sterol glucoside: daucosterol, ten phenolic compounds: salicylic acid, ferulic acid, pluchoic acid, ethyl caffeate, methyl caffeate, protocatechuic acid, rosmarinic acid, *p*-coumaric acid, tyrosol, protocatechuic aldehyde was isolated from ethyl acetate and *n*-

butanol extracts. Isolated compounds were characterized by using 1D-2D-$^{1}$H-$^{13}$C NMR and MS spectroscopic methods.
**Discussion and Conclusion:** The extracts and isolated compounds exhibited excellent total antioxidant and DPPH$^{\bullet}$ scavenging activities. According to results, the isolated phenolics and extracts had potency as a potential antioxidant and to be applied to different pharmacological investigations.
**Key words:** *Thymus munbyanus* subsp. *ciliatus*, phenolics, isolated compounds, antioxidant activity

**ÖZET**
**Amaç:** Bu çalışmanın amacı, HPLC-TO/MS kullanılarak *Thymus munbyanus* subsp. *ciliatus*'un suda çözünür ekstraktından fenolik bileşiklerin belirlenmesi, bileşiklerin izolasyonu, karakterizasyonu ve antioksidan aktivitelerinin belirlenmesidir.
**Yöntem ve gereçler:** Thymus munbyanus subsp. siliatus bitkisi küçük parçalar halinde kesildi ve üç kez metanol / su ile ekstraksiyon yapıldı. Konsantre edilen hidrometanolik özüt, saf su içerisinde çözündürüldü, daha sonra süzüldü ve art arda kloroform, etil asetat ve n-bütanol ile ekstraksiyon yapıldı. Ekstreler kolon kromatografisi yöntemi ile saflaştırıldı. *In vitro* antioksidan testler çalışıldı.

**Bulgular:** Daha önce belirlenmemiş 2 bileşik, metil-2,3,5,6-tetrahidroksibenzoat ve 4- hidroksi-5-metoksi-2-okso-2H-piran-3-karboksilik asit ile birlikte, 3 flavonoid olarak bilinen 14 bileşikle 3: 5 ', 5 ', 7-tetrahidroksiflavanon, luteolin ve izorhamnetin-3-*O*-beta-glukozit, 1 sterol glukozit: daucosterol, 10 fenolik bileşik: salisilik asit, plukhoik asit, metil kafeat, etil kafeat, protokateşik asit, ferulik asit, *p*- kumarik asit, rosmarinik asit, tirosol, protokateknik aldehit etil asetat ve *n*-bütanol ekstrelerinden izole edildi. Tüm bileşikler ilk kez bu bitkiden izole edildi ve NMR deneyleri ve LC-ESI-TOF / MS analizi dahil spektroskopik yöntemlerle aydınlatıldı.
**Tartışma ve sonuç:** Ekstraktlar ve izole edilmiş bileşikler yüksek toplam antioksidan ve DPPH• temizleme aktiviteleri sergiledi. Sonuçlara göre, izole edilmiş fenolikler ve ekstraktların kapasitesi, antioksidan potansiyele sahip ve farklı farmakolojik araştırmalara uygulanabilir.

**Anahtar kelimeler:** *Thymus munbyanus* subsp. *ciliatus*, fenolikler, izole bileşikler, antioxidant aktivite

## INTRODUCTION

*Lamiaceae* family covers numerous medicinal and aromatic plants, one of which is *Thymus* genus with biological activities like antiviral, antifungal antioxidant activities[1-3]. *Thymus* genus is represented by around 400 perennial, aromatic plant species and subshrubs growing mostly in the Southern Europe, Mediterranean region, North Africa and Asia[4]. Fifteen species are distributed in Algeria, including *T. munbyanus* subsp. *ciliatus* (Desf.) Greuter & Burdet [Synonym: *T. ciliatus* (Desf.) Benth.] (Arabic 'Zaatar'). The volatile chemical composition (essential oil) and antioxidant activity of *T. munbyanus* extracts were previously reported, but phytochemical investigations have never been reported [5-11].
In this study, above-ground parts of *Thymus munbyanus* subsp. *ciliatus* species' secondary metabolites were analyzed, isolated compounds and revealed *in vitro* antioxidant activities of compounds. The best of our knowledge, all isolated compounds were reported first time from this specie.

## MATERIALS AND METHODS

### *Chemicals and Plant Material*

The chemicals and reagents were analytical grade purchased from Sigma or obtained from other commercial sources. The above ground parts of *Thymus munbyanus* subsp. *ciliatus* were collected in May 2013 from Babor near Setif city, Algeria. This plant was identified by Dr. W. Nouioua based on Algerian flora[12]. A voucher specimen was deposited in the laboratory herbarium unit of the University of Constantine1, VARENBIOMOL Research (TC/123/05-13). Plant material was kept in a freezer until the extraction.

### *Extraction and isolation for compounds*

Dried parts (9.5 kg) of *Thymus munbyanus* subsp. *ciliatus* were powdered with bander and extracted three times with MeOH/water (80/20, v/v). The hydromethanolic extracts were concentrated and dissolved in $H_2O$ (1000 mL), then filtered. After that, the water soluble part was extracted three times as desired with organic solvents of chloroform, ethyl acetate, finally

with n-butanol, and chloroform (17.7 g), ethyl acetate (33 g) and n-butanol (59.2 g) extracts were obtained.
32 g of ethyl acetate extract was fractionated on column chromatography with Sephadex LH-20 eluting with isocratic system of $CHCl_3$/MeOH/hexane (7/2/1) to obtain 26 fractions. The precipitate from fraction 9 (155 mg) showed one spot contaminated by chlorophyll, which was washed with diethyl ether and acetone to give compound 5 (15 mg) and the fraction 10 (2.5 g). The fractiones were again fractionated by column chromatography with Sephadex LH-20 eluting with isocratic hexane/MeOH/$CHCl_3$ (1/2/7) and subsequently by preparative of silica gel eluted with toluene/ethylacetate/formic acid (10/6/1) to yield compounds 6 (3.1 mg), 11 (2.4 mg), 7 (5.5 mg) and 14 (2.7 mg). Moreover, fraction 11 (145 mg) and fraction 12 (124.18 mg) were purified on preparative plates of silica gel eluted with toluene/ethyl acetate/formic acid (10/4/1) to obtain compound 9 (1.7 mg) and compound 8 (7.3 mg) respectively. Also, the fraction 14 (345 mg) was purified by preparative of silica gel eluted with the same solvent mixture and ratio, to afford compounds 15 (5.5 mg),12 (1.7 mg) and compound 1 (20 mg) and also the fraction 15 (3.12 g) was separated with column chromatography using Sephadex LH-20 as the eluting isocratic chloroform/methanol (7/3) and subsequently by preparative silica gel eluted with toluene/ethyl acetate/formic acid (10/2/1) to yield compounds 2 (3.2 mg), 3 (1.3 mg), 10 (3.4 mg) and 16 (9.1 mg).
The butanoic extract (10 g) was subjected to the polyamide column (SC6) and fractionated with a gradient of toluene–MeOH with further increased solvent polarity to obtain 21 fractions. The fraction 5 (0.37 g) was applied to column chromatography with Sephadex LH-20 using isocratic $CHCl_3$/MeOH (6/4) to obtain 10 subfractions. The subfraction 7 (61.2 mg) was purified by thin-layer chromatography (TLC) with EtOAc/MeOH/$H_2O$ (18/1/1) to yield compound 13 (11 mg) and also the fraction 6 (76.3 mg) was further separated by column chromatography with methanol to afford compound 4 (17 mg) using Sephadex-LH-20 as stationary phase.

***HPLC-TOF/MS Analysis***

Phenolic contents of the organic solvent extracts, chloroform, ethyl acetate and *n*-butanol were analyzed by high pressure liquid chromatography coupled with time-of-flight mass spectrometry (HPLC-TOF/MS). The HPLC was Agilent Technology (1260 model) coupled with 6210 Time of Flight (TOF) LC/MS detector on ZORBAX SB-C18 (4.6×100 mm, 3.5 µm) column. The ultra-pure water with 0.1% formic acid (A) was used as mobile phase. B was 100% acetonitrile. The separation program of HPLC was 0-1 min 10% B, 1-20 min 50% B, 20-23 min 80% B and 23-30 min 10% B with a flow rate of 0.6 mL, respectively. The column was set at 35 □C of temperature and also the injection volume were and 10 µL. The phenolic compounds were determined by comparison with the standart components with retention times and evaluated m/z values. Crude extracts were dissolved in methanol to obtain a concentration of 200 ppm at 25 ºC and used 0.45 µm PTFE filters.[13]

***In vitro antioxidant assays***
***Total antioxidant capacity***
The activities of the extracts and isolated chemicals were performed by the ammonium phosphomolybdenum assay applied by Prieto et al [14]. Sample solutions (25, 50, 100) prepared in at different concentrations (each of 0.3 ml) and at the main reagent solution (3 ml, ammonium molybdate-sodium phosphate- sulfuric acid) were vortexed homogeneously. The closed tubes containing the reaction solutions were left in a hot water bath for 90 minutes. Then, the mixtures were cooled in ice bath and the absorbance of each resulting solution was further measured using a UV-visible spectrophotometer (at 695 nm with Thermo Scientific

Evaluation Array Uv-Vis Spectrophotometer). Solvent (0.3 mL) was used instead of the sample as the blank.

***Free radical scavenging capacity***

The activities were determined from samples according to the previously reported by Blois [15]. In this study, 1.5 mL of different concentrations of extracts and isolated compounds and $DPPH^{\cdot}$ solution (0.5 mL) were mixed in a test tube, homogeneously. Each mixture was incubated in the dark at 25 °C for 30 minutes. The absorbances of the solutions were measured using a UV-visible spectrophotometer (at 517 nm, Thermo Scientific Evaluation Array UV-Vis Spectrophotometer). The activity was calculated using the below formula; The percentage activity = $[(A_{1\,(517\,nm)} - A_{2\,(517\,nm)})/A_{1\,(517\,nm)}]$ x100 where $A_1$ is control absorbance and $A_2$ is absorbance of sample.

***Statistical analysis***

The antioxidant activity tests were analysed in triplicate and obtained averages of the activities. Data were analyzed by SPSS 20.0 of variance, $p < 0.05$.

**RESULTS AND DISCUSSION**

***Characterization of isolated components***

Two compounds previously undescribed named [methyl 2,3,5,6-tetrahydroxybenzoate (1)] and [4-hydroxy-5- methoxy-2-oxo-2*H*-pyran-3-carboxylic acid (16)], along with 14 known compounds-three flavonoids: 3',5,5',7-tetrahydroxyflavanone (2), luteolin (3) and isorhamnetin-3-*O*-*β*-glucoside (4), a sterol glucoside: daucosterol (5), 11 phenolic compounds: salicylic acid (6), pluchoic acid (7), methyl caffeate (8), ethyl caffeate (9), protocatechuic acid (10), ferulic acid (11), *p*-coumaric acid (12), rosmarinic acid (13), tyrosol (14), protocatechuic aldehyde (15) was isolated from *n*-butanol and ethyl acetate extracts of *T. munbyanus* subsp. *ciliatus*. The structures of isolated compounds from *T. munbyanus* were given in Figure 1.

Compound (1): It was obtained as powder of colorless amorphous. The molecular formula of the compound 1 was determined as $C_8H_8O_6$ by EI-MS negative ion m/z 198.08 $[M-H]^-$, which indicated that the molecule had five degrees of unsaturation. The $^1$H-NMR spectrum in acetone-d6 exhibited just two singlets: First at $\delta_H$ 7.33 (s, 1H, H-4) indicated penta substituted benzene ring and the second at $\delta_H$ 3.88 (s, 3H ) corresponds to the presence of methoxy groups.

The $^{13}$C NMR, HSQC and DEPT spectra exhibited 6 carbon signals including one methoxy group at δc 55.73 (C-8), one methine at δc 107.23 (C-4), four quaternary carbons including three aromatic carbons at δc 120.59 (C-1), 147.39 (C-2/C-6), 140.39 (C-3/C-5) and one at δc 166.55 (C-7) characterized as carbonyl carbon of an ester group.

The long-range C-H correlation of C-4 aromatic proton was observed in HMBC spectrum ($^2J_{CH}$ and $^3J_{CH}$ correlations) with C-3/C-5 and C-2/C-6. The structure was further confirmed

by HMBC spectrum. Thus, compound **1** was identified as methyl 2,3,5,6-tetrahydroxybenzoate.

As compounds 2-15 were isolated and characterized before, their data were given in Supplementary Information.

Compound (16): Compound 16 was obtained as a brown yellowish solid. The molecular formula for it was found as $C_7H_6O_6$, implying five degrees of unsaturation. The $^{13}C$ NMR and DEPT spectra of this compound confirmed the presence of the 7 carbon atoms consisting of one methoxy group was observed at δc 55.31, four vinyl carbons including one methine at δc 106.57 (C-6), quaternary carbon atoms at δc 128.11 (C-3), 137.54 (C-5), and 146.91 (C-4); and the carbonyl region showed two peaks at δc 173.81 and 168.83 were indicative for the presence of carbonyl carbon of the carboxylic acid and carbonyl carbon of the α-pyrone ring respectively. The 1H NMR and HSQC spectra confirmed the previous suggestion. It displayed one singlet of one proton at $\delta_H$
7.33 assigned to H-6, besides three proton singlets at $\delta_H$ 3.87 for $OCH_3$. The HMBC measurements showed long-range correlations between the protons signal at $\delta_H$ 7.33 and two quaternary carbons at δc 137.54 (C-5) and δc 146.91 (C-4). The HMBC experiments also showed the connectivity between the methoxy protons at $\delta_H$ 3.87 and the quaternary carbon at δc 146.91(C-4). These findings clearly indicated that the carboxylic acid was connected to C-3, while a methoxy function was connected to C-5. Therefore, compound 16 was finally assigned the structure of 4-hydroxy-5methoxy-2-oxo-2H-pyran-3-carboxylic acid.

***Quantification of polar constituents of Thymus munbyanus* subsp. *ciliatus* Extracts**

The extracts of *T. munbyanus* were analyzed by high pressure liquid chromatography (TOF/MS) method. The identifications of the extracts had been analysed with their retention times and m/z values by comparison with those of authentic samples. Overall, the spectral results exhibited the presence of 29 compounds including 11 phenolic acids and 18 flavonoids and phenolics (Table 1). These compounds were found in very small quantity in chloroform extract. The highest concentration of scutellarin, baicalin and fumaric acid were observed in butanoic extract but very low in ethyl acetate extract. On the contrary, in ethylacetate extract: the quercetin-3-*β*-D-glucoside, caffeic and 4-hydroxybenzoic acid were found as the majority. As it can be seen, the analyzed extracts (ethyl acetate and butanoic) comprises complex mixtures of plant secondary metabolites. These active ingredients are flavonoids and phenolic acids known for their antioxidant activity properties[16, 17].

***Total antioxidant capacity***

The activities of isolated and extracted samples were measured and expressed as the absorbance values. This assay was determined by the reduction of $Mo^{+6} \rightarrow Mo^{+5}$ as the antioxidants and the formation of a green phosphate/$Mo^{5+}$ complex with maximum absorption at 695 nm at acidic pH. This assay is widely used to evaluate the total antioxidant capacity of extracts and isolates. The results were exhibited in Figure 2. Total antioxidant activity indicates higher absorbance of the antioxidant meant to possess antioxidant property. It was observed that ethyl acetate extract and compound 8 exhibit similar activities at 25, 50 and 100 µg/mL when compared to BHA and BHT, $p<0.05$. The phenolic compounds have been researched about the prevention of cancer due to antioxidant potency [18]. It has also been reported that compounds are related to the antioxidant activity and play an important role in inhibition of lipid peroxidation [19]. On the base of the results, isolated compounds and different

solvent extracts were regarded as promising natural sources of nontoxic and natural antioxidants obtained from *T. munbyanus*.

***Free radical scavenging activity***

This activity is a generally applied assays to determine the antioxidant levels of extracts in a relatively short period of time to deliver hydrogen and also compared to other experiments based on their ability [20]. It has been decelerated that natural chemicals reduce DPPH$^{\bullet}$ due to their -H donating ability[21]. When an antioxidant and DPPH$^{\cdot}$ (a synthetic radical) are mixed, the antioxidant gives an electron to DPPH and the purple color turns into a yellow color.

The free radical scavenging activities of natural contents were significantly exhibited an increase to be in a dose-dependent manner, $p<0.05$. The activity was found more effective in compound 2, 7, 8, 10 and 15 than BHA and BHT at high dose (Figure 3). It may be concluded that the case of these results obtained from two different –OH substitutions favor the DPPH$^{\bullet}$ scavenging activity [22]. The stabilization of radicals by the two subsequent -OH substitutions of phenolic groups for compounds 3, 8 and 15 were shown in Figure 4. The compounds 3, 8 and 15 showed their ability to remove radical radicals due to their resonance stability and the hydroxyl groups they carry. It can stop the radical reaction as a radical inhibitor. Plant phenolics include superoxide radicals, lipid alkoxyl radicals, lipid peroxyl radicals, nitric oxide radical cleansing, metal chelating, functions as well as antiallergic, estrogenic and antiviral effects [23]. In the study, polymeric polyphenols are more effective antioxidants than monomeric phenolics [24]. The OH group in the ortho or para position of phenol increases the antioxidant activity.

## CONCLUSION

The present word carried out for the first time on Algerian *Thymus munbyanus* subsp. *ciliatus* species, resulted in the two new compounds named methyl 2,3,5,6-tetrahydroxybenzoate and 4-hydroxy-5 -methoxy-2-oxo-2*H*-pyran-3-carboxylic acid and also **14** known compounds from different chemical classes (flavonoids, sterol and phenolic derivatives). The ethyl acetate extract exhibited excellent free radical-scavenging activity (*in vitro*) correlated with the content of polyphenol derivatives (**3**, **8** and **15**).
In conclusion, the finding clearly demonstrates a scientific information to the use of *Thymus munbyanus* subsp. *ciliatus* derivatives as functional ingredients for Algerian traditional medicine**.**

## Disclosure statement

The authors declared that no conflict of interest, financial or otherwise.

## Acknowledgments

This work was supported by a grant from State Planning Organization of Turkey (DPT:2010K120720).

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# Table

**Table 1.** Phenolic compounds of *Thymus ciliatus* extracts determined by HPLC-TOF/MS

| Phenolic compounds, mg of phenolic comp./kg | $CHCl_3$ extract | Ethyl acetate extract | *n*-BuOH extract |
|---|---|---|---|
| Fumaric acid | nd | 0.44 | 9.07 |
| Gentisic acid | 0.14 | 3.82 | 0.72 |
| Chlorogenic acid | 0.09 | 0.73 | 2.14 |
| 4-hydroxybenzoic acid | 1.04 | 21.03 | 0.91 |
| Protocatechuic acid | nd | 0.78 | 1.06 |
| Caffeic acid | 0.11 | 24.96 | 0.50 |
| Vanillic acid | 0.26 | 1.62 | 0.39 |
| Syringic acid | 0.99 | 3.55 | 1.25 |
| Rutin | nd | 0.11 | 1.00 |
| 4-hydroxybenzaldehyde | 0.02 | tr | tr |

| | | | |
|---|---|---|---|
| Polydatine | tr | 0.90 | tr |
| Scutellarin | 0.39 | 0.64 | 40.29 |
| Quercetin-3-*β*-D-glucoside | tr | 17.14 | 5.52 |
| Naringin | 1.09 | 1.50 | 2.12 |
| Diosmin | 0.65 | 2.45 | 2.35 |
| Taxifolin | tr | 0.12 | tr |
| Neohesperidin | tr | 0.06 | tr |
| Baicalin | tr | tr | 15.58 |
| *p*-coumaric acid | tr | 0.13 | tr |
| Morin | 0.23 | 2.36 | 0.70 |
| Salicylic acid | tr | 0.33 | tr |
| Quercetin | tr | 1.48 | tr |
| Cinnamic acid | 0.32 | 0.30 | 0.51 |
| Apigenin | 0.05 | 4.05 | tr |
| Naringenin | tr | 0.29 | tr |
| Kaempferol | tr | 1.13 | tr |
| Diosmetin | tr | 4.37 | nd |
| Eupatorin | 0.32 | tr | tr |
| Wogonin | 0.90 | tr | nd |

# Figures

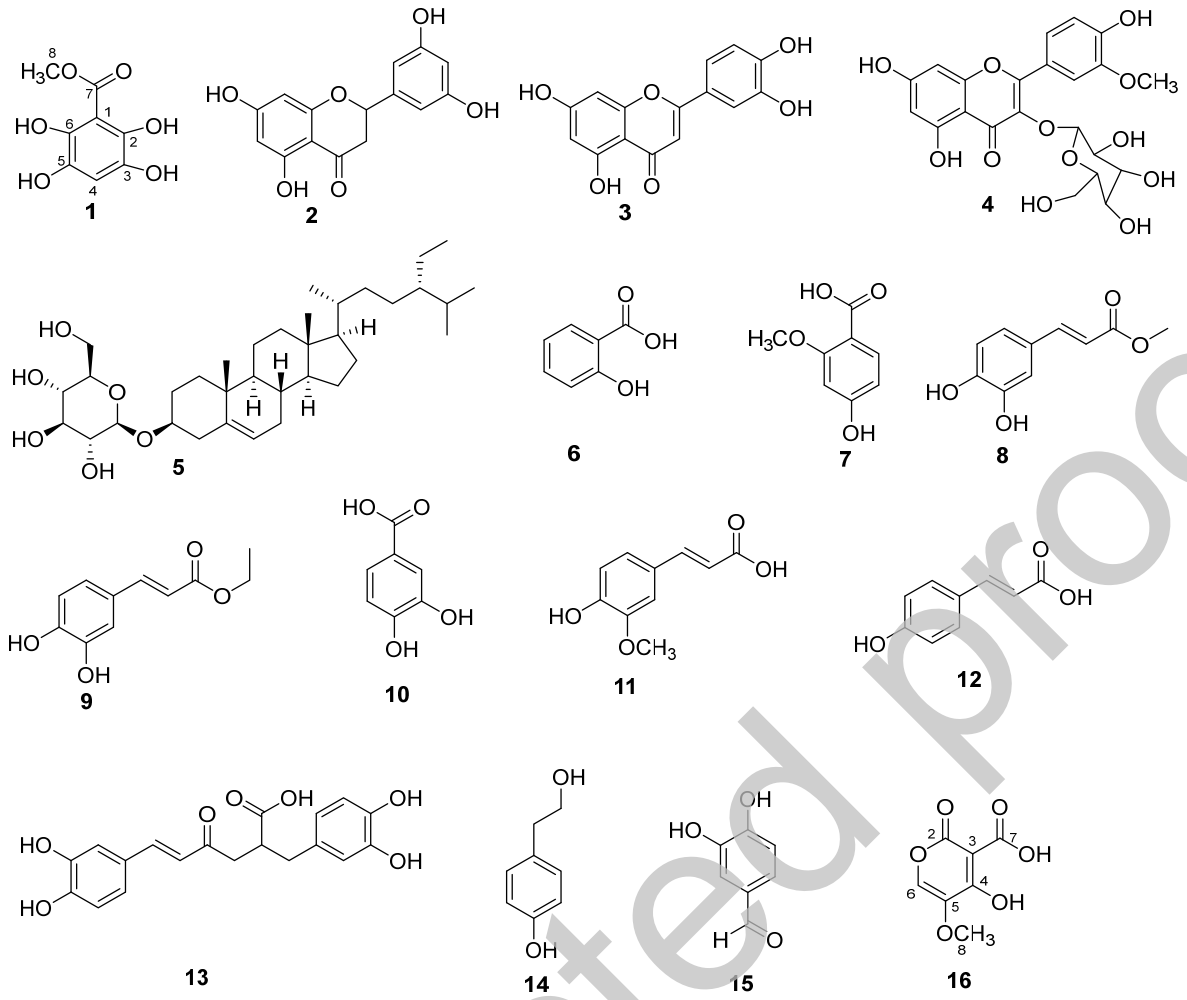


**Figure 1.** Structures of compounds isolated from *Thymus munbyanus* subsp. *ciliatus* as methyl 2,3,5,6-tetrahydroxybenzoate (1), 3',5,5',7-tetrahydroxyflavanone (2), luteolin (3), isorhamnetin-3-O-*β*-glucoside (4), daucosterol (5), salicylic acid (6), pluchoic acid (7), methyl caffeate (8), ethyl caffeate (9), protocatechuic acid (10), ferulic acid (11), *p*-coumaric acid (12), rosmarinic acid (13), tyrosol (14), protocatechuic aldehyde (15) and 4-hydroxy-5-methoxy-2-oxo-2*H*-pyran-3-carboxylic acid (16).

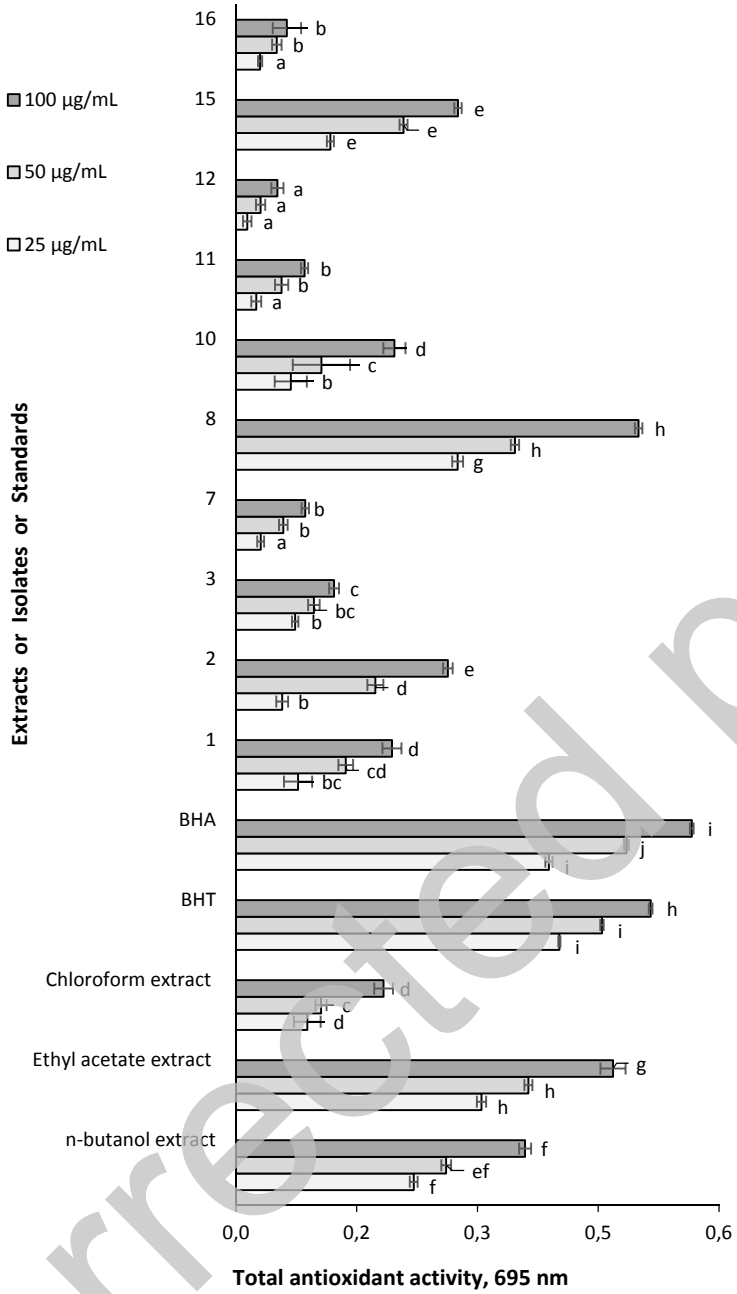


**Figure 2**. The total antioxidant activity of isolated and extracted samples.

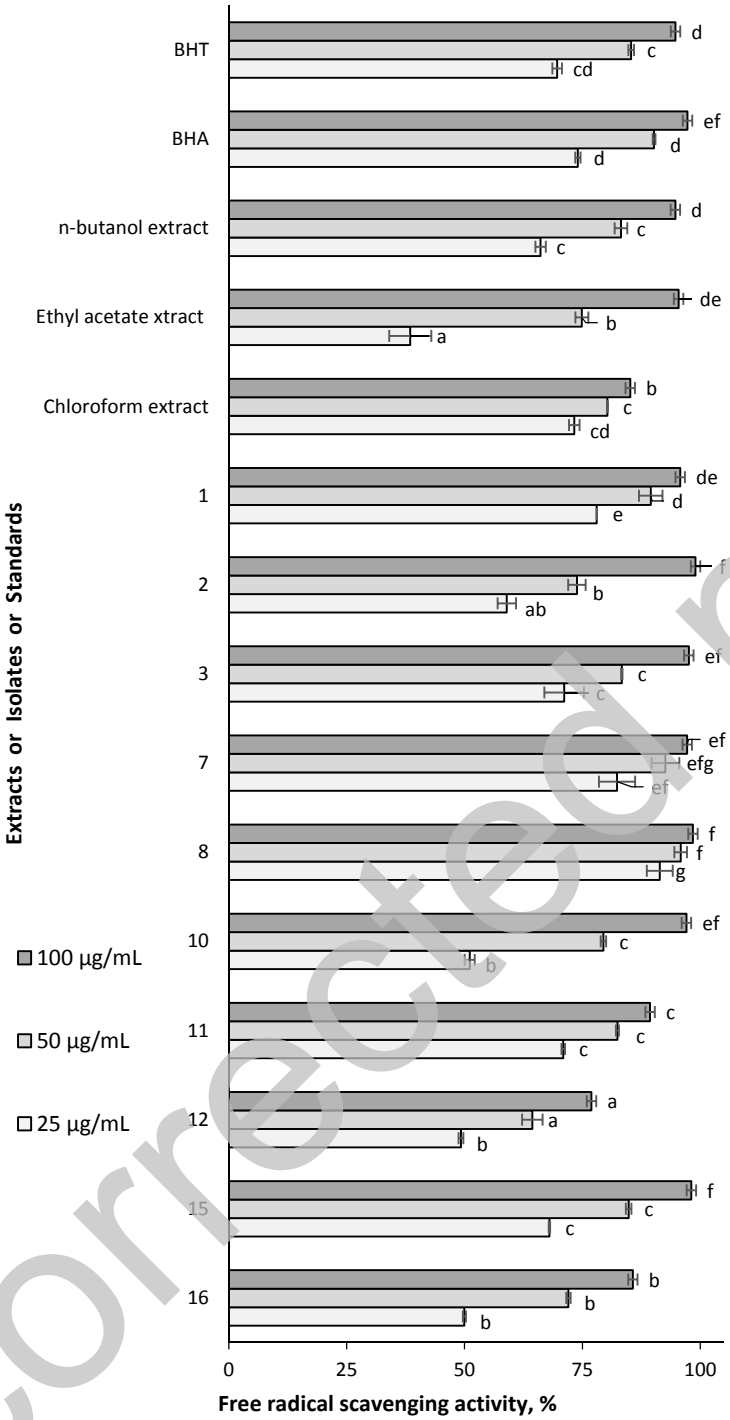


**Figure 3.** The free radical (DPPH•) scavenging activities of isolates and extracts.

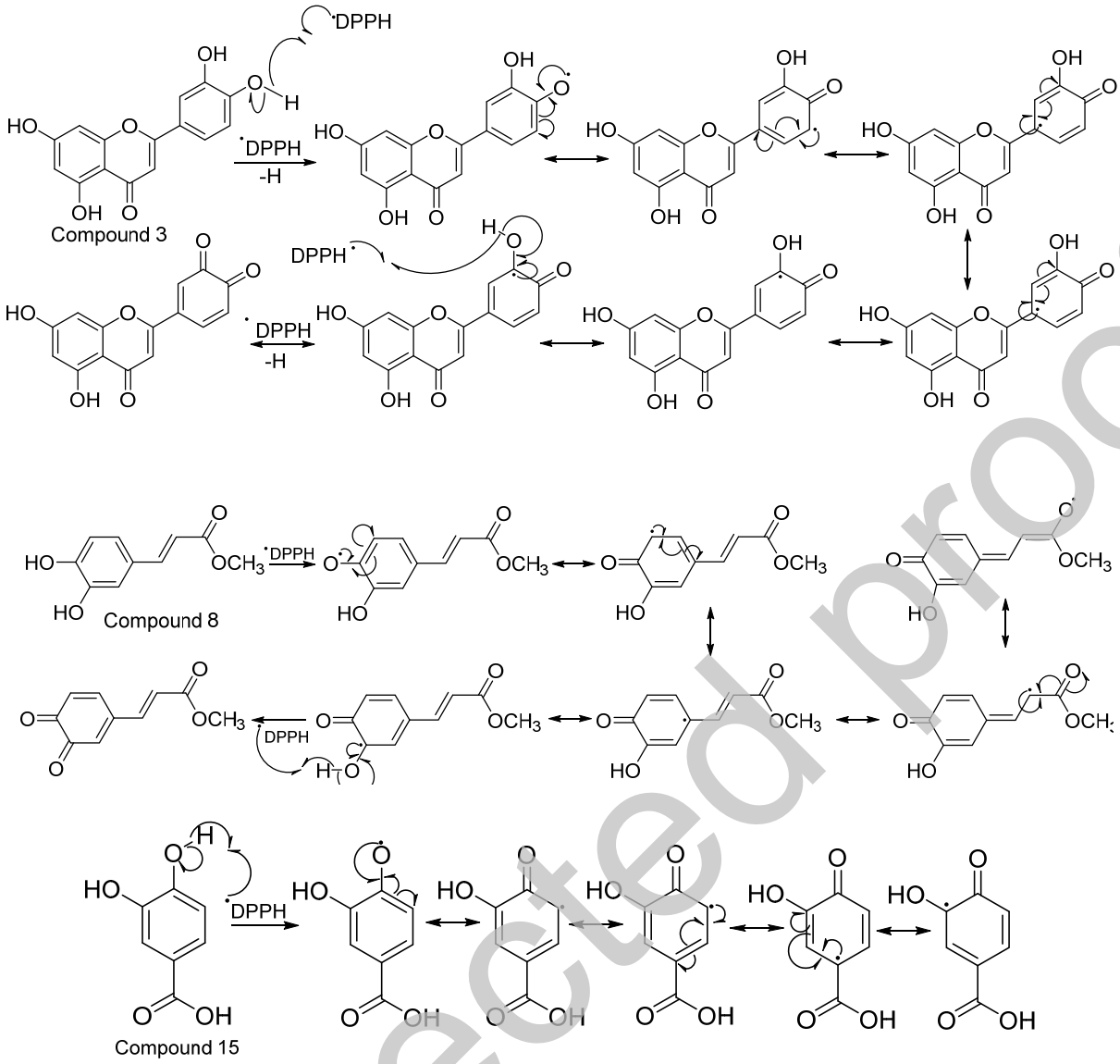


**Figure 4.** The reactions of free radical (DPPH·) scavenging with compound 3, 8 and 15.