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ORIGINAL ARTICLE

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 İzole Edilen Bileşenleri Üzerine Fitokimyasal Çalışmalar ve Antioksidan Aktiviteleri

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ABSTRACT

Objectives: The present study aimed to determine the phenolic compounds present in the water-soluble extracts of *Thymus munbyanus* subsp. *ciliatus* using high pressure liquid chromatography-time-of-flight mass spectrometry (MS). These phenolic compounds were further isolated and characterized for their antioxidant activities.

Materials and Methods: The aerial parts of *T. munbyanus* subsp. *ciliatus* were air dried, powdered, and extracted using water:methanol three times. The concentrated hydromethanolic extract was further dissolved in H_2O, filtered, and successively extracted using ethyl acetate, chloroform, and *n*-butanol. *T. munbyanus* extracts were further purified using column chromatography, and the purified extracts were subjected to *in vitro* antioxidant assays.

Results: Two previously undescribed compounds, namely methyl 2,3,5,6-tetrahydroxybenzoate and 4-hydroxy-5-methoxy-2-oxo-2H-pyran-3-carboxylic acid, and 14 known compounds, including 3 flavonoids; namely 3',5,5',7-tetrahydroxyflavanone, luteolin, and isorhamnetin-3-O-β-glucoside; a sterol glucoside named daucosterol; and 10 phenolic compounds, namely salicylic acid, ferulic acid, pluchoic acid, ethyl caffeate, methyl caffeate, protocatechuic acid, rosmarinic acid, p-coumaric acid, tyrosol, and protocatechuic aldehyde, were isolated from ethyl acetate and *n*-butanol extracts. The isolated compounds were characterized using 1D-2D $^{-1}H^{-13}C$ nuclear magnetic resonance and MS methods.

Conclusion: The compounds isolated from ethyl acetate and *n*-butanol extracts exhibited excellent antioxidant and 2,2-diphenyl-1-picrylhydrazyl scavenging activities. All these results highlighted the antioxidant potential of the isolated phenolic compounds and extracts, which could be further utilized for different pharmacological applications.

Key words: *Thymus munbyanus* subsp. *ciliatus*, phenolics, isolated compounds, antioxidant activity

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ÖZ

Amaç: Bu çalışmanın amacı, yüksek basınçlı sıvı kromatografisi-uçuş zamanlı-kütle spektrometresi (MS) kullanılarak *Thymus munbyanus* subsp. *ciliatus*'un suda çözünür ekstraktından fenolik bileşiklerin belirlenmesi, bileşiklerin izolasyonu, karakterizasyonu ve antioksidan aktivitelerinin belirlenmesidir.

Gereç ve Yöntemler: *Thymus munbyanus* subsp. *ciliatus* bitkisi küçük parçalar halinde kesilmiş ve üç kez metanol/su ile ekstre edilmiştir. Konsantre hidrometanolik ekstrakt saf su içerisinde tekrar çözünmüş; daha sonra süzülmüş ve art arda kloroform, etil asetat ve *n*-bütanol ile ekstre edilmiştir. *T. munbyanus* ekstreleri, kolon kromatografisi kullanılarak daha da saflaştırılmış ve saflaştırılmış ekstrelerde *in vitro* antioksidan deneyler yapılmıştır.

Bulgular: Önceden tanımlanmamış iki bileşik olan metil 2,3,5,6-tetrahidroksibenzoat ve 4-hidroksi-5-metoksi-2-okso-2H-piran-3-karboksilik asit ve 3 flavonoid dahil 14 bilinen bileşik; yani 3',5,5',7-tetrahidroksiflavanon, luteolin ve isorhamnetin-3-0-p-glukozit; daucosterol adlı bir sterol glikozit; ve etil asetat ve *n*-bütanol ekstraktlarından salisilik asit, ferulik asit, plukoik asit, etil kafeat, metil kafeat, protokateşik asit, rosmarinik asit, p-kumarik asit, tirosol ve protokateşik aldehit olmak üzere 10 fenolik bileşik izole edilmiştir. İzole edilen bileşikler, 1D-2D-1H-13C nükleer manyetik rezonans ve MS yöntemleri kullanılarak karakterize edilmiştir.

Sonuç: Etil asetat ve *n*-bütanol ekstraktlarından izole edilen bileşikler, mükemmel antioksidan ve 2,2-difenil-1-pikrilhidrazil süpürme aktiviteleri sergilemiştir. Tüm bu sonuçlar, farklı farmakolojik uygulamalar için daha fazla kullanılabilecek izole fenolik bileşiklerin ve ekstraktların antioksidan potansiyelini vurgulamıştır.

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

INTRODUCTION

Lamiaceae family of plants comprises of a variety of medicinal and aromatic plants. Among these, the members of *Thymus* genus are known to exhibit numerous biological activities, including antiviral, antifungal, and antioxidant activities.[1-3] *Thymus* genus mainly comprises of approximately 400 species of aromatic perennial plants and subshrubs, which grow mostly in Southern Europe, the Mediterranean region, North Africa, and Asia.[4] Among these, 15 species, including *Thymus munbyanus* subsp. *ciliatus* (Desf.) Greuter & Burdet, are known to be distributed in Algeria. This subspecies is also known as *Thymus ciliatus* (Desf.) Benth., and it is addressed as "Zaatar" in Arabic. Several previous studies have reported the volatile chemical composition (essential oil) and antioxidant activities of *T. munbyanus* extracts; however, no information regarding the phytochemical nature of *T. munbyanus* extracts is available.[5-11]

In this study, the secondary metabolites present in the aerial parts of *T. munbyanus* subsp. *ciliatus* were analyzed, isolated, and assessed for their *in vitro* antioxidant activities. To the best of our knowledge, this was the first study to identify and characterize compounds isolated from this plant.

MATERIALS AND METHODS

Chemicals and plant material

Most of the chemicals and reagents used in this study were purchased from Sigma (St. Louis, MO, USA). Some chemicals of analytical grade quality were procured from other commercial companies Roche (Darmstadt, Germany), Panreac Quimica (Spain) and MERCK (Germany). The aerial parts of *T. munbyanus* subsp. *ciliatus* were collected from Babor near Setif City, Algeria in May 2013. This plant was identified by Dr. W. Nouioua based on the information regarding Algerian flora.[12] A voucher specimen was deposited in the laboratory herbarium unit of the University of Constantine 1, VARENBIOMOL Research Unit (TC/123/05-13). The plant materials were stored at -20°C in a freezer until used for extraction.

Extraction and isolation of compounds

Air-dried parts (9.5 kg) of *T. munbyanus* subsp. *ciliatus* were powdered using a blender and extracted three times with MeOH/water (80/20, v/v). The hydromethanolic extracts were concentrated, dissolved in H_2O (1000 mL), and filtered. Further, the water-soluble part was extracted three times sequentially with chloroform, ethyl acetate, and *n*-butanol. Following extraction, chloroform (17.7 g), ethyl acetate (33 g), and *n*-butanol (59.2 g) extracts were collected.

Then, 32 g of ethyl acetate extract was fractionated using column chromatography, with a Sephadex LH-20 column. For elution, an isocratic system of $CHCl_3$/MeOH/hexane (7/2/1) was used and 26 fractions were collected. The precipitate from fraction 9 (155 mg) showed one spot contaminated with chlorophyll, which was washed with diethyl ether and acetone to give compound 5 (15 mg) and fraction 10 (2.5 g). The fractions were again fractionated using column chromatography, with a Sephadex LH-20 column, and elution was performed with an isocratic system of hexane/MeOH/$CHCl_3$ (1/2/7). This was subsequently followed by fractionation using a preparative column comprising silica gel, wherein elution with toluene/ethyl acetate/formic acid (10/6/1) yielded 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, and elution with toluene/ethyl acetate/formic acid (10/4/1) yielded compound 9 (1.7 mg) and compound 8 (7.3 mg), respectively. Further, fraction 14 (345 mg) was purified using a preparative column comprising silica gel, and elution with the same solvent mixture, at same ratio, resulted in the isolation of compounds 15 (5.5 mg), 12 (1.7 mg), and 1 (20 mg). Besides this, fraction 15 (3.12 g) was separated via column chromatography using a Sephadex LH-20 column, wherein samples were eluted isocratically with chloroform/methanol (7/3). This was followed by subsequent fractionation using a preparative silica gel, and elution with toluene/ethyl acetate/formic acid (10/2/1) yielded compounds 2 (3.2 mg), 3 (1.3 mg), 10 (3.4 mg), and 16 (9.1 mg).

The butanoic extract (10 g) was further subjected to fractionation on a polyamide column (SC6) with a gradient of toluene-

MeOH, which further increased solvent polarity and yielded 21 fractions. Further, fraction 5 (0.37 g) was subjected to column chromatography on a Sephadex LH-20 column using isocratic $CHCl_3$/MeOH (6/4), which yielded 10 subfractions. Subfraction 7 (61.2 mg) was purified using thin-layer chromatography with EtOAc/MeOH/H_2O (18/1/1) to yield compound 13 (11 mg). Additionally, fraction 6 (76.3 mg) was separated using column chromatography, with Sephadex LH-20 as a stationary phase and methanol as a solvent phase, which resulted in the isolation of compound 4 (17 mg).

High-pressure liquid chromatography coupled with time-of-flight mass spectrometry (HPLC-TOF/MS) analysis

Phenolic contents present in organic solvent extracts, i.e., chloroform, ethyl acetate, and *n*-butanol extracts, were analyzed using HPLC-TOF/MS. HPLC analysis was performed using an Agilent 1260 Infinity Binary System (Agilent Technologies, Santa Clara, CA, USA) coupled with a 6210 TOF LC/MS detector on a ZORBAX SB-C18 (4.6x100 mm, 3.5 µm) column. Ultrapure water with 0.1% formic acid was used in mobile phase A, whereas mobile phase B contained 100% acetonitrile. The chromatographic separation was performed as per the following gradient: 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. The column temperature was set at 35°C, and the injection volume was 10 µL. Retention times and m/z values were recorded for the phenolic compounds and in terms ofcompared with those of standard components. The crude extracts were dissolved in methanol at 25°C to obtain a concentration of 200 ppm and filtered using 0.45 µm PTFE filters.[13]

In vitro antioxidant assays

Total antioxidant capacity

To evaluate the antioxidant activities of the extracts and isolated compounds, ammonium phosphomolybdenum assay was performed, as previously described by Prieto et al.[14] To prepare reaction solutions, sample solutions were prepared at different concentrations, 25, 50, and 100 µg/mL (0.3 mL each), mixed with 3 mL of reagent solution (ammonium molybdate-sodium phosphate-sulfuric acid), and vortexed to obtain homogeneous solutions. Further, the closed tubes containing the reaction solutions were incubated in a hot water bath for 90 min. Following this, the mixtures were cooled in an ice bath, and the absorbance of each solution was measured at 695 nm using a ultraviolet-visible spectrophotometer (UV-Vis) (Thermo Scientific Evaluation Array UV-Vis Spectrophotometer). For blank, 0.3 mL of solvent was used.

Free radical scavenging capacity

The scavenging activity of the samples was evaluated according to the method previously reported by Blois.[15] In the present study, 1.5 mL of different concentrations of extracts and isolated compounds were mixed with 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\cdot}$) solution (0.5 mL) in a test tube to get a homogeneous solution. Each mixture was incubated at 25°C for 30 min in a dark environment. The absorbance of resulting solutions was measured at 517 nm using a UV-Vis spectrophotometer (Thermo Scientific Evaluation Array UV-Vis spectrophotometer). The scavenging activity was calculated as per the following formula:

% activity = $[(A_{1\,(517\ nm)} - A_{2\,(517\ nm)}) / A_{1\,(517\ nm)}] \times 100$; where A_1 denotes control absorbance and A_2 denotes absorbance of the sample.

Statistical analysis

The antioxidant activity of the samples was evaluated in triplicates, and the results were presented as an average of the three experiments. Data were analyzed using SPSS 20.0 software, and $p<0.05$ was considered to be statistically significant.

RESULTS AND DISCUSSION

Characterization of isolated compounds

The present study aimed to detect, isolate, and characterize the phenolic compounds present in the aerial extracts of *T. munbyanus* subsp. *ciliatus*. Two previously undescribed compounds, namely methyl 2,3,5,6-tetrahydroxybenzoate (1) and 4-hydroxy-5- methoxy-2-oxo-2H-pyran-3-carboxylic acid (16), and 14 known compounds, including 3 flavonoids; 3',5,5',7-tetrahydroxyflavanone (2), luteolin (3), and isorhamnetin-3-O-β-glucoside (4); a sterol glucoside named daucosterol (5); and 11 phenolic compounds, namely 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), and protocatechuic aldehyde (15), were isolated from *n*-butanol and ethyl acetate extracts of *T. munbyanus* subsp. *ciliatus*. The structures of all compounds isolated from *T. munbyanus* are shown in Figure 1.

Compound (1) was obtained as colorless amorphous powder. The molecular formula of compound 1 was determined to be $C_8H_8O_6$ using EI-MS with negative ion m/z 198.08 $(M-H)^-$, which indicated that the molecule had five degrees of unsaturation. The ^{1}H- nuclear magnetic resonance (NMR) spectrum in acetone-d6 exhibited just two singlets: first at δ_H 7.33 (s, 1H, H-4), which indicated the presence of penta substituted benzene ring, and second at δ_H 3.88 (s, 3H), corresponding to the presence of methoxy groups.

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

A long-range C-H correlation was observed for C-4 aromatic proton in the HMBC spectrum ($^2J_{CH}$ and $^3J_{CH}$ correlations) with C-3/C-5 and C-2/C-6. The structure was further confirmed by the HMBC spectrum. Thus, compound 1 was identified as methyl 2,3,5,6-tetrahydroxybenzoate.

Compounds 2-15 have been previously isolated and characterized, and data for these compounds are given in Supplementary Information.

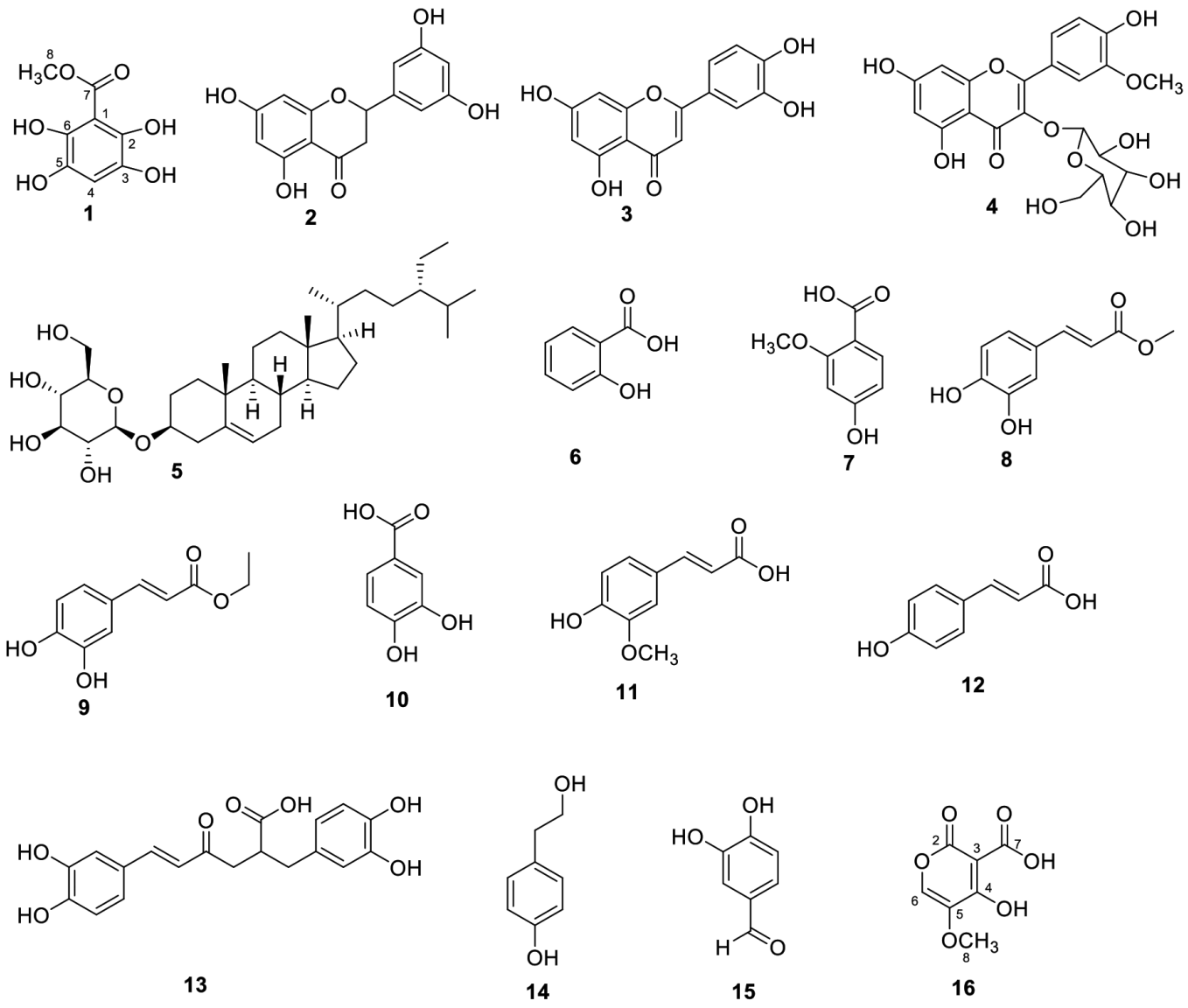

Figure 1. Structures of compounds isolated from *Thymus munbyanus* subsp. *ciliatus* as methyl 2,3,5,6-tetrahydroxybenzoate (1), 3',5,5',7-tetrahydroxyflavanone (2), luteolin (3), isorhamnetin-3-O-β-glucoside (4), daucosterol (5), salicylic acid (6), pluchoic acid (7), methyl caffeate (8), ethyl caffeate (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-2H-pyran-3-carboxylic acid (16)

Compound 16 was obtained as a yellowish-brown solid. The molecular formula for this compound was found to be $C_7H_6O_6$, which suggested the presence of five degrees of unsaturation. The ^{13}C NMR and DEPT spectra further confirmed the presence of seven carbon atoms that included one methoxy group at δ_c 55.31; four vinyl carbons with one methine at δ_c 106.57 (C-6); quaternary carbon atoms at δ_c 128.11 (C-3), 137.54 (C-5), and 146.91 (C-4); and the carbonyl region with two peaks at δ_c 173.81 and 168.83, which were indicative of the presence of a carbonyl carbon of the carboxylic acid and carbonyl carbon of the α-pyrone ring, respectively. The ^1H NMR and HSQC spectra further confirmed the hypothesis. In particular, it displayed one singlet of one proton at δ_H 7.33 that was assigned to H-6. Besides this, three proton singlets were reported at δ_H 3.87 for OCH_3. The HMBC measurements showed long-range correlations between the protons at δ_H 7.33 and two quaternary carbons at δ_c 137.54 (C-5) and δ_c 146.91 (C-4). The HMBC experiments also showed connectivity between the methoxy protons at δ_H 3.87 and the quaternary carbon at δ_c 146.91 (C-4). These findings indicated that the carboxylic acid was connected to C-3, whereas a methoxy functional group was connected to C-5. Therefore, compound 16 was assigned the structure of 4-hydroxy-5-methoxy-2-oxo-2H-pyran-3-carboxylic acid.

Quantification of the polar constituents of T. munbyanus subsp. ciliatus extracts

The extracts of *T. munbyanus* were analyzed using HPLC-TOF/MS. The compounds isolated from the extracts were identified in terms of their retention times and m/z values, and these values were compared with those of standard samples. Altogether,

the results of spectral analysis revealed the presence of 29 compounds, including 11 phenolic acids and 18 flavonoids and phenolics (Table 1). Interestingly, the concentration of these compounds was found to be very less in the chloroform extract. The highest concentrations of scutellarin, baicalin, and fumaric acid were reported in the butanoic extract; however, these compounds were present in very low amounts in the ethyl acetate extract. Whereas, quercetin-3-β-D-glucoside, caffeic, and 4-hydroxybenzoic acid were found to be the major consitutents of the ethyl acetate extract.

Thus, these results highlight that the analyzed extracts of *T. munbyanus* subsp. *ciliatus*, i.e., ethyl acetate and butanoic extracts, comprised of complex mixtures of plant secondary metabolites. These active ingredients, particularly flavonoids and phenolic acids, are previously known to exhibit antioxidant properties.[16,17]

Total antioxidant capacity

The antioxidant activities of the isolated and extracted samples were measured and expressed as absorbance values. This assay involved the reduction of $Mo^{+6} \rightarrow Mo^{+5}$, and the antioxidant activity was confirmed if a green phosphate/Mo^{5+} complex was formed, which showed maximum absorption at 695 nm at acidic pH.[14] This assay has been widely used to evaluate the total antioxidant capacity of various extracts and isolates. The results for total antioxidant activity of the isolates and extracts obtained from *T. munbyanus* subsp. *ciliatus* are shown in Figure 2. Here, higher absorbance of the antioxidant denoted the presence of antioxidant property. It was observed that ethyl acetate extract and compound 8 exhibited similar antioxidant activities at concentrations of 25, 50, and 100 µg/mL as compared to butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), $p<0.05$. Phenolic compounds have long been explored as a therapeutic option to prevent cancer, owing to its effective antioxidant potency.[18] It has also been reported that compounds having antioxidant activity play an important role in the inhibition of lipid peroxidation.[19] These results highlighted that the isolated compounds and different solvent extracts, obtained from *T. munbyanus*, acted as promising natural sources of nontoxic and natural antioxidants.

Free radical scavenging activity

DPPH˙ free radical scavenging assay is generally applied to determine the antioxidant levels of extracts in a relatively short period of time. In particular, the ability of compounds to deliver hydrogen is assessed. The utility of this assay has been established and compared with that of other experiments.[20] It has been shown that natural chemicals reduce DPPH˙ due to their ability to donate hydrogen atom.[21] When an antioxidant and DPPH˙ (a synthetic radical) are mixed, the antioxidant gives an electron to $DPPH_2$ and the purple color is converted into yellow color.

Interestingly, the free radical scavenging activitiy of natural contents was found to increase in a dose-dependent manner, $p<0.05$. In particular, compounds 2, 7, 8, 10, and 15 displayed a higher scavenging activity at high doses than BHA and BHT (Figure 3). This might be attributed to two different -OH substitutions that favor DPPH˙ scavenging activity.[22] The stabilization of radicals by two subsequent -OH substitutions of phenolic groups for compounds 3, 8, and 15 are shown in Figure 4. In particular, compounds 3, 8, and 15 showed their ability to remove radicals, which is attributed to their resonance stability and presence of hydroxyl groups. It can act as a radical inhibitor and stop the radical reaction. Plant

Table 1. Phenolic compounds isoalted from *Thymus munbyanus* subsp. *ciliatus* extracts, as determined using high-pressure liquid chromatography coupled with time-of-flight mass spectrometry

Phenolic compounds, mg of phenolic compound/kg	$CHCl_3$ extract	Ethyl acetate extract	*n*-butanol 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

tr: Trace; nd: Not detected

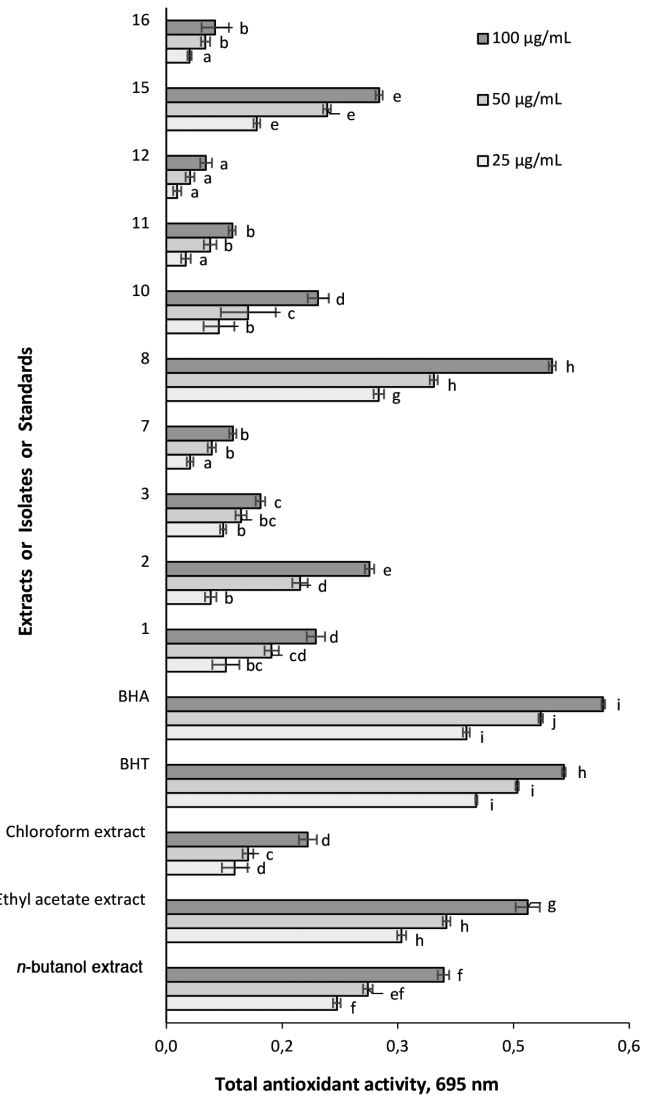

Figure 2. Total antioxidant activity of isolated and extracted samples
BHA: Butylated hydroxyanisole, BHT: Butylated hydroxytoluene

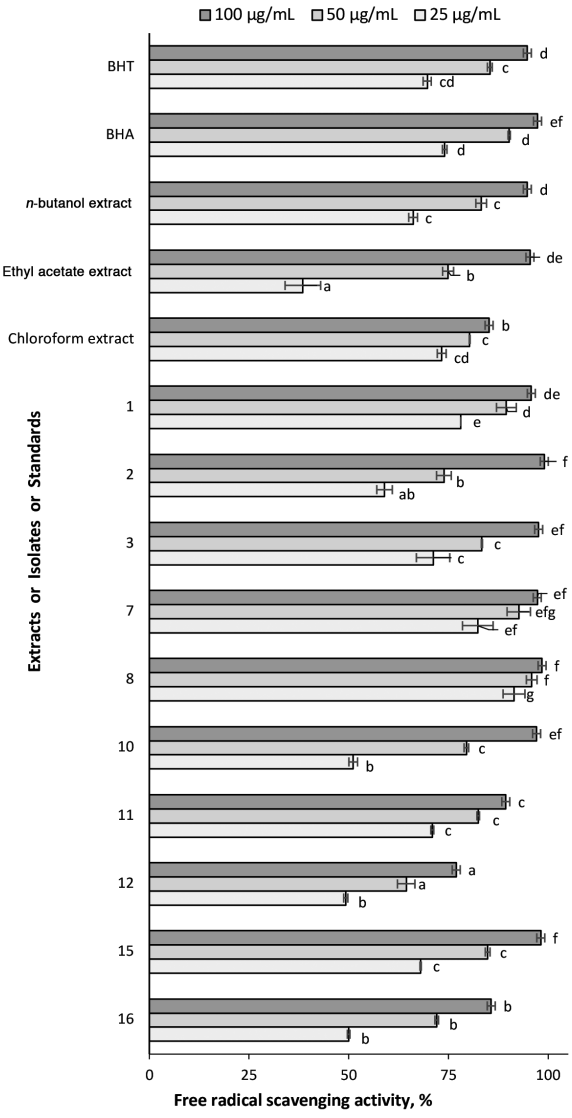

Figure 3. Free radical (DPPH˙) scavenging activities of isolates and extracts
BHA: Butylated hydroxyanisole, BHT: Butylated hydroxytoluene

phenolics generally include superoxide radicals, lipid alkoxyl radicals, lipid peroxyl radicals, and nitric oxide radical, which are known to exhibit cleansing, metal chelating, antiallergic, estrogenic, and antiviral effects.[23] In general, polymeric polyphenols are known to be more effective antioxidants than monomeric phenolics.[24] The presence of -OH group in the ortho or para position of phenol increases the antioxidant activity of a compound.

CONCLUSION

This is the first study to report the isolation and characterization of phenolic compounds from *T. munbyanus* subsp. *ciliatus* species grown in Algeria. In particular, the study reported the isolation of two new compounds, named methyl 2,3,5,6-tetrahydroxybenzoate and 4-hydroxy-5-methoxy-2-oxo-2H-pyran-3-carboxylic acid, and 14 previously known compounds that belonged to different chemical classes, namely flavonoids, sterol, and phenolic derivatives. The ethyl acetate extract exhibited excellent free radical scavenging activity *in vitro*, which correlated well with the presence of polyphenol derivatives in compounds 3, 8, and 15.

Thus, all these findings provide scientific basis for the use of *T. munbyanus* subsp. *ciliatus* derivatives as functional ingredients in Algerian traditional medicine.

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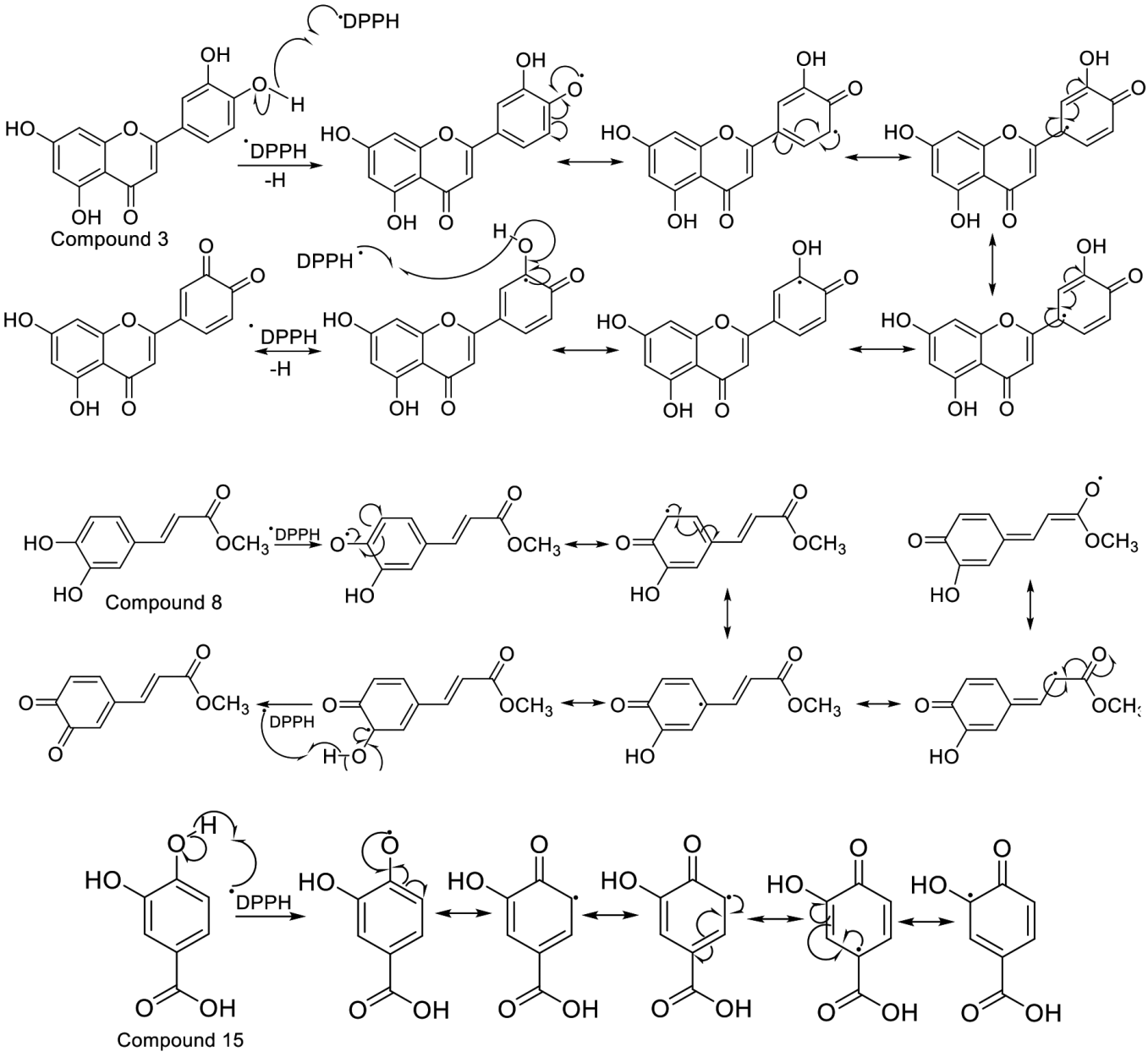

Figure 4. Free radical (DPPH˙) scavenging reaction for compounds 3, 8, and 15

Conflict of interest: No conflict of interest was declared by the authors. The authors are solely responsible for the content and writing of this paper.

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