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Chemical Constituents of Two Endemic *Sideritis* Species from Turkey with Antioxidant Activity

Sema Çarıkçı[1],Turgut Kılıç[1], Akın Azizoğlu[1] and Gülaçtı Topçu[2*]

[1] *Balıkesir University, Faculty of Arts and Science, Department of Chemistry, 10145,Balıkesir, Türkiye*

[2] *Bezmialem Vakıf University, Faculty of Pharmacy, Deparment of Pharmacognosy 34093, Fatih-Istanbul, Türkiye*

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Abstract: In this study, two *Sideritis* species, endemic to Turkey, *S. niveotomentosa* Huber – Morathii, *S. brevidens* P.H. Davis have been studied for their diterpenic compounds and the antioxidant properties. Eight known diterpenoids, which have *ent*-kaurene skeleton, were isolated from acetone and methanol extracts of these species. The structures of the isolated diterpenes were determined by using the NMR (^{1}H-NMR, ^{13}C-NMR, COSY, HMQC, and HMBC) spectroscopy. The analysis of the phenolic compounds of the extracts was performed by high performance liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). Also the antioxidant capacity of the extracts was investigated namely by two methods; free radical scavenging and β-carotene bleaching activities.

Keywords: Lamiaceae; *Sideritis niveotomentosa*; *Sideritis brevidens*; diterpenoids; kaurane; antioxidant potential.

1. Introduction

A derivate of the Greek word “Sideron” was used to name the genus *Sideritis*, due to its alleged ability to aid in curing wounds caused by iron blades [1]. *Sideritis* L. belongs to the family of Lamiaceae (Labiatae) which is one of the most common and diverse plants of the world. Over 150 species of the genus *Sideritis* are mainly found in the Mediterranean area [2]. There are 44 *Sideritis* species (55 taxa) in Turkey and, endemism rate of this genus is high (almost 80%) [3]. The Aerial parts of *Sideritis* species have been widely used in folk medicine to threat some diseases such as cough, common cold, gastrointestinal disorders [3], and their constituents showed antiseptic, anti-inflammatory, anti-rheumatic, antimicrobial activities and insecticidal properties [4,5], and therefore, the species are used as herbal tea in Turkey as well as in the other Mediterranean countries [6].The essential oil composition of *S. niveotomentosa* and *S. brevidense* were reported from the flora of Turkey. The main components of the essential oils were determined as *β*-pinene and *β*-caryophyllene, which are the main chemotaxonomic marker of the genus of *Sideritis* [7]. On the diterpenoids of *S. niveotomentosa* and *S. brevidens,* a single study was found in the literature

* Corresponding author: E-Mail: gulacti_topcu@yahoo.com; Phone: +90-212-4531823 *Fax:* +90-212-5332326

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[8]. Only the diterpenoids linearol, epicandicandiol, foliol and sidol which are the main components of the *Sideritis* genus, were reported from the species.

In our previous studies on the species of *Sideritis,* eight new and twenty-five known diterpenoids have been reported [5, 9-19]. In continuation of our studies, we have identified the diterpenoid and phenolic constituents of *Sideritis niveotomentosa* Huber – Morathii and *S. brevidens* P.H. Davis, and assessed their antioxidant capacity.

2. Materials and Methods

2.1. Plant material

The aerial parts of both plants were collected from Mersin which is located in south of Turkey. *Sideritis niveotomentosa* collected from Sertavul subway between Mut and Karaman at 1600 m altitude, while *S. brevidens* was collected between Gülnar and Mut, twenty kilometers from Gülnar. The species were identified by Dr. Tuncay Dirmenci, at Balıkesir University. Voucher specimens were deposited at the Herbarium of Faculty of Education, Balıkesir University, Balıkesir, Turkey (TD 3266, TD 3264-b, respectively).

2.2. General

^{1}H- and ^{13}C-NMR spectra were obtained in $CDCl_3$ at 600 and 150 MHz, respectively, using a Varian 600 NMR, HMQC and HMBC experiments were recorded on the same spectrometer, using the standard pulse sequence programs. The mass measurements were obtained on a Thermo Polaris Q Ion Trap Mass Spectrometry LC-MS/MS. The LC-MS/MS measurements were performed on Zivak® HPLC and Zivak® Tandem Gold Triple quadrupole mass spectrometry. Flash chromatography was performed using Silica gel Merck 60 (70-230 mesh), Preparative TLC, and Merck Silica gel 60 F_{254} 20x20 cm Aluminum sheets.

2.3. Extraction and isolation

The ground aerial parts of *Sideritis* species were shade-dried and cut into small pieces, and extracted with acetone and methanol respectively, for two weeks. *Sideritis niveotomentosa* gave 31.7 g of acetone extract and 97.0 g of methanol extract (dry plant weight is 1.75 kg, yields are 1.81 and 5.54 %,respectively), and *S. brevidens* afforded 50 g of acetone and 93 g of methanol extracts (dry plant weight is 1.50kg, yields are 3.33 and 6.20 %, respectively).

The crude extract was adsorbed on silicagel (Silicagel 60) and subjected to preparative column chromatography using same adsorbent (Silicagel 60) on the column. Elution was started with hexane and continued with gradients of dichloromethane, acetone and methanol. Fractions were controlled via TLC techniques and similar fractions were combined. These fractions were subjected to further mini column chromatography, controlled via TLC again.

For purification of the isolated diterpenoids, preparative TLC on pre-coated silica gel F_{254} aluminum plates was applied and the following solvent systems were used: Compound **1,** from *S. niveotomentosa* (185.0 mg), from *S. brevidens* (24.3 mg) on the solvent system CH_2Cl_2: Acetone (90:10; 95:5; v/v); both acetone and methanol extracts of the plants. Compound **2**, from the acetone extract of *S. niveotomentosa* (4.5 mg) on CH_2Cl_2: Acetone (90:10; v/v), compound **3,** from *S. niveotomentosa* (3.6 mg), compound **4** from *S. niveotomentosa* (4.0 mg) and *S. brevidens* (7.5 mg) CH_2Cl_2: Acetone (85:15; 83:17; v/v); from acetone extracts of both plants. Compound **5,** from the acetone extract of *S. niveotomentosa* (2.3 mg); CH_2Cl_2: Acetone (70:30; v/v), compound **6**, from the acetone extract of *S. niveotomentosa* (3.1 mg); CH_2Cl_2: Acetone (70:30; v/v). Compound **7** from the acetone extract of *S. niveotomentosa* (2.1 mg); $CHCl_3$: Ethylacetate (60:40; v/v); and (**8**), from the acetone extract of *S. brevidens* (690.0 mg); CH_2Cl_2: Acetone (83:17; v/v).

2.4. Antioxidant activity

2.4.1. Free-radical-scavenging activity

The free-radical-scavenging activity of the extracts was determined by the $DPPH^{\cdot}$ assay as described by M. S. Bloiss [20]. In the radical form, $DPPH^{\cdot}$ absorbs at 517 nm, but upon reduction by an antioxidant or a radical species its absorption decreases. Briefly, 0.1 mM solution of $DPPH^{\cdot}$ in methanol was prepared and 4 mL of this solution was added to 1 mL of sample solution in methanol at different concentrations. Thirty minutes later, the absorbance was measured at 517 nm. Lower absorbance of the reaction mixture indicates higher free radical scavenging activity. The capability to scavenge the DPPH radical was calculated using the following equation:

$$DPPH^{\cdot} \text{ Scavenging Effect} = [(A_{control} - A_{sample}) / A_{control}] \times 100$$

2.4.2. Determination of the antioxidant activity with the β-carotene bleaching method

The antioxidant activity of the extracts was evaluated using the β-carotene-linoleic acid model system [21]. β- Carotene (0.5 mg) in 1 mL of chloroform was added to 25 µL of linoleic acid, and 200 mg of Tween 40 emulsifier mixture. After evaporation of chloroform under vacuum, 100 mL of distilled water saturated with oxygen, was added by vigorous shaking. Four thousand microlitres of this mixture were transferred into different test tubes containing different concentrations of the sample. As soon as the emulsion was added to each tube, the zero time absorbance was measured at 470 nm using a spectrophotometer. The emulsion system was incubated for 2 h at 50°C. A blank, devoid of β-carotene, was prepared for background subtraction. Butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and α-tocopherol (α-TOC) were used as standards.

2.5. LC-MS/MS measurements

2.5.1. Chemicals

The following compounds were used as standards in LC–MS/MS analysis: ascorbicacid (99%, Sigma–Aldrich), caffeicacid (98%, Sigma–Aldrich), catechol (99% Sigma–Aldrich), ellagicacid (95%, Fluka), ferulicacid (98% Sigma–Aldrich), gallicacid (98%, Sigma–Aldrich), p-coumaricacid (98%, Sigma–Aldrich), p-hydroxybenzoicacid (99%, Merck), pyragallol (98%, Sigma–Aldrich), quercetin (98%, Sigma–Aldrich), syringicacid (97%, Fluka), vanillin (99% Merck), α-tocopherol (98%, Fluka)

Stock solutions were prepared as 5 mg/L in ethanol, except for catechol and ascorbic acid, which were prepared as 50 and 25 mg/L, respectively, in the same solvent. ^{13}C Labelled benzoic acid (98%) and HPLC grade methanol were purchased from Merck (Darmstadt, Germany). Calibration solutions were prepared in ethanol–water (50:50, v/v) in a linear range (Table 1). Dilutions were performed using automatic pipettes and glass volumetric flasks (A class), which were stored at -20 °C in glass containers. Thousand micrograms per liter curcumin solution was freshly prepared, from which 100 µL was used as an Internal Standard (IS) in all LC–MS/MS experiments.

The compounds used for antioxidant activity such as 1,1-diphenyl- 2-picryl-hydrazyl ($DPPH^{\cdot}$), β-carotene, linoleic acid and methanol were obtained from Sigma (Sigma–Aldrich GmbH, Steinheim, Germany). All other chemicals used were of analytical grade and obtained from either Sigma–Aldrich or Merck.

2.5.2. Preparation of test solution and LC–MS/MS conditions

A hundred milligram of each extract was dissolved in 5 mL of ethanol–water (50:50 v/v) in a volumetric flask, from which 1 mL was transferred into another 5 mL of volumetric flask. The detailed description of method was given in literature [22]. Experiments were performed by a Zivak® HPLC and Zivak® Tandem Gold Triple quadrupole (Istanbul, Turkey) mass spectrometer equipped with a Macherey–Nagel Nucleoder C18 Gravity column (125 x 2 mm i.d., 5 µm particle size). Since optimization of HPLC methods and LC–MS/MS procedure have

already done in our previous studies showed that ionization by ESI source is better than APCI source for this kind of small and relatively polar molecules [22]. The optimum MS paramaters are given in supporting information.The mobile phase was composed of methanol (A, 0.1% formic acid) and water (B, 0.1% formic acid), the gradient programme of which was 0–3.00 min 100% B, 3.01– 13.00 min 30 % A - 70 % B and finally 13.01–20.00 min 100% B. The flow rate of the mobile phase was 0.3 mL/min, and the column temperature was set to 25 ^{o}C. The injection volume was 10 µL.

2.6. Validation

In validation experiments of all the compounds, ^{13}C *p*-hydroxybenzoic acid was used as an internal standard. The validation was done according to the following parameters such as linearity, recovery, repeatability, LOD and LOQ experiments.

2.6.1. Linearity

The linearity of the reported LC-MS/MS method for compounds **11-22** was assessed by analyzing of standard solutions. The linearity ranges and correlation coefficients (r^2) of each individual compounds are given in supporting information.

2.6.2. Recovery, repeatability and precision

The recovery of the experiments was determined by three fortification levels. The detailed information for the recovery, repeatability and precision evaluation was given in our previous study [22]. The recoveries of the method for related compounds were evaluated for each fortification level employing the following formula. The recoveries were found to be in the range of 98% to 101.5 %.

Recovery (100%) = [Measured concentration - Endogenous concent.)/Spiked concentration] x 100

2.6.3. LOD and LOQ

LOD and LOQ of the LC–MS/MS methods for the reported compounds were given in supporting information. The limits of the quantification (LOQs) were assigned to be 10 x LOD.

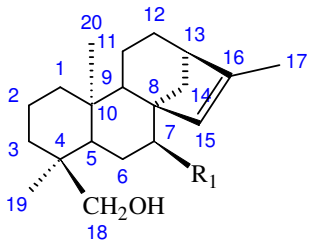
2.7. Estimation of uncertainty

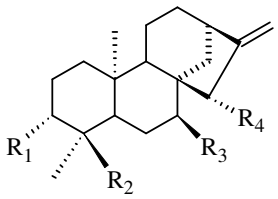
Identification of uncertainty sources and the calculation of uncertainties of each compound by LC-MS/MS were described in the literature [22-23]. The sources and quantification of the uncertainty for the applied method were evaluated and calculated by using EURACHEM/CITAC Guide, 2000[24]. The sources of uncertainty of experiments were assigned as the impurity of reference standard, the sample weighing, calibration curve and dilution of the solutions. Detailed procedures of uncertainty evaluation have been previously reported in the literature [22,25]. The percent relative uncertainties [U_{95}(%)] of the reported compounds were found range between 0.6 % and 6.5 % at 95% confidence level (k: 2) (Table 1).

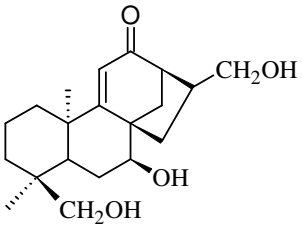
3. Results and Discussion

Eight kaurane diterpenoids were isolated from *S. niveotomentosa* Huber-Morathii and *S. brevidens* P.H. Davis. Structures of these diterpenoids were confirmed as (**1**) siderol (*ent*-7α-acetyl-18-hydroxykaur-15-ene) [26-27], (**2**) sideridiol (*ent*-7α,18-dihydroxykaur-15-ene) [28], (**3**) 7-epicandicandiol (*ent*-7α-,18-dihydroxykaur-16-ene) [29,30], (**4**) sidol (*ent*-3β-acetyl-7α,18-dihydroxykaur-16-ene) [31, 32], (**5**) eubotriol (*ent*-7α,15β,18-

trihydroxykaur-16-ene) [33], (**6**) eubol (*ent*-7α-acetyl-15β,18-dihydroxykaur-16-ene) [32], (**7**) athonolone (*ent*-7α,17,18-trihydroxy-9,(11)-en-12-one) [9], (**8**) linearol (*ent*-3β,7α-dihydroxy-18-acetylkaur-16-ene) [31, 33] (Figure 1).







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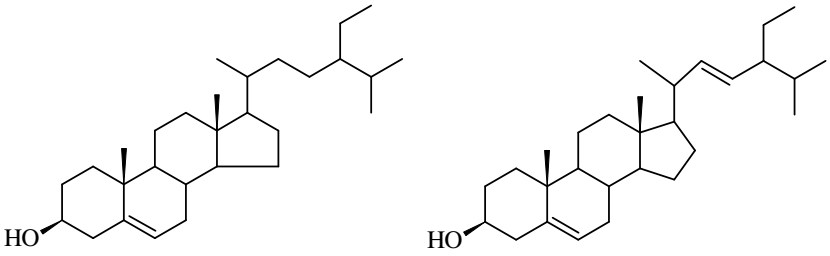
	R$_1$
1	OAc
2	OH

	R$_1$	**R$_2$**	**R$_3$**	**R$_4$**
3	H	CH_2OH	OH	H
4	OAc	CH_2OH	OH	H
5	H	CH_2OH	OH	OH
6	H	CH_2OH	OAc	OH
8	OH	CH_2OAc	OH	H

Figure 1. Structures of the isolated diterpenoids

While siderol was isolated from all species, linearol was isolated only from *S. brevidens* (690 mg). When regarding our previous studies, we have obtained the linearol as a main component only from *S. athoa* [9]. Siderol was isolated from *S. niveotomentosa* as a main compound (185 mg). The rest of the isolated compounds were in small amounts in both species. In contrast to, Spanish ad Italian *Sideritis* species, 95 % of isolated diterpenoids from *Sideritis* species of Anatolia are kaurane diterpenoids [5]. However, the studies carried out on the collected *Sideritis* plants from other Mediterranean countries, labdane and pimaranes were reported as the main compounds. The present study supports this information. Labdane and pimarane type of diterpenoids were not previously isolated from *S. niveotomentosa* or *S. brevidens*. Apart from the diterpenoids of species further steroidal compounds isolated such as, β-sitosterol (**9**) and stigmasterol (**10**) (Figure 2). These steroids are fairly common all Lamiaceae species.



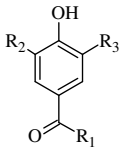
9 **10**

Figure 2. Structures of the isolated steroids

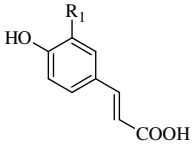
Phenolic acids and flavonoids have been implicated to many reported bioactivities [22]. The existence of antioxidants compounds in plants is important, and those compounds play crucial roles in the scavenging and inhibition of free radicals. So, investigation and discovery of new sources of these compounds have become important. The content of phenolic compounds in the extracts of *S. niveotomentosa* and *S. brevidens* was determined by LC/MS-MS and the amounts of compounds (mg/ 100g extract) summarized in Table 1.

Table 1. The amount of phenolic compounds determined by LC-MS/MS in acetone extract of the *Sideritis* species (mg/100 g extract)

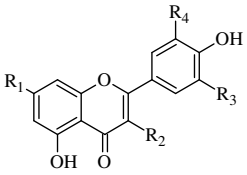
Phenolics	***S.niveotomentosa***	***S.brevidens***	**U*95*± %**
p-Hydroxybenzoic acid (**11**)	605.2	614.1	0.6
Vanillin (**12**)	44.2	44.2	2.4
p-Coumaric acid (**13**)	141.4	153.0	3.1
Gallic acid (**14**)	47.5	46.2	2.8
Caffeic acid (**15**)	42.8	40.1	6.5
Ferulic acid (**16**)	68.1	66.2	6.1
Ellagic acid (**17**)	<LOD	37.1	2.4
Syringic acid (**18**)	52.4	53.0	1.8
Quercetin (**19**)	<LOD	16.2	2.3
Apigenin (**20**)	81.2	88.2	1.7
Kaempferol 3-*O*-glucoside (**21**)	123.4	122.1	2.8
Luteolin 7-*O*-glucoside (**22**)	102.6	90.2	1.4



	R_1	R_2	R_3
11	OH	H	H
12	H	OCH_3	H
14	OH	OH	OH
18	OH	OCH_3	OCH_3



	R_1
13	H
15	OH
16	OCH_3



	R_1	R_2	R_3	R_4
19	OH	OH	H	OH
20	OH	H	H	H
21	OH	OGlucoside	H	H
22	OGlucoside	H	OH	H

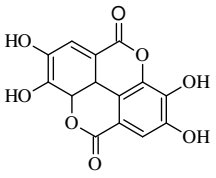


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Figure 3. Structures of the determined phenolic compounds by LC-MS/MS

Antioxidant activity tests were carried out by the $DPPH^{\cdot}$ free radical and lipid peroxidation inhibitory activity. Both of species have similar results, and they showed weak activity. The results are given in Table 2.

Table 2. Antioxidant Activity Results [a]

Samples	$DPPH^{\cdot}$ assay IC_{50} (µg/mL)	β- Carotene-linoleic acid assay IC_{50} (µg/mL)
Acetone extract of *Sideritis niveotomentosa*	50.98 ± 0.57	148.60 ± 1.15
Methanol extract of *Sideritis niveotomentosa*	42.04 ± 0.22	245.96 ± 3.13
Acetone extract of *Sideritis brevidens*	56.26 ± 0.61	107.10 ± 1.04
Methanol extract of *Sideritis brevidens*	41.75 ± 0.54	196.84 ± 2.12
α- TOC[b]	25.35 ± 0.10	2.89 ±0.01
BHT[b]	41.77 ± 1.1	4.43 ±0.07
BHA[b]	15.36 ± 0.06	5.11 ± 0.09

[a] IC50 values represent the means ± standart deviation of three parallel measurement ($p<0.05$)
[b] Reference compound

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Supporting Information

Supporting Information accompanies this paper on http://www.acgpubs.org/RNP

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