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Ent-kaurane diterpenoids isolated from *Sideritis congesta*

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ABSTRACT

A new *ent*-kaurane diterpenoid, together with eight known *ent*-kauranes, were isolated from the petroleum ether and acetone extracts of the whole plant of *Sideritis congesta* P.H. Davis & Hub.-Mor. and their structures were elucidated as the new compound *ent*-7α-acetoxy-16β,18-dihydroxy-kaurane (7-acetyldistanol) (1) and the known compounds *ent*-3β,7α-dihydroxy,18-acetoxy-15β,16β-epoxykaurane (epoxyisolinearol) (2), sideroxol (3), sideridiol (4), siderol (5), 7-epicandicandiol (6), foliol (7), linearol (8) and sidol (9). Characterization of compounds 1–9 was based on spectral analyses and comparison with reported data, particularly the new compound 1 was identified by 1D- and 2D-NMR and mass spectroscopic analyses. The antioxidant potential of the extracts, and also of the *ent*-kauranes except for 7, was investigated by three methods including β-carotene bleaching method, free radical scavenging activity and superoxide anion scavenging activity. The anticholinesterase activity was also evaluated for the *ent*-kauranes except for 7, and most of the diterpenes exhibited weak acetylcholinesterase inhibitory activity. However, almost all diterpenes exhibited some inhibitory activity against butyrylcholinesterase; particularly, compounds 3 and 6 exhibited better BChE inhibitory activity than the standard compound galanthamine.

1. Introduction

As a member of the Lamiaceae plant family the *Sideritis* genus is represented by about 150 species in the world occurring mainly in the Mediterranean region. The *Sideritis* genus is represented by 55 taxa in Turkey (Duman, 2000; Mill, 1982). *Sideritis* plant extracts and constituents were found to have anti-inflammatory, antirheumatic, antiulcer, insecticidal, antifeedant, antimicrobial, antioxidant and cytotoxic activities (Topçu and Gören, 2007). *Sideritis* species are commonly used for medicinal purposes and also as herbal teas and flavouring agents in Turkey. In rural areas they are even more favoured than the *Salvia* species, as teas. As a continuation of our studies towards the isolation of biologically active compounds from the *Sideritis* species, *Sideritis congesta* which is endemic to Anatolia was investigated and a new *ent*-kaurane diterpenoid together with eight known *ent*-kauranes were isolated. This paper describes the isolation and characterization of 1 and the biological activity evaluation of the extracts and the isolated compounds, except for foliol (7).

2. Results and discussion

Compound 1 was obtained as a white crystalline solid. It showed a molecular ion peak at *m/z* 364.2825 (calculated 364.2834) in the HR-EIMS. The resulting molecular formula was determined to be $C_{22}H_{36}O_4$, representing five degrees of unsaturation.

The ^{13}C (APT) NMR spectrum confirmed the presence of 22 carbons consisting of four methyls, nine methylenes, four methines and five quaternary carbons (Table 1). The ^{1}H NMR spectrum exhibited four methyl signals at δ 0.71 (s, 3H), δ 1.07 (s, 3H), and δ 1.36 (s, 3H, corresponding to a methyl neighboring an oxygenated moiety), and δ 2.02 (s, 3H, an acetyl methyl function). By the gHSQC experiment these were assigned to C-19, C-20, C-17 and an acetyl methyl, respectively. The ^{13}C NMR signals at δ_C 80.4, 79.0 and 71.3 indicated the presence of three distinct oxygenated carbons. The signal at δ_C 71.3 belongs to a hydroxymethylene. An AB system was observed at δ 3.00 (d, J = 10.28, H-18$_a$) and δ 3.26 (d, J = 10.28, H-18$_b$) characteristic of the hydroxymethylene group, probably at C-4. The COSY spectrum demonstrated a correlation between δ 3.00 and H$_3$-19 at δ 0.71, thus the position of the primary

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Table 1
^{1}H and ^{13}C NMR spectral data for compound 1 (in $CDCl_3$).

Positions	δ_C	δ_H (J in Hz)
1	39.7	1.78 m 0.84 m
2	17.4	1.42–1.60 m
3	36.2	1.86 m 1.74 m
4	37.0	
5	40.0	1.68 m
6	24.2	1.78 m 1.53 m
7	80.4	4.76 dd (2.5, 4.0)
8	47.8	–
9	52.0	1.42 m
10	38.8	–
11	17.9	1.66 m 1.48 m
12	27.0	1.50–1.64 m
13	48.6	1.88 m
14	35.2	1.48 m 1.23 m
15	54.2	1.66–1.58 m
16	79.0	–
17	24.4	1.36 s
18	71.3	3.00 d (10.28) 3.26 d (10.28)
19	17.4	0.71 s
20	17.9	1.07 s
Ac-Me	21.4	2.02 s
Ac-CO	170.9	–

hydroxyl group was verified to be at C-18. The presence of two additional oxygenated groups followed from the ^{13}C spectrum. In the HSQC experiment the methine signal at δ_C 80.4 correlates with the proton signal at δ_H 4.76 (dd, J = 2.5, 4.0 Hz). This belongs to an equatorial proton geminal to an axial acetyl group at C-7. Observation of couplings between H-7 and the signals at δ_H 1.78 and 1.53 in the COSY spectrum indicated that the latter signals should be the C-6 methylene protons. The third oxygenated carbon peak at δ_C 79.0 belongs to a quaternary carbon atom, and is assigned to C-16. All other methylene and methine proton signals extended between 0.8 and 1.8 ppm in the ^{1}NMR spectrum (Table 1). The ^{13}C NMR assignments for 1 were confirmed by HSQC and HMBC correlations (Table 1). All the data indicated the structure to be *ent*-7α-acetoxy-16β,18-dihydroxykaurane (7-acetyldistanol) for compound (1) (Fig. 1).

The known *ent*-kauranes 2–9 (Fig. 1) were identified based on spectral data and by comparison with data from the literature, as *ent*-3β,7α-dihydroxy,18-acetoxy-15β,16β-epoxykaurane (epoxyisolinearol) (2) (Topçu et al., 2002; Venturella et al., 1975), sideroxol (3) (Halfon et al., 2011; Kılıç et al., 2005; Piozzi et al., 1968), sideridiol (4) (Algarra et al., 1983), siderol (5) (Cabrera et al., 1983; Piozzi et al., 1968), 7-epicandicandiol (6) (Aljancic et al., 1996; Gonzalez et al., 1981), foliol (7) (Başer et al., 1996; Topçu et al., 1999), linearol (8) (Başer et al., 1996; Quesada et al., 1972) and sidol (9) (Gonzalez et al., 1981; Quesada et al., 1972).

Antioxidant activity tests were carried out by lipid peroxidation inhibitory activity, DPPH free radical and superoxide anion radical scavenging activity tests on the petroleum ether and acetone extracts. Antioxidant activity and scavenging activity assays were also carried out on all the individual compounds 1–6 and 8–9, except for 7. Although the acetone extract was found to be more promising as an antioxidant source, the isolated *ent*-kauranes were not found to be active. Only the new compound (1), epoxyisolinearol (2) and 7-epicandicandiol (6), exhibited marginal activity in β-carotene-linoleic acid assay indicating their lipid inhibitory properties. The antioxidant activity test results are given in Table 2. The anticholinesterase activity tests were carried out against two enzymes, acetylcholinesterase and butyrylcholinesterase by the Ellman method (Table 3). Although the tested diterpenoids were found to be weakly active against AChE enzyme, the *ent*-kauranes sideroxol (3) and 7-epicandicandiol (6) exhibited better activity against BChE than the standard galanthamine.

3. Experimental

3.1. General

The spectra were recorded with the following instruments: NMR: Varian-400, 400 MHz and 100 MHz for ^{1}H- and ^{13}C-NMR in $CDCl_3$; MS: HRMS (HEJ Research Institute of Chemistry, Karachi University) and GC-MS (Istanbul University); melting points were measured on Reichert-Kofler; Silicagel 60 was used for column chromatography, and precoated Kieselgel 60F_{254} (E. Merck) plates were used for preparative TLC.

3.2. Plant material

S. congesta P.H. Davis & Hub.-Mor. was collected from Antalya, Manavgat Province, on the road to Akseki, in June 2003. The plant was identified by Tuncay Dirmenci (Special Collection, No: 2296).

3.3. Extraction and isolation

The powdered whole plant (1.5 kg) was extracted successively with petroleum ether and acetone to give 63 g (yield: 1.77%) and

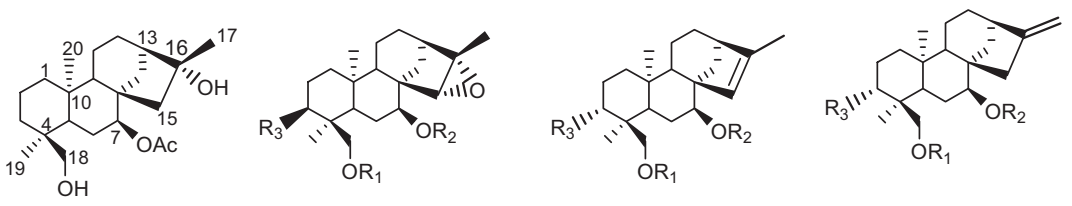

Fig. 1. Chemical structures of *ent*-kaurane diterpenoids 1–9.

Table 2
Antioxidant activities of the extracts and compounds (1–6, 8, 9) by the β-carotene-linoleic acid, assay, and $O_2^{\bullet -}$, and DPPH$^\bullet$ assays[a].

Samples	β-Carotene-linoleic acid assay	$O_2^{\bullet -}$ assay	DPPH$^\bullet$ assay
	IC_{50} (μM)	IC_{50} (μM)	IC_{50} (μM)
Petroleum ether extract[b]	303.58 ± 3.15	124.04 ± 2.21	79.44 ± 1.00
Acetone extract[b]	4.50 ± 0.22	107.27 ± 1.90	25.47 ± 0.27
Ent-7α-acetoxy-16β,18-dihydroxy-kaurane (1)	94.52 ± 0.07	303.53 ± 1.22	NA
Epoxyisolinearol (2)	195.17 ± 1.11	337.52 ± 2.09	NA
Sideroxol (3)	NA	388.30 ± 3.26	NA
Sideridiol (4)	NA	273.85 ± 1.53	NA
Siderol (5)	NA	298.95 ± 2.55	NA
7-epicandicandiol (6)	53.82 ± 0.59	514.38 ± 4.65	NA
Linearol (8)	271.70 ± 2.05	571.05 ± 2.19	NA
Sidol (9)	355.82 ± 2.12	548.14 ± 3.29	NA
α-Tocopherol[c]	4.88 ± 0.38	17.27 ± 1.88	41.33 ± 1.03
BHT[c]	6.08 ± 0.09	144.27 ± 1.50	84.41 ± 1.05

NT: Not tested.
NA: Not active.
[a] IC_{50} values represent the means ± standard deviation of three parallel measurements ($p < 0.05$).
[b] In μg/mL concentration.
[c] Reference compounds.

Table 3
Anticholinesterase activity of the compounds (1–6, 8, 9) from *S. congesta*[a].

Samples	AChE assay	BChE assay
	IC_{50} (mM)	IC_{50} (mM)
Ent-7α-acetoxy-16β, 18-dihydroxy-kaurane (1)	1.89 ± 0.08	1.19 ± 1.67
Epoxyisolinearol (2)	0.87 ± 0.01	0.43 ± 0.02
Sideroxol (3)	1.27 ± 0.80	0.024 ± 0.00
Sideridiol (4)	8.04 ± 0.87	3.67 ± 0.99
Siderol (5)	0.69 ± 0.81	0.65 ± 0.09
7-Epicandicandiol (6)	0.23 ± 0.09	0.022 ± 0.01
Linearol (8)	2.66 ± 1.27	0.15 ± 0.01
Sidol (9)	0.92 ± 0.01	0.05 ± 0.00
Galanthamine[b]	0.0037 ± 0.00	0.041 ± 0.01

[a] IC_{50} values represent the means ± standard deviation of three parallel measurements ($p < 0.05$).
[b] Standard drug.

45 g (yield: 3.75%) of extract, respectively. Each extract was fractionated on a silica gel column. The petroleum ether extract was eluted with petroleum ether, dichloromethane, acetone and methanol gradients, respectively. From the petroleum ether extract, five diterpenoids, *ent*-7α-acetoxy-16β,18-dihydroxy-kaurane (7-acetyldistanol) (1) (20 mg), sideroxol (3) (14 mg), sideridiol (4) (80 mg), siderol (5) (21 mg) and 7-epicandicandiol (6) (97 mg) were isolated. The new compound (1) was isolated during elution with dichloromethane-acetone (8:2) and was purified by preparative TLC using a dichloromethane-acetone (9:1) solvent system. The new compound was isolated previously from *Sideritis arguta* (Ertaş et al., 2009). Due to its small quantity its structure could not then be elucidated. The acetone extract was first eluted with petroleum ether and dichloromethane, acetone and methanol gradients were used with 5–10% increments. From the acetone extract, six diterpenoids were isolated. They were identified as *ent*-3β,7α-dihydroxy,18-acetoxy-15β,16β-epoxykaurane (epoxyisolinearol) (2) (14 mg), siderol (5) (28 mg), 7-epicandicandiol (6) (97 mg), foliol (7) (7 mg), linearol (8) (250 mg) and sidol (9) (6 mg).

3.4. Ent-7α-acetoxy-16β,18-dihydroxykaurane (7-acetyldistanol), (1)

White crystals, m.p.: 128–129 °C; IR ($CHCl_3$) 3420–3300, 1722, 1459, 1375, 1260, 1179, 1125, 1072, 1036, 1020, 991, 879 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$); ^{13}C NMR (100 MHz, $CDCl_3$), see Table 1. HR-EIMS: *m*/*z* 364.2825 (calculated 364.2834 for $C_{22}H_{36}O_4$)

3.5. Antioxidant activity

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

The antioxidant activity of samples of *S. congesta* was evaluated using β-carotene-linoleic acid model system (Miller, 1971). 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. BHA and α-tocopherol were used as standards.

3.5.2. Free radical scavenging activity

The free radical scavenging activity of samples of *S. congesta* was determined by the DPPH assay described by M. S. Bloiss (Bloiss, 1958). In its radical form, DPPH absorbs at 517 nm, but upon reduction by an antioxidant or a radical species its absorption decreases. Briefly, 0.1 mM solution of DPPH in methanol was prepared and 4 mL of this solution was added to 1 mL of sample solutions 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.

$$\text{DPPH Scavenging Effect}(\%) = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100$$

3.5.3. Superoxide anion radical scavenging activity

Measurement of superoxide anion radical scavenging activity of samples of *S. congesta* was based on the method described by Liu et al. (1997) with slight modification. Superoxide radicals are generated in PMS-NADH systems by oxidation of NADH and assayed by the reduction of NBT. In this experiment, superoxide radicals were generated in 3 mL of Tris–HCl buffer (16 mM, pH 8.0) containing 1 mL of NBT (50 μM) solution, 1 mL NADH (78 μM) solution and sample solutions. The reaction started by adding 1 mL of PMS solution (10 μM) to the mixture. The reaction mixture was incubated at 25 °C for 5 min, and the absorbance at 560 nm was measured against blank samples. Decreased absorbance of the

reaction mixture indicates increased superoxide anion scavenging activity. The percentage inhibition of superoxide anion radical generation of three parallel measurements was calculated using the following formula:

Inhibition $(\%) = [(A_{control} - A_{sample})/A_{control}] \times 100$, where $A_{control}$ is the absorbance of control and A_{sample} is the absorbance in the presence of the extracts or standards.

3.5.4. Anticholinesterase activity

Acetyl- and butyrylcholinesterase inhibitory activities were measured by slightly modifying the spectrophotometric method developed by Ellman (Ellman et al., 1961). Acetylthiocholine iodide and butyrylthiocholine chloride were used as substrates of the reaction and DTNB was used for the measurement of the anticholinesterase activity. 160 μL of 100 mM sodium phosphate buffer (pH 8.0), 10 μL of test compound solution and 10 μL AChE or BChE solution were mixed and incubated for 15 min at 25 °C, and 10 μL of DTNB is added. The reaction was then initiated by the addition of 10 μL acetylthiocholine iodide and butyrylthiocholine chloride, respectively. The hydrolysis of these substrates was monitored spectrophotometrically by the formation of yellow 5-thio-2-nitrobenzoate anion as the result of the reaction of DTNB with thiocholine, released by the enzymatic hydrolysis of acetylthiocholine iodide or butyrylthiocholine chloride, at a wavelength of 412 nm. Methanol was used as a solvent to dissolve test compounds and the controls.

3.6. Statistical analysis

All data on all antioxidant and anticholinesterase activity tests are the average of triplicate analyses. The data were recorded as mean ± standard deviation. Analysis of variance was performed by ANOVA procedures. Significant differences between means were determined by student's-*t* test and p values <0.05 were regarded as very significant.

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References

Algarra, J., Garcia-Granadas, A., Bruaga, A.S.D., Bruaga, J.M.S., 1983. Diterpenoids from *Sideritis varoi*. Phytochemistry 22, 1779–1782.

Aljancic, I., Macura, S., Juranic, S., Andjelkovic, N., Randjelovic, N., Milosavljevic, S., 1996. Diterpenes from *Achilea clypeolata*. Phytochemistry 43, 169–172.

Başer, K.H.C., Bondi, M.L., Bruno, M., Kırımer, N., Piozzi, F., Tümen, G., Vasallo, N., 1996. An *ent*-kaurane from *Sideritis Huber–Morathii*. Phytochemistry 43, 1293–1295.

Bloiss, M.S., 1958. Antioxidant determinations by the use of a stable free radical. Nature 181, 1199–1200.

Cabrera, E., Garcia-Granadas, A., Buruaga, A.S.D., De Buruaga, J.M.S., 1983. Diterpenoids from *Sideritis hirsuta* subsp. *nivalis*. Phytochemistry 22, 2779–2781.

Duman, H., 2000. *Sideritis* L. In: Güner, A., Özhatay, N., Ekim, E., Başer, K.H.C. (Eds.), Flora of Turkey and East Aegean Islands (Supplement 2), University Press, Edinburgh, Vol. 11, pp. 201–205.

Ellman, G.L., Courtney, K.D., Andres, V., Featherston, R.M., 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem. Pharmacol. 7, 88–95.

Ertaş, A., Öztürk, M., Boğa, M., Topçu, G., 2009. Antioxidant and anticholinesterase activity evaluation of *ent*-kaurane diterpenoids from *Sideritis arguta*. J. Nat. Prod. 72, 500–502.

Gonzalez, A.G., Fraga, B.M., Hernandez, M.G., Hanson, J.R., 1981. The ^{13}C-NMR spectra of some *ent*-18-hydroxykaur-16-enes. Phytochemistry 20, 846–847.

Halfon, B., Gören, A.C., Ertaş, A., Topçu, G., 2011. Complete ^{13}C NMR assignments for *ent*-kaurane diterpenoids from *Sideritis* species. Magn. Reson. Chem. 49, 291–294.

Kılıç, T., Yıldız, Y.K., Topçu, G., Gören, A.C., Ay, M., Bodige, S.G., Watson, W.H., 2005. X-ray analysis of sideroxol from *Sideritis leptoclada*. J. Chem. Crystallogr. 35, 647–650.

Liu, F., Ooi, V.E.C., Chang, S.T., 1997. Free radical scavenging activities of mushroom polysaccharide extracts. Life Sci. 60, 763–771.

Mill, M., 1982. *Sideritis* L. In: Davis, P.H. (Ed.), Flora of Turkey and East Aegean Islands, University Press, Edinburgh, Vol. 7, pp. 193.

Miller, H.E., 1971. A simplified method for the evaluation of antioxidants. J. Am. Oil Chem. Soc. 45, 91–98.

Piozzi, F., Venturella, P., Bellino, A., Mondelli, R., 1968. Diterpenes from *Sideritis sicula*. Tetrahedron 24, 4073.

Quesada, T., Rodriguez, B., Valverde, S., Huneck, S., 1972. Six new diterpenes from *Sideritis leucantha* Cav. and *Sideritis linearifolia* Lam. Tetrahedron Lett. 13, 2187–2190.

Topçu, G., Gören, A.C., 2007. Biological activity of diterpenoids isolated from Anatolian Lamiaceae plants. Rec. Nat. Prod. 1, 1–16.

Topçu, G., Gören, A.C., Yıldız, Y.K., Tümen, G., 1999. *Ent*-kaurane diterpenes from *Sideritis athoa*. Nat. Prod. Lett. 14, 123–129.

Topçu, G., Gören, A.C., Kılıç, T., Yıldız, Y.K., Tümen, G., 2002. Diterpenes from Sideritis sipylea and Sideritis dichotoma. Turk. J. Chem. 26, 189–194.

Venturella, P., Bellino, A., Piozzi, F., 1975. Diterpenes from *Sideritis theezans*. Phytochemistry 14, 1451–1452.