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A Novel Isopimarane Diterpenoid with Acetylcholinesterase Inhibitory Activity from *Nepeta sorgerae*, an Endemic Species to the Nemrut Mountain

Anıl Yılmaz[a,b], Pınar Çağlar[c], Tuncay Dirmenci[d], Nezhun Gören[c] and Gülaçtı Topçu[a,b*]

[a]*Department of Chemistry, Faculty of Science and Letters, Istanbul Technical University, Istanbul 34469, Turkey*
[b]*Department of Pharmacognosy, Faculty of Pharmacy, Bezmialem Vakif University, Istanbul 34093, Turkey*
[c]*Department of Biology, Faculty of Arts & Science, Yildiz Technical University, Istanbul 34210, Turkey*
[d]*Department of Science and Mathematics for Secondary Education, Necatibey Faculty of Education, Balikesir University, Balıkesir 10145, Turkey*

gulacti_topcu@yahoo.com

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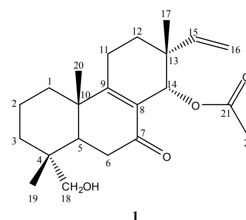
From the dichloromethane extract of *Nepeta sorgerae*, the isolation and structure elucidation are now reported of a new isopimarane diterpenoid, named sorgerolone, and two known triterpenoids, oleanolic acid and ursolic acid. Antioxidant activity of the extracts and the isolated terpenoids was determined by the DPPH free radical scavenging and lipid peroxidation inhibition (β-carotene bleaching) methods. Anticholinesterase activity of the extracts and isolates was investigated by Ellman's method against AChE and BChE enzymes. Although the antioxidant activity results were low, the AChE enzyme inhibition of the extracts and terpenoids was very promising.

Keywords: *Nepeta sorgerae*, Isopimarane diterpene, Triterpenoids, Antioxidant activity, Anticholinesterase activity.

Nepeta species, family Lamiaceae, are either annual or perennial plants with a pleasant aroma. The genus *Nepeta* has a worldwide distribution with over 250 species widely growing in temperate Europe, Asia, North America, North Africa and in the Mediterranean region [1]. In Turkey, *Nepeta* species are represented by 41 taxa, of which 18 are endemic [2]. The endemic and non-endemic species mostly grow in East Anatolia and the Taurus Mountains [3]. Some *Nepeta* species are used in folk medicine as a diuretic, diaphoretic, antitussive, antispasmodic, antiasthmatic, febrifuge, emmenagogue, sedative, spice and herbal tea [4-6]. Secondary metabolites of *Nepeta* species [7-12] are mainly terpenes (monoterpenes, diterpenes, triterpenes), iridoids and their glucosides [9,13] and flavonoids [10]. Essential oils of *Nepeta* species have been extensively studied [13-15]. Nepetalactones are characteristic monoterpenes for *Nepeta* species [8], which act as feline attractant and insect repellent [15].

There are a number of publications related to the isolation of non-volatile constituents from *Nepeta* species, which include many diterpenes, particularly abietane diterpenoids [16] and pimarane diterpenoids [17], some with biological activity. From *N. parnassica*, besides the pimarane diterpene parnapimarol, two dimeric nepetalactones, nepetaparnone and nepetanudone, were isolated [18]. In fact, nepetanudone was first isolated from *N. tuberosa* ssp. *tuberosa*, and then X-ray analysis of the compound was carried out by our group with a sample obtained from *N. nuda* ssp. *albiflora* [19]. Triterpenoids [8,10,12,20-23] were isolated from several *Nepeta* species. *Nepeta* extracts, essential oils and their constituents exhibited various activities [15,24-26]. *N. latifolia* and *N. sibthorpii* have a sedative effect [24] due to the presence of non-glycosylated iridoids, specifically nepetalactones. The methanolic extract of *N. sibthorpii* has anti-inflammatory activity [25]. The antimicrobial activity of methanol extracts of *N. rtanjensis*, *N. sibirica* and *N. nervosa* against eight bacteria and eight fungi was evaluated and all the extracts showed significant antibacterial and strong antifungal activity [26]. In the present study, a local endemic species, *N. sorgerae*, collected from the Nemrut Mountain, has been examined. Its dichloromethane extract afforded a new isopimarane diterpene, named sorgerolone (**1**), along with two triterpenoids, oleanolic acid (**2**) [27] and ursolic acid (**3**) [28]. Structures of the isolates were established on the basis of spectral analyses, particularly the analysis of the new diterpene based on 1D and 2D-NMR spectra consisting of ^{1}H and ^{13}C NMR (APT), ^{1}H-^{1}H-COSY, gHSQC and gHMBC experiments, as well as mass spectra. Their antioxidant activity was investigated by two assay methods, DPPH free radical scavenging and lipid peroxidation inhibitory activity (β-carotene bleaching method). Their anticholinesterase activity was also detected by Ellman's method *in vitro* against AChE and BChE enzymes.



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Compound **1** was obtained as a yellow amorphous solid. The APCI (+)-MS exhibited a molecular ion peak $[M+1]^+$ at m/z 361.1 corresponding to the molecular formula $C_{22}H_{32}O_4$ (calculated 360.3), which has seven double bond equivalents. The ^{1}H NMR spectrum of compound **1** ($CDCl_3$, 500 MHz) showed four methyl signals as singlets at δ 0.88, 0.90, 1.18, 1.94; the signal at δ 1.94 belongs to methyl protons of an acetyl group. The ^{13}C NMR (APT) spectrum showed twenty two carbon atom signals (four methyl carbons at δ 17.38, 19.65, 21.26, 21.42; eight methylene carbons at

Table 1: ^{13}C NMR and ^{1}H NMR (in $CDCl_3$) spectral assignments of sorgerolone (**1**).

	H	^{13}C	^{1}H (J in Hz)	gHMBC
1	α β	35.62	1.16 m 1.83 m	C-3, C-2 C-9, C-3
2	α β	17.97	1.66 m 1.70 m	C-4, C-1, C-3 C-4, C-10
3	α β	34.38	1.35 m 1.56 m	C-18, C-19 C-18, C-1
4		37.76	-	-
5		41.83	2.19 dd (4.25, 5.60)	C-7, C-3, C-18
6	α β	35.14	2.39 m 2.46 m	C-7, C-9 C-9
7		197.52	-	-
8		128.66	-	-
9		170.58	-	-
10		39.48	-	-
11	α β	22.02	2.52 m 2.57 m	C-13, C-8 C-8
12	α β	25.94	1.48 m 1.85 m	C-14 C-14
13		38.51	-	-
14		68.34	5.78 s	C-7, C-8, C-9, C-12 C-13, C-15, C-21
15		143.93	5.91 dd (10.93, 17.65)	C-12, C-14, C-16
16	*trans* *cis*	112.63	4.99 dd (0.90, 17.65) 5.02 dd (0.90, 10.93)	C-13, C-15 C-13, C-15
17		19.65	0.88 s	C-14, C-15
18	a b	70.60	3.15 d (10.93) 3.43 d (10.93)	C-3, C-5, C-19 C-3, C-5, C-19
19		17.38	0.90 s	C-3, C-18
20		21.42	1.18 s	C-1, C-5, C-9
21		170.02	-	-
22		21.26	1.94 s	C-21

δ 17.97, 22.02, 25.94, 34.38, 35.14, 35.62, 70.60, 112.63; three methine carbons at δ 41.83, 68.34, 143.93 and seven quaternary carbons at δ 37.76, 38.51, 39.48, 128.66, 170.02, 170.58, 197.52), these data showed compound **1** to have a diterpene structure.

The presence of an ethylenic side chain in the structure, with characteristic *cis* and *trans* coupling constants for H-15 (δ 5.91, dd, J= 10.93, 17.65 Hz), together with a pair of methylene signals at δ 4.99 (dd, J= 0.90, 17.65 Hz) and δ 5.02 (dd, J= 0.90, 10.93 Hz) bound to C-16 (δ 112.63) can be assigned to a pimarane-type skeleton of the diterpenoid. In the ^{13}C NMR (APT) spectrum, olefinic methine and methylene carbon atoms of the side chain were observed at δ 143.93 and 112.63, and their assignments were made based on gHSQC experiments as C-15 and C-16, respectively. Another methylene pair of signals were observed as doublets at δ 3.15 and δ 3.43 (J= 10.95 Hz) attached to the carbon atom at δ 70.60, which should have a hydroxyl group. In the HMBC experiment, both protons exhibited three-bond away correlations with C-3, C-5 and C-19 signals, which indicated the location of a hydroxymethylene group at C-4. The location of the hydroxymethylene group at C-18 was deduced by the observation of a three-bond away correlation from a methyl proton signal at δ 0.90 to the hydroxymethylene carbon signal at δ 70.60 (C-18) in the gHMBC experiment, and the determination of the C-19 signal at δ17.38 by gHSQC experiment. The signal resonating at δ 197.52 in the ^{13}C NMR spectrum was assigned to the presence of a carbonyl group, which interacted with the characteristic H-5 signal, observed at δ 2.19 (dd, J= 4.25, 5.60 Hz), via three bonds in the gHMBC spectrum; this indicated that the carbonyl carbon should be placed at C-7. In addition, the presence of a double bond in the structure followed the observation of the quaternary carbon atoms signals at δ 170.58 and 128.66. The double bond was deduced to be either between C-8 and C-9 or between C-8 and C-14, which was conjugated to the carbonyl group. The downfield signal resonating at δ 170.58 was indicative of C-9 being in the β position relative to the carbonyl group at C-7. In addition, a signal observed at δ 5.78 as a singlet attached to a methine carbon (δ 68.34) indicated that this carbon probably bears either an acetate or an ester moiety. The observation of an acetoxy group with signals at δ 170.02 and δ 21.26, and δ 1.94 in the ^{13}C and ^{1}H NMR spectra, respectively, as well as H-C correlations via three bonds in the gHMBC experiment from the proton at δ 5.78 (H-14) to the carbonyl carbon at δ 170.02, clearly support the assignment to C-14 of an acetyl group. This location for the acetate group was also verified based on observation of two and three-bond away correlations from H-14 to C-7, C-8, C-9, C-12, C-13 and C-15. Therefore, the double bond must be placed between C-8 and C-9. Furthermore, the Me-17 signal was assigned based on the three-bond correlation between its proton signal at δ 0.88 and C-14 in the gHMBC experiment. Observation of a 2D NOESY cross peak between the methyl proton signals at δ 0.88 (Me-17) and Me-20 protons at δ 1.18 assigned the compound to the isopimarane series [29]. In the APCI-MS, the observed fragment ions at m/z 342.6 $[M-H_20]^+$ (22), 318.2 $[M+1-COCH_3]^+$ (38), 301.1 $[M-OCOCH_3]^+$ (75), 334.4 $[M+1-CH=CH_2]^+$ (25), and the base peak at 263.1 (100), besides the molecular ion peak at m/z 361.1 $[M+1]^+$ (44), were in good agreement with the substituents of the compound.

All the spectral data indicated the structure of compound **1** to be 14α-acetoxy-18-hydroxyisopimara-8,15-diene-7-one, named sorgerolone. Although similar compounds have been isolated in previous studies [18,30], sorgerolone **(1)** has been isolated from nature for the first time.

In this study, the antioxidant and anticholinesterase activities of the dichloromethane and methanol extracts, and the terpenoids were investigated. The results showed the DPPH free radical scavenging and lipid peroxidation inhibition activities of both extracts and isolates to be very weak (Table 2). Although the BChE inhibition of the extracts and isolates was not high, their AChE inhibition was promising (Tables 2-3).

Table 2: Bioactivity results (IC_{50}) of the extracts, isolated compounds and standards (IC_{50} values are given in µg/mL)

Sample	DPPH scavenging activity	Lipid peroxidation inhibition	AChE Inhibition	BChE Inhibition
1	NA*	59.5	17.8	120.0
2	NA	NA	28.37	NA
3	NA	NA	39.19	NA
NSD	NA	NA	NT	NT
NSM	NA	NA	NT	NT
BHT	36.1	1.3	NT	NT
BHA	23.8	14.3	NT	NT
α-Toc	26.0	2.1	NT	NT
Galanthamine	NT**	NT	1.4	4.0

NA*: Not active, NT**: Not tested

Table 3: Anticholinesterase activity of the extracts at 200 µg/mL[a]

Extract	Inhibition % against AChE	Inhibition % against BChE
NSD[b]	55.9 ± 1.2	13.9 ± 0.4
NSM[c]	66.7 ± 0.7	5.3 ± 0.4
Galanthamine[d]	74.1 ± 0.1	74.5 ± 0.6

[a]Values expressed are mean ± SD of three parallel measurements (p<0.05)
[b]NSD: Dichloromethane extract of *N. sorgerae*
[c]NSM: Methanol extract of *N. sorgerae*
[d]Standard drug

In conclusion, the new compound exhibited high AChE inhibition, but moderate lipid peroxidation inhibition and weak BChE inhibition.

Experimental

Chemicals: Silica gel 60 (1.07734), preparative TLC plates (1.05554), ethanol, chloroform, dichloromethane, diethyl ether,

HPLC grade methanol, acetonitrile, potassium acetate, butylated hydroxytoluene, cerium (IV) sulfate tetrahydrate, aluminum nitrate nonahydrate, and sulfuric acid were purchased from Merck (Darmstadt, Germany); β-carotene, linoleic acid, Tween 40, copper (II) chloride dihydrate, DTNB {5,5-dithiobis- (2-nitro benzoic acid)}, acetylcholinesterase, and butyrylcholinesterase from Sigma (Steinheim, Germany); acetylthiocholine iodide, Folin Ciocalteu Phenol reagent from Applichem (Darmstadt, Germany); α-tocopherol from Aldrich (Steinheim, Germany); neocuproine, DPPH (2,2-diphenyl-1-picrylhydrazyl), galantamine hydrobromide, and butylated hydroxyanisole from Sigma-Aldrich (Steinheim, Germany); light petroleum, methanol, acetone, sodium carbonate, sodium hydrogen phosphate, and sodium dihydrogen phosphate from Reidel de Haen (Germany); and butyrylthiocholine iodide from Fluka (Steinheim, Germany).

General experimental procedures: Shimadzu HPLC CBM-20A, Bruker Avance III NMR (^{1}H NMR: 500 MHz, ^{13}C NMR: 125 MHz), Moleculer Devices ELISA (USA).

Plant material: The aerial parts of *Nepeta sorgerae* Hedge et Lamond, endemic to East Anatolia, was collected from between Tepehan Town and Nemrut Mountain, near Büyük Uz village, Malatya, Turkey in July 2009, , at 1645 m altitude, and identified by Dr. Tuncay Dirmenci (3705-a). The voucher specimen Dirmenci & Akçiçek was deposited in the Herbarium of Necatibey Education Faculty at Balikesir University.

Extraction and isolation: The dried and powdered aerial parts (2800 g) of *Nepeta sorgerae* were extracted with 10 L of dichloromethane at room temperature (7 days x 2 times), and then extracted with 10 L of methanol, successively, at room temperature (7 days x 2 times). After filtration, the solvents were evaporated to dryness under vacuum, and 121 g of dichloromethane extract and 70 g of methanol extract were obtained. The methanol extract has not yet been studied. The crude dichloromethane extract was fractionated on a silica gel column (5 x 150 cm), eluted with light petroleum (40-60°C), followed by a gradient of dichloromethane, acetone and then methanol up to 100%. In total, 152 fractions were obtained and similar fractions were combined after checking by TLC. In order to isolate pure compounds from the fractions, further purifications (preparative TLC, CC, and HPLC) was applied to the fractions.

14α-Acetoxy-18-hydroxyisopimara-8,15-diene-7-one (1): The fraction from the dichloromethane extract corresponding to the gradient of dichloromethane/acetone (80:20) was chromatographed using HPLC (Shim-pack PREP-ODS (H) kit, C18 column, 250 x 4.6 mm, 5 μm) eluted with acetonitrile/methanol 90/10, flow rate 7 mL/min, to afford pure 14α-acetoxy-18-hydroxyisopimara-8,15-diene-7-one.
$[\alpha]_D$: -21.04 (*c* 0.3 g/100 mL, $CHCl_3$)
^{1}H NMR and ^{13}C NMR: Table 1.
MS (APCI (+)-MS): *m/z* (%) = 361.1 [M + H^+] (% 100).

Oleanolic acid (2) and ursolic acid (3): The fractions collected during elution by dichloromethane/acetone (80:20) and (90:10) were chromatographed on silica gel prep. TLC plates, developed with dichloromethane/acetone (90:10), to obtain oleanolic acid and ursolic acid, respectively.

Antioxidant Activity

DPPH free radical scavenging activity: The free radical scavenging activity of the extracts and isolated pure terpenoids was determined by the DPPH assay described by Blois [31]. Each of the extracts (40 μL), isolated pure compounds and standards (BHT, BHA, α-Toc), at different concentrations, were added to a 96 well plate and 160 μL 0.1 mM DPPH solution was added. After 30 minutes incubation in the dark, the absorptions were measured at 517 nm.

Lipid peroxidation inhibitory activity: The antioxidant activity of the extracts and isolated pure terpenoids was evaluated by the β-carotene-linoleic acid model system [32,33]. β-Carotene (1 mg) in 2 mL chloroform was added to 100 μL of linoleic acid, and 800 μL of Tween 40 emulsifier mixture. After evaporation of the chloroform from the extracts, the isolated pure compounds and standards at different concentrations were transferred into a 96-well plate and 160 μL of emulsion mixture. Two hundred mL of distilled water saturated with oxygen was added by vigorous shaking, followed by 40 μL each emulsion. The zero time absorbance was measured at 490 nm using a spectrophotometer. The emulsion system was incubated for 2 h at 50°C and the absorptions were measured at 490 nm. A blank, devoid of β-carotene, was prepared for background subtraction. BHT, BHA and α-tocopherol were used as standards.

Anti-cholinesterase activity: The inhibition of AChE and BChE of samples was determined by the Ellman method [34]. To a 96-well plate, 130 μL pH 8 phosphate buffer, 10 μL sample at different concentrations, and 20 μL of either AChE or BChE were added, and the zero time absorbance was measured at 412 nm. After 15 minute-incubation at room temperature, 20 μL DTNB and 20 μL either AcI (acetylthiocholine iodide) or BuCI (butyrylthiocholine iodide) were added, and 10 minutes later the second measurement was carried out at 412 nm. Galanthamine was used as a standard and ethanol as a blank.

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