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Novel Terpenoids with Potential Anti-Alzheimer Activity from *Nepeta obtusicrena*

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Abstract: Dichloromethane extract of *Nepeta obtusicrena* Boiss. Et Kotschy Ex Hedge afforded two novel terpenoids, a diterpenoid and a triterpenoid in addition to two known triterpenoids, oleanolic acid and ursolic acid. Purification of the diterpenoid was carried out by HPLC, and its structure was elucidated as 14α-acetoxy-6-oxo-abieta-7-ene, and structure of the triterpenoid was elucidated as 2α,3β,19α,24-tetrahydroxy-11-oxo-olean-12-ene. Both of the novel terpenes were obtained from nature for the first time and named as obtusicrenone and nemrutolone, respectively. Anticholinesterase (anti-Alzheimer) and antioxidant activities [DPPH free radical scavenging activity, ABTS cation radical scavenging activity, lipid peroxidation inhibitory activity, CUPRAC (Cupric Reducing Antioxidant Capacity)] of the dichloromethane and methanol extracts and the isolated four terpenoids were investigated. Both of the extracts and the isolated four terpenoids exhibited high anticholinesterase activity, particularly against acetylcholinesterase (AChE) enzyme. None of the samples tested showed high antioxidant activity.

Keywords: *Nepeta obtusicrena*; abietane diterpenoid; triterpenoids; anticholinesterase activity; antioxidant activity.

1. Introduction

The genus *Nepeta* is a member of Lamiaceae family and has a worldwide distribution with over 250 species which widely grow in Europe, Asia, North America, North Africa and in the Mediterranean region [1]. In Turkey, *Nepeta* species are represented by 41 taxa (18 of 41 taxa are endemic) [2] and mostly distributed in East Anatolia and the Taurus Mountains [3]. Some *Nepeta* species have been traditionally used as diuretic, diaphoretic, antitussive, antispasmodic, antiasthmatic, febrifuge, sedative, spice and herbal tea such as other members of Lamiaceae [4-11].

Secondary metabolites of *Nepeta* species are particularly terpenes (monoterpenes, diterpenes, triterpenes), iridoids and their glucosides as well as flavonoids [12-18]. Essential oils of *Nepeta*

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species have been extensively studied [18-20]. Nepetalactones are characteristic monoterpenes for *Nepeta* species [13], which act as feline attractant and insect repellent [20]. There are several studies on the isolation of nonvolatile constituents from *Nepeta* species, which include many diterpenes, particularly abietane [21] and pimarane diterpenoids [22]. A pimarane diterpene, parnapimarol and two dimeric Nepetalactones, Nepetaparnone and Nepetanudone, were isolated from *Nepeta parnassica* [23]. Nepetanudone was first isolated from *Nepeta tuberosa* ssp. *tuberosa*, and then X-ray analysis of the compound was carried out by our group with a sample obtained from *Nepeta nuda* ssp. *albiflora* [24]. Triterpenoids with ursane, oleanane or lupane skeleton were isolated from several *Nepeta* species [25-27].

Nepeta extracts, essential oils, and their constituents exhibited various activities besides their usage as food and food supplements. *Nepeta racemosa* is used as an ingredient of vegetable soup [28] and *Nepeta caesarea* is used as a traditional food [29]. The leaves and shoots of *Nepeta italica* L. and the aerial parts of *Nepeta flavida* Hub.-Mor. are used for cold (stomach cold), flu and bronchitis [30,31]. The antimicrobial activity of methanol extracts of *Nepeta rtanjensis*, *Nepeta sibirica* and *Nepeta nervosa* against eight bacteria and eight fungi was evaluated, and all the extracts showed significant antibacterial and strong antifungal activity [32].

Alzheimer disease (AD) is a progressive neurodegenerative disorder [33], and it has been reported that the protein level of acetylcholine receptors which is responsible for memory is reduced in AD and that dysfunction of cholinergic signal transmission could be responsible for the symptoms of AD. Acetylcholinesterase enzyme breaks acetylcholine down after acetylcholine is used in the brain. Therefore, one of the approaches for the treatment of AD is reducing the amount of acetylcholinesterase enzyme using cholinesterase inhibitors in order to keep acetylcholine level in the brain up. In present, there are four medicines for the treatment of AD on the market, three of them are cholinesterase inhibitors including donepezil, rivastigmine and galanthamine and also an NMDA receptor antagonist, memantine. Among the cholinesterase inhibitors, galanthamine is a natural compound which was obtained from *Galanthus nivalis*, *G. woronowii* and some other Amaryllidaceae family plants while rivastigmine is a natural derived compound. Donepezil is a synthetic compound. All of the approved treatments for AD have disturbing side effects. Recently, in searching better cholinesterase inhibitors from natural sources, we have studied Lamiaceae family plants [34], and one of the endemic Anatolian *Nepeta* species, *Nepeta sorgerae* was investigated by our group [22], which afforded three highly active terpenoids against AChE enzyme consisting of a pimarane diterpene, sorgerolone and triterpenoids, oleanolic acid and ursolic acid. There are some similar studies in the literature demonstrating that some ursane and oleanane triterpenoids and some diterpenoids exhibit strong cholinesterase inhibitory activity [35]. In fact, neurodegeneration is associated with inflammation. Oxidative stress is another factor contributing the neurodegeneration [36]. Some *Nepeta* species such as *Nepeta latifolia* and *Nepeta sibthorpii* also showed a sedative effect on the central nervous system [37]. The latter also exhibited anti-inflammatory activity [38]. Furthermore, *Nepeta* species, especially *Nepeta cataria*, has been known to have insect repellent and feline attractant effect [20] and there is a correlation between insect repellent activity and anticholinesterase activity. All these effects suggest their cholinesterase inhibitors may represent a multi-targeted drug class for the treatment of AD and some related neurological disorders. Thus, we have investigated the extracts of *N. obtusicrena* in anticholinesterase activity-directed isolation and purification in the present study.

A local endemic species, *N. obtusicrena*, collected from the Nemrut Mountain in the South-Eastern Anatolia, has been studied for bioactive constituents. For this purpose, dichloromethane extract was fractionated on a si-gel column by the elution of petroleum ether, dichloromethane, acetone and methanol in increasing amounts. A fraction obtained during elution of dichloromethane/acetone (7:3) was purified by eluting with methanol 100 % on a HPLC C-18 column to afford a new pure abietane diterpene 14α-acetoxy-6-oxo-abieta-7-ene named obtusicrenone **(1)**. In addition, a new triterpene, 2α,3β,19α,24-tetrahydroxy-11-oxo-olean-12-ene named nemrutolone **(2)** along with two known triterpenoids, oleanolic acid **(3)** [39] and ursolic acid **(4)** [40] were obtained from the dichloromethane extract of *Nepeta obtusicrena*. Structures of the isolated terpenoids were

elucidated by using spectral analyses, particularly 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 for the two new terpenes.

The antioxidant potential of the extracts and isolated pure compounds were investigated by four complementary antioxidant assay methods; DPPH free radical scavenging activity, ABTS cation radical scavenging activity, lipid peroxidation inhibitory activity (β-carotene bleaching method) and CUPRAC (cupric ion reducing antioxidant capacity). Their anticholinesterase activity was detected by Ellman method *in vitro* against AChE and BChE enzymes.

2. Materials and Methods

2.1. General experimental procedures

Heidolph and Buchi Rotary Evaporators, Shimadzu HPLC CBM-20A, Bruker Avance III-500 MHz NMR (^{1}H-NMR: 500 MHz, ^{13}C-NMR: 125 MHz), Varian VNMRS-600 MHz NMR (^{1}H-NMR: 600 MHz, ^{13}C-NMR: 150 MHz), Mass Spectrometer Zivak Tandem Gold LC-MS/MS (Triple Quadrupole Mass Analyzer), Moleculer Devices ELISA (USA), Optical Activity AA-55 Series Automatic Polarimeters.

2.2 Plant material

N. obtusicrena Boiss. et Kotschy ex Hedge is a perennial species, and its flowering and fruit season is July-August and grows on steppes and volcanic banks. Stems are 40-70 cm, glabrascent to pilose or scabridulous, with or without glandular papillae or sessile glands. Corollas are lavender blue with a lenght 12-16 mm, tube somewhat curved, exserted from calyx teeth. *N. obtusicrena* is an endemic species to East Anatolia, and aerial parts of the plant was collected from inside the crater of Nemrut Mountain, Bitlis, Turkey in July 2009, at 2450 m altitude, and identified by Dr. Tuncay Dirmenci. The voucher specimen Dirmenci & Akçiçek (2723) was deposited in the Herbarium of Necatibey Education Faculty at Balıkesir University, Turkey.

2.3 Extraction and isolation

The dried and powdered aerial parts of 950 g *N. obtusicrena* were extracted with 4 L of dichloromethane and methanol (7 days x 2 times) at room temperature, successively. After filtration, the solvents were evaporated to dryness under vacuum, and 27.5 g of dichloromethane extract and 22.0 g of methanol extract were obtained. The crude dichloromethane extract was fractionated on a silica gel column (5 x 150 cm). The column was eluted with petroleum ether (40-60 °C), followed by a gradient of dichloromethane, acetone and then methanol up to 100%. Totally, 162 fractions were obtained and similar fractions were combined by checking their spots on thin-layer chromatography plates. In order to isolate pure compounds from fractions, further purifications (preparative TLC, column chromatography and HPLC) were carried out on the fractions.

2.4 Compound 1

Compound **1** was obtained as a yellow-brown amorphous solid. $[\alpha]_D^{20}$: +13.3 (*c* 0.3 g/100 mL, $CHCl_3$). The fraction from the dichloromethane extract corresponding to the gradient of dichloromethane/acetone (7:3) was chromatographed using HPLC (Shim-pack PREP-ODS (H) kit, C18 column, 250 x 4.6 mm, 5 µm) and eluted with methanol 100%, flow rate 4 mL/min to afford pure compound **1** (10 mg).

2.5 Compound 2

Compound **2** was obtained as a yellow-brown amorphous solid. $[\alpha]_D^{20}$: +14.3 (*c* 0.13 g/100 mL, CH_3OH). The fraction from the dichloromethane extract corresponding to the gradient of dichloromethane/acetone (4:6) was purified using column chromatography (4 cm x 120 cm) to afford pure compound **2** (15 mg) named nemrutolone.

2.6 DPPH free radical scavenging activity assay

The free radical scavenging activity of the extracts and isolated pure terpenoids was determined by DPPH free radical scavenging activity assay method described by Blois [41]. DPPH (2,2-diphenyl-1-picrylhydrazyl) is a stable free radical which has a characteristic absorption at 517 nm. When DPPH radical meets an antioxidant, its absorption decreases and the antioxidant capacity of the extracts and isolated pure compounds are then determined along with the decreasing of absorption by this method. 40 µL of each of the extracts, isolated pure compounds and standards (BHT, BHA, α-Toc) at different concentrations were added to a 96 well plate and 160 µL of 0.1 mM DPPH solution was added. After 30 minute-incubation in the darkness, the absorptions were measured at 517 nm. BHT (Butylated hydroxytoluene), BHA (Butylated hydroxyanisole) and α-tocopherol were used as standards.

2.7 ABTS cation radical scavenging activity assay

This method was used to evaluate the ABTS cation radical scavenging activity potential of the samples, based on the protocol of Re et al. [42] with some modifications. ABTS [2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)] cation radical were prepared by reacting a 7.4 mM ABTS stock solution with 2.45 mM potassium persulphate (1:1, v/v), and then the mixture was kept overnight (12-16 h) in the dark at room temperature. The ABTS radical solution was diluted with distilled water till it gave an absorbance value between 1.0 and 1.2. A 1 ml of aliquot of the diluted sample was added to 50 ml of each of the above-prepared radical solutions and protected from light for 60 min. Absorbance were measured at 734 nm using a spectrophotometer. BHT, BHA and α-tocopherol were used as standards.

2.8 Lipid peroxidation inhibitory activity assay

The antioxidant activity of the extracts and isolated pure terpenoids was evaluated by β-carotene-linoleic acid model system [43,44]. β-Carotene (1 mg) in 2 mL of chloroform was added to 100 µL of linoleic acid, and 800 µL of Tween 40 emulsifier mixture. After evaporation of chloroform of the extracts, isolated pure compounds and standards at different concentrations were transferred into a 96-well plate and 160 µL of emulsion mixture was added. 200 mL of distilled water saturated with oxygen were added by vigorous shaking, and 40 µL of each emulsion was added, the zero time absorbance was measured at 490 nm using a spectrophotometer. The emulsion system was incubated for 2 h at 50 ^oC 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.

2.9 CUPRAC (Cupric reducing antioxidant capacity) assay

The antioxidant capacity of the samples were determined along with the reducing of copper (II) by this method [45]. 67 µL of each samples (extracts, isolated pure compounds and standards) at

different concentrations were added to a 96 well plate and 61 µL of 0.01 M $CuCl_2.2H_2O$, 61 µL of alcohol solution of $7.5x10^{-3}$ M neocuproine and 61 µL of ammonium acetate aqueous buffer at pH 7 were added and after 30-60 minutes absorbances were measured at 450 nm. BHT, BHA and α-tocopherol were used as standards.

2.10 Anticholinesterase activity assay

The inhibition of AChE and BChE enzymes of samples was determined by the Ellman method [46]. 130 µL of phosphate buffer solution at pH 8, 10 µL of sample at different concentrations and 20 µL of either AChE or BChE enzymes solutions were added to a 96-well plate and the zero time absorbance was measured at 412 nm. After 15 minute-incubation at room temperature, 20 µL of DTNB and 20 µL of either AcTChI (acetylthiocholine iodide) or BuTChI (butyrylthiocholine iodide) solutions 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. Both antioxidant and anticholinesterase activity tests were carried out as triplicate and each at four concentrations, except for only the anticholinesterase activity of the extracts.

3. Results and Discussion

3.1. Structure elucidation

The APCI (+)-MS of compound **1** exhibited a quasi molecular ion peak $[M+1]^+$ at *m/z* 347.3 corresponding to the molecular formula $C_{22}H_{34}O_3$ (calculated 346.3), with six double bonds equivalency. The ^{13}C-NMR (APT) spectrum showed twenty two carbon atom signals consisting of six methyl carbons at δ 15.3, 17.4, 20.7, 20.8 (x2), 21.3; five methylene carbons at δ 17.6, 22.5, 23.9, 37.5, 37.8; six methine carbons at δ 28.6, 47.4, 49.2, 59.1, 73.3, 129.1; five quaternary carbons at δ 39.4, 42.7, 155.6, 169.6, 198.9. The ^{1}H-NMR spectrum of compound **1** ($CDCl_3$, 600 MHz) exhibited six methyl signals, appearing as four singlets at δ 0.93, 1.26, 1.46, 2.04 and two doublets at δ 0.92 (*J*=6.6 Hz), 0.94 (*J*=6.6 Hz) which revealed a diterpene framework of compound **1** together with the ^{13}C-NMR data (Table 1).

An isopropyl group in the structure followed by the ^{1}H and ^{13}C-NMR spectra with the signals at δ 1.51, m (H-15) and δ 28.6 (C-15) together with two methyl protons at δ 0.92 (H-16) and 0.94 (H-17) as doublets with a *J* value of 6.6 Hz and their carbons observed at δ 20.8 exhibited the presence of an abietane type skeleton of the diterpenoid.

Considering the nature of carbon signals determined by APT, the presence of a carbonyl group [at δ 198.9 (Cq)] and a double bond [at δ 129.1 (CH), 155.6 (Cq)] was deduced in the structure. Interestingly, the C-5 proton was shifted to a more downfield region than expected, resonating at δ 3.02 as a singlet. Its carbon signal followed by HMQC experiment at δ 59.1 indicating an oxo should be located on its neighboring carbon atom (C-6).

An olefinic proton signal was resonated at δ 6.06 which corresponded to a signal at δ 129.1 by HMQC experiment. The location of the double bond should be between C-1 and C-2, or C-2 and C-3, or C-7 and C-8. Based on the HMBC experiment, three bond away correlations were observed between the olefinic proton at δ 6.06 (H-7) and C-5 (at δ 59.1) as well as H-7 and C-9 (δ 49.2). Therefore, the presence of the double bond was deduced to be between C-7 and C-8. Considering the all above relationships, followed by HMQC and HMBC experiments, the enone moiety should be located on ring B.

In the ^{1}H-NMR spectrum, a proton signal appeared at δ 5.68 (brs) attributing to an oxygenated carbon's proton. Its carbon signal was observed at δ 73.3 following a direct correlation in the HMQC experiment. The presence of an acetate group in the structure was verified by observation of a methyl signal [δ ^{1}H 2.04 and δ ^{13}C 21.3] and carbon signal at δ 169.6 for acetyl carbonyl carbone. The location and stereochemistry of the acetate group was determined to be on C-14α based on its multiplicity as a broadened singlet. All the spectral data, particularly HMQC and two and/or three

bonds away HMBC correlations were supported abietane structure of compound **1** determined as 14α-acetoxy-6-oxo-abieta-7-ene, and named as obtusicrenone.

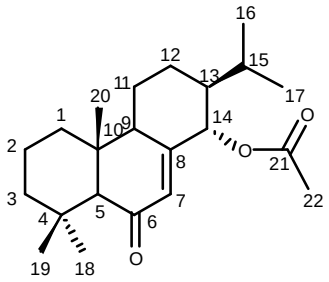

Figure 1. Obtusicrenone **(1)**

Table 1. ^{13}C (150 MHz) and ^{1}H (600 MHz) NMR data of compound 1[a]

Position	δ_C	δ_H, multiplicity (*J* in Hz)
1	37.8	1.83, ddd (13.6, 12.8, 3.0) 1.86, ddd (13.6, 11.4, 3.6)
2	17.6	not obs.[b]
3	37.5	1.63, brdd (14.2, 2.8) 1.76, brdd (14.2, 3.4)
4	39.4	-
5	59.1	3.02, s
6	198.9	-
7	129.1	6.06, d (3.0)
8	155.6	-
9	49.2	2.34, d (12.2)
10	42.7	-
11	22.5	1.28, m 1.32, brddd (13.6, 12.2, 3.9)
12	23.9	not obs.[b]
13	47.4	2.48, ddd (12.8, 9.6, 3.6)
14	73.3	5.68, brs
15	28.6	1.51, m
16	20.8	0.92, d (6.6)
17	20.8	0.94, d (6.6)
18	20.7	1.26, s
19	15.3	0.93, s
20	17.4	1.46, s
21	169.6	-
22	21.3	2.04, s

[a]In $CDCl_3$, [b]Not observed

The ESI (+)-MS of compound **2** exhibited a quasi molecular ion peak $[M-1]^+$ at *m/z* 487.6 corresponding to the molecular formula $C_{30}H_{48}O_5$ (calculated 488.4) with seven double bond equivalency. Thirty carbon signals were found in the ^{13}C-NMR (APT) spectrum consists of seven methyl carbons at δ 17.0, 22.9, 24.2, 24.4, 25.0, 26.0, 28.2, eight methylene carbons at δ 18.4, 22.9, 23.6, 32.2, 33.3, 34.4, 46.2, 65.5, seven methine carbons at δ 47.0, 51.6, 56.0, 69.0, 73.5, 85.3, 118.5 and eight quaternary carbons at δ 29.2, 37.8, 38.3, 41.6, 43.2, 49.0, 174.0, 210.0. Observation of seven methyl singlet signals at δ 0.93, 0.94, 0.95, 1.07, 1.08, 1.30, 1.31 in the ^{1}H-NMR spectrum revealed that an oleanane-type triterpene framework of compound **2** (Table 2).

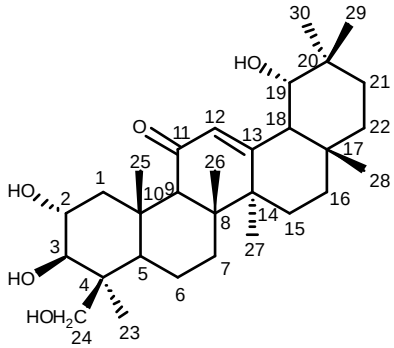

Figure 2. Nemrutolone **(2)**

Table 2. ^{13}C (125 MHz) and ^{1}H (500 MHz) NMR data of compound 2[a]

Position	δ_C	δ_H, multiplicity (*J* in Hz)
1	46.2	1.96, dd (12.5, 11.5) (H-1α) 0.92, dd (12.5, 3.8) (H-1β)
2	69.0	3.92 ddd (11.5, 9.6, 3.8)
3	85.3	3.20, d (9.6)
4	49.0	-
5	56.0	0.98, d (10.4)
6	18.4	0.94, brddd (13.6, 10.4, 9.4) (H-6α) 0.80, brddd (13.6, 12.8, 3.4) (H-6β)
7	33.3	not obs.[b]
8	41.6	-
9	51.6	2.60, brs
10	38.3	-
11	210.0	-
12	118.5	5.70, brs
13	174.0	-
14	43.2	-
15	22.9	not obs.[b]
16	23.6	not obs.[b]
17	29.2	-
18	47.0	1.70, brs
19	73.5	3.83, brs
20	37.8	-
21	32.2	1.42, brdd (14.1, 11.3) (H-21α) 2.08, m (H-21β)
22	34.4	2.17, m (H-22β) not obs.[b]
23	26.0	1.30, s
24	65.5	4.15, d (11.2) 3.41, d (11.2)
25	22.9	1.31, s
26	24.4	0.93, s
27	17.0	0.95, s
28	25.0	1.07, s
29	28.2	1.08, s
30	24.2	0.94, s

[a]In $CDCl_3$, [b]Not observed

The signal at δ 5.70 as a broadened singlet indicates the presence of an unsaturation which was proved by the one bond interaction between the proton at δ 5.70 and the carbon at δ 118.5 in the HSQC spectrum. The quaternary carbon of the trisubstituted double bond was observed at δ 174.0.

Such a downfield resonance is indicative of C-13 being in the β position to the carbonyl group at C-11, appeared at δ 210.0.

In the ^{1}H-NMR spectrum of compound **2** ($CDCl_3$, 500 MHz), in addition to the seven methyl protons, three methine protons were observed at δ 3.92 (*J*=ddd, 11.5, 9.6, 3.8 Hz), 3.83 (brs), 3.20 (d, *J*=9.6 Hz) which corresponded to the carbons at δ 69.0, 73.5, 85.3, respectively in the HSQC spectrum. These correlations verified that the three carbon atoms are hydroxylated. An observation of interaction via three bond away between the protons at δ 3.92 and 3.20 indicated their vicinity. Their possible locations should be considered as C-1 and C-2; C-2 and C-3; C-6 and C-7; C-15 and C-16; or C-21 and C-22. However, coupling values of the two protons clearly eliminated the last three possibilities except for (C-1 and C-2) or (C-2 and C-3) locations on ring A. Due to biogenetic consideration, the first hydroxyl group is located at C-3 in triterpenes, in general. Moreover, the chemical shift at δ 3.20 of H-3 signal with a *J* coupling value of 9.6 Hz as a doublet assigned to an axial-axial coupling with C-2 proton at δ 3.92 verifying their vicinity, and no presence of any substituent at C-1 based on coupling values of C-2 methine with C-1 methylene protons as *J*=3.8 and 11.5 Hz. The location of the secondary hydroxyl group at C-3 was further verified by the observation of a three-bond away correlations between H-3 signal and C-1 (δ 46.2) as well as H-9 signal (δ 2.60) and C-1 signal in the HMBC experiment. The presence of a hydroxymethylene group was observed by the proton signals at δ 4.15 (d, *J*=11.2 Hz, 1H) and 3.41 (d, *J*=11.2 Hz, 1H) corresponding to the same carbon signal, resonated at δ 65.5 followed by the HSQC spectrum. The location of hydroxymethylene group was deduced to be at C-24 by the observation of the two-bond away HMBC correlation between one of the methylene protons (δ 3.41) and C-4 (δ 49.0) signal. The third secondary hydroxyl group could be located on ring E rather than rings A, B or D due to its appearance as a broadened singlet at δ 3.83. In the location on either B or D rings, its methine proton signal would be observed as a doublet of doublet or a multiplet, therefore, its location on B and D rings were eliminated. Its location on ring A was also not possible due to its appearance as a broadened singlet rather than a dd or multiplet. Thus, the only possibility was the location on ring E at C-19. Its stereochemistry was determined as α based on the angle (84.0^{o}) between H-18β and H-19β which corresponds to almost 0 Hz according to the Karplus curve.

Compound **2** is a C-3β-hydroxylated oleanane triterpene with a hydroxymethylene group attached to C-4 either positioned on C-23 or C-24. However, the appearance of C-3α signal at δ 3.20 was found to be relatively higher field resonance as compared with 23-hydroxy derivatives, assigning to its location at C-24 rather than C-23, because 3β-hydroxylated oleanane triterpenes with 23-hydroxymethylene group, such as 23-hydroxyerythrodiol and hederagenin showed more downfield signals (δ 3.63 and 3.67, respectively) for their C-3α protons due to C-23 hydroxymethylene. This upfield shift may be due to the loss of the deshielding effect that the 23-CH_2OH function exerts in 23-hydroxyerythrodiol and hederagenin. The ^{13}C NMR data of **2** (Table 2) showed positive and lower γ-anti effects for C-3 and C-5 because in this 24-hydroxy derivative, the 1,3-diaxial proton interactions are not possible, and so C-3 and C-5 are more deshielded (δ 85.3 and δ 56.0, respectively). These values are comparable to δ 85.8 and 85.7 for the 24-hydroxy derivatives hyptatic acid-B and sericoside, respectively, and lower than in the corresponding 23-hydroxy derivative [47]. Thus, the structure of compound **2** was deduced to be 2α,3β,19α,24-tetrahydroxy-11-oxo-olean-12-ene.

The antioxidant activities of the extracts and the isolated pure terpenoids were investigated by using four antioxidant assay methods [DPPH free radical scavenging activity, ABTS cation radical scavenging activity, lipid peroxidation inhibition activity, CUPRAC (Cupric Reducing Antioxidant Capacity)], and none of the samples exhibited significant antioxidant activity (Tables 4-5). The anticholinesterase activities of the extracts and the isolated pure terpenoids were investigated by Ellman method. The BChE inhibition of the extracts and isolates was not strong. However, their AChE inhibition values were very promising (Tables 3-4), particularly for obtusicrenone [IC_{50}=13.78 μg/mL].

Table 3. Anticholinesterase activity of the extracts at 200 µg/mL

Samples	Inhibition % against AChE	Inhibition % against BChE
NOD[a]	71.03	12.06
NOM[b]	73.59	4.89
Galantamine	74.12	74.48

[a]NOD: Dichloromethane extract of *Nepeta* obtusicrena.
[b]NOM: Methanol extract of *Nepeta* obtusicrena.

Table 4. IC_{50} values of the samples (µg/mL)

Samples	DPPH	ABTS	β-Caroten	Anti-AChE	Anti-BChE
NOD[a]	NA[c]	NA	139.12	*	*
NOM[b]	162.21	**69.16**	NA	*	*
Obtusicrenone	NA	173.15	140.91	**13.78**	NA
Nemrutolone	NA	116.67	107.96	**54.36**	NA
Oleanolic acid	NA	NA	171.20	**27.88**	NA
Ursolic acid	NA	NA	197.30	**36.09**	NA
BHA	16.19	12.66	5.06	-	-
BHT	74.59	18.35	6.24	-	-
α- Tocopherol	33.09	19.35	0.53	-	-
Galantamine	-	-	-	5.01	53.90

[a]NOD: Dichloromethane extract of *Nepeta* obtusicrena.
[b]NOM: Methanol extract of *Nepeta* obtusicrena.
[c]NA: Not active (IC_{50}≥200).

Table 5. CUPRAC assay results

Samples	10 µg/mL	25 µg/mL	50 µg/mL	100 µg/mL
NOD[a]	0.13	0.17	0.22	0.30
NOM[b]	0.13	0.21	0.32	0.49
Obtusicrenone	0.11	0.19	0.26	0.41
Nemrutolone	0.14	0.20	0.28	0.36
Oleanolic acid	0.90	0.18	0.24	0.31
Ursolic acid	0.12	0.16	0.23	0.30
BHA	1.32	1.74	2.04	2.57
BHT	1.51	1.84	2.24	2.69
α- Tocopherol	0.32	0.49	0.75	1.47

[a]NOD: Dichloromethane extract of *Nepeta* obtusicrena.
[b]NOM: Methanol extract of *Nepeta* obtusicrena.

4. Conclusion

A novel diterpenoid, 14α-acetoxy-6-oxo-abieta-7-ene (obtusicrenone) and a novel triterpenoid, 2α,3β,19α,24-tetrahydroxy-11-oxo-olean-12-ene (nemrutolone) were isolated from the dichloromethane extract of *N. obtusicrena* in addition to two known triterpenoids, oleanolic acid and ursolic acid. The antioxidant and anticholinesterase activities of the extracts and isolated terpenoids were investigated. Although AChE inhibition of the extracts and four isolates was very significant, their antioxidant activities were found not to be good. The antioxidant activity and BChE inhibiton results of the methanol extract were found to be better compared to dichloromethane extract, probably due to presence of phenolic constituents and other flavonoids in the methanol extract which has not been studied yet. In the previous studies, although most of the tested abietane diterpenes exhibited high cholinesterase inhibitory activity particularly against BChE enzyme, obtusicrenone as an abietane diterpene with a non-aromatic ring C showed high acetylcholinesterase inhibitory activity. It is significant that the abietane diterpene and three triterpenes exhibited selective activity against AChE enzyme and found to be no activity against BChE enzyme.

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Supporting Information

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

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