Cholinesterase Inhibitory Triterpenes from *Perovskia atriplicifolia*

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Received: 7 January 2014;

Accepted: 20 February 2014;

Published online: 1 September 2014;

AJC-15875

Nine compounds including β -amyrin (1), erythrodiol (2), oleanolic acid (3), 3 β -hydroxy-11,13(18)-oleanadien-28-oic acid (4), glycyrrhetic acid (5), 2 α ,3 β -dihydroxyolean-12-en-28-methyl ester (6), 2 α ,3 β -dihydroxyolean-12-en-28-oic acid (7), 2 α ,3 β ,24-trihydroxyolean-12-en-28-oic acid (8) and 2 α ,3 β ,19 β -trihydroxyurs-12-en-28-oic acid (9), have been isolated from *Perovskia atriplicifolia*. Their structures have been established with the help of different spectral data. All of these compounds were tested for cholinesterase inhibitory activity. Among the tested compounds, 8 and 9 were found to be the most active against both enzymes, with a significant butyrylcholinesterase (BChE) inhibitory activity demonstrating IC₅₀ values 9.50 and 13.52 mM, respectively, compared to galanthamine standard (IC₅₀: 8.51 mM).

Keywords: Cholinesterase inhibition, Lamiaceae, *Perovskia atriplicifolia*, Triterpenes.

INTRODUCTION

Alzheimer's disease (AD) is a chronic neurological disorder characterized by memory impairment, cognitive dysfunction and behavioral disturbances^{1,2}. Alzheimer's disease has been found to be associated with a cholinergic deficit in the post-mortem brain characterized by a significant decrease in acetylcholine content^{3,4}. Acetylcholine is a neurotransmitter inhibited primarily by acetylcholinesterase (AChE) and secondly by butyrylcholinesterase (BChE) and is considered to play a role in the pathology of Alzheimer's disease⁵. Despite the unknown etiology of Alzheimer's disease, elevation of acetylcholine amount through AChE enzyme inhibition has been accepted as the most effective treatment strategy against Alzheimer's disease⁶. It has been found that BChE is found in significantly higher quantities in Alzheimer plaques than in plaques of normal age-related non-demented brains. It is generally viewed as a backup for the homologous acetylcholinesterase and to act as a scavenger for anticholinesterase compounds⁷. Therefore, AChE and BChE inhibitors have become the remarkable alternatives in treatment of Alzheimer's disease. However, the present drugs with AChE inhibitory activity have some side effects and are effective only against the mild type of Alzheimer's disease⁸. Consequently, it is compulsory to develop new drugs in order to combat Alzheimer's disease.

Genus *Perovskia* belongs to family Lamiaceae and comprises seven species, one of which is *Perovskia atriplicifolia*, commonly known as Russian sage. The plant is shrubby, found in Central Asia, Pakistan, Afghanistan and Iran. *P. atriplicifolia* has antibacterial activity and is also used as cooling medicine in the treatment of fever⁹. Many compounds have been previously reported from this species¹⁰. The ethnopharmacological and chemotaxonomic importance of the genus *Perovskia* prompted us to do further investigation of the chemical constituents of *P. atriplicifolia*. Moreover, pharmacological screening revealed significant cholinesterase inhibitory activity in the chloroform soluble fraction of the plant. Bioassay directed isolation studies on this fraction resulted in the isolation of triterpenes 1-9.

EXPERIMENTAL

General procedure: Column chromatography (CC): Silica gel 70-230 mesh; TLC: pre-coated silica gel 60 F₂₅₄ (20 × 20 cm, 0.2 mm thick; E-Merck) plates; UV: detection at 254 nm and using ceric sulphate reagent. Optical rotations: Jasco-DIP-360 digital polarimeter. IR spectra: Shimadzu IR-460 spectrophotometer. ¹H NMR spectra: Bruker spectrometers operating at 300, 400 and 500 MHz. Chemical shift δ in ppm relative to SiMe₄ as internal standard and coupling constants

J in Hz. EI-MS, HR-EI-MS: JEOL JMS-HX-110 and JMS-DA-500 mass spectrometers, *m/z*: (rel. int). The purity of the isolated compounds was checked on pre-coated thin layer chromatography (TLC) plates of E. Merck.

The aerial parts of *Perovskia atriplicifolia* (8.0 kg) were collected from Quetta (Pakistan) and identified by Prof. Rasool Bakhsh Tareen, Department of Botany, University of Baluchistan, where a voucher specimen has been deposited (BU-68).

Isolation: The shade dried, powdered aerial parts (8 kg) were extracted with methanol (3 × 20 L) at room temperature. The extract was evaporated to yield the residue (620 g) which was divided into *n*-hexane (130 g), chloroform (90 g), ethyl acetate (95 g), *n*-butanol (150 g) and water (140 g) soluble fractions. Part of the chloroform soluble sub-fraction (75 g) was subjected to column chromatography over silica gel eluting with chloroform, chloroform-methanol and methanol in increasing order of polarity to give nine sub-fractions C1-C9. Sub-fractions C2-C7 were rich in triterpenes. Sub-fraction C2 (chloroform, 100 %) was rechromatographed over silica gel using chloroform-methanol (9.8:0.2) as eluent to afford (**1**, 15 mg) and (**2**, 12 mg). Sub-fraction C₃ (chloroform-methanol, 9.7:0.3) was flash chromatographed on silica using chloroform-methanol 9.7:0.3, as eluent to obtain a mixture of two compounds. These were finally purified by preparative TLC using the solvent system chloroform-methanol 9.5:0.5, providing (**4**, 23 mg) and (**5**, 20 mg), respectively. Compound (**3**, 14 mg) was obtained from fraction C₇ by direct crystallization with acetone. The sub-fraction C₄ (chloroform-methanol, 9.5:0.5) was purified by further chromatography eluting with chloroform-methanol (9.4:0.6) to furnish compound (**6**, 18 mg). Sub-fraction C₅ (chloroform-methanol, 9.4 : 0.6) was rechromatographed over silica gel using solvent system chloroform-methanol 9.1:0.9, to obtain (**7**, 15 mg) and (**8**, 10 mg). Sub-fraction C₆ (chloroform-methanol, 9.2:0.8) was rechromatographed over silica gel using solvent system chloroform-methanol 9.0:1.0 to give (**9**, 10 mg).

in vitro Cholinesterase inhibition assay: Electric eel acetylcholinesterase (EC 3.1.1.7, type-VI-S), horse butyrylcholin-esterase (EC 3.1.1.8), acetylthiocholine iodide, butyrylthiocholin chloride and 5,5'-dithio-*bis*-nitrobenzoic acid (DTNB) were purchased from the Sigma (St. Louis, MO). Buffers and other chemicals were of extra pure analytical grade. Galanthamine (Reminyl® Johnson & Johnson) was used as the standard drug. Acetylcholinesterase inhibition was determined spectrophotometrically using acetylthiocholine as substrate by modifying the method of Ellma and coworkers^{11,12}. In this method, 140 µL of 0.1 mM sodium phosphate buffer (pH 8.0), 20 µL enzyme preparation and 10 µL test compound solution dissolved in ethanol were mixed and incubated for 0.5 h. 10 µL of 5,5'-dithio-*bis*-nitrobenzoic acid was added and the reaction was then started by adding 10 µL of acetylthiocholine. Ten microliters of butyrylthiocholine chloride was used as a substrate to assay butyrylcholinesterase enzyme, while all the other reagents and conditions were the same. The hydrolysis of acetylthiocholine or butyrylthiocholine was determined by monitoring the formation of the yellow 5-thio-2-nitrobenzoate anion as a result of the reaction with 5,5'-dithio-*bis*-nitrobenzoic acid with thiocholines, catalyzed by

enzymes at a wavelength of 412 nm. Ethanol was used as negative control. All the reactions were performed in triplicate in 96-well microliter plates on a Spectra Max 384 (Molecular Devices, USA). The IC₅₀ values were calculated using according to Michaelis-Menten model by using "EZ-Fit. Enzyme Inhibition Kinetic Analysis (EZ-Fit:Enzyme Kinetics MS Windows Software, Perrella Scientific, Inc., Amshert, USA)" program.

Statistical method: The assays were conducted in triplicate and all tabulated results were expressed as means ± S.E.M. and were compared using Student's t-test. A P value of less than 0.05 was considered significant.

RESULTS AND DISCUSSION

The chloroform soluble fraction has been subjected to a series of column chromatographic separations to obtain nine triterpenoids reported for the first time from *P. atriplicifolia* (Fig. 1). These were identified as β-amyrin (**1**)¹³, erythrodiol (**2**)¹⁴, oleanolic acid (**3**)¹⁵, 3β-hydroxy-11,13(18)-oleanadien-28-oic acid (**4**)¹⁶, glycyrrhetic acid (**5**)¹⁵, 2α,3β-dihydroxy-olean-12-en-28-methyl ester (**6**)¹⁵, 2α,3β-dihydroxyolean-12-en-28-oic acid (**7**)¹⁷, 2α,3β,24-trihydroxyolean-12-en-28-oic acid (**8**)¹⁸ and 2α,3β,19β-trihydroxyurs-12-en-28-oic acid (**9**)¹⁹.

Compounds **1-9** were evaluated for their cholinesterase inhibitory activities and results are shown in Table-1.

TABLE-1
in vitro QUANTITATIVE INHIBITION OF
CHOLINESTERASE BY TRITERPENES **1-9**

Compounds	AChE IC ₅₀ [µM]	BChE IC ₅₀ [µM]
1	-	-
2	-	-
3	-	-
4	-	-
5	54.53 ± 0.05 ^a	34.52 ± 0.04 ^a
6	46.51 ± 0.05 ^a	28.06 ± 0.06 ^a
7	33.50 ± 0.05 ^a	19.04 ± 0.05 ^a
8	24.57 ± 0.05 ^a	9.50 ± 0.03 ^a
9	29.54 ± 0.05 ^a	13.52 ± 0.03 ^a
Galanthamine	5.20 ± 0.05	8.51 ± 0.05

Values were expressed as mean ± S.E.M. (n = 3). ^aP < 0.001, compared to reference drug. IC₅₀: concentration of test compound that inhibited the hydrolysis of substrate by 50 %.

According to the results, triterpenes **1-4** did not show any cholinesterase inhibitory activity, while compounds **5-7** were moderately active. Among the tested compounds, **8** and **9** were found to be the most active against both enzymes. Previous reports on cholinesterase inhibitory activity of structurally related triterpenes could be found in literature²⁰. Compounds **8** and **9** possessed potent inhibitory activity against acetylcholinesterase, with IC₅₀ values 24.57 and 29.54 µM, respectively. Moreover, they showed significant and more selective butyrylcholinesterase inhibitory activity with IC₅₀ values 9.50 and 13.52 µM compared to galanthamine standard (IC₅₀: 8.51 µM). Upon examining the structures of the isolated compounds, it could be seen that the number and the position of the hydroxyl groups are the controlling factors for the cholinesterase inhibitory activities and not the ursane or oleanane skeleton of

Fig. 1. Structures of triterpenes 1-9

the triterpene. Compounds **8** and **9** possess three hydroxyl groups instead of two and the presence of all three groups on the same ring seems to increase the activity even more, as is the case with compound **8**. In the light of these findings, both compounds could be considered for further studies in the treatment of Alzheimer's disease.

β -Amyrin (1) (36 mg) was obtained as colorless needles from CH_3OH ; m.p. 185–186 °C; $[\alpha]_{\text{D}}^{25} + 100$ ($c = 0.20$, CHCl_3); IR (KBr, ν_{max} , cm^{-1}): 3430, 3045, 1600, 815; $^1\text{H NMR}$ (CDCl_3 , 300 MHz) δ : 5.16 (1H, t, 3.2 Hz, H-12), 3.18 (1H, dd, $J = 10.4$, 4.2 Hz, H-3), 1.17, 1.03, 1.05, 0.98, 0.93, 0.87, 0.80 and 0.79 (3H, each s, CH_3); HR-EI-MS showed $[\text{M}]^+$ at m/z 426.3853 (calcd. for $\text{C}_{30}\text{H}_{50}\text{O}$, 426.3860). The physical and spectral data coincided with the literature values.

Erythrodiol (2) (21 mg) was obtained as white crystals; m.p. 228–229 °C; $[\alpha]_{\text{D}}^{27} + 45.0$ ($c = 0.87$, CHCl_3); IR (KBr, ν_{max} , cm^{-1}): 3580, 3430 and 1610; $^1\text{H NMR}$ (CDCl_3 , 300 MHz) δ : 5.12 (1H, t, $J = 3.5$ Hz, H-12), 3.52 (1H, m, H-3), 3.19 (2H, m, H-28), 1.08, 0.98, 0.98, 0.97, 0.93, 0.91, 0.77 (3H, each s, Me); HR-EI-MS showed $[\text{M}]^+$ at m/z 442.3798 (calcd. for $\text{C}_{30}\text{H}_{50}\text{O}_2$, 442.3811). The physical and spectral data corresponded to the reported values.

Oleanolic acid (3) (21 mg) was obtained as an amorphous powder; m.p. 305–306 °C; $[\alpha]_{\text{D}}^{27} + 78.9$ ($c = 0.07$, CHCl_3); IR

(KBr, ν_{max} , cm^{-1}): 3400, 1700, 1660 and 820; $^1\text{H NMR}$ (CDCl_3 , 300 MHz) δ : 5.25 (1H, t, $J = 3.5$ Hz, H-12), 3.18 (1H, dd, $J = 4.7$, 10.2 Hz, H-3), 1.11, 0.96, 0.90, 0.89, 0.88, 0.75 and 0.73 (3H, each s, Me); HR-EI-MS showed $[\text{M}]^+$ at m/z 456.3545 (calcd. for $\text{C}_{30}\text{H}_{48}\text{O}_3$, 456.3603). The physical and spectral data corresponded to the reported values.

3β -Hydroxy-11,13(18)-oleanadien-28-oic acid (4) (32 mg) was obtained as colourless needles from CH_3OH ; m.p. 237–240 °C; $[\alpha]_{\text{D}}^{25} - 51$ ($c = 0.08$, MeOH); IR (KBr, ν_{max} , cm^{-1}): 3400–2640, 1700, 1660 and 820; $^1\text{H NMR}$ (CD_3OD , 300 MHz) δ : 6.65 (d, $J = 10.5$ Hz, H-12), 6.30 (dd, $J = 10.5$, 2.6 Hz, H-11), 3.21 (m, H-3), 0.98, 0.98, 0.93, 0.91, 0.84, 0.79 and 0.75 (each, s, CH_3); HR-EI-MS showed $[\text{M}]^+$ at m/z 454.3441 ($\text{C}_{30}\text{H}_{46}\text{O}_3$ (calcd. for $\text{C}_{30}\text{H}_{46}\text{O}_3$ 454.3447). The physical and spectral data showed complete resemblance with the reported values.

Glycyrrhetic acid (5) (21 mg) was obtained as an amorphous powder; m.p. 300–302 °C; $[\alpha]_{\text{D}}^{27} + 161^\circ$ ($c = 0.07$, CHCl_3); IR (KBr, ν_{max} , cm^{-1}): 1730, 1630 and 1550; $^1\text{H NMR}$ (CD_3OD , 300 MHz) δ : 5.72 (1H, s, H-12), 2.94 (ddd, $J = 11.0$, 4.0 Hz, H-3), 2.61 (m, H-18), 2.42 (s, H-9), 0.83, 1.04, 1.08, 1.14, 1.20, 1.25 and 1.35 (each, s, CH_3); HR-EI-MS showed $[\text{M}]^+$ at m/z 470.3275 (calcd. for $\text{C}_{30}\text{H}_{46}\text{O}_4$, 470.3396). The physical and spectral data corresponded to the reported values.

2 α ,3 β -Dihydroxyolean-12-en-28-methyl ester (**6**) (55 mg) was obtained as gummy solid, 298-300 °C; $[\alpha]_D^{25} + 67.5^\circ$ (c = 0.07, CHCl₃); IR (CHCl₃) (KBr, $\nu_{\max}$, cm⁻¹): 3450, 1725 and 1650 cm⁻¹; ¹H NMR (CD₃OD, 300 MHz) δ : 5.13 (1H, s, H-12), 3.56 (m, H-2), 3.23 (s, OMe), 2.72 (d, *J* = 10.7 Hz, H-3), 0.77, 0.78, 0.79, 0.97, 1.01, 1.13 and 1.17 (each, s, CH₃); HR-EI-MS showed [M]⁺ at *m/z* 486.3519 C₃₁H₅₀O₄ (calcd. for C₃₁H₅₀O₄; 486.3709). The physical and spectral data coincided with the reported values.

2 α ,3 β -Dihydroxyolean-12-en-28-oic acid (**7**) (21 mg) was obtained as an amorphous powder; 305-306 °C; $[\alpha]_D^{27} + 78.9^\circ$ (c = 0.07, CHCl₃); IR (KBr, $\nu_{\max}$, cm⁻¹): 3400-2640, 1700, 1660 and 820; ¹H NMR (CD₃OD, 300 MHz) δ : 5.24 (t, *J* = 3.45 Hz, H-12), 3.61 (m, H-2), 2.88 (d, *J* = 9.5 Hz, H-3), 0.79, 0.81, 0.89, 0.93, 0.99, 1.00 and 1.15 (each, s, CH₃); HR-EI-MS showed [M]⁺ at *m/z* 472.3410 C₃₀H₄₈O₄ (calcd. for C₃₀H₄₈O₄; 472. 3552). The physical and spectral data corresponded to the reported values.

2 α ,3 β ,24-Trihydroxyolean-12-en-28-oic acid (**8**) (32 mg) was obtained as colorless needles from CH₃OH; m.p. 305-306 °C; $[\alpha]_D^{25} + 78.9^\circ$ (c = 0.07, CHCl₃); IR (KBr, $\nu_{\max}$, cm⁻¹): 3400-2640, 1700, 1660 and 820; ¹H NMR (CD₃OD, 300 MHz) δ : 5.24 (1H, t, *J* = 3.5 Hz, H-12), 3.76 (dd, *J* = 9.6, 4.2 Hz, H-2), 3.02 (d, *J* = 9.6 Hz, H-3), 4.00 (d, *J* = 11.2 Hz, H-24) and at 3.35 (d, *J* = 11.2 Hz, H-24), 2.82 (dd, *J* = 14.1, 4.1 Hz, H-18), 0.78, 0.89, 0.93, 0.97, 1.15 and 1.12 each, s, CH₃); HR-EI-MS showed [M]⁺ at *m/z* 488.3416 C₃₀H₄₈O₅ (calcd. for C₃₀H₄₈O₅; 488. 3501). The physical and spectral data showed complete resemblance with the reported values.

2 α ,3 β ,19 β -Trihydroxyurs-12-en-28-oic acid (**9**) (32 mg) was obtained as colorless needles from CH₃OH; m.p. 270-272 °C; $[\alpha]_D^{25} + 12^\circ$ (c = 1.0, MeOH); IR (KBr, $\nu_{\max}$, cm⁻¹): 3400-2640, 1700, 1660 and 820; ¹H NMR (CD₃OD, 300 MHz) δ : 5.27 (1H, t, *J* = 3.3 Hz, H-12), 3.60 (dd, *J* = 3.3, 9.6 Hz, H-2), 2.89 (d, *J* = 9.6 Hz H-3), 2.50 (s, H-18), 0.79, 0.80, 0.99, 1.01, 1.18 and 1.33 each, s, CH₃), 0.91 (d, *J* = 6.7 Hz, CH₃-30); HR-EI-MS showed [M]⁺ at *m/z* 488.3419 C₃₀H₄₈O₅ (calcd. for C₃₀H₄₈O₅ 488.3501).

The physical and spectral data showed complete resemblance with the reported values.

Conflict of interest: The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

The authors extended their sincere appreciation to the Deanship of Scientific Research at King Saud University for its funding of this research through the Research Group Project no. RGP-VPP-221.

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