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A New Ursane-type Triterpenoid from *Salvia santolinifolia*

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Santolinoic acid (**1**), a new ursane-type triterpene was isolated from the chloroform soluble fraction of *Salvia santolinifolia*. Its structure was established as 2α,3β,9α-trihydroxyurs-12-en-28-oic acid on the basis of spectroscopic analysis, including high resolution mass spectroscopy, and 1- and 2-dimensional NMR techniques. The compound (**1**) showed inhibitory activity against the enzymes acetylcholinesterase and butyrylcholinesterase.

Key Words: *Salvia santolinifolia*, Labiatae, triterpenoid, enzyme inhibition.

Introduction

The genus *Salvia* belongs to the family Labiatae, which comprises 24 species. It is characterized by branched herbs and shrubs that are distributed in Pakistan, India, and Afghanistan. *Salvia santolinifolia* Boiss. is a branched, scabrid, and hispid straggling undershrub occurring in Peshawar, Baluchistan, and Karachi. Various species of this genus are widely used for treatment of coronary heart diseases, particularly angina pectoris, amenorrhea, dysmenorrhea, and insomnia. Most species, including *S. santolinifolia*, additionally possess antiseptic, carminative, diuretic, hemostatic, and spasmolytic properties.[1−3]

Previously, a variety of compounds, including diterpenes, aromatic ethers, phenolic glycosides, triterpenes, and abietane type pigments, have been reported from this plant.[4] The methanolic extract of this plant revealed a strong toxicity in the brine shrimp lethality test.[5,6] During a search for new bioactive compounds from indigenous medicinal plants, we isolated santolinoic acid (**1**), as well as oleanolic acid (**2**) and 1β, 3β, 23-trihydroxyolean-12-en-28-oic acid (**3**), from the chloroform-soluble fraction, which is reported for the first time from this plant source.

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Experimental

General and instrumentation

The ^{1}H-NMR, ^{13}C-NMR, HMQC, and HMBC spectra were recorded on Bruker spectrometers, operating at 400 MHz for ^{1}H-NMR and 100.6 MHz for ^{13}C-NMR. EIMS and HR-FAB-MS were recorded on a Jeol JMS-HX-110 with a data system and on JMS-DA 500 mass spectrometers. IR spectra were recorded on a 460 Shimadzu spectrometer (ν in cm^{-1}). Optical rotations were measured on a JASCO DIP-360 polarimeter. Visualization of the TLC plates was achieved under UV at 254 and 366 nm, and by spraying with ceric sulfate reagent solution (with heating). For TLC, pre-coated aluminum sheets, silica gel 60 F_{254}(20 $\times$ 20 cm, 0.2 mm thick; Merck), and for column silica gel (230-400 mesh) were used. The chemical shift values are reported in ppm (δ) units and the coupling constants (J) are in Hz. For enzyme inhibition assay, all chemicals and butyrylcholinesterase were purchased from Sigma (USA).

Plant material

Whole plants of *Salvia santolinifolia* Boiss. (24 kg) were collected from Karachi (Pakistan) in July 2002 and identified by Dr. Surriya Khatoon, Plant Taxonomist, Department of Botany, University of Karachi, where a voucher specimen (LS 831) is deposited.

Extraction and isolation

Salvia santolinifolia plants were shade dried, ground, and extracted 3 times with ethanol. The combined ethanolic extract was partitioned between hexane and MeOH. The MeOH fraction was further partitioned with $CHCl_3$ and then with EtOAc. The $CHCl_3$ fraction (35 g) was subjected to column chromatography over silica gel eluting with hexane-$CHCl_3$, $CHCl_3$, $CHCl_3$ -MeOH, and MeOH, in increasing order of polarity. The fractions that were obtained from the column through elution with $CHCl_3$-MeOH (9.5:0.5) were a mixture of 2 components, which could be resolved through preparative TLC using a solvent system ($CHCl_3$: MeOH; 9.2:0.8) to obtain oleanolic acid (**2**) (18 mg) and 1β, 3β, 23-trihydroxyolean-12-en-28-oic acid (**3**) (20 mg), respectively.

The identity of these compounds was confirmed through comparison of their physical and spectral data with those reported in the literature.[7,8]

The fraction obtained from $CHCl_3$-MeOH (9.6:0.4) was subjected to column chromatography over silica gel using a solvent system $CHCl_3$-MeOH (9.3:0.7) and final purification was performed by preparative TLC using a solvent system $CHCl_3$-MeOH (8.8:1.2) to afford the new triterpene, santolinoic acid (**1**) (28 mg).

Santolinoic Acid (1)

Santolinoic acid (**1**) was obtained as a colorless amorphous solid. $[\alpha]_D^{26}$ – 78.2 $^\circ$ (c = 0.064, MeOH) IR (KBr) ν_{max} cm^{-1}: 3450, 1700, 1655. ^{1}H-NMR and^{13}C-NMR (Table 1). HREIMS [M$^+$] m/z 488.3490 (calcd. for $C_{30}H_{48}O_5$: 488.3501). EIMS m/z (rel. int. %): 488 (10), 456 (28.5), 442 (5.0), 396 (8.4), 248 (100), 203 (82), 187 (33.8), 149 (15), 105 (44) 69 (41.3), 55 (52.6).

Acetylation of santolinoic acid (1)

Compound **1** (14.0 mg) was acetylated with Ac_2O (2 mL) in pyridine (2 mL) at room temperature for 24 h. Usual workup provided the corresponding acetyl derivative (**1a**). Amorphous solid $[\alpha]_D^{26}$ – 45.4 ° (c = 0.058, MeOH): IR (KBr) ν_{max} cm^{-1}: 3410, 1720, 1660. ^{1}H-NMR (400 MHz, CD_3OD) δ : 0.79 (3H, s, Me-24), 0.98 (3H, d, J = 5.8 Hz, H-29), 1.09 (3H, s, Me-23), 1.11 (3H, d, J = 6.7 Hz, H-30), 1.18 (3H, s, Me-25), 1.29 (3H, s, Me-27), δ 2.03 (6H, s, 2xOAC), 3.27 (1H, d, J = 9.5 Hz, H-3), 3.97 (1H, ddd, J = 10.8, 9.5, 4.0 Hz, H-2), 5.25 (1H, t, J = 3.4 Hz, H-12),. HR-FAB-MS [M^+] m/z 572.3761 (calcd. for $C_{34}H_{52}O_7$: 572.3713). EIMS m/z (rel. int %): 440 (5.8), 375 (10), 248 (100), 219 (18), 187 (33).

Methylation of 1a

To an ethereal solution of **1a** (10 mg) was added freshly prepared CH_2N_2 in excess, followed by storage at room temperature overnight. Usual workup of the reaction mixture afforded **1b**, which crystallized in white needles from concentrated methanolic solution in the cold. ^{1}H-NMR (500 MHz, CD_3OD) δ: 0.78 (3H, s, Me-24), 0.92 (3H, d, J = 6.7Hz, H-30), 1.01 (3H, d, J = 5.8Hz, H-29), 1.03 (3H, s, Me-23), 1.15 (3H, s-Me-25), 1.31 (3H, s, Me-27), 2.02 (6H, s, 2× OAc) 3.12 (3H, s, OCH_3), 3.29 (1H, d, J = 9.5 Hz, H-3), 3.99 (1H, ddd, J = 10.8, 9.5, 4.0 Hz, H-2), 5.25 (1H, t, J = 3.4 Hz, H-12). HR-FAB-MS [M^+] m/z: 586.3895 (calcd. for $C_{35}H_{54}O_7$: 586.3869) EIMS m/z (rel. int %): 442 (5), 248 (100), 187 (33).

Cholinesterase inhibition assay

The enzyme activities were determined by a modified method of Ellman et al.[9,10] Electric eel acetylcholinesterase (AChE, 3.1.1.7), horse serum butyrylcholinesterase (BChE, 3.1.1.8), acetylthiocholine iodide, butyrylthiocholine chloride, 5,5‘-dithiobis [2-nitrobenzoic acid (DTNB), and galanthamine were purchased from Sigma (USA). The reaction mixture contained 150 μL of phosphate buffer, pH 8.0 (100 mM), 10 μL of DTNB (0.25 mM), 10 μL of test compound in ethanol, and 20 μL of enzyme. The contents were mixed and pre-incubated at 25 ° C for 10 min. The reaction was initiated by the addition of 10 μL of the substrate (30 mM) of the respective enzyme. The hydrolysis of the substrate was measured at 412 nm after 15 min by the formation of the yellow 5-thio-2-nitrobenzoate anion. All the reactions were performed in triplicate on a Spectra Max 384 Plus 96-well plate reader (Molecular Devices, USA). The IC_{50} values were calculated using the EZ-Fit Enzyme Kinetics program (Perrella Scientific Inc., Amherst, USA).

Results and Discussion

The IR spectrum of **1** displayed absorptions for hydroxyl groups (3450 cm^{-1}), acid carbonyl (1700 cm^{-1}), and a double bond (1655 cm^{-1}).[11] The HREIMS showed [M^+] a peak at m/z 488.3490 corresponding to the molecular formula $C_{30}H_{48}O_5$ (calcd. for $C_{30}H_{48}O_5$, 488.3501). Acetylation of **1** provided diacetate (**1a)**, which still showed the hydroxyl absorption in its IR spectrum, confirming the presence of one or more tertiary hydroxyls. Methylation of **1** a afforded the monomethyl ester (**1b**), confirming the presence of one carboxyl group. In EIMS, the peaks at m/z 240.1720 ($C_{14}H_{24}O_3$, 240.1725) and 248.1768 ($C_{16}H_{24}O_2$, 248.1776) could be assigned to the fragments of the A/B rings and D/E rings formed by retro-Diels-Alder

fragmentation of the C-ring. These fragment ions confirmed the presence of 2 oxygen functionalities in rings D/E and the remaining 3 oxygen functionalities in rings A/B. The fragment at 248, being common to that of ursolic acid, allowed us to assign the carboxylic group at the angular C-17 position.[8] This was further authenticated by a peak at m/z 203 due to the loss of the carboxylic group from the fragment ion at m/z 248. In addition, the ^{13}C-NMR spectrum showed signals for 7 methyls, 8 methylenes, 7 methines, and 8 quaternary carbons, together with 1 carboxyl group at δ 181.0. While the signals of olefinic carbons were observed at δ 138.2 and δ 128.5, respectively, 2 oxymethine carbons were observed at δ 83.4 and δ 68.4, while a downfield quaternary carbon at δ72.9 indicated the presence of a tertiary hydroxyl group. The ^{1}H-NMR spectrum showed a trisubstituted double bond at δ 5.25 (t, J = 3.4 Hz), and 2 oxymethine protons at δ 3.61 (1H, ddd, J = 10.8, 9.5, 4.0 Hz) and δ 2.92 (1H, d, J = 9.5 Hz). In addition, 5 tertiary methyls were observed as singlets at δ 1.31, 1.16, 1.03, 0.99, and 0.78, while doublets of 2 additional secondary methyls were observed at δ 1.00 (3H, d, J = 5.8 Hz) and 0.91 (3H, d, J = 6.7Hz). The above data was consistent with an ursane-type triterpene carrying a double bond at Δ^{12}.[12]

Acetylation of **1** to the corresponding diacetyl derivative (**1a**) showed that 2 out of the 3 remaining oxygens are accounted for by secondary hydroxyls, providing evidence for a tertiary hydroxyl. It was supported by the presence of the corresponding carbons signals at δ68.4 (d), 83.4 (d), and 72.9 (s) in the ^{13}C-NMR spectrum. As the mass fragmentation pattern of **1** indicated that all the hydroxyl groups were present in rings A and/or B, their positions at C-2, C-3, and C-9 were conclusively established through HMQC and HMBC experiments in which H-2 (δ3.61) showed ^{2}Jcorrelation to C-3 (δ83.4) and ^{3}J to C-4 (δ41.1), while H-3 (δ2.92) showed ^{2}Jcorrelation to C-2 (δ68.4) and C-4 (δ41.1), and ^{3}J correlation to C-5 (δ53.1), C-23 (δ28.3), and C-24 (δ16.4). The remaining tertiary hydroxyl group at C-9 could further be confirmed by HMBC experiments in which Me-25 and Me-26 (δ1.16, 1.31) showed ^{3}J correlations to C-9 (δ72.9) supported by ^{3}J correlation of H-12 (δ5.25) to C-9 (δ72.9).

The oxymethine proton at δ2.92 showed ^{1}H-^{1}H correlations to the oxymethine proton at δ3.61, while the latter also showed further ^{1}H-^{1}H correlation with 2 other protons. This allowed us to assign the hydroxyl groups to the C-2 and C-3 positions. The ^{1}H-NMR spectrum showed ddd at δ 3.61 ($J_{a,a}$= 10.8, 9.5 Hz and $J_{a,e}$= 4.0 Hz) and a doublet at δ2.92 ($J_{a,a}$= 9.5 Hz) for H-2 and H-3, allowing us to assign α and equatorial configuration for OH at 2-position, and β and equatorial configuration for OH at 3-position. The α configuration was assigned to OH-9 on the basis of biogenetic grounds and experimental results. Re-recording of the ^{1}H-NMR spectrum of **1a** in pyridine-d_5 did not show an anisotropic effect of OH-9 on Me-25 and Me-26, ruling out its β-configuration. The stereochemistry at C-2 and C-3 positions was precisely confirmed through 2D-NOESY correlations in which H-2 (δ3.61) showed correlations with Me-24 (δ0.78) and Me-25 (δ1.16), Me-25 (δ1.16) with Me-26 (δ0.99), and H-3 (δ2.92) with Me-23 (δ1.03). Therefore, the structure of santolinoic acid (**1**) was elucidated as 2α,3β,9α-trihydroxyurs-12-en-28-oic acid. Santolinoic acid (**1**) is the first naturally occurring ursane-type triterpene (from this plant source) with a hydroxyl function at C-9.

The inhibitory activity of santolinoic acid (**1**) (Table 2) observed in the present study revealed that this compound was active against the enzymes acetylcholinesterase and butyrylcholinesterase. Alzheimer's disease (AD) is a chronic neurological disorder, particularly affecting the elderly in developing countries. Despite the unknown etiology of AD, increasing the level of acetylcholine through AChE and BChE enzyme inhibition has been accepted as an effective treatment strategy for AD.[13] Therefore, AChE and BChE

inhibitors have become good alternatives in the treatment of AD. The data showed that the newly isolated compound has moderate anti-cholinesterase activity.

Table 1. ^{1}H-NMR, ^{13}C-NMR, and HMBC correlations for santolinoic acid (**1**).

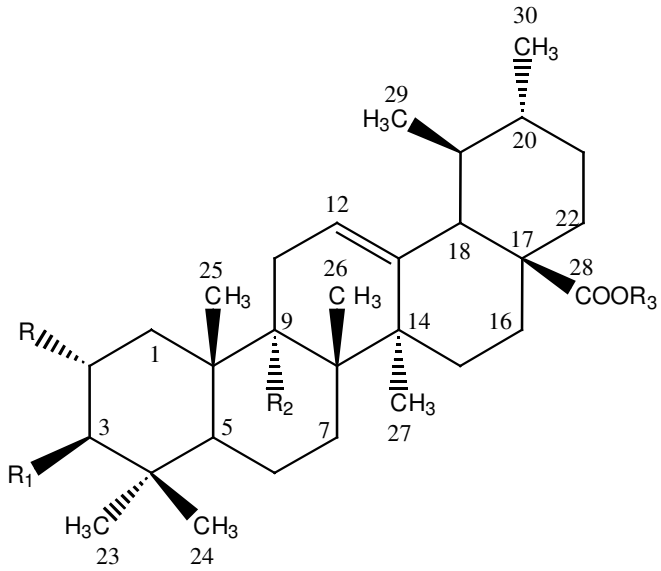
1 (CD_3OD)			
Position	δ_C	δ_H	HMBC
1	46.1	0.88m	C-2, 10
2	68.4	3.61 ddd (10.8, 9.5, 4.0)*	C-3, 4
3	83.3	2.92 d (9.5)	C-2, 4, 5, 23, 24
4	41.1	-	
5	53.3	0.87 m	
6	18.3	1.34 m	
7	32.5	1.49 m	C-8, 5, 9, 14
8	39.0	-	
9	72.9	-	
10	37.9	-	-
11	25.3	1.91 m	
12	128.5	5.25 t (3.4)	C-9, 14
13	138.2	-	
14	39.8	-	
15	28.0	1.53 m	
16	23.5	1.62 m	
17	47.5	-	
18	55.0	2.58 d (6.64)	C-28
19	41.0	1.89 m	
20	47.0	1.99 m	
21	37.4	1.34 m	
22	25.3	1.66 m	
23	28.3	1.03 s	C-3, 5
24	16.4	0.78 s	C-3, 5
25	26.9	1.16 s	C-1, 5, 9
26	15.8	0.99 s	C-8, 9
27	24.1	1.31 s	C-13, 15
28	181.0	-	C-18
29	26.9	1.00 d (5.8)	C-19, 20
30	16.2	0.91 d (6.7)	C-20, 19, 21

*J(Hz) in parentheses.

Table 2. AChE and BChE activity of santolinoic acid (**1**) (n = 3).

Compound	AChE Activity (μM)	BChE Activity (μM)
1	62.5 ± 0.52	54.8 ± 1.8
***Galanthamine**	0.52 ± 0.05	8.5 ± 0.05

*Positive control.



(1) R = R_1 = R_2 = OH, R_3 = H
(1a) R = R_1 = OAc, R_2 = OH, R_3 = H
(1b) R = R_1 = OAc, R_2 = OH, R_3 = CH_3

Figure 1. Structure of santolinoic acid **(1)**.

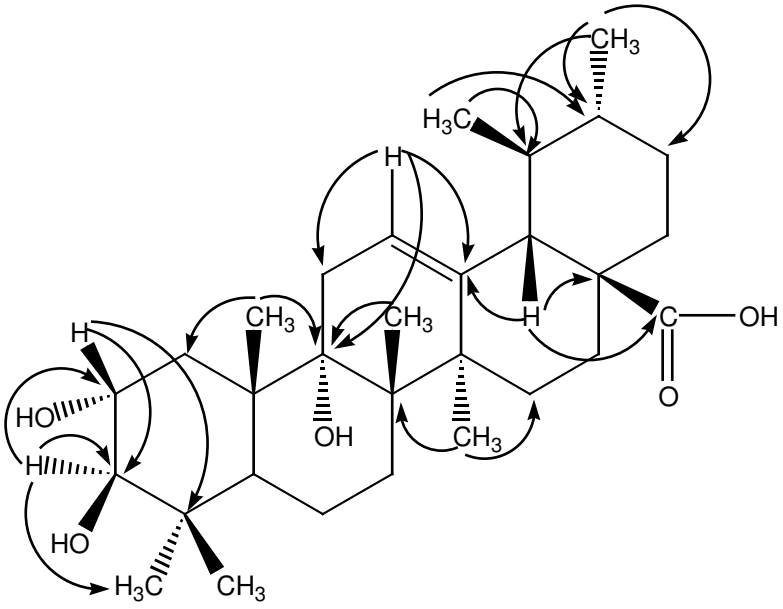


Figure 2. Selected HMBC correlations of santolinoic acid **(1)**.

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