Phytochemical Studies on *Salvia santolinifolia*

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Summary: The chloroform and ethyl acetate soluble fractions of *Salvia santolinifolia* showed antileishmanial activity. Studies on the $CHCl_3$ soluble fraction yielded seven compounds namely, lupeol (**1**), *3β, 6α*-dihydroxylup-20(29)-en (**2**), *β*-amyrin (**3**), erythrodiol (**4**), dillenic acid *E* (**5**), *6α*-hydroxy-3-epi-oleanolic acid (**6**) and *β*-sitosterol 3-*O*-*β*-D-glucoside (**7**), respectively. Their structures have been elucidated by spectroscopic techniques. All of these compounds are reported for the first time from *S. santolinifolia*.

Introduction

Salvia santolinifolia Boiss belongs to the family Labiatae which is widely distributed in rocky arid areas of Pakistan. It occurs in Peshawar, Baluchistan and Karachi. Various species of this genus are widely used for treatment of coronary heart diseases, particularly angina pectoris, myocardial infarction, amenorrhea, dismenorrhea and insomnia [1, 2]. These also possess antiseptic, carminative, diuretic, hemostatic and spasmolytic activities [3]. Previously butyrylcholinesterase inhibitory triterpenes have been reported from this species [4]. The medicinal importance of *S. santolinifolia* prompted us to carry out phytochemical investigations on this species. We now report the isolation and structure elucidation of lupeol (**1**), *3β, 6α*-dihydroxylup-20 (29)-ene (**2**), *β*-amyrin (**3**), erythrodiol (**4**), dillenic acid E (**5**), *6α*-hydroxy-3-epi-oleanolic acid (**6**) and *β*-sitosterol 3-*O*-*β*-D-glucoside (**7**), respectively, from the chloroform soluble fraction.

Results and Discussion

The methanolic extract of the whole plant showed strong toxicity in brine shrimp lethality test. It was subsequently divided into *n*-hexane, chloroform, ethyl acetate, *n*-butanol and water soluble fractions. Out of these the chloroform and ethyl acetate soluble fractions showed major toxicity in the brine shrimp lethality test. Further screening revealed antileishmanial activity in both these fractions. The chloroform soluble fraction has been subjected to a series of column chromatographic techniques as described in the experimental to obtain seven known compounds reported for the first time from *Salvia santolinifolia*. These could be identified as lupeol (**1**), *3β, 6α*-dihydroxylup-20 (29)-ene (**2**), *β*-amyrin (**3**), erythrodiol (**4**), dillenic acid E (**5**), *6α*-hydroxy-3-epi-oleanolic acid (**6**) and *β*-sitosterol 3-*O*-*β*-D-glucoside (**7**), respectively, on the basis of spectral data. None of the isolated compounds showed antileishmanial activity leading to the conclusion that the activity of the chloroform soluble fraction is due to combine effect of various constituents present therein.

Experimental

General

Column chromatography (CC): Silica gel 70-230 mesh; TLC: pre-coated silica gel 60 F_{254} (20 x 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. UV and IR spectra: Hitachi-UV-3200 and Shimadzu IR-460 spectrophotometer, respectively. ^{1}H- and ^{13}C-NMR spectra: Bruker spectrometers operating at 300 MHz, 400 MHz or 500 MHz. Chemical shift δ in ppm relative to $SiMe_4$ as internal standard and coupling constants *J* in Hz. EIMS, HREIMS, 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 high performance thin layer chromatography (HPTLC) plates of E. Merck.

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Plant Material

The whole plant of *Salvia santolinifolia* Boiss. (14 kg) was 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.

Isolation

The whole plant of *Salvia santolinifolia* (14 kg) was shade dried ground and extracted thrice with methanol (3×50 L). The combined methanolic extract (550 g) was partitioned between *n*-hexane and water. The water fraction was further extracted out with chloroform, ethylacetate and *n*-butanol. The chloroform soluble fraction (65 g) was subjected to column chromatography eluting with *n*-hexane, *n*-hexane-$CHCl_3$, $CHCl_3$, $CHCl_3$ - MeOH and MeOH in increasing order of polarity to give five fractions F_1-F_5. The fraction F_2 which eluted from *n*-hexane – $CHCl_3$ (8:2) was again chromatographed over silica gel using *n*-hexane-$CHCl_3$ (9:1) as eluent to afford two successive fractions F_2A and F_2B. Column chromatography of F_2A using *n*-hexane-$CHCl_3$ (9:1) as eluent provided compound **1**. On the other hand, column chromatography of fraction F_2B and elution with *n*-hexane-$CHCl_3$ (8: 5: 1.5) afforded compound **3**. The fraction F_3 obtained from *n*-hexane-$CHCl_3$ (7: 3) was rechromatographed over silica gel to afford two successive fractions F_3A and F_3B using solvent system *n*-hexane-$CHCl_3$ (7: 3-5: 5). The fraction F_3B was rechromatographed and eluted with *n*-hexane-$CHCl_3$ (6: 4) to afford compounds **2** and **4** from the top and the tail fractions, respectively. The fraction F_4 which eltuted from *n*-hexane-$CHCl_3$ (4:6) was again chromatographed over silica gel using *n*-hexane-$CHCl_3$ (4:6-2:8) as eluents to provide two successive fractions F_4A and F_4B, respectively. Column chromatography of F_4A successively provided compounds **5** and **6**, respectively using *n*-hexane-$CHCl_3$ (4: 6) as eluent. Column chromatography of fraction F_4B and elution with *n*-hexane $CHCl_3$ (2:8) afforded compound **7**. The ethyl acetate soluble fraction was not worked up due to paucity of material.

Lupeol (1)

Compound **1** (32 mg) was obtained as colourless needles from CH_3OH; m.p. 215-216 °C ; $[\alpha]_D^{25}$ + 26.4° (c = 0.26, MeOH); IR (KBr): ν_{max} cm^{-1}: 3455 (OH), 3075, 1645 and 880 (C=CH_2); ^{1}H-NMR ($CDCl_3$, 300 MHz) δ: 4.62, 4.75 (1H, each, brs, CH_2-29), 3.21 (1H, dd, $J_{ax, ax}$ = 9.9, $J_{ax, eq}$ = 4.5 Hz, H-3), 2.36 (J = 10.5, 10.5, 5.4 Hz, H-19), 1.65 (3H, br s, CH_3-30), 1.05 (3H, s, CH_3-26), 0.97 (6H, s, CH_3-25, CH_3-27), 0.94 (3H, s, CH_3-24), 0.85 (3H,s, CH_3-28), 0.79 (3H, s, CH_3-23); HREIMS showed $[M]^+$ at *m/z* 426.3835 (calcd. for $C_{30}H_{50}O$, 426.3861). The physical and spectral data showed complete resemblance with the reported values [5].

Table-1: *In vitro* antileishmanial activities of the fractions of *S. santolinifolia*.

	Fraction	IC_{50} (μg/ ml)
1.	Chloroform fraction	54.347 ± 1.05
2.	Ethylacetate fraction	58.823 ± 0.6
3.	Amphotericin B[a]	0.19 ± 0.05

[a]Standard drug

3β, 6α-Dihydroxylup-20 (29)-ene (2)

Compound **2** (55 mg) was obtained as gummy solid, $[\alpha]_D^{25}$ + 14° (c = 0.4, $CHCl_3$); IR ($CHCl_3$) ν_{max} cm^{-1}: 3400, 3045, 1650 and 888. ^{1}H-NMR ($CDCl_3$, 300 MHz) δ: 4.67 (1H, d, J = 2.3 Hz, H-29), 4.57 (1H, d, J = 2.2 Hz, H-29), 3.81 (1H, dt, J = 4.8, 10.5 Hz, H-6), 3.16 (1H, dd, J = 5.46, 10.8 Hz, H-3), 1.68 (3H, s, CH_3-30), 1.21 (3H, s, CH_3-26), 1.02 (3H, s, CH_3-23), 0.97 (3H, s, CH_3-27), 0.96 (3H, s, CH_3-25), 0.80 (3H, s, CH_3-28), 0.75 (3H, s, CH_3-24); HREIMS showed $[M]^+$ at *m/z* 442.6404 (calcd. for $C_{30}H_{50}O_2$, 442.64019). The physical and spectral data coincided with the reported values [6].

β-Amyrin (3)

Compound **3** (36 mg) was obtained as colourless needles from CH_3OH, m.p. 197-198 °C; $[\alpha]_D^{25}$ + 100 (c = 0.21, $CHCl_3$); IR ($CHCl_3$) ν_{max} cm^{-1}: 3430, 3045, 1600, 815; ^{1}H-NMR ($CDCl_3$, 300 MHz) δ: 5.11 (1H, m, H-12), 3.21 (1H, dd, J = 10.0, 4.5 Hz, H-3), 1.08, 1.02, 1.01, 0.99, 0.95, 0.88, 0.85 and 0.80 (3H, each s, CH_3); HREIMS showed $[M]^+$ at *m/z* 426.3825 (calcd. for $C_{30}H_{50}O$, 426.3861). The physical and spectral data coincided with the literature values [7, 8].

Erythrodiol (4)

Compound **4** (21 mg) was obtained as an amorphous powder; $[\alpha]_D^{27}$ + 45.0° (c = 0.87,

$CHCl_3$): IR (KBr) ν_{max} cm^{-1}: 3580, 3430 and 1610 cm^{-1}; ^{1}H-NMR ($CDCl_3$, 300 MHz) δ: 5.77 (1H, t, J = 3.6 Hz, H-12), 3.21 (1H, dd, $J_{ax,\ ax}$ = 11.2 Hz, $J_{ax,\ eq}$ = 4.5 Hz, H-3), 1.15 (3H, s, CH_3-27), 0.98 (3H, s, CH_3-23), 0.93 (3H, s, CH_3-26), 0.92 (3H, s, CH_3-25), 0.87 (3H, s, CH_3-30), 0.86 (3H, s, CH_3-29), 0.78 (3H, s, CH_3-24); HREIMS showed $[M]^+$ at *m/z* 442.3798 (calcd. for $C_{30}H_{50}O_2$, 442.3812). The physical and spectral data corresponded to the reported values [9].

Dillenic acid E (5)

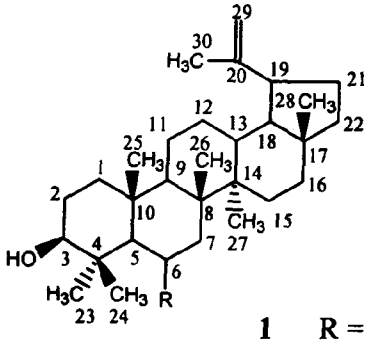
Compound **5** (16 mg) was obtained as amorphous solid ; $[\alpha]_D^{24}$ + 88° (c = 0.05, MeOH); IR (KBr) ν_{max} cm^{-1}: 3412, 2960, 1730 and 1700 cm^{-1}: ^{1}H-NMR (CD_3OD, 400 MHz) δ: 5.15 (1H, br s, H-12), 3.47 (1H, dd, J = 12.3, 3.6 Hz, H-3), 3.35 (1H, brs, H-1), 1.74, 1.52 (1H each, m, H-2), 1.20 (3H, s, CH_3-27), 1.05 (3H, s, CH_3-29), 0.92 (3H, s, CH_3-23), 0.90 (3H, s, CH_3-26), 0.84 (3H, s, CH_3-25), 0.73 (3H, s, CH_3-28) and 0.67 (3H, s, CH_3-24); HREIMS showed $[M]^+$ at *m/z* 472.3535 (calcd. for $C_{30}H_{48}O_4$, 472.3544). The physical and spectral data were similar to those reported previously in literature [10].

6α-Hydroxy-3-epi-oleanolic acid (6)

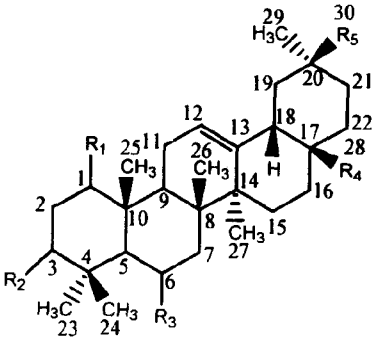
Compound **6** (32 mg) was obtained as colourless needles from CH_3OH; m.p. 235-237 °C; $[\alpha]_D^{20}$ + 7.14° (c = 0.07, $CHCl_3$); IR ($CHCl_3$) ν_{max} cm^{-1}: 3600, 3400, 2650, 1700, 1660 and 820; ^{1}H-NMR (CD_3OD, 500 MHz) δ: 5.24 (1H, t, J = 3.6 Hz, H-12), 3.60 (1H, ddd, J = 10.0 and 4.4 Hz, H-6), 3.40 (1H, *t*, J = 2.6 Hz, H-3), 1.12, 1.01, 0.96, 0.89, 0.87, 0.85 and 0.80 (3H, each s, Me); HREIMS showed $[M]^+$ at *m/z* 472.3527 (calcd. for $C_{30}H_{48}O_4$, 472.3520). The physical and spectral data showed complete agreement with the reported values [11].

β-Sitosterol 3-O-β-D-glucoside (7)

Compound **7** (26 mg) was obtained as amorphous solid, m.p. 280 °C , $[\alpha]_D^{25}$ – 14.5° (c = 0.4, MeOH); IR (KBr) ν_{max} cm^{-1}: 3460, 3050, 1650, 815; ^{1}H-NMR ($CDCl_3$ + CD_3OD, 400 MHz) δ: 0.66 (3H, s, CH_3-18), 0.77 (3H, d, J = 6.2 Hz, CH_3-27), 0.80 (3H, d, J = 6.3 Hz, CH_3-26), 0.79 (3H, t, J = 7.1 Hz, CH_3-29), 0.89 (3H, d, J = 6.5 Hz, CH_3-21), 0.99 (3H, s, CH_3-19), 3.43-



1 R = H
2 R = α-OH



	R_1	R_2	R_3	R_4	R_5
3	H	β-OH	H	CH_3	CH_3
4	H	β-OH	H	CH_2OH	CH_3
5	α-OH	β-OH	H	CH_3	COOH
6	H	α-OH	α-OH	COOH	CH_3

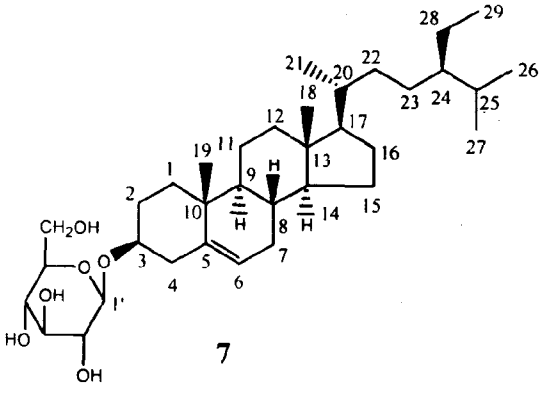


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Fig. 1: Structures of compounds **1-7**

3.82 (m, Glu-H), 4.35 (1H, d, J = 7.7 Hz, H-1'), 5.32 (1H, brs, H-6) and HREIMS showed $[M]^+$ at *m/z* 576.3051 (calcd. for $C_{35}H_{60}O_6$, 576.3021). The physical and spectral data coincided with the literature values [12].

In vitro antileishmanial assay

Leishmania promastigotes are grown in bulk early in modified NNN biphasic medium using normal physiological saline. Leishmania parasite promastigotes were cultured with RPMI 1640 medium supplemented with 10 % heat inactivated foetal bovine serum (FBS). Parasites at log phase are centrifuged at 2000 rpm for 10 minutes and washed thrice with saline at same speed and time. Parasites are diluted with fresh culture medium to a final density of 10^6cells/ ml.

In a 96-well micro titer plate, 180 μl of medium was added in different wells. 20 μl of the experimental compound was added in medium and serially diluted. 100 μl of parasite culture was added in all wells. Two rows should be left for negative and positive controls. Negative controls receive medium while the positive control contains varying concentrations of standard antileishmanial compound e.g. Amphotericin B, pantamidine. The plate was incubated between 21-22 °C for 72 h. The culture was examined microscopically on an improved neubauer counting chamber and IC_{50} values of compounds possessing antileishmanial activity were calculated by software Ezfit 5.03 perella scientific [13,14]. All assays were run in duplicate.

The chloroform and ethyl acetate fractions of *S. santolinifolia* displayed interesting antileishmanial activities against leishmania major, as compared to standard drug amphotericin B (IC_{50} = 0.19 μg/ml). Our findings of antileishmanial activity in these fractions may encourage further exploration of their use in the development of antileishmanial drugs. However, none of the isolated pure compounds showed antileishmanial activity.

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