Recurvosides A and B, Antifungal Novel Steroidal Glucosides from *Haloxylon recurvum*

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Recurvosides A (**1**) and B (**2**), new steroidal glucosides, have been isolated from the ethyl acetate soluble fraction of *Haloxylon recurvum* and their structures elucidated by spectroscopic techniques including two-dimensional NMR. Both the compounds exhibited potent antifungal activity.

Key words: *Haloxylon recurvum*, Chenopodiaceae, Recurvoside A, Recurvoside B, Antifungal Activity

Introduction

Haloxylon recurvum Bunge ex Boiss belongs to the family Chenopodiaceae which comprises 100 genera and 1200 species [1]. In Pakistan this family is represented by 35 genera. Only five species of *Haloxylon* are found in Pakistan [2]. *H. recurvum* is perennial shrub with glabrous leaves. It is widely distributed in Turkey, Syria, Iraq, Iran, Afghanistan, Kashmir, India and Central Asia [3]. The plant is traditionally reported to apply externally on insect stings. The ash of the plant is used for internal ulcers while a decoction of the whole plant is used for the treatment of various viral disorders [4, 5]. The methanolic extract of plant showed strong toxicity in brine shrimp lethality test, revealing the presence of bioactive compounds. Further pharmacological screening revealed significant antifungal activity which was most pronounced in EtOAc fraction. This prompted use to carry out bioassay-guided isolation of bioactive compounds from this fraction. As a result of these studies we have isolated two new steroidal glucosides namely recurvosides A (**1**) and B (**2**). Both the compounds showed potent antifungal activity.

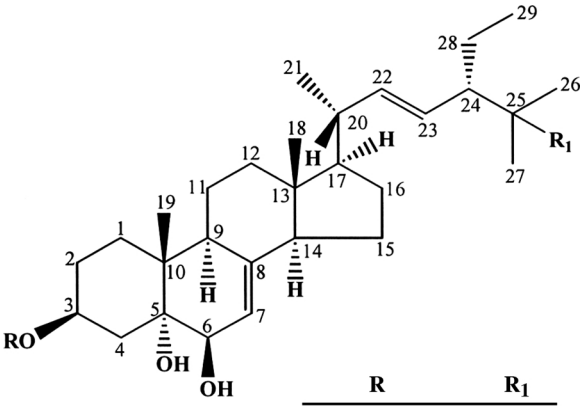
Results and Discussion

The methanolic extract of *H. recurvum* was divided into *n*-hexane, $CHCl_3$, EtOAc, *n*-BuOH and water soluble fractions. The EtOAc soluble fraction which showed major antifungal activity was further subjected to chromatographic techniques to afford two new steroidal glucosides named as recurvosides A (**1**) and B (**2**), respectively. Both the compounds gave positive Salkowski and Lieberman Burchard tests for steroids as well as Molish's test for glycosides [6].

Recurvoside A (**1**) was obtained as amorphous powder, m. p. 258 – 260 °C (dec), [α_D^{20} $-22°$ (c = 1.0, MeOH). The IR spectrum showed bands for hydroxyl (3380 – 3421 cm^{-1}), sugar C-O stretching (1072, 1055 and 1025 cm^{-1}) and double bond (3025 – 3075, 1634, 650 cm^{-1}). The FABHRMS showed $(M+H)^+$ peak at *m/z* 623.4128 corresponding to molecular formula $C_{35}H_{59}O_9$ (calcd. 623.4159). The loss of hexose moiety from the pseudomolecular ion peak gave on intense peak at *m/z* 461.3594 (calcd. for $C_{29}H_{49}O_4$, 461.3593). The HREIMS showed characteristic fragments of unsaturated steroidal nucleus at *m/z* 306.4001 (M^+-sugar-side chain) and *m/z* 265 (M^+-sugar-ring D fission [7].

The ^{13}C NMR spectrum (BB and DEPT) showed thirtyfive signals including six methyl, nine methylene, fifteen methine and five quaternary carbons. It showed signals of four olefinic bonds at δ = 142.0, 133.5, 131.1 and 119.9 and anomeric carbon at δ = 101.1. It further showed four oxygenated carbons at δ = 79.8, 74.4 74.3 and 72.7, besides the hydroxyl containing carbons of the sugar moiety ranging from δ = 76.6 to δ = 61.0. The signals of six methyl carbons were observed at δ = 25.5, 23.9, 19.5, 19.2, 12.4 and 12.0.

The ^1H NMR spectrum showed an anomeric proton as doublet at δ = 4.59 (J = 7.4 Hz). The larger coupling constant inferred the β configuration of the



	R	R_1
1	β-D-Glu	OH
1a	H	OH
2	β-D-Glu	H
2a	H	H

Fig. 1. Structures of recurvosides A (**1**) and (**2**).

Table 1. ^{13}C NMR (100 MHz, C_5D_5N) chemical shifts of compounds **1** and **2**.

Position	1	2	Position	1	2
1	34.0	34.2	2	26.8	26.5
3	74.4	74.4	4	35.5	35.4
5	79.8	79.8	6	72.7	72.5
7	119.9	119.8	8	142.0	142.0
9	48.0	48.0	10	42.1	41.9
11	22.5	22.4	12	38.4	38.6
13	42.0	42.0	14	55.7	55.7
15	24.8	24.5	16	28.9	28.9
17	57.2	57.6	18	12.4	12.6
19	19.5	19.5	20	39.8	40.5
21	19.2	21.1	22	133.5	138.4
23	131.1	129.4	24	55.5	51.3
25	74.3	32.0	26	25.5	19.4
27	23.9	21.2	28	22.6	25.4
29	12.0	12.2			
1’	101.1	100.7	2’	70.0	70.0
3’	76.7	76.6	4’	73.4	73.2
5’	76.6	76.4	6’	61.0	61.1

sugar moiety. It further showed signals of four tertiary methyls at $\delta = 0.78$, 1.30, 1.32, 1.59, one secondary methyl at $\delta = 0.88$ ($J = 6.8$ Hz) and primary methyl as triplet at $\delta = 0.86$ ($J = 7.1$ Hz). The proton of a trisubstituted double bond was observed as broad doublet at $\delta = 5.32$ ($J = 4.8$ Hz) while signals for a trans disubstituted double bond were observed at $\delta = 5.01$ (1H, dd, $J = 8.3$, 15.2 Hz) and 5.10 (1H, dd, $J = 7.9$, 15.2 Hz). Two oxymethine protons resonated at $\delta = 4.11$ (1H, d, $J = 4.8$ Hz) and 4.09 (1H, m). Two double doublets at $\delta = 2.10$ ($J = 12.6$ and 11.8 Hz) and 1.98 ($J = 12.6$ and 5.1 Hz), mutually coupled and coupled with the 3α-proton at 4.09, were assigned, respectively, to the 4-H_{ax} and 4-H_{eq}. In a double resonance experiment, irradiation at 5.32 ppm transformed the broad doublet ($J = 4.8$ Hz) at 4.11 ppm into a singlet, thus indicating that the olefinic proton is located next to a hydroxyl, the latter being adjacent to a quaternary carbon. The multiplet centered around 4.09 ppm had a complexity normally observed for a 3α carbinol proton, and the unusually low field shift suggested the additional tertiary hydroxyl group at C-5 [8–13]. These data indicated either a $\Delta^7 - 3\beta$,5,6-triol or a $\Delta^{9(11)}$-3, 5,12-triol structure. Examination of the NMR signals of C-18 and C-19 protons, were in better agreement with a $\Delta^7 - 3\beta$, 5α, 6β-triol structure. The downfield shift of the C-19 methyl signal at $\delta = 1.59$ in the spectrum recorded in pyridine-d_5 was indicative of the β-orientation of the C-6 hydroxyl group [14].

Oxymethine protons of the sugar moiety were observed from $\delta = 3.40$ to 3.05 and the oxymethylene

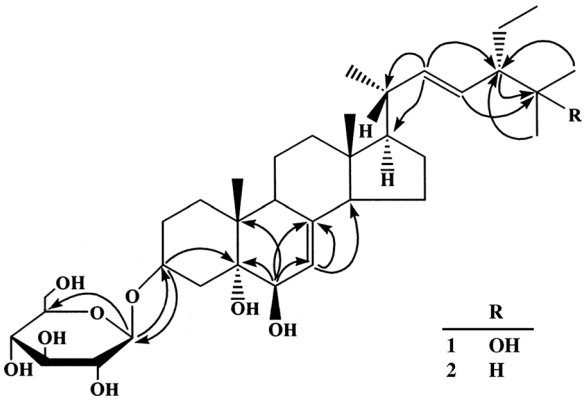


Fig 2. Important HMBC correlations of recurvosides A (**1**) and B (**2**).

protons resonated at $\delta = 3.61$ (1H, dd, $J = 11.1$ and 4.7 Hz) and 3.58 (1H, dd, $J = 11.1$ and 5.8 Hz). The oxymethine proton at $\delta = 4.09$ showed ^{1}H-^{1}H COSY correlations with four neighbouring protons providing further evidence for the attachment of glycoside moiety at the more usual C-3 position. The sugar moiety was identified as D-glucose by comparing its ^{13}C NMR with the reference data [15, 16] and further confirmed through acid hydrolysis which provided aglycone (**1a**) and the sugar moiety. The latter could be confirmed as D-glucose through sign of its optical rotation ($[\alpha]_D + 52.7°$) and Co-TLC with an authentic sample. In ^{1}H-^{1}H COSY spectrum the olefinic protons at $\delta = 5.10$ (H-22) and $\delta = 5.01$ (H-23) showed cross peaks with each other as well as with the neighbouring protons at $\delta = 1.51$ (H.20) and $\delta = 1.94$

(H-24), respectively. The tertiary hydroxyl group must, therefore, be at C-25. This was confirmed by downfield shifts of both C-26 and C-27 in both ^{1}H and ^{13}C NMR spectra, as well as the observed correlations in HMBC experiments (Fig. 2). The α (*S*) configuration at C-24 was established by the comparison of the chemical shifts of the signal of the side chain with compounds having identical side chain at C-17 [17–23]. Based on these evidences the structure of recurvoside A (**1**) was assigned as (24*S*)-24-ethyl-cholesta-7-22*E*-diene, 5α,6β,25 triol 3-O-β-D-glucoside. The ^{13}C NMR spectrum, ^{1}H-^{1}H COSY, HMQC, HMBC and NOESY correlations were in complete agreement to the assigned structure.

Recurvoside B (**2**) was obtained as amorphous powder, m. p. 240–242 °C (dec), $[\alpha]_D^{25}$ $-39°$ ($c = 0.75$, CH_3OH). The high resolution FABMS (Positive mode) gave $[M+H]^+$ peak at *m/z* 607.4252 corresponding to molecular formula $C_{35}H_{59}O_8$, (calcd. 607.4210) indicating seven degrees of unsaturation, while the other intense peak at *m/z* 445.3662 (calcd. for $C_{29}H_{49}O_3$, 445.3681) indicated the loss of hexose moiety. The ^{1}H NMR spectrum of **2** displayed the characteristic signal for an anomeric proton as a doublet at $\delta = 4.40$ ($J = 7.1$ Hz). The larger coupling constant value showed the β configuration of the sugar moiety. The ^{1}H NMR further showed the signals of two tertiary methyl at $\delta = 0.77$ and 1.60 as singlet, three methyl doublet at $\delta = 0.79$, 0.82 and 0.90 with J values in range of 6.8–7.1 Hz, and the methyl triplet was observed at $\delta = 0.85$ ($J = 7.1$ Hz). The ^{1}H and ^{13}C NMR spectra were very similar to **1** except the absence of tertiary hydroxyl group in the side chain. This was evident by the loss of side chain in the HREIMS to provide the peak at *m/z* 305.3951 ($C_{19}H_{29}O_3$) (M^+-sugar-side chain). The presence of isopropyl moiety was further inferred by the presence of intense peak of isopropyl group at *m/z* 43.0521 (C_3H_7). The sugar was identified as D-glucose by comparison of its carbon signals in the ^{13}C NMR spectrum with the reference data [15, 16] and further confirmed through acid hydrolysis which provided (24*S*)-ethyl-cholesta-7,22E-diene-3β, 5α, 6β-triol (**2a**) [24] and the sugar which could be identified as D-glucose through Co-TLC with an authentic sample and optical rotation ($[\alpha]_D + 52.3°$). Thus recurvoside B (**2**) could be assigned the structure (24*S*)-ethyl-cholesta-7,22*E*-diene-5α,6β-diol-3-O-β-D-glucoside.

The antifungal activity was determined by agar tube dilution method [25]. Recurvosides A (**1**) and B (**2**) showed significant to moderate activities against *Trichophyton longifusus*, *Candida albicans*, *Aspergillus flavus*, *Microsporum canis*, *Candida glabrata* and *Fusarium solani*. Recurvoside A was slightly more potent probably due to the presence of additional hydroxyl group at C-25 (Table 2).

Table 2. Antifungal activity of recurvosides A (**1**), and B (**2**) in Agar Tube Dilution Method.

Microorganisms	Compound Zone of inhibition		Standard antifungal drug	MIC μg/ml
	1	**2**		
Trichophyton longifusus	80	77	Miconazole	70
Candida albicans	75	60	Miconazole	110
Aspergillus flavus	47	41	Amphotericin B	20
Microsporum canis	79	66	Miconazole	98.4
Candida glabrata	82	70	Miconazole	110.8
Fusarium solani	60	52	Miconazole	73.25

Values are % inhibition of radial growth.

Experimental Section

General

IR spectra were recorded on Jasco-320-A spectrometer. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AM-400 spectrometer with tetramethylsilane (TMS) as an external standard. The 2D NMR spectra were recorded on a Bruker AMX 500 NMR spectrometer. Optical rotations were measured on a Jasco DIP-360 digital polarimeter using a 10 cm tube. Mass spectra (EI and HR-EI-MS) were measured in an electron impact mode on Finnigan MAT 12 and MAT 312 spectrometers, and ions are given in *m/z* (%). TLC was performed on precoated silica gel F_{254} plates; the detection was done at 254 nm and by spraying with ceric sulphate reagent. Silica gel (E. Merck, 230–400 mesh) was used for column chromatography. Melting points were determined on a Gallenkemp apparatus and are uncorrected. For antifungal bioassay miconazole and amphotericin B were purchased from Sigma Chemical Company (St. Louis, MO, USA), Sabouraud dextrose agar was purchased from Oxoid Ltd (Basingstoke, Hampshire, England) and DMSO was purchased from BDH Laboratory Supplies (Poole, England).

Collection and identification

The whole plant *Haloxylon recurvum* Bunge ex Boiss was collected from the Cholistan desert near the Bahawalpur district, Pakistan in October, 2001 and identified by Dr. Muhammad Arshad, Plant Taxonomist, Cholistan Institute of Desert Studies, Islamia University Bahawalpur, where a voucher specimen (020/CIDS/IUB/PK) has been deposited.

Extraction and isolation

The air dried whole plant (20 kg) was exhaustively extracted with methanol (50 l × 3) at r. t. The extract was evap-

orated to yield the residue (700 g) which was divided into *n*-hexane (60 g), chloroform (75 g), ethyl acetate (40.5 g), *n*-butanol (70 g) and water (38 g) soluble fractions. The EtOAc-soluble fraction was subjected to column chromatography over silica gel eluting with *n*-hexane-$CHCl_3$, $CHCl_3$, $CHCl_3$-MeOH in increasing order of polarity. The fractions which were obtained from $CHCl_3$-MeOH (7.5 : 2.5), were combined and rechromatographed over silica gel eluting with $CHCl_3$-MeOH in increasing order of polarity. The fractions obtained from $CHCl_3$: MeOH (8.0 : 2.0) were subjected to preparative TLC ($CHCl_3$: MeOH; 7.8 : 2.2) to afford **1** (15 mg) and **2** (18 mg), respectively.

Recurvoside A (**1**): Colorless amorphous powder. M. p. 258 – 260 degC. $[\alpha]_D^{20}$ $-22°$ (MeOH, $c = 1.0$ mg/ml). IR ($CHCl_3$) $\upsilon = 3380$, 3421, 3025, 3075, 1634, 1650, 1072, 1055, 1025, 870, 725 cm^{-1}. ^1H NMR (400 MHz, C_5D_5N): $\delta = 0.78$ (s, 3H, 18-H), 0.86 (t, $J = 7.1$ Hz, 3H, 29-H), 0.88 (d, $J = 6.8$ Hz, 3H, 21-H), 1.30 (s, 3H, 26-H), 1.32 (s, 3H, 27-H), 1.59 (s, 3H, 19-H), 2.10 (dd, $J = 12.6$, 11.8 Hz, 1H, 4_{ax}-H), 1.98 (dd, $J = 12.6$, 5.1 Hz, 1H, 4_{eq}-H), 4.09 (m, 1H, 3-H), 4.11 (brd, $J = 4.8$ Hz, 1H, H-6), 5.01 (dd, $J = 8.3$, 15.2 Hz, 1H, 22-H), 5.10 (dd, $J = 7.9$, 15.2 Hz, 1H, 23-H), 5.32 (brd, $J = 4.8$ Hz, 1H, 7-H), glucose moiety: $\delta = 4.59$ (d, $J = 7.4$ Hz, 1H, 1'-H), 3.05 (m, 1H, 2'-H), 3.40 (m, 1H, 3'-H), 3.12 (m, 1H, 4'-H), 3.07 (m, 1H, 5'-H), 3.58 (dd, $J = 11.1$, 5.8 Hz, 1H, 6b'-H), 3.61 (dd, $J = 11.1$, 4.7 Hz, 1H, 6a'-H). ^{13}C NMR (100 MHz, C_5D_5N): see Table 1. HRFABMS $[M+H]^+$: *m/z* 623.4128 (calcd. for $C_{35}H_{59}O_9$, 623.4159): EIMS: *m/z* 442 (M^+-sugar-H_2O) (18), 424 (M^+-sugar-$2H_2O$) (13), 409 (22), 303 (100), 275 (39), 157 (49), 55 (70).

Recurvoside B (**2**): Colorless amorphous powder. M. p. 240 – 242 °C $[\alpha]_D^{20}$ $-39.0°$ (MeOH, $c = 0.75$ mg/ml). IR ($CHCl_3$) $\upsilon = 3390$, 3425, 3025, 3088, 1630, 1652, 1075, 1060, 875, 720 cm^{-1}. ^1H NMR (400 MHz, C_5D_5N): $\delta = 0.77$ (s, 3H, 18-H), 0.81 (d, $J = 6.5$ Hz, 3H, 27-H), 0.82 (d, $J = 6.8$ Hz, 3H, 26-H), 0.85 (t, $J = 7.1$ Hz, 3H, 29-H), 0.90 (d, $J = 7.1$ Hz, 3H, 21-H), 1.60 (s, 3H, 19-H), 4.06 (m, 1H, 3-H), 4.10 (brd, $J = 4.6$ Hz, 1H, 6-H), 5.02 (dd, $J = 15.2$, 8.8 Hz, 23-H), 5.15 (dd, $J = 15.2, 8.4$ Hz, 22-H). 5.32 (brd, $J = 4.6$ Hz, 1H, 7-H), glucose moiety: $\delta = 4.52$ (d, $J = 7.0$ Hz, 1H, 1'-H), 3.05 (m, 1H, 2'-H), 3.41 (m, 1H, 3'-H), 3.12 (m, 1H, 4'-H), 3.08 (m, 1H, 5'-H), 3.91 (dd, $J = 11.6$, 6.5 Hz, 1H, 6a'-H), 3.67 (dd, $J = 11.6$, 5.2 Hz, 1H, 6b'-H), ^{13}C NMR (100 MHz, C_5D_5N): see Table 1. HRFABMS $[M+H]^+$: *m/z* 607.4252 (calcd. for $C_{35}H_{59}O_8$, 607.4210).

Acid hydrolysis of compounds **1** *and* **2**

A solution of **1** or **2** (8 mg) in methanol (4 ml) and 1 M HCl (4 ml) was refluxed for 4 h. The solution was concentrated under reduced pressure and diluted with H_2O (4.5 ml). It was extracted with ethyl acetate and the sugar recovered from the aqueous phase was identified as D-glucose by sign of its optical rotation ($[\alpha]_D + 52.6$) and co-TLC with an authentic sample of D-glucose using solvent system *n*-BuOH-EtOAc-HOAc-H_2O (12 : 2 : 2 : 2). TLC was run three times in the same direction and spots were visualized with aniline phthalate reagent. The aglycone from 1 was a new sterol, $[\alpha]_D^{20}$ $-42.5°$ (MeOH, $c = 0.75$ mg/ml). The aglycone in the case of **2** was identified as (24*S*)-ethylcholesta-7, 22*E*-diene-3β,5α,6β-triol (**2a**) by comparing optical rotation ($[\alpha]_D$ -4.2) and ^{13}C NMR reported in literature [23].

In vitro antifungal bioassay

Antifungal activity was performed using agar tube dilution method [24]. Each compound (1.5 mg) dissolved in 1 ml of sterile dimethylsulphoxide (DMSO) served as stock solution. Sabouraud dextrose agar (SDA) (4 ml) was added into screw caped tubes and autoclaved at 121 °C for 15 min and then cooled to 50 °C. The non-solidified SDA media was poisoned with 66.6 μl of the stock solution to give 200 μg compound/ml of SDA. Tubes were then allowed to solidify in slanting position at r. t. Each tube was inoculated with 4 mm diameter piece of the inoculum removed from a seven days old culture of fungi. For non-mycelial growth, an agar surface streak was employed. Inhibition of fungal growth was observed after seven days of incubation at 28 ± 1 °C. Negative and positive control experiments were also carried out with DMSO and reference antifungal drugs, respectively.

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