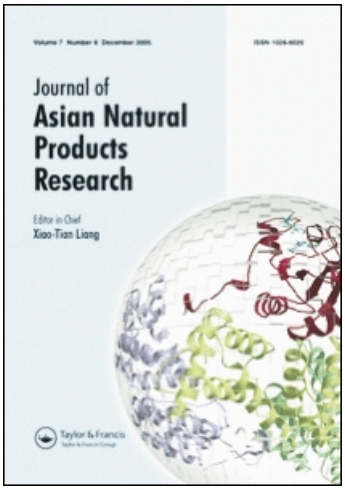

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Saponins from the seeds of *Achras sapota*

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A new pentacyclic triterpenoid saponin (**2**) along with one known was isolated from the cotyledons of *Achras sapota*. Their structures were determined with the help of ^{1}H NMR, ^{13}C NMR and MS spectral data. The isolated compounds were named as 3-*O*-β-D-glucopyranosyl-(1 → 6)-β-D-glucopyranosyl-28-*O*-α-L-rhamnopyranosyl-(1 → 3)-β-D-xylopyranosyl-(1 → 4)-α-L-rhamnopyranosyl-(1 → 2)-α-L-arabinopyranosyl protobassic acid (**1**) and 3-*O*-β-D-glucopyranosyl-(1 → 3)-β-D-glucopyranosyl-28-*O*-α-L-rhamnopyranosyl-(1 → 3)-β-D-xylopyranosyl-(1 → 4)-α-L-rhamnopyranosyl-(1 → 2)-α-L-arabinopyranosyl-16α-hydroxy protobassic acid (**2**). Compound **2** showed antibacterial activity against Gram positive and negative bacteria.

Keywords: *Achras sapota*; Seeds; Saponins; 3, 28-*O*-Bisdesmoside

1. Introduction

Achras sapota (family Sapotaceae) is an evergreen tree, widely distributed in the West Indies and Tropical America. It is also cultivated in Malaysia, India, and Pakistan [1]. Different parts of the plants are reported to have curative properties in folk medicine [2–4]. *Achras sapota*'s bark (Aq. Extract) contains tuberculostatic principles [5]. The plant has been reported to include triterpenoids and saponins that have been found to possess anti-inflammatory [6] and spermicidal activities [7]. The mixture of saponins isolated from *n*-BuOH extract has been found to exhibit antibacterial and antifungal activities and brine shrimp cytotoxicity. In the present paper we report the isolation and structure elucidation of a new triterpenoid saponin (**2**) along with a known saponin (**1**) from the cotyledons of *A. sapota*.

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2. Results and discussion

Repeated column chromatography of the crude BuOH extract afforded two pure saponins different in the genin parts, which were predicted to be 3, 28-di-*O*-glycoside of *oleanane* type terpenoid series.

Compound **1** was obtained as an amorphous powder. It was shown to have the molecular formula $C_{64}H_{104}O_{32}$ by negative FAB-MS that gave the molecular ion peak at *m/z* 1383.32 due to $[M - H]^-$. The infrared spectrum showed absorption bands at 3430 cm^{-1} due to hydroxyl group, 1730 cm^{-1} due to ester, and 1640 cm^{-1} indicating C═C bonds. Its ^1H NMR spectrum showed signals for six methyl groups at δ 0. 89, 1.0, 1.22, 1.53, 1.83, and 2.05 (each 3H, assignable to C-30, C-29, C-27, C-25, C-24, C-26, respectively), one olefinic proton and six anomeric protons. The ^{13}C NMR spectrum showed the presence of a pair of olefinic carbons at δ 123.5 and 143.7, an ester carbonyl at δ 176.4, and six anomeric carbons at δ 94.0, 101.0, 102.0, 104.8, 105.0, and 106.0. The ^{13}C NMR spectral data indicated the presence of 64 carbon atoms in the molecule, in which 34 were assigned to the carbons of sugar moieties and 30 to those of aglycone. The presence of a peak at *m/z* 503 in the mass spectrum indicated the aglycone part was protobassic acid. This was subsequently confirmed as follows.

Acid hydrolysis of **1** gave an aglycone **1a** along with arabinose, glucose, rhamnose, and xylose. The EI-MS spectrum of **1a** showed the molecular ion peak $[M]^+$ at *m/z* 504 indicating a possible molecular formula $C_{30}H_{48}O_6$. It was identified as protobassic acid by comparing its ^1H and ^{13}C signals (tables 1 and 2) with those of the authentic compound as reported in the literature [5]. Also the comparison of ^{13}C chemical shifts of **1a** with those of **1** showed a downfield shift for the C-3 signal from δ 73.2 to 83.4, which indicated that the C-3 position was glycosylated. Similarly the downfield shift of the C-28 carbon of **1** to δ 180.0 from δ 177.1 of **1a** indicated that the C-28 position was also glycosylated.

The alkaline hydrolysis of **1** selectively cleaved the saponin to give compound **1b** along with arabinose, rhamnose, and xylose in the hydrolysate. Subsequent hydrolysis of **1b** afforded glucose and the aglycone **1a**. The exact structure of the 28-oxygenated tetrasaccharide unit was resolved by detailed scrutiny of the proton and carbon resonances of every monosaccharide unit of **1** and a series of 2D NMR experiments such as COSY, NOESY, DEPT and also by the detailed examination of its negative FAB-MS spectrum. Signals at δ 78.0 and 78.3 were assigned to C-3 of inner glucose and outer glucose, respectively. The downfield shift of C-6 resonance of glucose at δ 69.8 instead of δ 62.4 suggested that two glucose units were linked together via 1 → 6 linkage. The other oligosaccharide at C-28 had the same arrangement as that of mimusopsin reported in the literature [8].

The presence of six sugar units in the compound was also confirmed from six anomeric protons at δ 4.82, 4.89, 4.96, 5.59, 5.98, and 6.20. The β-anomeric configuration for the glucose and xylose was based on their large coupling constants (7–8 Hz). The anomeric proton resonates as a doublet at δ 6.20 with a coupling constant $J = 2.8$ Hz, showing the α-configuration for an L-arabinose moiety, while the anomeric signals appearing at δ 4.89 (d, $J = 7.9$ Hz) and 4.82 (d, $J = 7.8$ Hz) implied β-configuration for the two glucose moieties. Similarly, resonance at δ 4.96 (d, $J = 7.8$ Hz) showed the β-configuration of xylose and those at δ 5.59 (d, $J = 1.7$ Hz), 5.98 (d, $J = 1.7$ Hz) implied the α-configuration for both the rhamnose units. ^1H NMR spectrum also showed the presence of an olefinic proton resonance as a broad singlet at δ 5.58, which is characteristic of the Δ^{12} proton in the pentacyclic triterpenes. ^1H NMR assignments were further confirmed by the help of

Table 1. ^{13}C NMR and ^{1}H NMR spectral data for aglycones of compounds **1** and **2**.

	*Aglycone of compound **1***		*Aglycone of compound **2***	
Position	^{13}C	^{1}H *(m, J)*	^{13}C	^{1}H *(m, J)*
1	46.5		46.6	
2	69.9	4.56 (br s)	71.4	4.58 (br s)
3	83.4	4.27 (d, 3.6)	83.4	4.28 (d, 4.4)
4	43.6		44.1	
5	48.6		49.0	
6	67.3	5.09 (br s)	68.0	5.20 (br s)
7	40.7		41.2	
8	39.8		39.8	
9	48.8		48.4	
10	37.1		37.3	
11	23.9		24.3	
12	123.7	5.58 (br s)	124.0	5.76 (br s)
13	143.6		143.3	
14	42.7		43.3	
15	28.0		36.3	
16	23.6		74.9	5.10 (br s)
17	47.3		50.3	
18	41.3	3.36 (m, 9.0)	42.3	3.66 (m)
19	46.1		46.5	
20	30.6		31.2	
21	34.3		36.1	
22	33.3		32.4	
23	67.0	4.31 (d, 10.4) 3.99 (d, 10.0)	65.7	4.30 (d, 10.3) 3.99 (d, 10.4)
24	16.3	1.83 (s)	17.0	2.02 (s)
25	17.7	1.53 (s)	19.2	2.25 (s)
26	17.9	2.05 (s)	18.9	1.65 (s)
27	26.20	1.22 (s)	27.2	1.81 (s)
28	177.1		177.0	
29	32.9	1.0 (s)	32.2	1.02 (s)
30	23.6	0.89 (s)	25.1	1.18 (s)

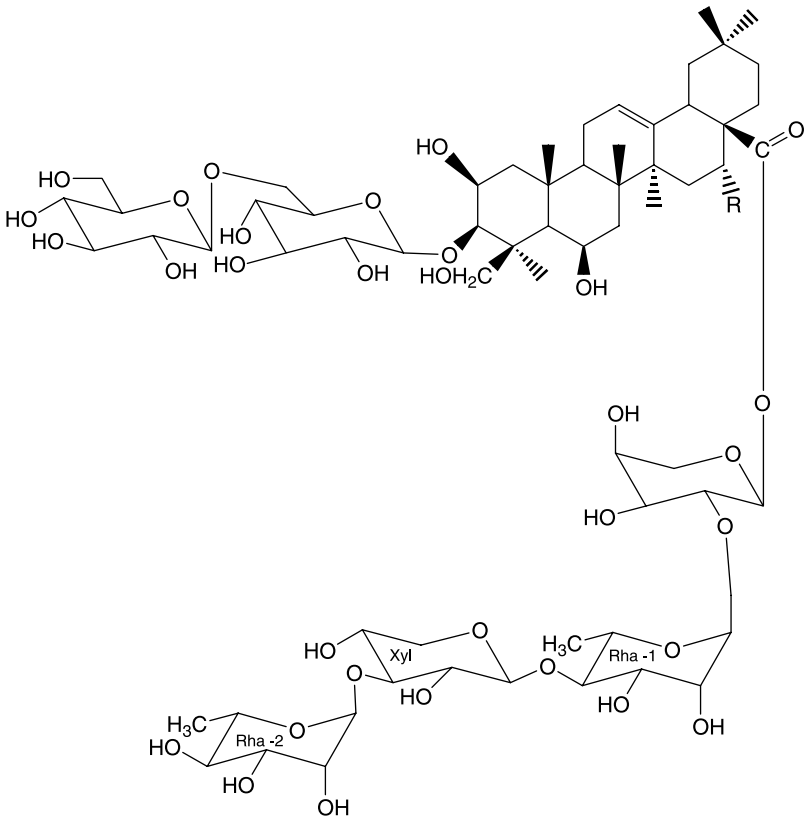
2D-J-resolved COSY and HETCOR experiments [9]. The points of attachment of sugar units were determined through ^{13}C chemical shifts in which the downfield shifts of α carbons and up field shifts of β carbons were characteristic for the establishment of inter glycoside linkage [10]. These observations were further supported by ROESY and HMBC experiments.

The NOE experiments showed the correlations between H-1 of glucose (δ 4.89) and H-3 (δ 4.27) of protobassic acid, between H-1 of terminal glucose (δ 4.89) and H-6 of inner glucose (δ 4.05), between H-1 of xylose (δ 4.96) and H-4 of inner rhamnose (δ 4.10), and between H-1 of inner rhamnose (δ 5.59) and H-2 of arabinose (δ 4.10). An NOE of weak intensity was also observed between H-1 of terminal rhamnose (δ 5.98) and H-3 of xylose (δ 4.03). Cross correlation peaks appeared between aglycone H-3 and glucosyl C-1, aglycone C-28 with arabinose H-1, rhamnosyl C-1 and arabinose H-2, and H-1 of terminal rhamnose and C-3 of xylose. This pattern of linkage of four sugars, namely, ara-rha-xyl-rha, is common to a large number of saponins in many plant families such as Sapotaceae, Rubiaceae, etc. Analysis of the ^{1}H NMR and ^{13}C NMR spectra and the data available in the literature fully define the structure of **1** as 3-*O*-β-D-glucopyranosyl-(1 → 6)-β-D-glucopyranosyl-28-*O*-α-L-rhamnopyranosyl-(1 → 3)-β-D-xylopyranosyl-(1 → 4)-α-L-rhamnopyranosyl-(1 → 2)-α-L-arabinopyranosyl protobassic acid as previously reported [11] (figure 1).

Table 2. ^{13}C NMR and ^{1}H NMR spectral data for the sugar parts of compounds **1** and **2**.

	1		***2***	
Sugar	^{13}C	^{1}H	^{13}C	^{1}H
Glucose-inner	104.8	4.82 (d, 7.8)	105.1	4.80 (d, 8.0)
	75.0	3.91 (t, 8.0)	74.6	3.89 (t, 8.0)
	78.0	4.01 (t, 8.0)	88.1	4.20 (t, 8.0)
	71.5	4.03 (dd, 9.7)	69.6	4.09 (dd, 9.7)
	76.4	3.90 (m)	77.4	3.90 (m)
	69.8	4.05 (dd, 12.0, 4.0)	62.7	4.05 (dd, 12.0, 4.5)
		4.75 (dd, 8.4, 2.0)		4.69 (dd, 8.4, 1.0)
Glucose-terminal	105.0	4.89 (d, 7.9)	105.2	4.82 (d, 7.9)
	74.9	4.09 (dd, 9.0, 7.5)	74.7	3.59 (dd, 9.0, 7.0)
	78.3	4.05 (m)	78.0	4.0 (m)
	71.6	3.80 (t, 9.0)	71.5	4.01 (t, 9.0)
	77.2	3.82 (m)	77.3	3.84 (m)
	62.4	4.05 (dd, 12.0, 6.0)	62.4	4.38 (dd)
		3.81 (dd, 12.2, 2.5)		4.20 (dd)
Arabinose	94.0	6.20 (d, 2.8)	94.0	6.40 (d, 2.8)
	75.4	4.10 (m)	75.6	4.42 (m)
	69.7	4.20 (m)	69.4	4.28 (m)
	65.4	4.27 (m)	66.7	4.30 (m)
	62.6	3.58 (d, 9)	62.5	3.85 (d, 9)
		3.90 (d, 9)		4.0 (d, 9)
Rhamnose-inner	101.0	5.59 (d, 1.7)	101.0	6.80 (br, s)
	72.1	4.30 (br, s)	72.2	4.62 (br, s)
	72.3	4.0 (d, 6)	72.3	4.40 (d, 6)
	83.5	4.10 (t, 9)	83.2	4.19 (t, 9)
	68.9	4.20 (m)	69.0	4.76 (m)
	18.3	1.59 (d, 6.2)	18.3	1.6 0 (d, 6)
Xylose	106.0	4.96 (d, 7.8)	106.4	4.96 (d, 7.5)
	75.6	3.90 (t, 8.0)	75.6	3.90 (t, 8.0)
	83.4	4.03 (t, 8.0)	83.4	4.03 (t, 8.0)
	68.9	4.0 (m)	69.0	4.10 (m)
	67.09	4.10 (dd, 12, 10)	67.9	4.12 (dd, 12, 10.5)
Rhamnose-terminal	102.0	5.98 (d, 1.7)	102.0	5.98 (d, 1.7)
	72.0	4.38 (dd, 3.5, 1.5)	72.0	4.40 (dd, 3.5, 1.4)
	71.7	4.40 (dd, 9.5, 3.0)	71.5	4.38 (dd, 9.5, 3.0)
	73.9	4.10 (t, 9.5)	73.9	4.17 (t, 9.5)
	69.7	4.27 (d, q, 9.5, 6)	69.9	4.27 (d, q, 9.5, 5.6)
	18.8	1.64 (d, 5.7)	18.8	1.64 (d, 5.8)

Compound **2** was isolated as a white solid compound, mp 227°C (decomposed). Its negative ion FAB-MS gave molecular ion peak at *m/z* 1399 $[M - H]^-$, corresponding to the molecular formula $C_{64}H_{104}O_{33}$. Stepwise loss of fragments in the FAB-MS (negative ion) indicated the presence of six sugars, including two units of pentoses, two rhamnoses, and two glucoses, respectively. The genin was found to be 16α-hydroxyprotobassic acid (*m/z* at 519). The fragment at *m/z* 1253 $[M - 146 - H]^-$ indicated the presence of a terminal rhamnose. Another fragment at *m/z* 843 showed the loss of $[M - 2 \times \text{rhamnose} - 2 \times \text{pentose} - H]^-$. The fragment ion at *m/z* 681 further showed a loss of $[M - 2 \times \text{rhamnose} - 2 \times \text{pentose} - \text{glucose} - H]^-$. A loss of 162 from 681 due to the second glucose unit yielded the fragment of 519, characterising the presence of a genin of 16 mass units heavier than protobassic acid, indicating that the genin was probably its analogue, 16α-hydroxyprotobassic acid. The ^{1}H NMR spectrum exhibited six methyl singlets at δ 1.02, 1.18, 1.65, 1.81, 2.02, and 2.25 (3H each) for C-29, C-30, C-26, C-27, C-24, and C-25, respectively. A multiplet at δ 3.66 was attributed to H-18 and a doublet at δ 3.99 ($J = 10.4$ Hz) was assigned to H-23. Another



1 R = H

2 R = OH

Figure 1. The structures of compounds **1** and **2**.

doublet at δ 4.28 was due to 3α-H. The presence of an α-hydroxyl group at C-16 was confirmed by the broad singlet at δ 5.10 due to H-16β. A broad singlet at δ 5.76 was due to H-12 of aglycone. Further evidence of glycosidic moiety and sugar was achieved through the examination of ^{13}C NMR spectrum and by COSY and HETEROCOSY analysis.

The ^{13}C NMR spectrum exhibited six anomeric signals at δ 94.0, 101.0, 102.0, 105.2, 105.1, and 106.4, corresponding to six anomeric protons at δ 6.40, 6.80, 5.98, 4.96, 4.82, and 4.80 in the ^{1}H NMR spectrum indicated the presence of two units of rhamnose. The ^{13}C NMR spectrum of the genin moiety showed a downfield shift of C-16 to δ 74.2 from δ 23.6 as found in **1**, indicating that the C-16 was substituted by an α-hydroxyl group. A close examination of ^{13}C NMR chemical shifts of the glucose units revealed that the two glucose units were joined together *via* 1 → 3 linkage, signals at δ 62.7 and 62.4 were assigned to C-6 of inner and outer glucose units, respectively. Downfield shift of C-3 resonance at δ 88.1, instead of δ 78.0, indicated that the two glucose units were linked together *via* 1 → 3 linkage. While in compound **1** the C-3 of inner and outer glucose was observed at δ 78.0 and 78.3, respectively (table 2). Close inspection of ^{13}C NMR spectrum also showed

that the sugar sequence of **2** at C-28 position was identical to that of **1**, as a very common mode of sequence of sugars in plants. The structure of **2** was thus established as 3-*O*-β-D-glucopyranosyl-(1 → 3)-β-D-glucopyranosyl-28-*O*-α-L-rhamnopyranosyl-(1 → 3)-β-D-xylopyranosyl-(1 → 4)-α-L-rhamnopyranosyl-(1 → 2)-α-L-arabinopyranosyl-16α-hydroxyprotobassic acid.

The pure compound **2** exhibited antibacterial activity against both Gram-positive and Gram-negative bacteria with MICs ranging between 125 and 500 μg/ml (tables 3 and 4). As indicated in table 3, **2** was found to be more effective against three of the Gram-positive organisms including *Bacillus subtilis*, *Corynebacterium xerosis*, and *Corynebacterium diphtheriae* with MIC value of 125 μg/ml. MIC values for the remaining Gram-positive and Gram-negative organisms tested were found to be 250 and 500 μg/ml. According to the current study, compound **2** was found to be active against various clinical and environmental microorganisms. It inhibits the growth of *Staphylococcus aureus*, which causes skin infections, bacteraemia, and toxic shock syndrome. Similarly, **2** also inhibited the Gram negative group of organisms including *Escherichia coli*, *Salmonella typhi*, *Shigella dysenteriae*, *Proteus* species and especially against *Pseudomonas aeruginosa*, which is a notorious organism found everywhere and produces infections with abnormal host defences.

The present study showed the antimicrobial activity of compound **2** against multi-drug resistant (MDR) group of bacteria. Though the concentration of **2** required to bring about the effective inhibition of the growth of microorganisms tested is quite high (MIC range 125–500 μg/ml) as compared to standard antibiotics used for treating infections

Table 3. ^{13}C NMR spectral data for compounds **1**–**2b** (CD_3OD 500 MHz).

C	***1***	***1a***	***1b***	***2***	***2a***	***2b***
1	46.5	47.5	46.5	46.6	47.5	46.6
2	69.9	72.0	70.9	71.4	71.9	70.8
3	83.4	73.2	83.3	83.4	73.4	83.1
4	43.6	43.4	44.0	44.1	43.4	43.9
5	48.6	49.0	49.2	49.0	49.1	49.0
6	67.3	67.9	67.8	68.0	67.9	67.8
7	40.7	41.3	41.1	41.2	41.3	41.3
8	39.8	39.4	39.3	39.8	39.5	40.8
9	48.8	49.2	48.8	48.4	48.3	48.5
10	37.1	37.2	36.9	37.3	37.1	37.0
11	23.9	24.1	24.1	24.3	24.2	24.2
12	123.7	123.2	123.3	124.0	123.0	122.6
13	143.6	144.0	144.3	143.3	144.6	144.8
14	42.7	43.1	42.9	43.3	42.9	43.0
15	28.0	28.4	28.3	36.3	36.2	36.2
16	23.6	23.2	23.8	74.9	74.6	74.9
17	47.3	46.5	46.8	50.3	49.5	49.5
18	41.3	42.3	42.2	42.3	41.7	42.1
19	46.1	46.5	46.6	46.5	47.4	47.4
20	30.6	31.1	31.0	31.2	31.0	31.0
21	34.3	34.4	34.3	36.1	36.2	36.2
22	33.3	33.3	33.3	32.4	32.7	31.8
23	67.0	67.8	65.4	65.7	67.8	65.5
24	16.3	16.7	16.8	17.0	16.0	16.8
25	17.7	18.9	19.1	19.2	19.0	19.1
26	17.9	18.7	18.7	18.9	18.6	18.9
27	26.20	26.5	26.5	27.2	27.5	27.7
28	177.1	180.6	180.2	177.0	180.5	181.9
29	32.9	33.4	33.3	32.2	33.4	33.5
30	23.6	24.0	23.8	25.1	24.9	25.7

Table 4. Antibacterial activity of compound **2** against Gram-positive bacteria.

No.	*Gram-positive bacteria*	*Minimum inhibitory concentration of **2** (μg/ml)*
1	*Staphylococcus aureus*	250
2	Methicillin-resistant *Staphylococcus aureus* (MRSA)	500
3	*Staphylococcus sapropyticus*	250
4	*Staphylococcus epidermidis*	250
5	*Streptococcus faecalis*	250
6	*Streptococcus pyogenes* 10403	250
7	*Micrococcus luteus*	250
8	*Bacillus subtilis*	125
9	*Corynebacterium xerosis*	125
10	*Corynebacterium diphtheriae*	125

caused. Our observations strongly suggest that the bioactive components of *Achras sapota*, especially **2**, can be a good source for developing effective, non-toxic new antibacterial drugs for the treatment of microbial diseases.

3. Experimental

3.1 General experimental procedures

All melting points were determined on a Gallenkamp melting point apparatus and are uncorrected. TLC was carried out on Merck TLC plates aluminium sheets (Silica gel 60 GF_{254} precoated plates) and on freshly prepared glass plates (5 × 20 cm, silica gel layer 0.1 mm). Precoated HPTLC plates were also employed for the determination of the purity of isolated compounds. Infrared spectra were recorded on a JASCO-302 spectrophotometer. EI-MS was scanned on a Varian MAT-12 mass spectrophotometer. The negative ion FAB-MS was recorded on a Varian MAT-312 spectrophotometer. ^{1}H NMR and ^{13}C NMR spectra were scanned in CD_3OD on a Bruker Aspect AM-300 spectrophotometer and AM-400 and 500 MHz. The HPLC consisted of a Schimadzu model LC-6A pump as a solvent delivery system, a Rheodyne sample injector within a 100 μl loop, a RP-18 column (25 cm × 10 mm i.d.) and a Schimadzu model RD-6A refractive index detector connected with a recorder.

3.2 Plant material

Plant material (5 kg), purchased from the fruit market Karachi, was confirmed by Botany Department, University of Karachi. After collection, the seeds were separated from flesh, and allowed to dry at ambient temperature for about 1 week. A voucher sample is deposited in the Department of Botany, University of Karachi, Ch-04-3192.

3.3 Extraction and isolation

After removing the kernel, *A. sapota* seeds (cotyledons) were crushed and soaked in methanol for 15 days at room temperature and were repeatedly extracted (15 days). The residue (375 g) obtained after removal of solvent from the combined methanolic extracts, were fractionated with n-hexane, ethyl acetate, and finally with n-butanol saturated with

water. Butanol was removed under high vacuum, yielding a white gummy solid (17 g). This crude mixture containing saponins was subjected to column chromatography on silica gel. The fractions eluted with chloroform/methanol/water (20:10:1) showed positive indication of saponins on TLC. All the fractions were combined and further separated and purified by HPLC using methanol/water (70:30) on a semi preparative RP-18 column at a flow rate of 1.0 ml/min to yield **1** (18 mg) and **2** (25 mg).

Compound **1** was obtained as an amorphous solid, mp 231°C (decomposed). HRFAB-MS *m/z* 1383.6021, (calcd for $C_{64}H_{104}O_{32}$ 1384.5064). FAB-MS *m/z* 1383.6 $[M - H]^-$ 1237 $[M - 146 - H]^-$, 1221 $[M - O$ glu $- H]^-$, 1105 $[M - H -$ pentose-rham$]^-$, 1059 $[M - H - 2 \times$ glu$]^-$, 827 $[M - 2 \times$ rhamnose-2 $\times$ pentose $- H]^-$, 665 $[M - 2 \times$ rhamnose $- 2 \times$ pentose $-$ glucose $- H]^-$; EI-MS *m/z* 503 [aglycone, M $-$ sugars]$^+$, 485 $[M - H_2O - H]$, 486 $[M - H_2O]^+$, 301, 248, 203. IR (KBr) ν_{max} (cm^{-1}): 3420, 2920, 1642, 1385, 1050, and 643. ^{1}H NMR in C_5D_5N at 500 MHz and ^{13}C NMR C_5D_5N at 125 MHz spectral data (tables 1 and 2, respectively).

3.4 Acid hydrolysis of compound 1

Compound **1** (10 mg) was refluxed with 2 M sulphuric acid in aqueous methanol on a boiling water bath for 4 h. The mixture was treated with water and hydrolysate was then extracted with ethyl acetate. The ethyl acetate layer, containing the aglycone, was kept aside for further work-up and the aqueous layer was neutralised with a weak base ($NaHCO_3$), evaporated under vacuum. The residue so obtained was compared with standard sugars on silica gel TLC (EtOAc/AcOH/H_2O/MeOH; 6:1:1:2). The sugars were found to be arabinose, glucose, rhamnose, and xylose. The identity of these sugars was further confirmed by comparison with standard sugars on PC and with data available in the literature [12].

The ethyl acetate layer was examined for the identification of aglycone, the compound was labelled as **1a**, re-crystallised from methanol, mp 306°C; lit. 310–312°C. FAB-MS (negative) *m/z* 503 $[M - H]^-$ equivalent to $C_{30}H_{48}O_6$. 485 $[M - H_2O - H]$; EI-MS *m/z* 486 $[M - H_2O]^+$, 301, 248, 203. ^{1}H NMR δ 0.93,1.02, 1.26, 1.61, 1.99, 2.20 (3H each, s, H-29, H-30, H-7, H-6, H-4, H-5 respectively, 3.36 (1H, m, $W_{1/2} = 9.0$ Hz; H-18), 3.99 1H,s, $J = 10.0$ Hz, H-23); 4.27 (1H s, $J = 3.6$ Hz, H-3a), 4.31 (1H, d, $J = 10.4$ Hz, H-23), 4.56 (1H, br s, H-2a), 5.09 (1H, br s, H-6a), 5.58 (1H, br s, H-12).

Compound **2** was obtained as white powder, mp 227°C (decomposed). HRFAB-MS *m/z* 1399.4809 (calcd for $C_{64}H_{104}O_{33}$ 1400.5059). FAB-MS *m\z* 1399.5 $[M - H]^-$ 1253 $[M - 146 - H]^-$, 1237 $[M - O$ glc $- H]^-$, 843 $[M - 2 \times$ rhamnose $- 2 \times$ pentose $-$ H$]^-$, 681 $[M - 2 \times$ rhamnose $- 2 \times$ pentose $-$ glucose $- H]^-$; EI-MS *m/z* 519 [aglycone, M $-$ sugars]$^+$, 501 $[M - H_2O]^-$, 339, 325, 264, 474, 458, 264, 255, 246, 238, 202, 187. IR (KBr) ν_{max} (cm^{-1}): 3400, 1733, 1225, and 1640. ^{1}H NMR in C_5D_5N at 500 MHz and ^{13}C NMR C_5D_5N at 125 MHz spectral data (tables 1 and 2, respectively).

3.5 Acid hydrolysis of compound 2

Compound **2** was hydrolysed in the same manner as that described for compound **1**. The aqueous layer was evaporated to dryness for the identification of sugars that were found to be glucose, rhamnose, xylose, and arabinose. The identification of these sugars was further confirmed by comparison with standard sugars on PC (Whatman filter paper No.1).

The ethyl acetate extract, obtained above through the acid hydrolysis of **2**, was examined for the identification of the aglycone (16α-hydroxy protobassic acid, **2a**). The compound so obtained was re-crystallised in MeOH to yield needles, mp 235–237°C, negative FAB-MS: *m/z* 519 $[M - H]^-$, equivalent to $C_{30}H_{48}O_7$.

3.6 Alkaline hydrolysis of compound 2

Solution of **2** (5 mg) in 5% aqueous-MeOH (5 ml) was refluxed with 10% aqueous KOH at 100°C for four hours. The reaction mixture was cooled at room temperature and neutralised with dilute HCl. The mixture was then treated with water and hydrolysate was extracted with ethyl acetate. The aqueous layer was evaporated under vacuum. The residue so obtained was compared with standard sugars on silica gel TLC (EtOAc/AcOH/H_2O/MeOH; 6:1:1:2). The sugars were found to be arabinose, glucose, rhamnose, and xylose. The identity of these sugars was further confirmed by comparison with standard sugars on PC and with data available in literature. The ethyl acetate layer was examined for the identification of aglycone, the compound was labelled as **2b**, re-crystallised from methanol. FAB-MS (negative) *m/z* 843 $[M - H]^-$ which is equivalent to $C_{42}H_{68}O_{17}$, 681 [M − H − glu]; 519 [aglycone, M − H − (2 × glu)]$^-$. ^{1}H NMR in C_5D_5N at 500 MHz exhibited signals at δ 1.02, 1.18, 1.65, 1.81, 2.02, 2.25 (3H each, due to six methyls at C-29, 30, 26, 27, 24, and 25, respectively), 3.66 (1H, m, H-18), 3.99 (1H, d, $J = 10.4$ Hz, H-23), 4.28 (1H, d, $J = 4.4$ Hz, H-3α), 4.58 (1H, br s, H-2α), 5.10 (1H, br. s, H-16 β), 5.20 (1H, br s, H-6α), 5.76 (1H, br H-12). The two anomeric protons of the sugars appeared as doublets at δ 4.81 ($J = 7.8$) and 4.87 ($J = 7.8$). **2b**, by acid hydrolysis gave the glucose (identified by comparing with standard sugars) [13].

3.7 Antibacterial assay

The antibacterial activity of compound **2**, isolated from *A. sapota* was determined against 10 Gram-positive and 14 Gram-negative bacteria. All the isolates were obtained from the Culture Collection of the Department of Microbiology, University of Karachi, and were identified and characterised by the conventional microbiological methods. Stock solution (10 mg/ml) was prepared by dissolving the compound in DMSO and filtered by passing

Table 5. Antibacterial activity of compound **2** against Gram-negative bacteria.

No.	*Gram-negative bacteria*	*Minimum inhibitory concentration of **2** (μg/ml)*
1	*Escherichia coli*	250
2	*Escherichia coli* ATCC	250
3	*Salmonella typhi*	250
4	*Salmonella typhi* ATCC	250
5	Multi-drug resistant *Salmonella typhi*	250
6	*Salmonella paratyphi* A	250
7	*Salmonella paratyphi* B	250
8	*Shigella flexeneri*	250
9	*Shigella dysenteriae*	250
10	*Klebsiella pneumoniae*	250
11	*Pseudomonas aeruginosa*	250
13	*Proteus mirabilis*	250
14	*Proteus vulgaris*	250

through 0.22 μm pore size syringe filter (Awaki Glass). Further serial dilutions were made in Mueller Hinton broth (Oxoid, UK). The antibacterial activity was determined by evaluating the minimum inhibitory concentrations (MICs) by Micro Broth Dilution method as described by the National Committee for Clinical Laboratory Standards (NCCLS) [14].

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References

[1] M.A. Ginai. *A Treatise on Horticulture*, 1st ed., p. 220, Bureau of Agriculture information and publication Division, Govt of Pakistan, Lahore (1968).
[2] K.R. Kritikar, B.D. Basu. *Indian Medicinal Plants*, **II**, p. 1493, Latil Mohan Basu Ltd., Allahabad, India (1978).
[3] G. Watt, M. John. *Dictionary of Commercial Products of India*, **II**, London, pp. 625 (1908).
[4] W. Dymock. *Pharmacographia Indica*, **II**, p. 362, Thaker Spink and Co., Calcutta, India (1989).
[5] A. Mirimanoff, M.L. Ihanoz. *Pharm. Acta Helv.*, **36**, 997 (1961).
[6] R. Banerji, A.K. Stravastava, G. Nigam, S.K. Sing, S. Nigam, R.C. Sexana. *Indian Drugs*, **17**, 6 (1979).
[7] K.P. Bhangar, M.B. Gupta, G.P. Gupta, C.R. Mitra. *Indian J. Med. Res.*, **58**, 724 (1970).
[8] S.K. Nigman, L.X. Cong, D.Z. Wang, G. Misra, C.R. Yang. *Phytochemistry*, **31**, 3169 (1992).
[9] P. Gariboldi, L. Verrottla, B. Gabetta. *Phytochemistry*, **29**, 2629 (1990).
[10] Y. Shao, B. Zhou, K. Ma, H. Wu, L. Lin, G.A. Cordell. *Phytochemistry*, **39**, 875 (1995).
[11] N.P. Sahu, K. Koike, Z. Jia, T. Nikaido. *Phytochemistry*, **44**, 1145 (1997).
[12] M. Srivastava, J. Singh. *Indian J. Pharmacog.*, **32**, 197 (1994).
[13] R. Banerji, G. Misra, S.K. Nigam. *Fitoterapia*, **49**, 104 (1978).
[14] Pa. National Committee for Clinical Laboratory Standards. *Methods for dilution antimicrobial susceptibility test for bacteria that grow aerobically*, NCCLS Publication M7-A4 and Supplement M100-S7, NCCLS. Wayne, (1998)