Polystachyasaponin with Adjuvant Activity from *Entada polystachya*

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A new complex triterpenoid saponin, polystachyasaponin, was isolated from leaves of *Entada polystachya* (L.) DC. (Leguminosae) by using chromatographic methods. Its structure was established as 15,16-dihydroxy-3-[[*O*-β-D-xylopyranosyl-(1→2)-*O*-α-L-arabinopyranosyl-(1→6)-2-(acetylamino)-2-deoxy-β-D-glucopyranosyl]oxy]-(3β,15α,16α)-olean-12-en-28-oic acid *O*-D-apio-β-D-furanosyl-(1→3)-*O*-β-D-xylopyranosyl-(1→2)-*O*-[β-D-glucopyranosyl-(1→4)]-6-*O*-[(2*E*,6*R*)-6-hydroxy-2,6-dimethyl-1-oxo-2,7-octadienyl]-β-D-glucopyranosyl ester. Structural elucidation was performed using detailed analyses of ^{1}H and ^{13}C NMR spectra including 2D NMR spectroscopic techniques and chemical conversions. The hemolytic activity of the saponin was evaluated using *in vitro* assays, and its adjuvant potential on the cellular immune response against ovalbumin antigen was investigated using *in vivo* assays.

Key words: Entada polystachya, Leguminosae, Polystachyasaponin, Complex Triterpenoid Saponin, Ovalbumin, Adjuvant Activity

Introduction

Entada polystachya (L.) DC. (Leguminosae) is a native and common plant in coastal thickets from western Mexico through Central America to Colombia and Brazil. The root decoction is taken to relieve urinary burning, especially in venereal diseases. It is also taken as a diuretic [1]. The seeds and roots of several related species, namely, *Entada abyssinica*, *E. africana*, *E. phaseoloides* and *E. scandens* are used in traditional medicine for the treatment of various ailments such as liver diseases, sleeping sickness, stomachache, and hemorrhoids [2, 3]. The occurrence of acacic acid, echinocystic acid, entagenic acid and oleanolic acid glycosides has been reported for *Entada* species [4 – 8].

From the present investigation on *E. polystachya* leaves, we report the structure of a new complex triterpenoid saponin, polystachyasaponin (**1**), having entagenic acid as aglycone. Taking into account the fact that some complex triterpenoid saponins, such as QS-21 [9], pulcherrimasaponin [10] and brevifoliasaponin [11] isolated from *Quillaja saponaria* Molina, *Calliandra pulcherrima* Benth. and *Calliandra brevifolia* Benth., respectively, have been found to cause substantial enhancement of immune response when given together with an antigen, compound **1** was tested for its adjuvant potential on the cellular immune response against ovalbumin antigen, and the results are reported herein.

Experimental Section

Plant material

Fresh leaves of *Entada polystachya* (L.) DC. were obtained from the Catolé Farm (Senador Pompeu, Ceará, Brazil) in August 2002, and a voucher specimen (no. LQPM-49) is maintained in the Laboratory of Chemistry of Medicinal Plants at this University.

General procedures

Melting points were determined by an Electrothermal 9200 micro-melting apparatus and are uncorrected. The optical rotations were measured on a Perkin Elmer 243B polarimeter. UV and IR spectra were measured on Shimadzu UV-1601 and Perkin Elmer 599B spectrometers, respectively. The MALDI-TOFMS was conducted using a Perseptive Voyager RP mass spectrometer. GC analyses were performed with a Shimadzu GCMS-QP5050A spectrometer using an ionization voltage of 70 eV and an ionization current of 60 μA for EI. GC was carried out with FID using a glass capillary column (0.25 micron × 25 m; J. & W. Scientific Inc., Folsom, CA, USA) DB-1. Mass spectra were taken on a VG Auto SpecQ spectrometer. NMR experiments were performed on a Bruker DRX-600 spectrome-

ter at 300 K. NMR spectra were acquired in [D_5]pyridine with tetramethylsilane (δ = 0.00) used as internal standard. Silica gel columns (230 – 400 mesh ASTM (Merck) and Sephadex LH-20 (Pharmacia)) were used for CC. TLC was performed on silica gel plates (Kieselgel 60F_{254}, Merck) using the following solvent systems: (A) $CHCl_3$/MeOH/H_2O (65 : 35 : 10, v/v/v, lower phase) for compounds **1**, **3**, **4**, and **6**, (B) *n*-BuOH/Me_2CO/H_2O (4 : 5 : 1, v/v/v), (C) *n*-BuOH/pyridine/H_2O (6 : 4 : 3, v/v/v) for monosaccharides and (D) $CHCl_3$/MeOH/H_2O (8 : 3 : 1, v/v/v) for compounds **5** and **5a**. The spray reagents were orcinol/H_2SO_4 for compounds **1**, **3**, **4**, and **6** and $CeSO_4$ for compounds **5** and **5a**.

*Extraction and isolation of compound **1***

Fresh leaves (500 g) were extracted with MeOH (3.0 L) for 72 h at r. t. The extract was concentrated under reduced pressure to remove most of the MeOH, and the resulting aqueous phase was shaken with *n*-BuOH [water/*n*-BuOH (1 : 1, v/v)]. This procedure was repeated three times, and the resulting organic phase was evaporated *in vacuo* to give a crude material (17.3 g), which was dissolved in MeOH (100 mL) and roughly chromatographed (1.73 g/10 mL each time) on Sephadex LH-20 (3.8 × 65 cm) with MeOH. The fractions were combined based on the TLC profiles to give crude triterpenoid saponin (930 mg). Further purification by chromatography on a silica gel column (2.8 × 90 cm) eluted with $CHCl_3$/MeOH/H_2O (65 : 35 : 10, v/v/v, lower phase) afforded a TLC homogeneous compound **1** (380 mg; R_f = 0.32) which gave a dark-blue color with orcinol/H_2SO_4.

*Polystachyasaponin (**1**)*

Colorless powder. – M. p. 215 – 218 °C (dec.). – UV/Vis (MeOH): λ_{max} (lg ε_{max}) = 220 nm (3.78). – $[\alpha]_D^{25} = -17$ (c = 0.60, MeOH). – IR (KBr): ν = 3420 (O-H), 2929 (C-H), 1713 (C=O), 1643 (C=O), 1553 cm^{-1}. – ^{1}H NMR (600 MHz, [D_5]pyridine, 25 °C, TMS): δ = 0.92 (s, 3 H, 25-C*Me*), 0.97 (s, 3 H, 29-C*Me*), 0.98 (s, 3 H, 24-C*Me*), 1.07 (s, 3 H, 30-C*Me*), 1.14 (s, 3 H, 23-C*Me*), 1.17 (s, 3 H, 26-C*Me*), 1.42 (s, 3 H, 10-C*Me*), 1.80 (s, 3 H, 27-C*Me*), 1.95 (s, 3 H, 9-C*Me*), 2.12 (s, 3 H, NHCO*Me*), 4.94 (d, J = 7.3 Hz, 1 H, Xyl 1′-H), 5.02 (d, J = 8.2 Hz, 1 H, GlcNAc 1-H), 5.12 (d, J = 5.4 Hz, 1 H, Ara 1-H), 5.23 (d, J = 7.6 Hz, 1 H, Xyl 1-H), 5.28 (d, J = 7.9 Hz, 1 H, Glc 1′-H), 5.98 (d, J = 7.1 Hz, 1 H, Glc 1-H), 6.05 (d, J = 3.0 Hz, 1 H, Api 1-H), 8.86 (br s, 1 H, *NH*COMe). – ^{13}C NMR data given in Table 1. – HRMS ((+)-MALDI-TOF): *m/z* = 1733.8737 (calcd. 1733.8778 for $C_{80}H_{127}NO_{38}Na$, $[M+Na]^+$).

*Acid hydrolysis of **1***

A solution of saponin **1** (20 mg) in 2 M HCl/H_2O (10 mL) was heated in a sealed tube for 4 h at 100 °C. The reaction mixture was diluted with H_2O (20 mL) and extracted with diethyl ether (30 mL). The organic layer was concentrated *in vacuo* to give a residue (5.2 mg), which was chromatographed on a silica gel column (1 × 30 cm) eluted with $CHCl_3$/MeOH (100 : 3, v/v) affording a colorless solid, which was crystallized twice from MeOH to give colorless rectangular plates [**2**; 3.4 mg; m. p. 313 – 315 °C; $[\alpha]_D^{20}$ = +33 (c = 0.8, EtOH)]. The aqueous solution was passed through an Amberlite IRA-410 column. The eluate was concentrated *in vacuo* to give a residue (11.3 mg). A sample of the sugar residue (1 mg) was dissolved in pyridine (100 μL) and analyzed by silica gel TLC in the above described solvent system (B). After spraying with orcinol/H_2SO_4, apiose gave a yellow spot at R_f = 0.78, xylose gave a blue spot at R_f = 0.57, arabinose gave a purple spot at R_f = 0.48, and glucose gave a blue spot at R_f = 0.40. 2-Amino-2-deoxy-glucose was analyzed by silica gel TLC in the above solvent system (C) and gave a blue spot at R_f = 0.10.

*Alkaline hydrolysis of compound **1***

Compound **1** (100 mg) was hydrolyzed with 1 N KOH/H_2O (5 mL) for 24 h at r. t. The reaction mixture was neutralized with 1 N HCl in water and extracted with *n*-BuOH. This procedure was repeated, and the resulting organic phase was evaporated *in vacuo* to give a residue (92 mg), which was chromatographed on Sephadex LH-20 with MeOH and afforded compound **3** (47 mg) as an amorphous powder. – M. p. 210 – 215 °C (dec.). – $[\alpha]_D^{25} = -23°$ (c = 0.60, MeOH). – HRMS ((+)-MALDI-TOF): *m/z* = 977.1124 (calcd. 977.1186 for $C_{48}H_{75}NO_{18}Na$, $[M+Na]^+$), and compound **4** (26 mg) as a colorless powder. – $[\alpha]_D^{25}$ = −54 (c = 0.60, MeOH). – HRMS ((+)-MALDI-TOF): *m/z* = 629.5223 (calcd. 629.5265 for $C_{22}H_{38}NO_{19}Na$, $[M+Na]^+$).

*Mild alkaline hydrolysis of compound **1***

To a solution of compound **1** (50 mg) in dioxane (6 mL) was added 1 % KOH (6 mL), and the mixture was stirred at 0 °C for 3 h under N_2 gas. The reaction mixture was acidified with 10 % HCl and extracted with $CHCl_3$. The $CHCl_3$ solution was washed with H_2O and evaporated to dryness. The residue (4.8 mg) was chromatographed on a silica gel column (1 × 30 cm) eluted with *n*-hexane/Me_2CO (4 : 1, v/v) affording two oily substances **5**; [2.7 mg; $[\alpha]_D^{25}$ = −24.5 (c = 0.65, $CHCl_3$)], and **5a** (1.4 mg) [12]. The aqueous phase resulting from the extraction with $CHCl_3$ was extracted with *n*-BuOH. The *n*-BuOH solution was washed with H_2O and evaporated *in vacuo* to give a residue (42 mg), which was dissolved in MeOH (5 mL) and chromatographed on Sephadex LH-20 (2 × 50 cm) with MeOH to afford compound **6** (35 mg) as an amorphous powder. – M. p. 202 – 208 °C (dec.). – $[\alpha]_D^{25} = -10°$ (c = 0.60, MeOH). – HRMS ((+)-MALDI-TOF): *m/z* = 1565.6347 (calcd. 1565.6400 for $C_{70}H_{111}NO_{36}Na$, $[M+Na]^+$).

*Reduction of compound **4***

Compound **4** (4 mg) was dissolved in water (2 mL), then sodium tetrahydroborate (20 mg) was added to the resulting solution. The reaction mixture was allowed to stand at r. t. for 12 h, then treated with 20 % acetic acid, and concentrated to give **4a**.

Molar carbohydrate composition and D,L configurations

The molar carbohydrate composition of compound **1** was determined by GC-MS analysis of its monosaccharides as their trimethylsilylated methylglycosides obtained after methanolysis (0.5 M HCl in MeOH, 24 h, 80 °C) and trimethylsilylation [13]. The configurations of the glycosides were established by capillary GC of their trimethylsilylated (–)-2-butylglycosides [14].

Methylation analysis

Compounds **1**, **3**, **4**, **4a** and **6** were methylated with dimethyl sulfoxide/lithium methylsulfinyl carbanion/methyl iodide [15]. The methyl ethers were obtained after hydrolysis (4 N trifluoroacetic acid, 2 h, 100 °C) and analyzed as partial alditol acetates by GC-MS [16].

Animals

Swiss albino male mice, weighing 30 – 35 g, were used. The animals were housed under standard environmental conditions and fed with standard rodent diet and water *ad libitum*.

Statistical analysis

The experimental data were tested for statistical differences using the Student's *t* test.

Hemolytic activity

Normal human red blood cell suspension (0.6 mL of 0.5 %) was mixed with 0.6 mL diluent containing 5, 10, 20, 30, 40, 50, 100, 250 and 500 μg mL^{-1} of compound **1**, aluminum hydroxide ($Al(OH)_3$), purified *Quillaja* saponin (QS-21), and 5 – 500 μL mL^{-1} of Freund's Complete Adjuvant (FCA) and Freund's Incomplete Adjuvant (FIA) in saline solution. Mixtures were incubated for 30 min at 37 °C and centrifuged at 70 *g* for 10 min. Saline and distilled water were included as minimal and maximal hemolytic controls. The hemolytic percents developed by the saline control were subtracted from all groups. The adjuvant concentration inducing 50 % of the maximal hemolysis was considered the HD_{50} (graphical interpolation). Experiments included triplicate at each concentration [17].

Immunization procedure

Male Swiss mice (three months old) were subcutaneously immunized twice at weekly intervals with 100 μg ovalbumin (OVA) in 100 μL saline (SAL) as the control group or with 100 μg ovalbumin mixed with 100 μg of each adjuvant dissolved in 100 μL of saline as vehicle. Delayed-type hypersensitivity (DTH) responses were assessed by measuring the increment in the right footpad thickness after subcutaneous challenge with 100 μg OVA in 100 μL saline a week after the second immunization. The footpad thickness was measured with a spring-loaded dial gauge (Mitutoyo Corp., Tokyo, Japan) before and 24, 48 and 72 h after injection. Injecting each animal with 100 μL saline in the left hind footpad served as control. The ovalbumin-specific responses

Table 1. ^{13}C NMR spectral data for polystachyasaponin (**1**)[a].

Position		^{13}C, δ	DEPT	Position		^{13}C, δ	DEPT
Agl	1	39.25	CH_2	GlcNAc	1	104.51	CH
	2	25.52	CH_2		2	57.77	CH
	3	89.92	CH		3	75.10	CH
	4	39.31	C		4	72.33	CH
	5	56.00	CH		5	76.11	CH
	6	19.11	CH_2		6	68.36	CH_2
	7	37.35	CH_2	$NHCOCH_3$		23.97	C=O
	8	40.08	C	$NHCOCH_3$		170.50	CO*Me*
	9	47.69	CH	Ara	1	103.47	CH
	10	37.25	C		2	80.96	CH
	11	24.25	CH_2		3	73.80	CH
	12	124.24	CH		4	68.65	CH
	13	144.60	C		5	66.39	CH_2
	14	47.85	C	Xyl	1′	107.75	CH
	15	68.41	CH		2′	76.43	CH
	16	79.05	CH		3′	78.12	CH
	17	48.77	C		4′	71.17	CH
	18	42.03	CH		5′	67.59	CH_2
	19	46.75	CH_2	Glc	1	93.08	CH
	20	30.94	C		2	78.35	CH
	21	36.13	CH_2		3	76.83	CH
	22	30.29	CH_2		4	79.96	CH
	23	28.38	C*Me*		5	74.78	CH
	24	17.32	C*Me*		6	63.34	CH_2
	25	16.03	C*Me*	Glc	1′	102.18	CH
	26	18.25	C*Me*		2′	74.94	CH
	27	21.08	C*Me*		3′	78.22	CH
	28	176.04	C=O		4′	71.77	CH
	29	33.59	C*Me*		5′	77.92	CH
	30	24.75	C*Me*		6′	62.46	CH_2
MT	1	167.20	C=O	Xyl	1	105.41	CH
	2	127.19	C		2	75.26	CH
	3	145.56	CH		3	84.97	CH
	4	24.55	CH_2		4	71.17	CH
	5	41.60	CH_2		5	67.18	CH_2
	6	73.68	C	Api	1	111.61	CH
	7	146.77	CH		2	78.72	CH
	8	112.06	CH_2		3	80.55	C
	9	12.85	C*Me*		4	75.44	CH_2
	10	28.84	C*Me*		5	65.74	CH_2

[a] ^{13}C (150 MHz) in [D_5]pyridine, δ in ppm. The following convention is used: Agl = aglycone, MT = monoterpene, GlcNAc = 2-(acetylamino)-2-deoxy-β-D-glucopyranosyl, Ara = α-L-arabinopyranosyl, Xyl = β-D-xylopyranosyl, Glc = β-D-glucopyranosyl, Api = β-D-apiofuranosyl.

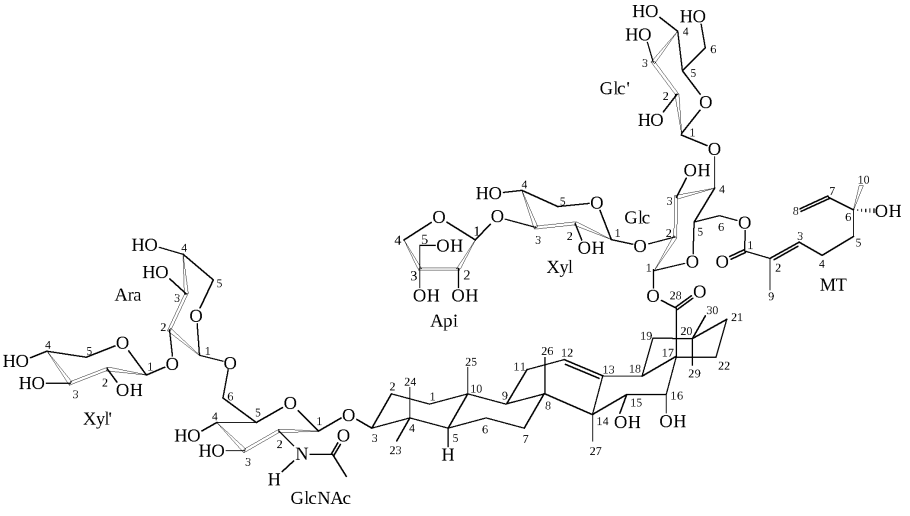

Fig. 1. Chemical structure of polystachyasaponin (**1**).

were obtained by subtracting the response to OVA challenge in unimmunized control mice [18].

Result and Discussion

Compound **1** was obtained as a colorless powder and gave a positive Liebermann-Burchard test for a triterpenoid saponin. It revealed a quasi-molecular ion peak at *m/z* = 1733.8737 $[M+Na]^+$ in the MALDI-TOF-MS. In the ^{13}C NMR spectrum, of the 80 carbon signals observed, there are ten methyl groups, nineteen methylene groups (eight of which were oxygenated), thirty-eight methine groups (thirty-two of which were oxygenated) and thirteen quaternary carbon atoms (five of which were oxygenated). The number of hydrogens attached to each individual carbon atom was determined by the DEPT spectrum. On the basis of the above mentioned MS and ^{13}C NMR spectral data (Table 1), compound **1** was assumed to be a saponin with the molecular formula of $C_{80}H_{127}NO_{38}$ (Fig. 1).

In addition to this, the UV spectrum of **1** featured an absorption at 220 nm of an α,β-unsaturated ester carbonyl group, confirmed by an absorption at 1643 cm^{-1} in the IR spectrum. The IR spectrum showed absorptions at 3420 and 1713 cm^{-1} attributed to the hydroxyl and ester-carbonyl groups, respectively. The presence of the NH group was proved by the ^1H NMR signal at δ = 8.86 (br s, *NH*COMe), and by the IR spectral absorptions at 1643 and 1553 cm^{-1} for a CONH group. The ^1H NMR spectrum showed signals ascribable to ten tertiary methyl groups at δ = 0.92 (s, 25-C*Me*), 0.97 (s, 29-C*Me*), 0.98 (s, 24-C*Me*), 1.07 (s, 30-C*Me*), 1.14 (s, 23-C*Me*), 1.17 (s, 26-C*Me*), 1.42 (s, 10-C*Me*), 1.80 (s, 27-C*Me*), 1.95 (s, 9-C*Me*), and 2.12 (s, NHCO*Me*). The ^1H NMR spectrum of **1** displayed signals for seven anomeric protons at δ = 4.94 (d, *J* = 7.3 Hz, 1 H), 5.02 (d, *J* = 8.2 Hz, 1 H), 5.12 (d, *J* = 5.4 Hz, 1 H), 5.23 (d, *J* = 7.6 Hz, 1 H), 5.28 (d, *J* = 7.9 Hz, 1 H), 5.98 (d, *J* = 7.1 Hz, 1 H), and 6.05 (d, *J* = 3.0 Hz, 1 H), which gave correlations in the HSQC spectrum with ^{13}C NMR signals (Table 1) at δ = 107.75, 104.51, 103.47, 105.41, 102.18, 93.08, and 111.61, respectively. Evaluation of chemical shifts and spin-spin couplings allowed the identification of two β-xylopyranosyl units, two β-glucopyranosyl units, one α-arabinopyranosyl unit, one β-apiofuranosyl unit and one 2-(acetylamino)-2-deoxy-β-glucopyranosyl unit. The ^{13}C NMR spectrum of **1** (Table 1) showed one ester-carbonyl carbon at δ = 176.04 (C=O, C-28), one amide-carbonyl carbon at δ = 170.50 (COMe), one α,β-unsaturated ester-carbonyl carbon of the monoterpenoid moiety (MT) at δ = 167.20 (C=O, MT C-1), one pair of the trisubstituted olefinic carbons of the aglycone moiety at δ = 124.24 (CH, C-12) and 144.60 (C, C-13), and two pairs of the trisubstituted and monosubstituted olefinic carbons at δ = 127.19 (C, MT C-2) and 145.56 (CH, MT C-3) and at δ = 112.06 (CH_2, MT C-8) and 146.77 (CH, MT C-7), respectively, and one sugar-linked methine carbon at δ = 89.92 (C-3). The upfield signal observed at δ = 176.04 suggests one sugar linkage at C-28. The attachment of the sugar moiety to the aglycone moiety was established by ^1H-^1H COSY, HMBC and HMQC experiments. The HMBC and HMQC spectra displayed long-range cou-

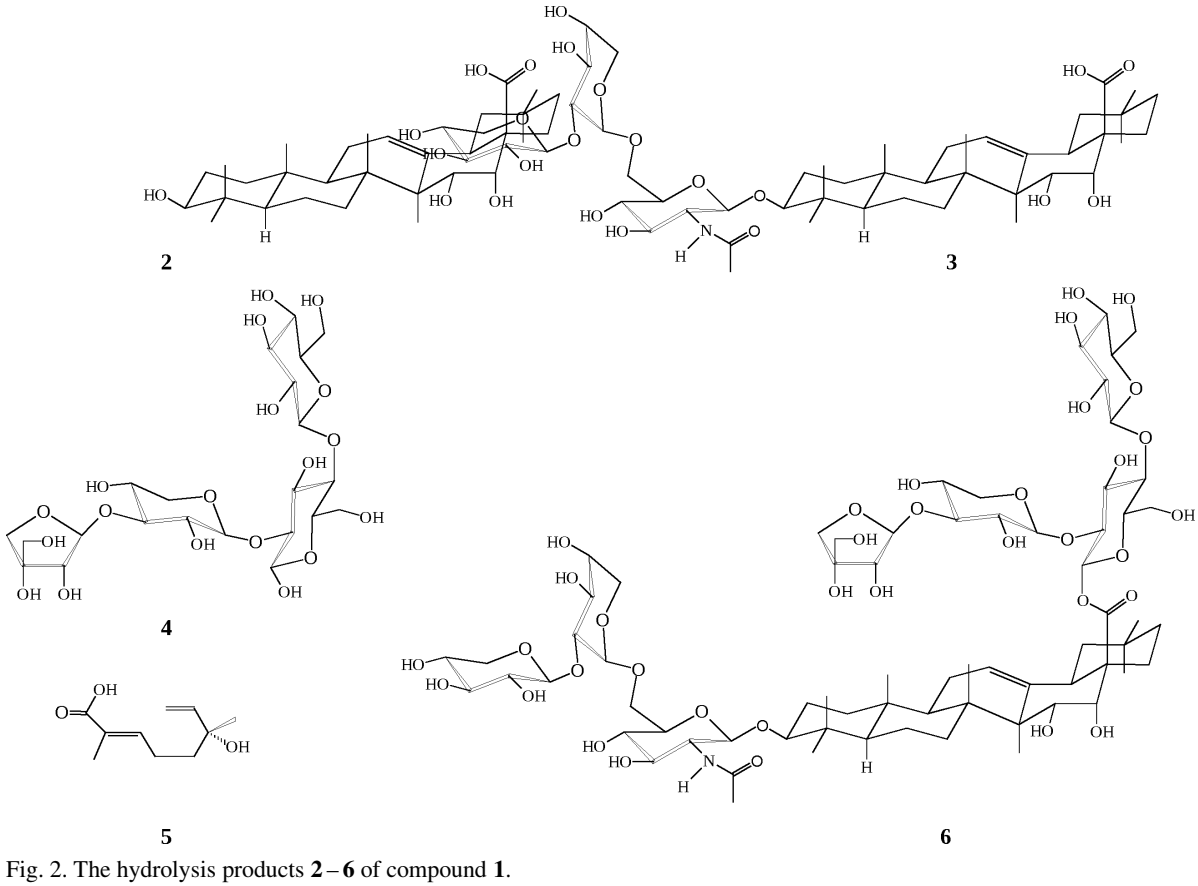

Fig. 2. The hydrolysis products **2** – **6** of compound **1**.

plings between 2-(acetylamino)-2-deoxy-glucose-H-1 at δ = 5.02 and aglycone-C-3 at δ = 89.92. The above facts suggest that compound **1** should be a 3,28-*O*-bis(desmoside) having seven monosaccharides and one monoterpenic acid.

On acid hydrolysis, compound **1** gave a sapogenin, 2-amino-2-deoxy-glucose, arabinose, xylose, apiose, and glucose. The structure of the sapogenin, compound **2**, was established as 3,15,16-dihydroxy-(3β,15α,16α)-olean-12-en-28-oic acid (entagenic acid; Fig. 2) by comparing its physical properties ([α]$_D$, and m. p.), IR, ^{1}H NMR and EIMS spectral data with those of entagenic acid [6]. The molar carbohydrate composition of **1** indicated the presence of one 2-(acetylamino)-2-deoxy-glucose, one arabinose, one apiose, two xyloses, and two glucoses [13]. Their absolute configurations were determined by GC of their trimethylsilylated (–)-2-butylglycosides [14].

The sequence of the sugar chain of **1** was confirmed by methylation analysis [15] which furnished 1,4-di-*O*-acetyl-2,3,5-tri-*O*-methyl apitol / 1,5-di-*O*-acetyl-2,3,4-tri-*O*-methyl xylitol / 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl glucitol / 1,3,5-tri-*O*-acetyl-2,4-di-*O*-methyl xylitol / 1,2,5-tri-*O*-acetyl-3,4-di-O-methyl arabinitol / 1,5,6-tri-*O*-acetyl-2-(*N*,*N*-dimethylamino)-2-deoxy-3,4-di-*O*-methyl glucitol / 1,2,4,5,6-penta-*O*-acetyl-3-mono-*O*-methyl glucitol with the ratio of 1 : 1 : 1 : 1 : 1 : 1 : 1. These results indicated that the sugar-aglycone linkage and the sequence of the sugar chains of compound **1** were as shown in Fig. 1.

Upon the alkaline hydrolysis of **1** with 1 N KOH in water, a prosapogenin **3** and an oligosaccharide **4** were obtained as the major products. By methylation analysis compound **3** furnished 1,5-di-*O*-acetyl-2,3,4 -tri-*O*-methyl xylitol / 1,2,5-tri-*O*-acetyl-3,4-di-*O*-methyl arabinitol / 1,5,6-tri-*O*-acetyl-2-(*N*,*N*-dimethylamino)-2-deoxy-3,4-di-*O*-methyl glucitol with the ratio of 1 : 1 : 1. On the basis of these data and the quasi-molecular ion peak at *m/z* = 977.1124 revealed by **3** in the MALDI-TOF-MS, the structure

of compound **3** was established as 15,16-dihydroxy-3-[[*O*-β-D-xylopyranosyl-(1→2)-*O*-α-L-arabinopyranosyl-(1→6)-2-(acetylamino)-2-deoxy-β-D-glucopyranosyl]oxy]-(3β,15α,16α)-olean-12-en-28-oic acid (Fig. 2). The sequence of the chain of compound **4** was established by methylation analysis which furnished 1,4-di-*O*-acetyl-2,3,5-tri-*O*-methyl apitol / 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl glucitol / 1,3,5-tri-*O*-acetyl-2,4-di-*O*-methyl xylitol / 1,2,4,6-tetra-*O*-acetyl-3,5-di-*O*-methyl glucitol. On the basis of these data and the quasi-molecular ion peak at *m/z* = 629.5223 revealed by **4** in the MALDI-TOF-MS, the structure of compound **4** was established as *O*-D-apio-β-D-furanosyl-(1→3)-*O*-β-D-xylopyranosyl-(1→2)-*O*-[*O*-β-D-glucopyranosyl-(1→4)]-D-glucose (Fig. 2). The reduction of **4** with sodium tetrahydroborate afforded compound **4a**. After methylation analysis **4a** gave 1,4-di-*O*-acetyl-2,3,5-tri-*O*-methyl apitol/ 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl glucitol/ 1,3,5-tri-*O*-acetyl-2,4-di-*O*-methyl xylitol/ 2,4-di-*O*-acetyl-1,3,5,6-tetra-*O*-methyl glucitol with the molar ratio of 1 : 1 : 1 : 1. This result indicated that **4a** is *O*-D-apio-β-D-furanosyl-(1→3)-*O*-β-D-xylopyranosyl-(1→2)-*O*-[*O*-β-D-glucopyranosyl-(1→4)]-D-glucitol. These results along with the ^{13}C NMR signals of **1** (Table 1) at δ = 63.34 (CH_2, Glc C-6), 93.08 (CH, Glc C-1), 167.20 (C=O, MT C-1), and 176.04 (C=O, C-28) confirmed that C-1 and C-6 of the internal glucose are linked with C-28 of the aglycone and C-1 of the monoterpenic acid, respectively.

On mild alkaline hydrolysis compound **1** afforded three compounds **5**, **5a** and **6**. By comparing UV, IR, ^{1}H NMR and MS spectral data of compounds **5** and **5a** with those reported in the literature [12, 19], **5** and **5a** were identified as (2*E*,6*R*)-6-hydroxy-2,6-dimethyl-2,7-octadienoic acid (Fig. 2) and its transformed product [12], respectively. The stereochemistry at C-6 of **5** was assigned to be *R* by comparing its optical activity, $[\alpha]_D^{25} = -24.5$ (c = 0.65, $CHCl_3$), with that reported in the literature [5], $[\alpha]_D^{25} = -24.6$ (c = 0.65, $CHCl_3$). After methylation analysis, compound **6** afforded 1,4-di-*O*-acetyl-2,3,5-tri-*O*-methyl apitol / 1,5-di-*O*-acetyl-2,3,4-tri-*O*-methyl xylitol / 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl glucitol / 1,3,5-tri-*O*-acetyl-2,4-di-*O*-methyl xylitol / 1,2,5-tri-*O*-acetyl-3,4-di-O-methyl arabinitol / 1,5,6-tri-*O*-acetyl-2-(*N*,*N*-dimethylamino)-2-deoxy-3,4-di-*O*-methyl glucitol / 1,2,4,5-tetra-*O*-acetyl-3,6-di-*O*-methyl glucitol with the ratio of 1 : 1 : 1 : 1 : 1 : 1 : 1. On the basis of this result together with the quasi-molecular ion peak at *m/z* = 1565.6347 revealed by **6** in the MALDI-TOFMS, compound **6** was established as 15,16-dihydroxy-3-[[*O*-β-D-xylopyranosyl-(1→2)-*O*-α-L-arabinopyranosyl-(1→6)-2-(acetylamino)-2-deoxy-β-D-glucopyranosyl]oxy]-(3β,15α,16α)-olean-12-en-28-oic acid *O*-D-apio-β-D-furanosyl-(1→3)-*O*-β-D-xylopyranosyl-(1→2)-*O*-[β-D-glucopyranosyl-(1→4)]-β-D-glucopyranosyl ester (Fig. 2).

Based on the above findings, the structure of polystachyasaponin (**1**) was established as 15,16-dihydroxy-3-[[*O*-β-D-xylopyranosyl-(1→2)-*O*-α-L-arabinopyranosyl-(1→6)-2-(acetylamino)-2-deoxy-β-D-glucopyranosyl]oxy]-(3β,15α,16α)-olean-12-en-28-oic acid *O*-D-apio-β-D-furanosyl-(1→3)-*O*-

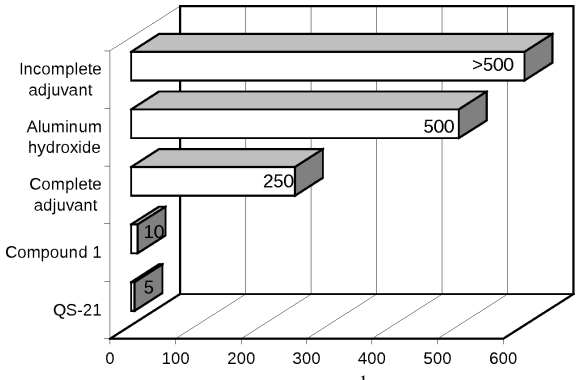

Fig. 3. Hemolytic activity (μg mL^{-1}) of compound **1** and adjuvants.

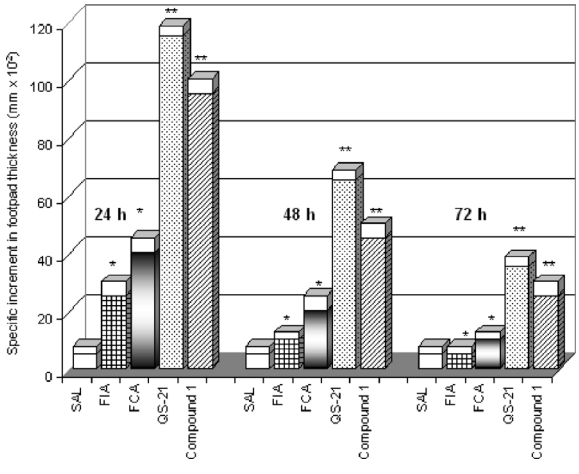

Fig. 4. Delayed-type hypersensitivity responses after two subcutaneous immunizations with 100 μg of ovalbumin and 100 μg of each adjuvant. Results are mean ± S.E.M. (n = 5); $^*p < 0.05$, $^{**}p < 0.01$ significantly different to the saline control. Student's t-test.

β-D-xylopyranosyl-(1→2)-*O*-[β-D-glucopyranosyl-(1→4)]-6-*O*-[(2*E*,6*R*)-6-hydroxy-2,6-dimethyl-1-oxo-2,7-octadienyl]-β-D-glucopyranosyl ester (Fig. 1).

Several important biological properties have been attributed to saponins. Because the original observation that certain saponins cause substantial enhancement of immune responses when given together with an antigen in a vaccine, their use as adjuvants received special attention [20 – 22]. Indeed, QS-21, a complex triterpenoid saponin isolated from *Quillaja saponaria* Molina [9] has been evaluated in a large number of vaccines in Phase I and Phase II human clinical trials and was recently synthesized [23]. In order to investigate the biological properties of polystachyasaponin (**1**), it was evaluated for hemolytic activity *in vitro* [17] and compared with adjuvants commonly used in animal and human experimental models (Fig. 3), showing a hemolytic potential similar to the purified saponin QS-21 obtained from commercial extracts of *Q. saponaria*. In addition to this, the immunoadjuvant property of compound **1** was evaluated against ovalbumin antigen (Fig. 4). The delayed-type hypersensitivity (DTH) reaction was measured as an *in vivo* assay of cellular immune response. In this study, mice immunized with ovalbumin conjugated with saponins showed remarkable responses greater than those when the antigen was combined with commercial adjuvants. This response developed rapidly after immunization and persisted at high levels for at least three days [18]. The structural similarities between polystachyasaponin (**1**) and QS-21 [24] may help to explain the potent adjuvant activity of these complex triterpenoid saponins [10]. The results obtained suggest the relevant adjuvant potential of the saponin **1** isolated from *Entada polystachya* in experimental vaccine formulations.

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