A new complex triterpenoid saponin, brevifoliasaponin, was isolated from leaves of *Calliandra brevifolia* Benth. (Leguminosae) by using chromatographic methods. Its structure was established as 3-[(O-α-L-arabinopyranosyl-(1 → 2)-O-α-L-arabinopyranosyl-(1 → 6)-2-(acetylamo)-2-deoxy-β-D-glucopyranosyl)oxy]-16-hydroxy-(3β,16α)-olean-12-en-28-oic acid O-β-D-xylopyranosyl-(1 → 3)-O-β-D-xylopyranosyl-(1 → 4)-O-β-D-glucopyranosyl-(1 → 3))-D-6-deoxy-α-L-mannopyranosyl-(1 → 2)-6-O-(2E,6S)-6-[(2-O-(2E,6S)-2,6-dimethyl-1-oxo-2,7-octadienyl)-β-D-xylopyranosyl]oxy]-2,6-dimethyl-1-oxo-2,7-octadienyl-β-D-glucopyranosyl ester. Its structural elucidation was performed using detailed analyses of 1H and 13C 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:** *Calliandra brevifolia*, Leguminosae, Brevifoliasaponin, Complex Triterpenoid Saponin, Ovalbumin, Adjuvant Activity

**Introduction**

Aqueous extracts of branches of *Calliandra anomala* (Kunth) Macbr. (Leguminosae) are used as an antimalarial and antifebrile agent in Mexico [1]. From this plant were reported fifteen complex triterpenoid saponins, called calliandra saponins (A–O) [2–5]. *Calliandra pulcherrima* Benth. and *Calliandra brevifolia* Benth. are related native species found in Tropical America. These evergreen plants are non invasive, but widespread ornamental plants often cultivated in gardens and parks [6]. In Brazil, the aqueous extract of the branches of *C. pulcherrima* is used as a remedy for malaria and leishmaniasis [7]. From this plant we reported a complex triterpenoid saponin, pulcherrimasaponin (CP05), with adjuvant activity for immunization in the murine model of visceral leishmaniasis [8]. Studies on the constituents of leaves of *Calliandra* species, in particular *C. brevifolia*, have been conducted in an attempt to isolate complex triterpenoid saponins. We report in this paper the structure of a new complex triterpenoid saponin, brevifoliasaponin (1) with adjuvant activity against ovalbumin antigen.

**Experimental Section**

**Plant material**

Fresh leaves of *Calliandra brevifolia* Benth. were obtained from the ornamental plant garden of the Federal University of Rio de Janeiro in September 2005, and a voucher specimen 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 × 25 m, 0.25 micron; J. & W. Scientific Incorporated, Folsom, CA, USA) DB-1. Mass spectra were taken on a VG Auto SpecQ spectrometer. NMR experiments were performed on a Bruker DRX-600 spectrometer at 300 K. NMR spectra
were acquired in [D$_2$]pyridine with tetramethylsilane ($\delta$ = 0.00 ppm) used as internal standard. Silica gel columns (230–400 mesh ASTM, Merck) and Sephadex LH-20 (Pharmacia) were used for column chromatography. TLC was performed on silica gel plates (Kieselgel 60F$_{254}$, Merck) using the following solvent systems: (A) CHCl$_3$/MeOH/H$_2$O (55 : 45 : 5) for compound 1, (B) CHCl$_3$/MeOH/H$_2$O (65 : 35 : 10, lower phase) for compounds 3–8, (C) n-BuOH/Me$_2$CO/H$_2$O (4 : 5 : 1), and (D) n-BuOH/pyridine/H$_2$O (4 : 3 : 3) for monosaccharides. The spray reagents were orcinol/H$_2$SO$_4$ for compounds 1, 3–8 and monosaccharides, and CeSO$_4$ for compound 2.

**Extraction and isolation of compound 1**

Fresh leaves (1.3 kg) were extracted with MeOH (4.0 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 (19.3 g), which was dissolved in MeOH (100 mL) and roughly chromatographed (1.93 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 (1.4 g). Further purification by chromatography on a silica gel column (2.8 × 90 cm) eluted with CHCl$_3$/MeOH/H$_2$O (55 : 45 : 5, v/v/v) afforded a TLC homogeneous compound 1 (319 mg, $R_f$ = 0.36) which gave a dark blue color with orcinol/H$_2$SO$_4$.

**Brevifoliasaponin (1)**

Amorphous solid. – M. p. 245 – 248 °C (dec.). – UV/Vis (MeOH): $\lambda_{max}$ (lg $\varepsilon_{max}$) = 220 nm (3.87). – [$\alpha$]$_D^{20}$ = –43 (c = 0.1, MeOH). – IR (KBr): v = 3242 (O–H), 2931 (C–H), 1707 (C=O), 1647 (C=O), 1560, 1449, 1415, 1337, 1311, 1281, 1259, 1204, 1164, 1077, 1044, 897, 865, 814 cm$^{-1}$. – $^1$H NMR (300 MHz, [D$_2$]pyridine, 25 °C, TMS): $\delta$ = 0.81 (s, 3 H, 25-C$_{Me}$), 0.85 (s, 3 H, 29-C$_{Me}$), 0.87 (s, 3 H, 24-C$_{Me}$), 0.95 (s, 3 H, 26-C$_{Me}$), 1.09 (s, 3 H, 23-C$_{Me}$), 1.38 (s, 3 H, 10'-C$_{Me}$), 1.43 × 2 (s, 6 H, 10'-C$_{Me}$ and 10''-C$_{Me}$), 1.58 (d, $J = 7.3$ Hz, 3 H, Rha-6-CH$_2$), 1.66 (s, 3 H, 27-C$_{Me}$), 1.78 (s, 3 H, 9'-C$_{Me}$), 1.81 × 2 (s, 6 H, 9'-C$_{Me}$ and 9''-C$_{Me}$), 2.08 (s, 3 H, NHCO$_{Me}$), 4.69 (d, $J = 7.8$ Hz, 1 H, Xyl$_{1}^{1'''}$-H), 4.75 (d, $J = 7.9$ Hz, 1 H, Xyl$_{1}^{1''''}$-H), 4.83 (d, $J = 7.9$ Hz, 1 H, Xyl$_{1}^{1'''}$-H), 4.85 (d, $J = 7.2$ Hz, 1 H, Ara$_{1}$-H), 4.94 (d, $J = 8.3$ Hz, 1 H, GlcNAc 1-H), 4.96 (d, $J = 4.7$ Hz, 1 H, Ara 1-H), 5.02 (d, $J = 7.2$ Hz, 1 H, Xyl$_{1}^{1'''}$-H), 5.21 (d, $J = 7.8$ Hz, 1 H, Glc 1-H), 5.31 (d, $J = 7.8$ Hz, 1 H, Xyl 1-H), 5.73 (br s, 1 H, Rha 1-H), 5.87 (d, $J = 7.2$ Hz, 1 H, Glc 1-H), – $^{13}$C NMR data given in Table 1. – HRMS ([+]+) = 2592.8138; calcd. 2592.8177 for C$_{123}$H$_{189}$NO$_{57}$Na, [M+Na]$^+$.

**Acid hydrolysis of 1**

A solution of saponin 1 (20 mg) in 2 M HCl/H$_2$O (10 mL) was heated in a sealed tube for 6 h at 100 °C. The reaction mixture was diluted with H$_2$O (20 mL) and extracted with diethyl ether (25 mL). The organic layer was concentrated in vacuo. The residue was crystallized from methanol to give colorless needles [2; 3.1 mg; m. p. 309 °C; [$\alpha$]$_D^{20} = +52$ (c = 0.1, MeOH)]. The aqueous solution was passed through an Amberlite IRA-410 column. The eluate was concentrated in vacuo to give a residue (13.2 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 (C). After spraying with orcinol/H$_2$SO$_4$, rhamnose gave a green spot at $R_f$ = 0.75, xylose gave a blue spot at $R_f$ = 0.57, arabinose gave a purple spot at $R_f$ = 0.48, 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 (D) 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$_2$O (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 (93 mg). The residue was chromatographed on Sephadex LH-20 with MeOH and afforded three fractions: an amorphous powder [3; 28 mg; m. p. 209 – 215 °C (dec.); [$\alpha$]$_D^{20} = +8.8$ (c = 0.3, MeOH)], another amorphous powder [4; 23 mg; m. p. 165 – 171 °C (dec.); [$\alpha$]$_D^{20} = −28.2$ (c = 0.5, H$_2$O)], and a colorless syrup [5; 24 mg; [$\alpha$]$_D^{20} = −25.6$ (c = 0.73, MeOH)].

**Mild alkaline hydrolysis of compound 1**

A solution of compound 1 (100 mg) in 5 % K$_2$CO$_3$/EtOH (1 : 1 v/v; 20 mL) was refluxed for 40 min. The reaction mixture was neutralized with Dowex 50W × 8, concentrated to about half the original volume and extracted with n-BuOH. The n-BuOH extract was evaporated to dryness, and the residue (77 mg) was purified by chromatography on silica gel using CHCl$_3$/MeOH/H$_2$O (13 : 7 : 2 v/v/v) as eluent and yielded four fractions: a colorless syrup [5; 9 mg: [$\alpha$]$_D^{20} = −25.6$ (c = 0.73, MeOH)], an amorphous powder [6; 6 mg; M. p. 227 – 235 °C (dec.); [$\alpha$]$_D^{20} = −14.3$ (c = 0.4, pyridine)], a second amorphous powder [7; 15 mg; m. p. 204 – 210 °C (dec.); [$\alpha$]$_D^{20} = −22.1$ (c = 0.7, MeOH)], and a third amorphous powder [8; 27 mg; m. p. 193 – 197 °C (dec.); [$\alpha$]$_D^{20} = +4.4$ (c = 0.3, MeOH)].

**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 [9]. The configurations of the glycosides were established by capillary GC of their trimethylsilylated (–)-2-butylglycosides [10].

Methylation analysis

Compounds 1, 3–8 and 4a were methylated with dimethyl sulfoxide/lithium methylsulfinyl carbanion/methyl iodide [11]. The methyl ethers were obtained after hydrolysis (4 N trifluoroacetic acid, 2 h, 100 °C) and analyzed as partial alditol acetates by GC-MS [12].

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 of compound 1, aluminum hydroxide (Al(OH)₃), purified Quillaja saponin (QS-21), and 5–500 µL/mL 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 percent developed by the saline control were subtracted from all groups. The adjuvant concentration inducing 50 % of the maximal hemolysis was considered the HD₅₀ (graphical interpolation). Experiments included triplicates at each concentration [13].

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 controls. The ovalbumin specific responses were obtained by subtracting the response to OVA challenge in unimmunized control mice [14].

Result and Discussion

Compound 1 was obtained as an amorphous solid and gave a positive Liebermann-Burchard test for a triterpenoidal saponin. It revealed a quasi-molecular ion peak at m/z 2592.8138 [M+Na]⁺ in the MALDI-TOFMS. In the ¹³C NMR spectra, the 121 carbon signals observed, there are fifteen methyl groups, twenty-eight methylene groups (ten of which were oxygenated), sixty methine groups (fifty of which were oxygenated) and eighteen quaternary carbon atoms (eight 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 ¹³C NMR spectral data (Table 1), compound 1 was assumed to be a saponin with the molecular formula of C₁₂₁H₁₈₉NO₅₇.

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 1647 cm⁻¹ in the IR spectrum. The IR spectrum showed absorptions at 3422 and 1707 cm⁻¹ attributed to the hydroxyl and ester-carbonyl groups, respectively. The ¹H NMR spectrum showed signals ascribable to fourteen tertiary methyl groups at δ = 0.81 (s, 25-CMe), 0.85 (s, 29-CMe), 0.87 (s, 24-CMe), 0.95 (s, 26-CMe), 0.99 (s, 30-CMe), 1.08 (s, 23-CMe), 1.38 (s, 10'-CMe), 1.43 × 2 (s, 10''-CMe and 10''-CMe), 1.66 (s, 27-CMe), 1.78 (s, 9'-CMe), 1.81 × 2 (s, 9''-CMe and 9''-CMe), 2.08 (s, COMe) and one secondary methyl group at δ = 1.58 (d, J = 7.3 Hz, Rha 6-CHMe). The ¹H NMR spectrum of 1 displayed signals for eleven anomeric protons at δ = 4.69 (d, J = 7.8 Hz, 1 H), 4.75 (d, J = 7.9 Hz, 1 H), 4.83 (d, J = 7.9 Hz, 1 H), 4.85 (d, J = 7.2 Hz, 1 H), 4.94 (d, J = 8.3 Hz, 1 H), 4.96 (d, J = 4.7 Hz, 1 H), 5.02 (d, J = 7.2 Hz, 1 H), 5.21 (d, J = 7.7 Hz, 1 H), 5.31 (d, J = 7.8 Hz, 1 H), 5.73 (br s, 1 H), and 5.87 (d, J = 7.2 Hz, 1 H) which gave correlations in the HSQC spectrum with ¹³C NMR signals (Table 1) at δ = 99.71, 97.47, 98.80, 105.73, 103.96, 101.78, 105.14, 104.35, 103.96, 101.47 and 94.50, respectively. Evaluation of chemical shifts and spin-spin couplings allowed the identification of five β-xylopyranosyl units, two α-arabinopyranosyl units, two β-gluopyranosyl units, one 2-(acetylamo)-1-deoxy-β-glucopyranosyl unit, and one α-rhamnopyranosyl unit. The ¹³C NMR spectrum of 1 (Table 1) showed one ester-carbonyl carbon at δ = 175.30 (C-28), one amide-carbonyl carbon at δ = 170.81 (COMe), three α, β-unsaturated ester-carbonyl carbons at δ = 166.78 (C-1″), 166.85 (C-1″) and 167.49 (C-1′), one pair of the olefinic carbon of the aglycone moiety at δ = 122.12 (CH, C-12) and 143.85 (C, C-13), three pairs of trisubstituted olefinic carbons at δ = 127.55 (C, C-2′), 142.68 (CH, C-3′), 127.69 × 2 (C, C-2″ and C-2″′) and 142.91 × 2 (CH, C-7″′ and C-7″′′) and 114.57 × 2 (CH₂, C-8″ and C-8″′), and one sugar-linked methine carbon at δ = 88.68 (C-3). The upfield signal observed at δ = 175.30 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 couplings between 2-amino-2-deoxy-glucose-H-
1 at δ = 4.94 and aglycone-C-3 at δ = 88.68. The above facts suggest that compound 1 should be a 3,28-O-bis(desmoside) having eleven monosaccharides and three monoterpenic acids.

On acid hydrolysis, compound 1 gave a sapogenin, 2-amino-2-deoxy-glucose, rhamnose, arabinose, glucose, and xylose. The structure of the sapogenin, compound 2, was established as 3,16-dihydroxy-(3β,16α)-olean-12-en-28-oic acid (echinocystic acid; Fig. 2) [15] by comparison with an authentic sample through m. p., [α]D, IR, 1H NMR and EIMS. The molar carbohydrate composition of 1 indicated the presence of one 2-(acetylamino)-2-deoxy-glucose, one rhamnose, two arabinoses, two glucoses, and five xyloses [9]. Their absolute configurations were determined by GC of their trimethylsilylated (−)-2-butylglycosides [10].

The sequence of the sugar chain of 1 was confirmed by methylation analysis [11] which furnished 1,5-di-O-acetyl-2,3,4-tri-O-methyl xylitol / 1,5-di-O-acetyl-2,3,4-tri-O-methyl arabinitol / 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl glucitol / 1,2,5-tri-O-acetyl-3,4-di-O-methyl xylitol / 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 / 1,3,4,5-tetra-O-acetyl-2-O-methyl rhamnitol / 1,2,5,6-tetra-O-acetyl-3,4-di-O-methyl glucitol with the ratio of 2:1:1:1:1:1:1:1:1. These results indicated that the sugar-aglycone linkage and the sequence of the sugar chains of compound 1 was 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 arabinitol / 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, m. p. and [α]D, the structure of compound 3 was established as 3-[[O-α-L-arabinopyranosyl-(1 → 2)-O-α-L-arabinopyranosyl-(1 → 6)-2-(acetylamino)-2-deoxy-β-D-glucopyranosyl] oxy]-16-hydroxy-(3β, 16α)-olean-12-en-28-oic acid (Fig. 2) [2, 3]. The sequence of the chain of compound 4 was established by methylation analysis which furnished 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 /
Fig. 2. The hydrolysis products 2–8 of compound 1.

1,2,5-tri-O-acetyl-3,4,6-tri-O-methyl glucitol / 1,3,4,5-tetra-O-acetyl-2-O-methyl rhamnitol with the ratio of 1:1:1:1:1:1. This result proves that 4 is the O-β-D-glucopyranosyl-(1→3)-O-[O-β-D-xylopyranosyl-(1→3)-β-D-xylopyranosyl-(1→4)]-O-6-deoxy-α-L-mannopyranosyl-(1→2)-D-glucose. The reduction of 4 with sodium tetrahydroborate afforded compound 4a. After methylation analysis 4a gave 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 / 2-O-acetyl-1,3,4,5,6-penta-O-methyl glucitol / 1,3,4,5-tetra-O-acetyl-2-O-methyl rhamnitol with the ratio of 1:1:1:1:1:1. This result indicated that 4a is O-β-D-glucopyranosyl-(1→3)-O-[O-β-D-xylopyranosyl-(1→3)-β-D-xylopyranosyl-(1→4)]-O-6-deoxy-α-L-mannopyranosyl-(1→2)-D-glucose. These results along with the $^{13}$C NMR signals of 1 (Table 1) at $\delta = 64.18$ (CH$_2$, Glc C-6), 94.50 (CH, Glc C-1), 167.49 (C=O, MT C-1'), and 175.30 (C=O, C-28) confirmed that C-1 and C-6 of the inter-
nal glucose are linked with C-28 of the aglycone and C-1’ of the monoterpenic acid, respectively.

Compound 5 was obtained from 1 by alkaline hydrolysis with 1 N KOH in water and 5 % K₂CO₃ in ethanol. By each reaction 5 was obtained as a colorless syrup. The identity was established by comparison of the IR, ¹H NMR and MS spectral data with those described in the literature [2, 16]. Compound 5 showed an optical rotation of [α]D²⁵ = -25.6 (c = 0.73, MeOH) [16] and was identified as 6-(β-D-xylopyranosyloxy)-2,6-dimethyl-[5-E]-2,7-octadienoic acid [2, 3, 16]. Compound 5 was methylated by Parente’s method [11] and afforded a syrup (5a) with [α]D⁰ = -33 (c = 0.7, CHCl₃), confirming that 5 is a 6α isomer. The methylated compound 5a was identified as [5S-(E)]-2,7-octadienoic acid 2,6-dimethyl-6-[(2,3,4-tri-O-methyl-β-D-xylopyranosyloxy]-methyl ester [2].

On mild alkaline hydrolysis compound 1 afforded compounds 6–8 together with 5. After methylation analysis, compound 6 afforded 1,5-di-O-acetyl-2,3,4-tri-O-methyl xylitol / 1,5-di-O-acetyl-2,3,4,tri-O-methyl arabinitol / 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 / 1,3,4,5-tetra-O-acetyl-2,6-dimethyl rhamnitol / 1,2,5,6-tetra-O-acetyl-3,4-di-O-methyl glucitol with the ratio of 1:1:1:1:1:1:1. On the basis of this result together with the m. p. and [α]D, compound 6 was established as 3-[O-α-L-arabinopyranosyl-(1 → 2)-O-α-L-arabinopyranosyl-(1 → 6)-2-(acetylamino)-2-deoxy-β-D-glucopyranosyl oxy]-16-hydroxy-(3β, 16α)-olean-12-en-28-oic acid O-β-D-glucopyranosyl-(1 → 3)-O-[β-D-xylopyranosyl-(1 → 3)]-β-D-xylopyranosyl-(1 → 4)]-O-6-deoxy-α-L-mannopyranosyl-(1 → 2)-β-D-glucopyranosyl ester [2, 3]. After methylation analysis, compound 7 furnished the same partial alditol acetates of 6 together with one 1,5-di-O-acetyl-2,3,4-tri-O-methyl xylitol. Therefore, the structure of 7 was characterized as 3-[O-α-L-arabinopyranosyl-(1 → 2)-O-α-L-arabinopyranosyl-(1 → 6)-2-(acetylamino)-2-deoxy-β-D-glucopyranosyl oxy]-16-hydroxy-(3β, 16α)-28(2E,6S)-olean-12-en-28-oic acid O-β-D-glucopyranosyl-(1 → 3)-O-[β-D-xylopyranosyl-(1 → 3)]-β-D-xylopyranosyl-(1 → 4)]-O-6-deoxy-α-L-mannopyranosyl-(1 → 2)-6-O-[2,6-dimethyl-1-oxo-6-(β-D-xylopyranosyloxy)-2,7-octadienyl]-β-D-xylopyranosyl ester [2, 3]. The m. p. and [α]D of compounds 7 and 8 were identical with the m. p. and [α]D of the calliandra saponin A [2] and calliandra saponin C [3], respectively.

Based on the above findings, the structure of brevifolisaponin (1) was established as 3-[O-α-L-arabinopyranosyl-(1 → 2)-O-α-L-arabinopyranosyl-(1 → 6)-2-(acetylamino)-2-deoxy-β-D-glucopyranosyl oxy]-16-hydroxy-(3β, 16α)-olean-12-en-28-oic acid O-β-D-xylopyranosyl-(1 → 3)-O-β-D-xylopyranosyl-(1 → 4)]-O-6-deoxy-α-L-mannopyranosyl-(1 → 2)-6-O-[2,6-dimethyl-1-oxo-6-(β-D-xylopyranosyloxy)-2,7-octadienyl]-β-D-xylopyranosyl ester [2].

Several important biological properties have been attributed to saponins. Since 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 [17–19]. Indeed, QS-21, a complex triterpenoid saponin isolated from *Quillaja saponaria* Molina [20], has been evaluated in a large number of vaccines in Phase I and Phase II human clinical trials and was recently synthesized [21]. In order to investigate the biological properties of brevifoliasaponin (I), it was evaluated for hemolytic activity *in vitro* [13] 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). 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 [14]. The structural similarities between brevifoliasaponin (I) and QS-21 [22] may help to explain the potent adjuvant activity of these complex triterpenoid saponins [8]. The results obtained suggest a relevant adjuvant potential of the saponin I isolated from *Calliandra brevifolia* in experimental vaccine formulations.

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