Phytochemistry Letters xxx (2012) xxx–xxx

Contents lists available at SciVerse ScienceDirect

Phytochemistry Letters

journal homepage: www.elsevier.com/locate/phytol

A new complex triterpenoid saponin from *Samanea saman* with haemolytic activity and adjuvant effect

Antony de Paula Barbosa, Bernadete Pereira da Silva, José Paz Parente*

Laboratório de Química de Plantas Medicinais, Núcleo de Pesquisas de Produtos Naturais, Centro de Ciências da Saúde, Universidade Federal do Rio de Janeiro, P.O. Box 68045, CEP 21941-971 Rio de Janeiro, Brazil

ARTICLE INFO

Article history:
Received 16 March 2012
Received in revised form 15 June 2012
Accepted 20 June 2012
Available online xxx

Keywords:
Samanea saman
Leguminosae
Complex triterpenoid saponin
Haemolytic activity
Adjuvant effect

ABSTRACT

A new complex triterpenoid saponin was isolated from the stem bark of *Samanea saman* by using chromatographic methods. Its structure was established as 3-[(2-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy]-2,23-dihydroxy-(2β,3β,4α)-olean-12-en-28-oic acid *O*-β-D-glucopyranosyl-(1 → 3)-*O*-[*O*-β-D-glucopyranosyl-(1 → 4)]-*O*-6-deoxy-α-L-mannopyranosyl-(1 → 2)-6-*O*-[4-*O*-[(2*E*,6*S*)-2,6-dimethyl-1-oxo-2,7-octadienyl]-6-deoxy-α-L-mannopyranosyl)oxy]-β-D-glucopyranosyl ester (**1**). Structural elucidation was performed using detailed analyses of ^{1}H and ^{13}C NMR spectra including 2D NMR spectroscopic techniques and chemical conversions. The haemolytic 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* models.

1. Introduction

Samanea saman (Jacq.) Merr. (Leguminosae), commonly known as raintree, is native to tropical America, extending from southern Mexico into northern South America. The raintree was long ago introduced to many other tropical countries as a street and shade tree. The pods ripen during the dry period and feed cattle and hogs. The leaves are used as a livestock forage supplement for cattle, sheep and goats. *S. saman* has been recognized as an important leguminous tree species for animal feeding (Durr, 2001).

S. saman has been used in traditional medicine as a remedy for the treatment of different diseases. In Venezuela, the root decoction is used in hot baths for stomach cancer. The leaf infusion is used as a laxative. In the West Indies, seeds are chewed for sore throat. The alcoholic extract of the leaves inhibits *Mycobacterium tuberculosis*. In Colombia, the fruit decoction is used as a sedative (ILDIS, 2005). Recently, the antimicrobial activity of the aqueous extract of this plant was investigated and showed inhibitory activity against *Staphylococcus aureus*, *Candida albicans* and *Escherichia coli*. Phytochemical screening of the plant revealed the presence of tannins, flavonoids, saponins, steroids, cardiac glycosides and terpenoids (Prasad et al., 2008).

According to the literature, complex triterpenoid saponins are shown to possess several physiological properties depending on their chemical structures, such as haemolytic activity and capacity for alteration of membrane permeability (Oda et al., 2000). Additionally, these compounds have been reported to possess therapeutic potential for immune system modulation through different mechanisms (Lacaille-Dubois and Wagner, 1996). As part of our ongoing efforts in discovering potentially bioactive componds from natural sources, we describe the structural elucidation and evaluation of the haemolytic activity and immunological adjuvant effect of a new complex triterpenoid saponin isolated from the bark of *S. saman*.

2. Results and discussion

The MeOH extract of *S. saman* was suspended in H_2O and partitioned with n-BuOH. The n-BuOH extract was subjected to chromatographic purification steps to afford compound **1**, a colorless amorphous powder, which was positive to Liebermann–Burchard test. It revealed a quasi-molecular weight ion peak at *m*/*z* 1780, 9013 $[M+Na]^+$ in the positive-ion mode MALDI-TOFMS. In the ^{13}C NMR spectrum, eighty-two carbon signals observed belong to ten methyl groups, eighteen methylene groups (six of which were oxygenated), forty-three methine groups (twenty-nine of which were oxygenated) and eleven quaternary carbon atoms (three of which were oxygenated). The number of hydrogen atoms attached to each individual carbon atom was calculated by DEPT-45, DEPT-90 and DEPT-135 spectra. On the basis of the above mentioned MS and ^{13}C NMR spectral data (Table 1), compound **1** was assumed to be a triterpenoid saponin

* Corresponding author. Tel.: +55 21 2562 6791; fax: +55 21 2562 6513.
E-mail address: parente@pq.cnpq.br (J.P. Parente).

1874-3900/$ – see front matter © 2012 Published by Elsevier B.V. on behalf of Phytochemical Society of Europe.
http://dx.doi.org/10.1016/j.phytol.2012.06.009

Table 1
^{13}C NMR data of compound **1** (75 MHz, pyridine-d_5).

Position	δ C	DEPT[a]	Position		δ C	DEPT[a]
1	44.5	CH_2	β-D-GlcI	1	103.0	CH
2	70.0	CH	2	83.6	CH	
3	83.2	CH	3	78.1	CH	
4	42.2	C	4	71.1	CH	
5	47.3	CH	5	78.0	CH	
6	18.9	CH_2	6	62.9	CH_2	
7	33.7	CH_2	β-D-GlcII	1	105.8	CH
8	39.5	C	2	76.7	CH	
9	48.3	CH	3	78.4	CH	
10	37.2	C	4	71.4	CH	
11	23.3	CH_2	5	78.4	CH	
12	123.4	CH	6	62.9	CH_2	
13	143.0	C	β-D-GlcIII	1	95.6	CH
14	41.8	C	2	76.8	CH	
15	27.1	CH_2	3	79.1	CH	
16	23.3	CH_2	4	71.4	CH	
17	47.4	C	5	77.3	CH	
18	41.1	CH	6	66.9	CH_2	
19	41.9	CH_2	β-D-GlcIV	1	105.7	CH
20	28.8	C	2	75.7	CH	
21	32.4	CH_2	3	78.6	CH	
22	30.3	CH_2	4	71.4	CH	
23	66.8	CH_2	5	78.4	CH	
24	14.6	CH_3	6	62.2	CH_2	
25	16.1	CH_3	β-D-GlcV	1	106.6	CH
26	17.2	CH_3	2	76.4	CH	
27	26.0	CH_3	3	78.6	CH	
28	174.7	C	4	71.4	CH	
29	29.5	CH_3	5	78.4	CH	
30	23.9	CH_3	6	62.9	CH_2	
MT	α-L-RhaI	1	101.9	CH		
1	168.0	C	2	70.7	CH	
2	128.6	C	3	82.2	CH	
3	144.1	CH	4	79.1	CH	
4	23.9	CH_2	5	69.2	CH	
5	41.8	CH_2	6	18.9	CH_3	
6	72.4	C	α-L-RhaII	1	99.5	CH
7	146.9	CH	2	72.9	CH	
8	112.0	CH_2	3	76.9	CH	
9	12.9	CH_3	4	75.8	CH	
10	28.8	CH_3	5	67.6	CH	
6	18.1	CH_3				

[a] Multiplicities were assigned from DEPT-45, DEPT-90 and DEPT-135 spectra.

with the molecular formula $C_{82}H_{132}O_{40}$, bearing one monoterpene and seven monosaccharide moieties. In addition to this, the IR spectrum showed an absorption at 1643 cm^{-1} which is typical of an α,β-unsaturated carbonyl group, supported by UV absorption at 220 nm.

On acid hydrolysis, compound **1** gave a sapogenin **1a**, glucose and rhamnose. The structure of **1a** (Fig. 1) was established as 2,3,23-trihydroxy-(2β,3β,4α)-olean-12-en-28-oic acid (bayogenin) by comparing its physical properties ($[\alpha]_D$ and m.p.), and ^{1}H and ^{13}C NMR spectra with those of known bayogenin (Eade et al., 1963; Fujioka et al., 1989; Mahato and Kundu, 1994; Tan et al., 1999). Analysis of the sugars by GC/MS indicated the presence of rhamnose and glucose in a ratio of 2:5, respectively (Kamerling et al., 1975). Their absolute configurations were determined by GC of their trimethylsilylated (−)-2-butylglycosides (Gerwig et al., 1978). D-Glucose and L-rhamnose were detected. The ^{1}H NMR spectrum of compound **1** displayed seven anomeric hydrogen atoms at δ 5.07 (d, J = 8.0 Hz), 5.20 (d, J = 8.0 Hz), 5.33 (d, J = 8.0 Hz), 6.09 (brs), 6.11 (d, J = 7.6 Hz), 6.26 (d, J = 8.4 Hz), 6.37 (brs) which gave correlations in the HSQC spectrum with seven anomeric carbon atoms at δ 103.0, 106.6, 105.8, 101.9, 95.6, 105.7, 99.5, respectively. Evaluation of chemical shifts and spin–spin couplings allowed the identification of two rhamnopyranosyl units (α-RhaI and α-RhaII) and five β-glucopyranosyl units (β-GlcI, β-GlcII, β-GlcIII, β-GlcIV and β-GlcV). The attachments of the sugar moieties to the aglycone moiety were established by ^{1}H–^{1}H COSY and HMBC experiments. The COSY spectrum was useful to establish couplings and determine the connectivity information in carbohydrate sequences. The HMBC spectrum displayed long range couplings between GlcI-H-1 at δ 5.07 and triterpenoid-C-3 at δ 83.2, between GlcIII-H-1 at δ 6.11 and triterpenoid-C-28 at δ 174.4, which accounted for two saccharide part linkages to the C-3 β-OH and C-28 COOH groups of bayogenin. In addition to this, long range couplings were observed between GlcII-H-1 at δ 5.33 and GlcI-C-2 at δ 83.6, between GlcIV-H-1 at δ 6.26 and RhaI-C-3 at δ 82.2, between GlcV-H-1 at δ 5.20 and RhaI-C-4 at δ 79.1, between RhaI-H-1 at δ 6.09 and GlcIII-C-2 at δ 76.8, between RhaII-H-1 at δ 6.37 and GlcIII-C-6 at δ 66.9, between RhaII-H-4 at δ 5.86 and monoterpenoid-C-1 at δ 168.0, which accounted for the elucidation of compound **1** (Fig. 1). The NMR signals of compound 1 were assigned by 2D NMR experiments including COSY, HSQC and HMBC and by comparing the NMR data of **1** (Table 1 and Exper. Part) with those reported in the literature (Zhang et al., 1995a,b; Castro et al., 1997; Zhang et al., 1999a,b,c; Tava et al., 2005).

On mild alkaline hydrolysis compound **1** afforded compound **1b**. By comparing UV, IR, ^{1}H and ^{13}C NMR and MS spectral data of compound **1b** with those reported in the literature (Okada et al., 1980; Zhang et al., 1999a), **1b** was identified as (2E,6S)-6-hydroxy-2,6-dimethyl-2,7-octadienoic acid (Fig. 1). The stereochemistry at

Please cite this article in press as: Barbosa, A.P., et al., A new complex triterpenoid saponin from *Samanea saman* with haemolytic activity and adjuvant effect. Phytochem. Lett. (2012), http://dx.doi.org/10.1016/j.phytol.2012.06.009

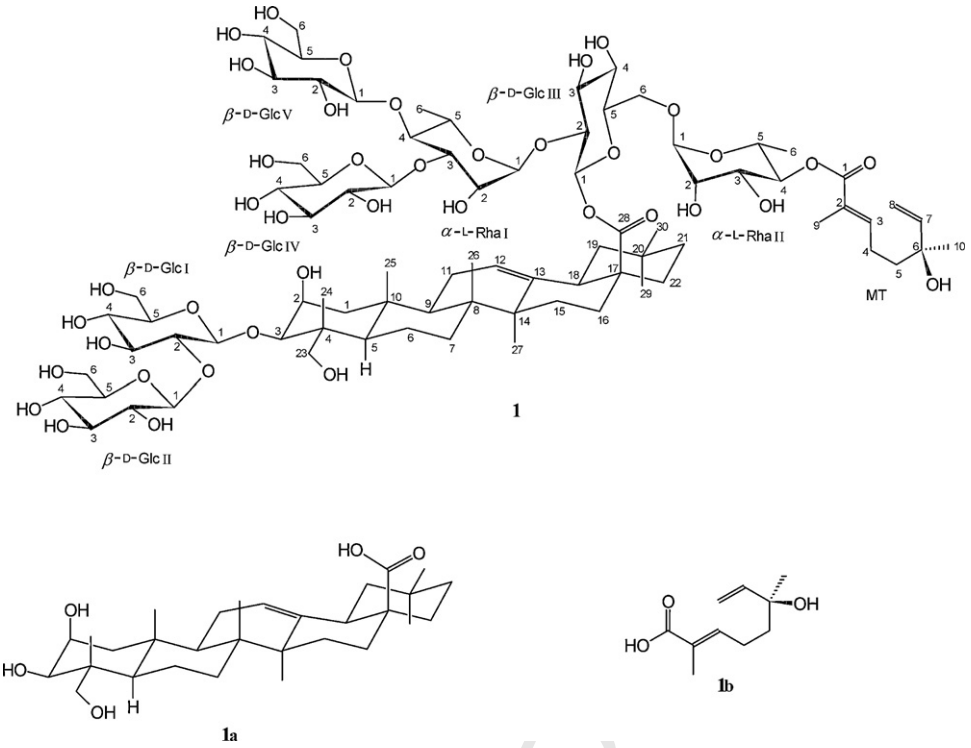

Fig. 1. Chemical structures of compounds **1**, **1a** and **1b**.

C-6 of **1b** was assigned to be *S* by comparing its optical activity, $[\alpha]_D^{25}$ +18 (c 0.65, $CHCl_3$) with that reported in the literature (Okada et al., 1980).

The sequence of sugar chain of compound **1** was confirmed by methylation analysis (Parente et al., 1985) which furnished 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl glucitol, 1,4,5-tri-O-acetyl-2,3-di-O-methyl rhamnitol, 1,2,5-tri-O-acetyl-3,4,6-tri-O-methyl glucitol, 1,3,4,5-tetra-O-acetyl-2-mono-O-methyl rhamnitol and 1,2,5,6-tetra-O-acetyl-3,4-di-O-methyl glucitol. Consequently, on the basis of the results described above, the structure of compound 1 was established as 3-[(2-O-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy]-2,23-dihydroxy-(2β,3β,4α)-olean-12-en-28-oic acid O-β-D-glucopyranosyl-(1 → 3)-O-[O-β-D-glucopyranosyl-(1 → 4)]-O-6-deoxy-α-L-mannopyranosyl-(1 → 2)-6-O-[4-O-[(2E,6S)-2,6-dimethyl-1-oxo-2,7-octadienyl]-6-deoxy-α-L-mannopyranosyl)oxy]-β-D-glucopyranosyl ester (**1**; Fig. 1).

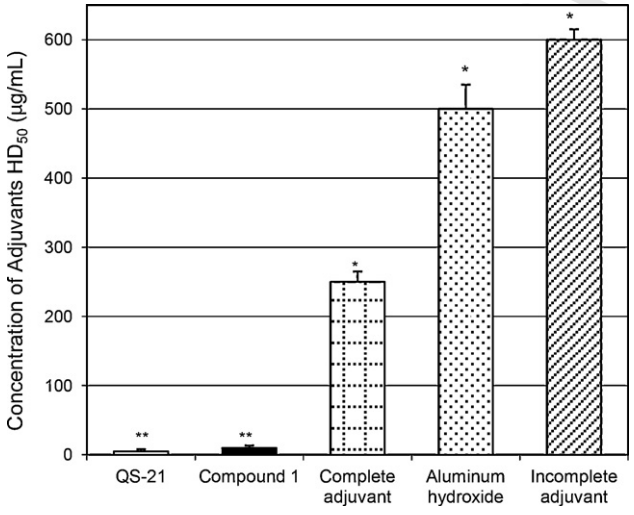

Fig. 2. Haemolytic activity ((g/mL) of compound **1** and commercial adjuvants commonly used in animal and human experimental models. The adjuvant concentration inducing 50% of the maximal haemolysis was considered the median haemolytic dose (HD_{50}; graphical interpolation). Each experiment included triplicates at each concentration. Results are mean ± S.E.M. ($n = 10$); $^{*}p < 0.05$, $^{**}p < 0.01$ significantly different from the control.

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 (Sun et al., 2009). Indeed, QS-21, a complex triterpenoid saponin isolated from the bark of the South American tree *Quillaja saponaria* shows an outstanding and specific adjuvant potential and was recently synthesized (Kim et al., 2006). It has been evaluated in a large number of vaccines in Phase I and Phase II human clinical trials. These vaccines include several cancer immunotherapeutics, formulations with recombinant glycoprotein vaccine against HIV-1 and with synthetic *Plasmodium falciparum* peptides against malaria (Fig. 2). It has been tested in more than 3000 individuals and appears to be a promising adjuvant for human vaccines (Waite et al., 2001). In order to investigate the biological properties of compound 1, it was evaluated for haemolytic activity *in vitro* and compared with adjuvants commonly used in animal and human experimental models (Fig. 3), showing a powerful haemolytic potential similar to the purified saponin QS-21 obtained from commercial extracts of *Quillaja saponaria* (Santos et al., 1997).

In addition, the immunological property of compound **1** was investigated and its adjuvant potential on the cellular immune response against ovalbumin (OVA) antigen was evaluated (Fig. 4). Delayed type hypersensitivity (DTH) reaction was measured as an *in vivo* assay of cellular immune response. This type of immunity is typically elicited by soluble protein antigens that are introduced with adjuvants. In the classical experimental model, the animal is first immunized by the administration of the antigen conjugated with adjuvants, which is called sensitization. After, in the elicitation stage, the animal is challenged subcutaneously with the same antigen and the subsequent reaction is analyzed. In this study, mice immunized with OVA conjugated with compound 1 showed response greater than those when the antigen was

Please cite this article in press as: Barbosa, A.P., et al., A new complex triterpenoid saponin from *Samanea saman* with haemolytic activity and adjuvant effect. Phytochem. Lett. (2012), http://dx.doi.org/10.1016/j.phytol.2012.06.009

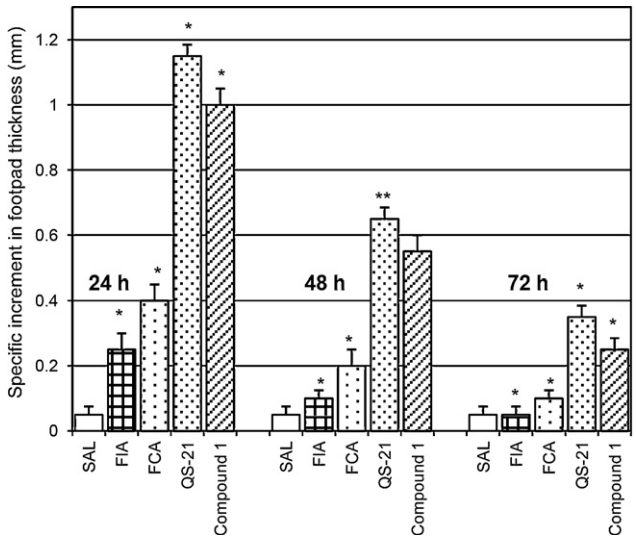

Fig. 3. Immunological adjuvant activity of compound **1** and commercial adjuvants on the cellular immune response against ovalbumin antigen. Delayed type hypersensitivity responses after two subcutaneous immunizations with 100 (g of ovalbumin and 100 (g of each adjuvant. Results are mean ± S.E.M. ($n = 5$); $^{*}p < 0.05$, $^{**}p < 0.01$ significantly different from the saline control. Student's *t*-test. *Abbreviations*: SAL, saline solution; FCA, Freund's complete adjuvant; FIA, Freund's incomplete adjuvant; QS-21, purified *Quillaja saponaria* saponin.

combined with commercial adjuvants. This response developed rapidly after immunization and persisted at high levels for at least three days (Mowat et al., 1991).

The structural similarities between compound **1** and other bioactive complex triterpenoid saponins isolated from medicinal plants may help to explain its immunological activity (Lacaille-Dubois and Wagner, 1996). For example, the sugar side chain at C-28 may be responsible for the activation of the cellular immune response, since this residue is shared by the adjuvant saponin isolated from *Calliandra pulcherrima* and its removal by hydrolysis reactions abolished this activity, indicating that the integrity of the carbohydrate moiety attached at that position is mandatory for the these functions (Sun et al., 2009). Moreover, it was proved that the remarkable property of *Quillaja saponaria* to stimulate lymphocyte proliferation appears to depend on their lipophilic acylated moiety in molecular structure, since this property was significantly diminished after the removal of the monoterpene units, which implies that these residues play a pivotal role in the adjuvant activity (Marciani, 2003). Specially, the overall conformation harmoniously constructed by both hydrophilic and hydrophobic functional groups, rather than each individual functional group itself, is the most essential element for the consideration of adjuvant activity (Sun et al., 2009). In conclusion, the investigation of the biological properties of compound **1** indicated that this substance may be the potential therapeutic agent involved in the immunomodulatory activity, justifying the use of *S. saman* in the traditional medicine.

3. Experimental

3.1. General experimental procedures

Carbohydrate content was analyzed by gas chromatography–electron impact mass spectrometry (GC–EIMS) of the alditol acetates (Sawardeker et al., 1985). The experimental data were tested for statistical differences using the Student's *t*-test. Melting points were determined by an Electrothermal 9200 micro-melting point apparatus and are uncorrected. The optical rotations were measured on a Perkin Elmer 243B polarimeter. IR spectra were measured on a Perkin Elmer FT-IR 1600 spectrometer. ^{1}H and ^{13}C NMR, DEPT, COSY, HSQC and HMBC experiments were performed in deuterated pyridine on a Mercury-300 NMR spectrometer (300 MHz for δ_H and 75 MHz for δ_C). All chemical shifts (δ) are given in ppm units with reference to tetramethylsilane (TMS) as the internal standard and the coupling constants (J) are in Hz. Gas chromatography (GC) was carried out with flame ionization detector (FID), using a glass capillary column WCOT SE-30 (0.31 mm × 25 m; 0.25 μm film thickness) using the following temperature programme for splitless injection mode: 60–250 °C (5 °C/min), and the detector temperature at 270 °C. GC–EIMS were taken on a VG Auto SpecQ spectrometer operating at 70 eV. The MALDI-TOFMS was obtained using a Perseptive Voyager RP mass spectrometer. Silica gel columns (230–400 mesh ASTM, Merck) and Sephadex LH-20 (Pharmacia) were used for column chromatography (CC). Thin-layer chromatography (TLC) was performed on silica gel coated plates (Merck) using the following solvent systems: (A) $CHCl_3$–MeOH–H_2O (65:35:10, v/v/v, lower phase) for triterpenoid saponin **1**, (B) $CHCl_3$–MeOH (95:5, v/v) for sapogenin **1a**, (C) $CHCl_3$–MeOH–H_2O (8:3:1, v/v/v) for monoterpenoid **1b** and (D) *n*-BuOH–acetone–H_2O (4:5:1, v/v/v) for monosaccharides. Spray reagents were orcinol/H_2SO_4 for triterpenoid saponin **1** and monosaccharides and $CeSO_4$ for sapogenin **1a** and monoterpenoid **1b**.

3.2. Plant material

Stem barks of *S. saman* (Jacq.) Merr. were collected from the Botanical Garden of the Federal University of Rio de Janeiro (Rio de Janeiro, Brazil) in February 2009.

3.3. Extraction and isolation

Fresh stem barks of *S. saman* (100 g), previously cut into small pieces, were extracted with MeOH (300 mL) for 72 h at r.t. and the extract was concentrated under reduced pressure. The residue (9.21 g) was suspended in water (200 mL), the suspension was extracted with *n*-BuOH (200 mL). The resulting organic phase was evaporated *in vacuo* to give a crude material (3.57 g). This residue was dissolved in MeOH (60 mL) and EtOAc (300 mL) was added to the MeOH solution to give a precipitate. After setting for 72 h at r.t., the supernatant was decanted off. The precipitate was suspended in MeOH (50 mL) and concentrated *in vacuo* to give a dry residue (1.54 g). It was dissolved in MeOH (20 mL) and chromatographed by column chromatography over Sephadex LH-20 (3.8 cm × 65 cm) using MeOH as eluent to yield 60 fractions (23 mL each one). The fractions containing saponin (19–21) were evaporated *in vacuo* to give an impure saponin (1.39 g). It was further purified by column chromatography over silica gel (2.8 cm × 95 cm), using $CHCl_3$–MeOH–H_2O (65:35:10, lower phase) as eluent to afford compound **1** (792 mg).

3.4. 3-[(2-O-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy]-2,23-dihydroxy-(2β,3β,4α)-olean-12-en-28-oic acid O-β-D-glucopyranosyl-(1 → 3)-O-[O-β-D-glucopyranosyl-(1 → 4)]-O-6-deoxy-α-L-mannopyranosyl-(1 → 2)-6-O-[4-O-[(2E,6S)-2,6-dimethyl-1-oxo-2,7-octadienyl]-6-deoxy-α-L-mannopyranosyl)oxy]-β-D-glucopyranosyl ester (**1**)

Colorless amorphous powder (792 mg); m.p. 284–288 °C (dec.); $[\alpha]_D^{25}$ +33 (*c* 0.6, MeOH); UV λ_{max} (nm): 220; IR (KBr) ν_{max} cm^{-1}: 3420 (OH), 2929 (CH), 1713 (C=O), 1643 (C=O); ^{1}H NMR (C_5D_5N, 300 MHz) δ 7.02 (1H, *dt*, $J = 7.6$, 1.5 Hz, MT-H-3), 6.37 (1H, *brs*, RhaII-H-1), 6.26 (1H, *d*, $J = 8.4$ Hz, GlcIV-H-1), 6.11 (1H, *d*, $J = 7.6$ Hz, GlcIII-H-1), 6.09 (1H, *brs*, RhaI-H-1), 6.07 (1H, *dd*, $J = 17.1$, 10.8 Hz, MT-H-7), 5.86 (1H, *t*, $J = 10.0$ Hz, RhaII-H-4), 5.53 (1H, *dd*, $J = 17.1$,

Please cite this article in press as: Barbosa, A.P., et al., A new complex triterpenoid saponin from *Samanea saman* with haemolytic activity and adjuvant effect. Phytochem. Lett. (2012), http://dx.doi.org/10.1016/j.phytol.2012.06.009

1.8 Hz, MT-H-8b), 5.41 (1H, *brs*, H-12), 5.33 (1H, *d*, *J* = 8.0 Hz, GlcII-H-1), 5.20 (1H, *d*, *J* = 8.0 Hz, GlcV-H-1), 5.15 (1H, *dd*, *J* = 10.8, 1.8 Hz, MT-H-8a), 5.07 (1H, *d*, *J* = 8.0 Hz, GlcI-H-1), 4.61 (1H, *brs*, H-2), 4.41 (1H, *d*, *J* = 11.0 Hz, H-23b), 4.33 (1H, *d*, *J* = 3.0 Hz, H-3), 3.72 (1H, *d*, *J* = 11.0 Hz, H-23a), 2.60 (2H, *m*, MT-H-4), 1.88 (3H, *s*, MT-Me-9), 1.70 (2H, *t*, *J* = 6.6 Hz, MT-H-5), 1.58 (3H, *s*, Me-25), 1.52 (3H, *s*, Me-24), 1.45 (3H, *s*, MT-Me-10), 1.24 (3H, *s*, Me-27), 1.10 (3H, *s*, Me-26), 0.95 (3H, *s*, Me-29), 0.88 (3H, *s*, Me-30). ^{13}C NMR (C_5D_5N, 75 MHz): see Table 1. MALDI-TOFMS *m*/*z*: 1780, 9013 $[M+Na]^+$ (calcd for $C_{82}H_{132}NaO_{40}^+$, 1780, 9106).

3.5. Acid hydrolysis of compound **1**

Compound **1** (100 mg) was dissolved in 1,4-dioxane/1 N H_2SO_4 1:1 (10 mL) and heated in a sealed tube at 100 °C during 1 h. After dilution with water (10 mL), the reaction mixture was extracted with diethyl ether. The ether layer was evaporated to dryness. The residue was recrystallized from MeOH–$CHCl_3$ to give the sapogenin (1a, 19 mg) as colorless needles, m.p. 320–322 °C (dec.), $[\alpha]_D^{25}$ +127 (*c* 0.67, pyridine). The water layer was passed through an Amberlite IRA-410 column. The eluate was concentrated to give a residue containing the monosaccharide mixture (52 mg). A sample of the monosaccharide mixture (1 mg) was dissolved in pyridine (100 μL) and analyzed by TLC and compared with standards of sugars.

3.6. 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 an inert atmosphere. 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 (4.3 mg) was chromatographed on a silica gel column (1 × 30 cm) eluted with *n*-hexane–acetone (4:1, v/v) to afford the oily monoterpene carboxylic acid (**1b**; 2.7 mg). $[\alpha]_D^{25}$ +18 (*c* 0.65, $CHCl_3$).

3.7. Molar carbohydrate composition and *D,L* configurations

The molar carbohydrate composition of compound **1** (1 mg) was determined by GC–MS analyses of their monosaccharides as their trimethylsilylated methylglycosides obtained after methanolysis (0.5 M HCl in MeOH, 24 h, 80 °C) and trimethylsilylation (Kamerling et al., 1975). The configurations of the glycosides were established by capillary GC and GC–EIMS of their trimethylsilylated (−)-2-butylglycosides (Gerwig et al., 1978).

3.8. Methylation analysis

Compound **1** (1 mg) was dissolved in dimethylsulfoxide (200 μL) in a Teflon-lined screw-cap tube. Lithium methylsulfinyl carbanion (200 μL) was added to the solution under an inert atmosphere and the mixture was sonicated for 60 min. After cooling to −4 °C, cold methyl iodide (400 μL) was added. Sonication was conducted in a sonication bath (20 °C) for 45 min. The methylation was terminated by addition of water (4 mL) containing sodium thiosulfate, and the permethylated product extracted with chloroform (3 × 2 mL) and evaporated (Parente et al., 1985). The methyl ethers were obtained after hydrolysis (4 N TFA, 2 h, 100 °C) and analyzed as alditol acetates by GC–EIMS (Sawardeker et al., 1965).

3.9. Haemolytic activity

Normal human red blood cell suspension (0.5 mL of 0.5%) was mixed with 0.5 mL of diluent containing 5, 10, 20, 30, 40, 50, 100, 250 and 500 μg/mL of compound **1**, $Al(OH)_3$, purified *Quillaja saponaria* saponin (QS-21), and 5–500 μg/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. The free haemoglobin in the supernatant was measured by absorbance at 412 nm. Saline and distilled water were included as minimal and maximal haemolytic controls, respectively. The haemolytic percents developed by the saline control were subtracted from all groups. The adjuvant concentration inducing 50% of the maximal haemolysis was considered the median haemolytic dose (HD_{50}; graphical interpolation). Each experiment included triplicates at each concentration (Santos et al., 1997).

3.10. Immunological adjuvant activity

Male Swiss mice (three months old) were subcutaneously immunized twice at weekly intervals with 100 μg ovalbumin antigen (OVA) dissolved in 100 μL sterile saline (SAL) as the negative control group or with 100 μg of OVA mixed with 100 μg of compound **1** or Freund's Complete Adjuvant (FCA) or Freund's Incomplete Adjuvant (FIA) as the positive control groups, each one dissolved in 100 μL of saline as vehicle. A reference compound, the commercial purified *Quillaja saponaria* saponin (QS-21) 100 μg was mixed with 100 μg of the antigen (OVA) and dissolved in 100 μL of saline, for comparison. The 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 were obtained by subtracting the response to OVA challenge in unimmunized control mice (Mowat et al., 1991).

Acknowledgements

This work was financially supported by FINEP, CAPES and CNPq.

References

Castro, V.H., Ramirez, E., Mora, G.A., Iwase, Y., Nagao, T., Okabe, H., Matsunaga, H., Katano, M., Mori, M., 1997. Structures and antiproliferative activity of saponins from *Sechium pittieri* and *S. talamancense*. Chem. Pharm. Bull. 45, 349–358.

Durr, P.A., 2001. The biology, ecology and agroforestry potential of the raintree, *Samanea saman* (Jacq.) Merr. Agroforest. Syst. 51, 223–237.

Eade, R.A., Simes, J.J.H., Stevenson, B., 1963. Extractives of Australian timbers IV. Castanogenin (medicagenic acid) and bayogenin, $C_{30}H_{48}O_5$, from *Castanospermum australe* Cunn. et Fras. Aust. J. Chem. 16, 900–905.

Fujioka, T., Iwamoto, M., Iwase, Y., Hachiyama, S., Okabe, H., Yamauchi, T., Mihashi, K., 1989. Studies on the constituents of *Actinostema lobatum* Maxim. V. Structures of lobatosides B, E, F and G, the dicrotalic acid esters of bayogenin bisdesmosides isolated from the herb. Chem. Pharm. Bull. 37, 2355–2360.

Gerwig, G.J., Kamerling, J.P., Vliegenthart, J.F.G., 1978. Determination of the *D* and *L* configuration of neutral monosaccharides by high-resolution capillary G.L.C. Carbohydr. Res. 62, 349–357.

ILDIS International Legume Database & Information Service, 2005. http://www.ildis.org/LegumeWeb?version~10.01&LegumeWeb&tno~158&genus~Albizia&species~saman#151.

Kamerling, J.P., Gerwig, G.J., Vliegenthart, J.F.G., Clamp, J.R., 1975. Characterization by gas-liquid chromatography–mass spectrometry and proton-magnetic-resonance spectroscopy of pertrimethylsilyl methyl glycosides obtained in the methanolysis of glycoproteins and glycopeptides. Biochem. J. 151, 491–495.

Kim, Y.-J., Wang, P., Navarro-Villalobos, M., Rohde, B.D., Derryberry, J.M., Gin, D.Y., 2006. Synthetic Studies of Complex Immunostimulants from *Quillaja saponaria*: Synthesis of the Potent Clinical Immunoadjuvant QS-21Aapi. J. Am. Chem. Soc. 128, 11906–11915.

Lacaille-Dubois, M.A., Wagner, H., 1996. A review of the biological and pharmacological activities of saponins. Phytomedicine 2, 363–383.

Mahato, S.B., Kundu, A.P., 1994. NMR spectra of pentacyclic triterpenoids – a compilation and some salient features. Phytochemistry 35, 1515–1575.

Marciani, D.J., 2003. Vaccine adjuvants: role and mechanisms of action in vaccine immunogenicity. Drug Discov. Today 8, 934–943.

Mowat, A.M., Donachie, A.M., Reid, G., Jarrett, O., 1991. Immune-stimulating complexes containing Quil A and protein antigen prime class I MHC-restricted T lymphocytes *in vivo* and are immunogenic by the oral route. Immunology 72, 317–322.

Oda, K., Matsuda, H., Murakami, T., Katayama, S., Ohgitani, T., Yoshikawa, M., 2000. Adjuvant and haemolytic activities of saponins derived from medicinal and food plants. Biol. Chem. 381, 67–74.

Okada, Y., Koyama, K., Takahashi, K., Okuyama, T., Shibata, S., 1980. Gleditsia saponins I. Structures of monoterpene moieties of Gleditsia saponin C. Planta Med. 40, 185–192.

Parente, J.P., Cardon, P., Leroy, Y., Montreuil, J., Fournet, B., Ricart, G., 1985. A convenient method for methylation of glycoprotein glycans in small amounts by using lithium methylsulfinyl carbanion. Carbohydr. Res. 141, 41–47.

Prasad, R.N., Viswanathan, S., Devi, J.R., Nayak, V., Swetha, V.C., Archana, B.R., Parathasarathy, N., Rajkumar, J., 2008. Preliminary phytochemical screening and antimicrobial activity of *Samanea saman*. J. Med. Plant. Res. 2, 268–270.

Santos, W.R., Bernardo, R.R., Peçanha, L.M.T., Palatnik, M., Parente, J.P., de Sousa, C.B.P., 1997. Haemolytic activities of plant saponins and adjuvants. Effect of *Periandra mediterranea* saponin on the humoral response to the FML antigen of *Leishmania donovani*. Vaccine 15, 1024–1029.

Sawardeker, J.S., Sloneker, J.H., Jeanes, A., 1965. Quantitative determination of monosaccharides as their alditol acetates by gas liquid chromatography. Anal. Chem. 37, 1602–1604.

Sun, H.-X., Xie, Y., Ye, Y.-P., 2009. Advances in saponin-based adjuvants. Vaccine 27, 1787–1796.

Tan, N., Zhou, J., Zhao, S., 1999. Advances in structural elucidation of glucuronide oleanane-type triterpene carboxylic acid 3, 28-*O*-bidesmosides (1962–1997). Phytochemistry 52, 153–192.

Tava, A., Mella, M., Avato, P., Argentieri, M.P., Bialy, Z., Jurzysta, M., 2005. Triterpenoid glycosides from leaves of *Medicago arborea* L. J. Agric. Food Chem. 53, 9954–9965.

Waite, D.C., Jacobson, E.W., Ennis, F.A., Edelman, R., White, B., Kammer, R., Anderson, C., Kensil, C.R., 2001. Three double-blind, randomized trials evaluating the safety and tolerance of different formulations of the saponin adjuvant QS-21. Vaccine 19, 3957–3967.

Zhang, D., Miyase, T., Kuroyanagi, M., Umehara, K., Ueno, A., 1995a. Studies on the constituents of *Polygala japonica* Hout. I. Structures of polygalasaponins I–X. Chem. Pharm. Bull. 43, 115–120.

Zhang, D., Miyase, T., Kuroyanagi, M., Umehara, K., Ueno, A., 1995b. Studies on the constituents of *Polygala japonica* Hout. II. Structures of polygalasaponins XI–XIV. Chem. Pharm. Bull. 43, 966–970.

Zhang, Z., Koike, K., Jia, Z., Nikaido, T., Guo, D., Zheng, J., 1999a. Four new triterpenoidal saponins acylated with one monoterpenic acid from *Gleditsia sinensis*. J. Nat. Prod. 62, 740–745.

Zhang, Z., Koike, K., Jia, Z., Nikaido, T., Guo, D., Zheng, J., 1999b. Gleditsiosides N–Q, new triterpenoid saponins from *Gleditsia sinensis*. J. Nat. Prod. 62, 877–881.

Zhang, Z., Koike, K., Jia, Z., Nikaido, T., Guo, D., Zheng, J., 1999c. Triterpenoidal saponins acylated with two monoterpenic acids from *Gleditsia sinensis*. Chem. Pharm. Bull. 47, 388–393.

Please cite this article in press as: Barbosa, A.P., et al., A new complex triterpenoid saponin from *Samanea saman* with haemolytic activity and adjuvant effect. Phytochem. Lett. (2012), http://dx.doi.org/10.1016/j.phytol.2012.06.009