

# Pulcherrimasaponin, from the leaves of *Calliandra pulcherrima*, as adjuvant for immunization in the murine model of visceral leishmaniasis

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## Abstract

A novel triterpenoidal saponin, called pulcherrimasaponin (CP05), isolated from the leaves of *Calliandra pulcherrima* Benth. shows remarkable similarities to the previously described potent adjuvant, QS21 saponin (*Quillaja saponaria* Molina). On the basis of chemical and physicochemical evidence, its structure was established as [3 $\beta$ ,16 $\alpha$ ,28[2E,6S[2E,6S(2E,6S)]]]-olean-12-en-28-oic acid 3-[[O- $\alpha$ -L-arabinopyranosyl-(1 $\rightarrow$ 2)-O- $\alpha$ -L-arabinopyranosyl-(1 $\rightarrow$ 6)-2-(acetylamino)-2-deoxy- $\beta$ -D-glucopyranosyl]oxy]-16-hydroxy-O- $\beta$ -D-glucopyranosyl-(1 $\rightarrow$ 3)-O-[O- $\beta$ -D-xylopyranosyl-(1 $\rightarrow$ 3)- $\beta$ -D-xylopyranosyl-(1 $\rightarrow$ 4)-O-6-deoxy- $\alpha$ -L-mannopyranosyl-(1 $\rightarrow$ 2)-6-O-[6-[[2-O-2,6-dimethyl-1-oxo-6-( $\beta$ -D-xylopyranosyloxy)-2,7-octadienyl]-[(6-deoxy- $\beta$ -D-glucopyranosyl)oxy]-2,6-dimethyl-1-oxo-2,7-octadienyl]- $\beta$ -D-xylopyranosyl]oxy]-2,6-dimethyl-1-oxo-2,7-octadienyl]- $\beta$ -D-glucopyranosyl ester. In vivo toxicity assays disclosed similar and transitory local swelling and loss of hair but no lethality for mice. The haemolytic index was higher for QS21 (5  $\mu$ g/ml) than for CP05 (13  $\mu$ g/ml). Mouse vaccination with either CP05 or QS21 in combination with the fucose-mannose ligand (FML) antigen of *Leishmania donovani* showed anti-FML responses, significantly enhanced over the saponin and saline controls, in IgM, IgG, IgG1, IgG2a, IgG2b and IgG3. Antibody levels were similar for both vaccines in most subtypes. However, QS21-FML vaccine showed a 1.5 to 2.1 proportional increase over the CP05-FML vaccine in IgG, IgG2a and IgG3 responses. The delayed type of hypersensitivity against leishmanial antigen was impressively increased for CP05-FML and for QS21-FML-treated animals over controls ( $p < 0.005$ ). Enhancement was similar for both vaccines ( $p < 0.05$ ). The safety analysis and the effect on humoral and cellular immune responses demonstrated that the novel *Calliandra pulcherrima* Benth. CP05 saponin is a potential candidate for a vaccine adjuvant.

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**Keywords:** *Leishmania donovani*; Kala-azar; Visceral leishmaniasis; *Calliandra pulcherrima*; Pulcherrimasaponin; FML-antigen; Adjuvant; *Quillaja saponaria* Molina; QS21 saponin

## 1. Introduction

Saponins are conjugates of triterpenes and carbohydrates that behave as very potent and specific adjuvants [1]. Saponins isolated from *Calliandra* species showed the

typical triterpene nucleus with glycidic moieties associated with C-3 and C-28. A major feature of this saponin is the presence of monoterpenes intercalated with sugars linked to the C-28 moiety [2–5]. Identification of normonoterpenes in saponins is very rare and was coincidentally described in the very potent saponins of the *Quillaja saponaria* Molina bark: QS21, 17, 18 [6]. The *Quillaja saponaria* saponins are currently used as outstanding adjuvants in vaccine formu-

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lations against intracellular pathogens, agents of very severe and lethal human and animals diseases [7–10]. They stimulate both the humoral and the cellular immune responses against the pathogens. In *Quillaja saponaria*, the normonoterpene moiety is associated with the potentiation of the cytotoxic T cell response (CTL) and to an undesirable slight toxicity [1,11]. On the other hand, the presence of an aldehyde group on C-23 of the triterpene of *Quillaja* fractions was associated also with the induction of a main TH1 response against exogenous proteins [1,11]. While QS21 shows two normonoterenes linked to a fucose that is attached to the C-28 of the quillaic acid triterpene [6], the *Calliandra anomala* saponins alternate two [2,3,5] or three [3,4] monoterpenes with sugar residues in the branched C-28 attached moiety. The similarities in chemical structure of the saponins of these two plants could point to similarities in their adjuvant potential. While no adjuvant activity was characterized yet for *Calliandra anomala*, aqueous extracts of its branches are used as an antimalarial and antifebrile agent in Mexico [12]. On the other hand, *Calliandra pulcherrima* Benth. (Leguminosae) is a related native species found in Tropical America. This evergreen plant is non-invasive, but a widespread ornamental plant often cultivated in gardens and parks [13]. In Brazil, the aqueous extract of the branches of *C. pulcherrima* is used as a remedy for malaria and leishmaniasis [14]. There is no report on the constituents making up this plant. In this work, we performed the isolation and structural elucidation of a *Calliandra pulcherrima* Benth. novel triterpenoidal saponin: the pulcherrimasaponin (CP05). We also assessed its potential adjuvant effect with the fucose–mannose ligand (FML) antigen of *Leishmania donovani* and compare it to the QS21 saponin of *Quillaja saponaria* Molina, in a previously described murine model of visceral leishmaniasis [15].

## 2. Materials and methods

### 2.1. Plant material

Fresh leaves of *Calliandra pulcherrima* were obtained from the ornamental plant garden of the Federal University of Rio de Janeiro in January 2002, and a voucher specimen is maintained in the Laboratory of Chemistry of Medicinal Plants at Federal University of Rio de Janeiro.

### 2.2. General procedures

Melting points were determined by an Electrothermal 9200 micro-melting point and are uncorrected. Optical rotations were measured on a Perkin-Elmer 243B polarimeter. UV and IR spectra were measured on a Shimadzu UV-1601 and on a Perkin-Elmer 599B, respectively. MALDI-TOFMS was conducted using a Perseptive Voyager RP mass spectrometer.

GC analyses were performed using a Shimadzu GCMS-QP5050A gas chromatograph mass spectrometer us-

ing an ionization voltage of 70 eV. GC was carried out with FID, using a glass capillary column (0.25 m × 25 m, 0.25 micron, J & W Scientific Incorporated, Folsom, CA, USA) DB-1.

NMR spectra were measured in pyridine-*d*<sub>5</sub> (100 mg of CP05 in 0.5 ml) at 25 °C with a Varian Gemini 200 NMR spectrometer, with tetramethylsilane ( $\delta = 0.00$ ) used as internal standard. <sup>1</sup>H NMR spectra were recorded at 200 MHz and <sup>13</sup>C NMR spectra at 50 MHz. 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 plates (Kieselgel 60F<sub>254</sub>, Merck) using the following solvent systems: (A) CHCl<sub>3</sub>–MeOH–H<sub>2</sub>O (55:45:5, v/v/v) for triterpenoidal saponin CP05; (B) CHCl<sub>3</sub>–MeOH (95:5 (v/v)) for sapogenin and (C) *n*-BuOH–C<sub>5</sub>D<sub>5</sub>N–H<sub>2</sub>O (60:40:30, v/v/v) for monosaccharides. Spray reagents were orcinol–sulfuric acid for triterpenoidal saponin CP05 and monosaccharides, and CeSO<sub>4</sub> for triterpenoidal sapogenin.

### 2.3. Extraction and isolation of CP05 saponin and QS21 saponin

The fresh leaves of the plant (1 kg) were extracted with methanol (4 l) followed by concentration under reduced pressure. The residue (36.3 g) was suspended in water (500 ml), the suspension was extracted with *n*-butanol (500 ml) and then the *n*-butanol soluble fraction was concentrated in vacuo to give a residue (14.3 g). This residue was dissolved in methanol (400 ml) and ethyl acetate (2 l) was added to the methanol solution to give a precipitate (4.3 g). It was roughly chromatographed on Sephadex LH-20 with MeOH to give crude triterpenoidal saponin (0.8 g). Further purification by chromatography on a silica gel column eluted with CHCl<sub>3</sub>–MeOH–H<sub>2</sub>O (55:45:5, v/v/v) afforded one homogeneous compound CP05 (285 mg), R<sub>f</sub> 0.50 which gave a dark blue color with orcinol–H<sub>2</sub>SO<sub>4</sub>.

The QS21 saponin was isolated from Riedel De Haen, Saponin pure<sup>®</sup> (8047-15-2) EINECE (West Germany). Saponin (1 g) was fractionated by ion exchange chromatography on DEAE–cellulose (Whatman DE 52) in a K 9/15 column (Pharmacia Fine Chemicals). The bed material was equilibrated with 0.1 M Tris–HCl buffer pH 7.5. The column was eluted either stepwise or by a linear salt gradient at a flow rate of 60 ml/h using a peristaltic pump. Gel exclusion chromatography was performed on Sephadex G 50 fine (Pharmacia Fine Chemicals) equilibrated with M/50 phosphate pH 7.5 in a K 16/70 column eluted at a flow rate of 10 ml/h. Desalting was carried out on Sephadex G-25 medium in a K 16/40 column to afford one TLC homogeneous saponin fraction (196 mg), R<sub>f</sub> 0.43 which gave a dark blue color with orcinol–H<sub>2</sub>SO<sub>4</sub>. The solvent system used to analyze QS21 was 96% *n*-butanol, 25% ethanol, aqueous ammonia (3:6:5). The saponin fraction was identified as QS21 by comparison of <sup>1</sup>H and <sup>13</sup>C NMR data with the literature [16].

#### 2.4. Acid hydrolysis of CP05

The triterpenoidal saponin CP05 (200 mg) was heated at 100 °C with 20 ml of 2 N H<sub>2</sub>SO<sub>4</sub> for 6 h. The reaction mixture was diluted with water and extracted with diethyl ether. The organic layer was concentrated in vacuo. The residue was recrystallized from methanol to give the triterpenoidal saponin. The aqueous solution was passed through an amberlite IRA-410 column. The eluate was concentrated to give a residue containing the monosaccharide mixture.

#### 2.5. Molar carbohydrate composition and D,L configurations

The molar carbohydrate composition of CP05 saponin 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 [17]. The configurations of the glycosides were established by capillary GC of their trimethylsilylated (–)-2-butylglycosides [18].

#### 2.6. Methylation analysis

CP05 saponin was methylated with dimethyl sulfoxide-lithium methylsulfinyl carbanion-methyl iodide [19]. The methyl ethers were obtained after hydrolysis (4N TFA, 2 h, 100 °C) and analyzed as partially alditol acetates by GC–MS [19,20].

#### 2.7. Haemolytic assay

Normal human red blood cell suspensions (0.6 ml of 0.5%) were mixed with 0.6 ml diluent containing 5, 10, 20, 30, 40, 50, 100, 250 and 500 µg/ml concentrations of the pulcherrimasaponin (CP05), in saline solution. Mixtures were incubated for 30 min at 37 °C and centrifuged at 70 × g for 10 min. Free hemoglobin in the supernatant was measured by absorbance at 412 nm [21]. Saline and distilled water were included as minimal and maximal haemolytic controls. The haemolytic percent developed by the saline control was subtracted from all groups. The adjuvant concentration inducing 50% of the maximum haemolysis was considered the HD<sub>50</sub> (graphical interpolation). Each experiment included triplicates at each concentration. Two independent experiments were performed for the analysis of each HD<sub>50</sub>.

Human red blood cells for the haemolytic assay were obtained from healthy adult blood bank donors (Hospital Universitário Clementino Fraga Filho, University of Rio de Janeiro, Brazil). Blood was collected with citrate phosphate dextrose adenine<sub>1</sub> (CPDA<sub>1</sub>) anticoagulant (7:1, v/v). Aliquots of 1.5 ml of blood in CPDA<sub>1</sub> were washed three times with sterile saline solution (0.9% (w/v) NaCl, pyrogen free) by centrifugation at 180 × g for 1 min. The red blood cell suspension was prepared by finally diluting the pellet to 0.5% in saline solution. All the human blood samples

used in this study, showed negative reactions in the assays for the presence of antibodies against Chagas' disease, HIV I and II viruses, syphilis and to the core of hepatitis B virus (anti-HBc), anti-hepatitis C virus (anti-HCV), and hepatitis B virus antigen (HBs Ag). All serological tests were done with ELISA kits (Abbott Diagnostics and Organon USA) while the Chagas' disease test was performed with the Biolab test (São Paulo, Brazil).

#### 2.8. Mice

Female outbred Balb/c mice (3-month-old) were obtained from the central animal care facilities, Centro de Ciências da Saúde, University of Rio de Janeiro, Brazil.

#### 2.9. In vivo toxic activities for saponins

Toxicity (assessed by lethality, local pain as assessed by vocal response to injection, local swelling, loss of hair and skin lesion) was tested in Balb/c female mice. 200 µg of CP05 saponin dissolved in 100 µl sterile saline were injected subcutaneously on the back of the mice ( $n=7$ ), as three doses at weekly intervals. The mice were monitored during 7 days after the last dose. Pyrogen free sterile saline solution was used as control.

#### 2.10. Immunization of mice

Animals were immunized with three doses at weekly intervals of the FML antigen of *L. donovani* (150 µg) [15] and 100 µg of CP05 saponin or QS21 saponin in 200 µl sterile saline solution through the subcutaneous (s.c.) route in the back of 2-month-old Balb/c mice. Saline and CP05 adjuvant-treated animals were included as controls. Two identical experiments were performed, each with  $n=7-8$  animals. Isolation and chemical characterization of the FML obtained from stationary-growth phase promastigotes of *L. donovani* Sudan (LD 1S/MHOM/SD/00-strain 1S) was performed as previously described [22]. Briefly, promastigotes were submitted to an aqueous extraction followed by heat inactivation and centrifugation. The aqueous supernatant was lyophilised and fractionated by gel filtration on a Bio-Gel P-10 column yielding the FML glycoproteic complex in void volume [22]. The FML-vaccine is registered as a Patent: INPI number: PI1100173-9 (18.3.97), Federal University of Rio de Janeiro, Brazil. Sera of animals were collected 7 days after complete immunization and analyzed by FML–ELISA.

#### 2.11. FML–ELISA assay

The anti-FML antibody levels were assayed in pools of all the vaccinated and control groups using the FML–ELISA as previously described [15], using 2 µg antigen per well and: goat anti-mouse IgG (Sigma) or goat anti-mouse IgG1, IgG2a horseradish peroxidase conjugated antibodies (Southern, Biotechnology Associates, Birmingham, AL, USA) in a

1:4000 dilution in blocking buffer. The reaction was developed with *O*-phenyldiamine (Sigma), interrupted with 1 N sulphuric acid, and monitored at 492 nm. Sera were analyzed by double-blind tests, in triplicate. Positive and negative control sera were included in each test. Results were expressed as absorbance values at 492 nm of the 1/100 diluted pool of sera obtained for each treatment (two experiments, each with  $n = 7$ –8), 7 days after the complete immunization.

### 2.12. Delayed type hypersensitivity (intra-dermal reaction to promastigote lysate)

This was determined by injecting mice intradermally, 10 days after complete immunization, in the right hind footpad, with  $10^7$  freeze-thawed stationary phase promastigotes of *L. donovani* in 0.1 ml sterile saline solution, measuring the footpad thickness with a Mitutoyo apparatus, both before and 0, 24, 48 and 72 h after injection. Injecting each animal with 0.1 ml saline in the left hind footpad served as controls. At each time, the values of the saline control were subtracted from the reaction due to *Leishmania* antigen. Previous experiments carried out in Balb/c mice and CB hamsters demonstrated that 24 h after inoculation saline-treated footpads returned to base levels [15,23,24].

### 2.13. Statistical analysis

Means were compared by a standard *t* test, ANOVA analysis, simple factorial test and by one way ANOVA, Student–Newman–Keuls method (SPSS for windows). Correlation coefficient analysis was determined on a Pearson bivariate, two tailed test of significance (SPSS for windows).

## 3. Results

### 3.1. Physical and spectral data of pulcherrimasaponin (CP05)

Pulcherrimasaponin (CP05) Amorphous powder, m.p. 240–246 °C (dec.);  $[\alpha]_D^{25} = -40^\circ$  ( $c$  0.1, MeOH); UV  $\lambda_{\max}$  MeOH nm: 220 ( $\log \epsilon$  3.87) ( $\alpha, \beta$ -unsaturated ester); IR  $\nu_{\max}$  (KBr)  $\text{cm}^{-1}$ : 3422 (OH), 2929, 1707 (COOR), 1453, 1425, 1379, 1314, 1260, 1159, 1074, 1038, 912, 895, 838, 812, 768. MALDI-TOFMS:  $m/z = 2606.9174$   $[M + Na]^+$  (high resolution).  $^{13}\text{C}$ - and  $^1\text{H}$  NMR spectral data are shown in Tables 1 and 2, respectively.

### 3.2. Acid hydrolysis of CP05

On acid hydrolysis with 2N sulphuric acid, pulcherrimasaponin (CP05) gave echinocystic acid [25], monoterpene carboxylic acid [26], arabinose, glucose, rhamnose, xylose, quinovose and *N*-acetylglucosamine as the component sugars.

### 3.3. Molar carbohydrate composition and D,L configurations

The molar carbohydrate composition of pulcherrimasaponin (CP05) indicated the presence of eleven monosaccharides: arabinose:glucose:rhamnose:xylose:quinovose:*N*-acetylglucosamine (2:2:1:4:1:1). Their absolute configurations were determined by GC of their trimethylsilylated (–)-2-butylglycosides and L-arabinose, D-glucose, L-rhamnose, D-xylose, D-quinovose and *N*-acetyl-D-glucosamine were identified.

### 3.4. Methylation analysis

The fully methylated products of pulcherrimasaponin (CP05) were hydrolyzed with acid, converted into the alditol acetates, and analyzed by GC and GC-EIMS. Pulcherrimasaponin (CP05) 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, 1,2,5-tri-*O*-acetyl-3,4-di-*O*-methyl quinovitol, 1,5,6-tri-*O*-acetyl-2-deoxy-*N,N*-dimethyl-3,4-di-*O*-methyl glucosaminitol, 1,3,4,5-tetra-*O*-acetyl-2-*O*-methyl rhamnitol, and 1,2,5,6-tetra-*O*-acetyl-3,4-di-*O*-methyl glucitol. Fig. 1 summarizes the chemical structure of CP05 saponin. The structure of QS21 saponin was also included for comparison.

### 3.5. Haemolytic and toxicity assays

The haemolytic activity of CP05 saponin was determined (Table 3). The  $\text{HD}_{50}$  was 13  $\mu\text{g}/\text{ml}$  for CP05 and 5  $\mu\text{g}/\text{ml}$  for QS21 saponin. No lethality was detected after treatment with either of the three doses of 200  $\mu\text{g}$  of the saponin, nor local pain. Swelling and/or loss of hair at the injection site were noted for both saponins after each injection dose, with complete spontaneous recovery after 15 days.

### 3.6. Humoral response

Mice were immunized with three weekly doses of the FML antigen of *L. donovani* and the CP05 or QS21 saponins. Seven days after complete immunization, sera of mice were collected in pools and analyzed by FML–ELISA in a 1:100 dilution. The total anti-FML antibody responses are represented in Fig. 2. Significant differences between treatments were detected in all types and subtypes of immunoglobulins ( $p < 0.005$ ) except IgA ( $p = 0.071$ ) (ANOVA analysis). Also, the CP05–FML- and the QS21–FML adjuvanted groups showed anti-FML responses which were significantly enhanced over the saponin and saline controls in all types of antibodies except IgA (Fig. 2). The highest absorbances were detected in the IgG class and its subtypes. While similar levels of humoral responses were achieved against both

Table 1  
<sup>13</sup>C NMR spectral data of pulcherrimasaponin (CP05)<sup>a,b</sup>

Carbon	CP05	Carbon	CP05	Carbon	CP05	Carbon	CP05
Aglycone		C-3 Sugar		C-28 Sugar		MTG	
1	38.40	GlcNAc-1	103.97	Glc-1''	104.33	MT-1''	166.84
2	25.88	2	57.05	2''	74.58	2''	127.69
3	88.88	3	74.63	3''	77.29	3''	143.02
4	38.67	4	71.77	4''	70.78	4''	23.19
5	55.46	5	75.19	5''	77.30	5''	40.56
6	18.07	6	68.77	6''	61.86	6''	79.12
7	32.98	NHCOCH <sub>3</sub>	170.83	Xyl-1'	103.73	7''	143.16
8	39.58	NHCOCH <sub>3</sub>	22.90	2'	73.78	8''	114.53
9	46.65	Ara-1'	101.67	3'	87.25	9''	12.00
10	36.51	2'	79.47	4'	68.79	10''	23.31
11	23.25	3'	71.70	5'	65.60	Qui-1	96.30
12	122.10	4'	68.78	Xyl-1''	105.06	2	74.92
13	143.72	5'	63.65	2''	74.40	3	75.07
14	41.51	Ara-1''	105.66	3''	76.73	4	76.20
15	35.44	2''	74.43	4''	69.90	5	72.12
16	73.57	3''	76.80	5''	66.20	6	17.99
17	48.82	4''	69.99	MTG		MT-1'''	167.30
18	40.85	5''	66.33	MT-1'	167.65	2'''	127.90
19	46.80	C-28 Sugar		2'	127.43	3'''	143.13
20	30.14	Clc-1''	94.20	3'	142.53	4'''	23.33
21	35.34	2'	77.68	4'	23.46	5'''	40.32
22	31.41	3'	76.87	5'	40.40	6'''	79.51
23	27.60	4'	70.67	6'	79.23	7'''	143.37
24	16.45	5'	75.08	7'	142.05	8'''	115.02
25	15.12	6'	63.81	8'	115.02	9'''	12.39
26	16.95	Rha-1	101.37	9'	11.90	10'''	23.52
27	26.59	2	69.76	10'	23.52	Xyl-1''''	99.67
28	175.40	3	82.82	Xyl-1'''	97.25	2''''	74.55
29	32.55	4	77.66	2''''	74.69	3''''	77.80
30	24.27	5	68.42	3''''	75.38	4''''	70.61
		6	18.20	4''''	70.46	5''''	66.25
				5''''	66.10		

<sup>a</sup> The assignments were made on the basis of DEPT, HETCOR, COLOC experiments and comparison of spectral data with the literature [3,5].

<sup>b</sup> The following convention is used: MTG = monoterpene glycoside, MT = monoterpene.

CP05 and QS21-saponin vaccines in most subtypes, antibodies against QS21-FML vaccine were specifically increased over the CP05FML vaccine in a 1.5–2.1 proportion, for IgG, IgG2a and IgG3 ( $p < 0.05$ , Student–Newman–Keuls method). As expected for a specific adjuvant, the absorbance levels of sera of animals treated with CP05 only were not different

from the saline controls for all types of antibodies except IgG (Student–Neuman–Keul method).

No correlation was found between anti-FML IgA antibodies and any other class or subclass. On the other hand, significant correlations were found between IgM, IgG, IgG1, IgG2a, IgG2b and IgG3 enhancements ( $p < 0.025$ ).

Table 2  
 Selected characteristic <sup>1</sup>H NMR data ( $\delta$ [ppm]),  $J$  [Hz] of CP05<sup>a</sup>

Sapogenin methyl groups		Monoterpene methyl groups		Sugar methyl groups	
23-Me	1.11 s	9'-Me	1.82 s	Rha-Me-6	1.56 d (6.1)
24-Me	0.90 s	10'-Me	1.40 s	Qui-Me-6	1.47 d (6.1)
25-Me	0.84 s	9''-Me	1.88 s	NHCOMe	2.10 s
26-Me	0.98 s	10''-Me	1.42 s		
27-Me	1.67 s	9'''-Me	1.87 s		
29-Me	0.90 s	10'''-Me	1.40 s		
30-Me	1.01 s				
Sugar anomeric protons					
GlcNAc-H-1	4.91 d (7.4)	Rha-H-1	5.71 br s	Xyl-H-1''''	4.76 d (8.6)
Ara-H-1'	4.93 d (5.5)	Glc-H-1''	5.18 d (7.3)	Qui-H-1	4.78 d (8.0)
Ara-H-1''	4.81 d (6.7)	Xyl-H-1'	5.28 d (7.3)	Xyl-H-1''''	4.66 d (7.9)
Glc-H-1'	5.84 d (7.3)	Xyl-H-1''	4.99 d (7.3)		

<sup>a</sup> The assignments were made on the basis of <sup>1</sup>H-<sup>1</sup>H-COSY, HETCOR, COLOC experiments and comparison of spectral data with the literature [3,5].

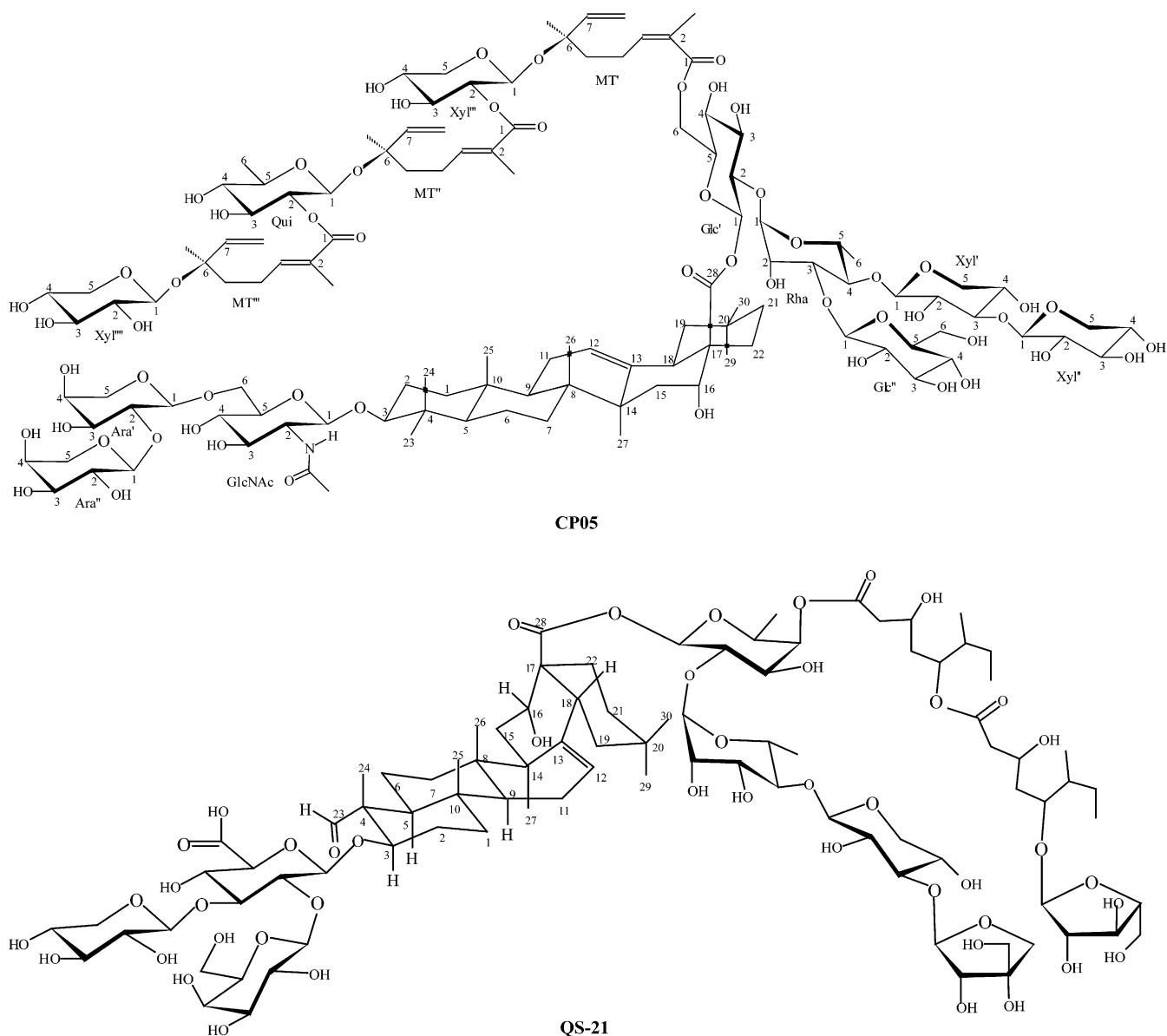


Fig. 1. Chemical structure of pulcherrimasaponin (CP05) isolated from *Calliandra pulcherrima* and QS21 saponin from *Quillaja saponaria* Molina.

### 3.7. Delayed type hypersensitivity (intradermal reaction to promastigote lysate)

The intradermal response to the *L. donovani* f/t lysate antigen is represented in Fig. 3. The footpad swelling was evaluated 0, 24 and 48 h after antigen injection. The in-

tradermal reaction (IDR) values were already subtracted from their respective saline control. An impressive increase in IDR was noted in vaccinated animals. ANOVA analysis disclosed highly significant differences among all different treatments ( $p < 0.0005$ ;  $F = 92.457$ ) at 24 and 48 h. Also, a significant decrease was noted in all IDR re-

Table 3  
Toxicity in vivo and haemolytic effect of pulcherrimasaponin (CP05) and QS21 saponin<sup>a</sup>

Adjuvant	First dose					Second dose					Third dose					HD <sub>50</sub> (μg/ml)
	P	s	l	H	sl	p	s	l	h	sl	p	s	L	h	sl	
CP05	0/7	7/7	0/7	3/7	2/7	0/7	7/7	0/7	5/7	0/7	0/7	7/7	0/7	4/7	0/7	13
QS21	0/7	7/7	0/7	2/7	2/7	0/7	7/7	0/7	5/7	0/7	0/7	7/7	0/7	5/7	0/7	5

<sup>a</sup> Results are expressed as number of mice per group, within 7 days after subcutaneous injection of 200 μg saponin in the back showing: (p) local pain; (s) local swelling; (l) lethality; (h) loss of hair; (sl) skin lesion at the site of injection.

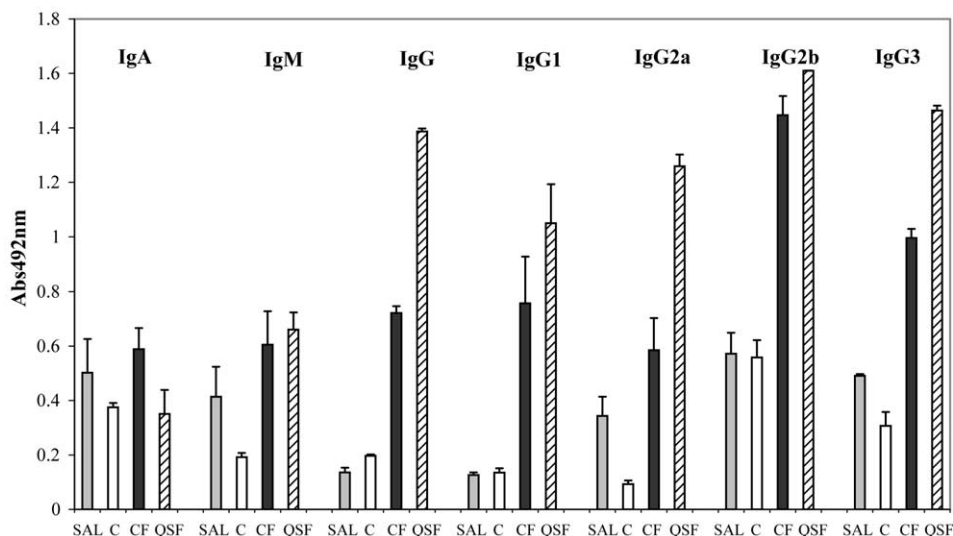


Fig. 2. Anti-FML antibodies in mice vaccinated with the FML antigen of *Leishmania donovani* and the pulcherrimasaponin (CF) or the QS21 saponin (QSF). From left to right: anti-FML total IgA, IgM, IgG, IgG1, IgG2a, IgG2b and IgG3 antibodies. Control animals received only saline (SAL) or pulcherrimasaponin adjuvant (C), as indicated. The y-axis represents the FML–ELISA absorbance values at 492 nm of the 1/100 diluted pool of sera obtained for each treatment (two experiments, each with  $n = 7$ –8 animals), 7 days after complete immunization. The results show the mean and standard errors of two identical experiments.

responses at 48 h (ANOVA,  $p < 0.001$ ;  $F = 12.954$ ). IDR responses were significantly higher for CP05–FML and for QS21–FML vaccines than for CP05 or saline-treated controls ( $p < 0.005$ ) (Student–Newman–Keuls method). The enhancement of IDR was similar for both vaccines ( $p > 0.05$ ). The increase in IDR response to promastigote lysate is highly correlated with the increase in IgG antibodies ( $p = 0.001$ ) and all its subtypes ( $p = 0.034$ ) but not with IgA ( $p = 0.095$ ) or IgM ( $p = 0.251$ ). Taken together, the safety analysis and the effect on humoral and cellular immune responses, the CP05–FML formulation shows a significant and spe-

cific adjuvant effect on the FML antigen of *Leishmania donovani*.

#### 4. Discussion

In the present investigation, we isolated and chemically characterized the CP05 saponin of *Calliandra pulcherima* Benth. and compared it with the QS21 saponin of *Quilaja saponaria* Molina regarding their toxicity and adjuvant potential, in formulation with the FML antigen of *Leish-*

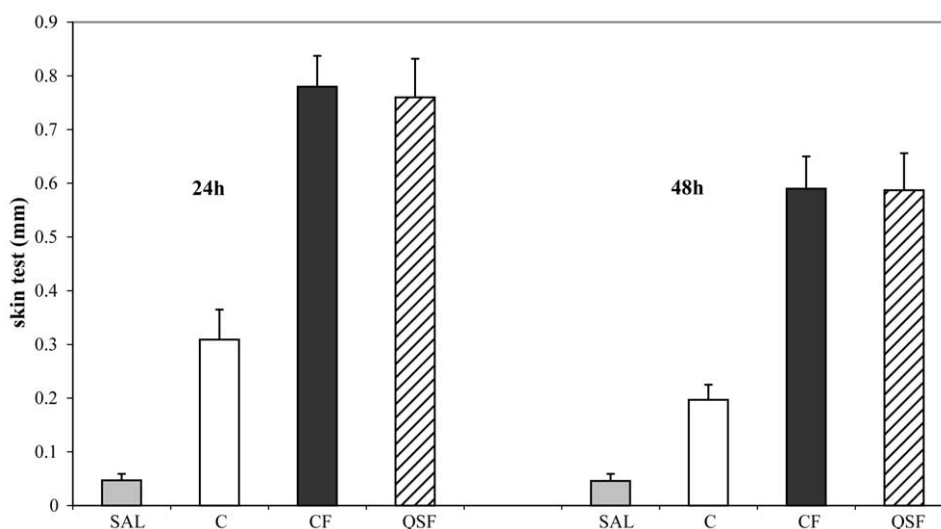


Fig. 3. Delayed type hypersensitivity in Balb/c mice immunized with the FML antigen of *Leishmania donovani* and the pulcherrimasaponin (CF) or the QS21 saponin (QSF). Control animals received only saline (SAL) or pulcherrimasaponin adjuvant (C), as indicated. The y-axis represents the thickness of skin test in mm, at 24 and 48 h after injection with  $10^7$  freeze-thawed stationary phase promastigotes of *L. donovani*. The results show the mean and standard errors of two identical experiments, each with seven to eight animals per group. At each time, the values of the saline control were subtracted from the reaction due to *Leishmania*.

*mania donovani*. The QS21 saponin isolated from *Quillaja saponaria* shows an outstanding and specific adjuvant potential [1,7–11]. It has already been used in clinical trials in humans with malaria peptide vaccine [9] and low dose HIV-1 gp120 [27] and it is currently under clinical evaluation for various vaccines, tested in more than 3000 patients in 60 clinical trials (reviewed in [30]).

Saponins of *Quillaja saponaria* in combination with FML, have already proved to be excellent adjuvants for immunoprophylaxis and immunotherapy of murine and canine visceral leishmaniasis. The analysis of the chemical structure of QS21 disclosed the presence of an aldehyde group at C-4 and oligosaccharide chains attached to positions C-3 and C-28 of their triterpene aglycone [11]. A unique feature of the *Quillaja* saponins appears to be the presence of two monoterpenic ester moieties linked linearly to a fucosyl residue attached at the C-28 position. The aldehyde group is crucial for saponin adjuvant effectiveness, mainly for stimulation of TH1 response [11]. On the other hand, the acylated C-28 linked hydrophobic moiety is responsible for stimulating the production of the cytotoxic T cell response (CTL) against exogenous proteins [1,11] and of some undesirable toxic effects after vaccine injection. Similar to QS21, the saponins from the genus *Calliandra* are also triterpenoids linked to two glycosidic chains. The one linked to C-28 is composed of variable number of monoterpenes [2–5]. The presence of normonoterpenes in both *Quillaja* saponins and *Calliandra* saponins could also point to some similarities in the induction of the immune response.

In the present investigation, we described the chemical structure of the CP05 saponin of *Calliandra pulcherrima* Benth. that shows three monoterpenes in its hydrophobic C-28 linked chain. Therefore, CP05 is similar to QS21 and even more enriched in its moiety related to CTL and mild toxicity. Indeed, Pulcherrimasaponin (CP05) isolated from the leaves of *C. pulcherrima* was obtained as a white powder. The MALDI-TOFMS quasimolecular ion at  $m/z$  2606.9174 [M+Na] combined with  $^{13}\text{C}$  NMR spectral data gave a molecular formula  $\text{C}_{122}\text{H}_{191}\text{NO}_{57}$  for CP05. The  $^{13}\text{C}$  NMR spectral data of compound CP05 revealed 122 carbon signals, 30 of which were assigned to the aglycone moiety, 15 to the three monoterpene units, while 77 were assigned to the carbohydrate part. The quasimolecular ion of CP05 on MALDI-TOFMS was 132 mass units larger than that of the known saponin I (3) [3]. The  $^{13}\text{C}$  NMR spectral data of the saponin part of CP05 were similar to those of saponin I (3) [3], the known oleanane-type triterpene echinocystic acid [24]. Also, the  $^{13}\text{C}$  NMR data of the sugar moiety at C-3 were similar to those of I (3) [3].

However, in pulcherrimasaponin (CP05), there is a signal at  $\delta$  79.51 due to the resonance of C-6''' of monoterpene (MT''') indicating that one sugar unit should be at C-6'''. The  $^1\text{H}$  NMR spectral data showed eleven anomeric signals with coupling constants characterizing anomeric configurations of the monosaccharides as  $\alpha$ -arabinose,  $\beta$ -glucose,  $\alpha$ -rhamnose,  $\beta$ -xylose,  $\beta$ -quinovose and *N*-

acetyl- $\beta$ -D-glucosamine. Also, the  $^{13}\text{C}$  NMR spectral data of CP05 showed eleven anomeric signals. The results of methylation analysis indicated that pulcherrimasaponin (CP05) contained xylose, arabinose and glucose substituted at C-1; xylose, arabinose and quinovose substituted at C-1 and C-2; xylose substituted at C-1 and C-3; 2-deoxy-*N*-acetyl-glucosamine substituted at C-1 and C-6; rhamnose substituted at C-1, C-3 and C-4; glucose substituted at C-1, C-2 and C-6. The sequence of the oligosaccharide chain was deduced from methylation analysis and  $^{13}\text{C}$  chemical shift comparisons between individual sugar residues and model compounds [3,5]. Based on the above findings, the UV and IR spectral data, the acid hydrolysis results, the molar carbohydrate composition obtained and the determination of absolute configurations of the monosaccharides, the structure of pulcherrimasaponin (CP05) was elucidated as [3 $\beta$ ,16 $\alpha$ ,28[2*E*,6*S*[2*E*,6*S*(2*E*,6*S*)]]]-olean-12-en-28-oic acid 3-[[*O*- $\alpha$ -L-arabinopyranosyl-(1 $\rightarrow$ 2)-*O*- $\alpha$ -L-arabinopyranosyl-(1 $\rightarrow$ 6)-2-(acetylamino)-2-deoxy- $\beta$ -D-glucopyranosyl]oxy]-16-hydroxy-*O*- $\beta$ -D-glucopyranosyl-(1 $\rightarrow$ 3)-*O*-[*O*- $\beta$ -D-xylopyranosyl-(1 $\rightarrow$ 3)- $\beta$ -D-xylopyranosyl-(1 $\rightarrow$ 4)-*O*-6-deoxy- $\alpha$ -L-mannopyranosyl-(1 $\rightarrow$ 2)-6-*O*-[6-[[2-*O*-2,6-dimethyl-1-oxo-6-( $\beta$ -D-xylopyranosyloxy)-2,7-octadienyl]-[(6-deoxy- $\beta$ -D-glucopyranosyl)oxy]-2,6-dimethyl-1-oxo-2,7-octadienyl]- $\beta$ -D-xylopyranosyl]oxy]-2,6-dimethyl-1-oxo-2,7-octadienyl]- $\beta$ -D-glucopyranosyl ester.

The comparative analysis of saponin in vivo toxicity showed that neither QS21 nor CP05 was lethal even using a higher dose than the one used for vaccine formulation. Both of them showed similar transitory side effects with a slightly higher haemolytic potential for QS21. The HD<sub>50</sub> reported here for QS21 isolated from Riedel de Haen saponin is even higher than that disclosed for QS21 isolated from QuilA mixture (18  $\mu\text{g}/\text{ml}$ ) [15].

The haemolytic activities of saponins are related to their chemical composition [21,28]. The haemolytic activities were abolished after chemical removal of the sugar moieties of the saponins [29]. Also, the steroidal saponins proved to be more haemolytic than the triterpenoidal ones [21,28]. The QS-21 saponin, although having a triterpenoidal nature, is strongly haemolytic [15,30,31]. In this case, the degree of haemolytic activity was shown to be related to the presence of side chains bearing aglycone (sugar chains) [21] or acyl residues or the epoxy framework system [31]. All QS-fractions isolated from *Quillaja saponaria* showed a monomer size consistent with the molecular weight predicted for a triterpene with 8–10 monosaccharide residues [32]. The haemolytic potential of QuilA mixture or QS-21 could be related then to their increased number of monosaccharides and the complexity of their glycidic moieties. Indeed, QS-21 consists of quillaic acid with one branched trisaccharide and one unbranched tetrasaccharide attached, and a dimeric normonoterpene acyl group attached to the first sugar of the tetrasaccharide by an ester linkage. An eighth sugar is attached to the normonoterpene acyl group [30]. The presence of normonoterpene acids could also favour in-

teractions between the saponin and membrane cholesterol promoting the haemolysis. In the case of CP05 saponin, the haemolytic activity could be then related as well to its complex structure: one triterpenoidal nucleus attached to eleven sugar units, distributed as two branched trisaccharide and pentasaccharide moieties in the extremities of the aglycone, and three monoterpene residues joined by ester linkages to sugar units. The structural similarities between CP05 saponin from *Calliandra pulcherrima* and QS-21 saponin from *Quillaja saponaria* may help to explain the similar haemolytic activity of both substances [15 and this investigation].

The comparative analysis of the antibody titers, after mouse vaccination with FML, showed a very potent induction of the humoral response by both QS21 and CP05 saponins. As expected for a systemic vaccination (sc route), the differences in IgA antibodies were not significant but the main response was obtained in the IgG class. In previous work, we showed that QS21 induced the highest serological response to FML in all subtypes, when compared to BCG, IL12, QuilA and Riedel de Haen mixtures [15]. Also, the protective response induced by QS21 in the murine model was maximal and compatible with that of a Riedel de Haen saponin and a QuilA mixture [15]. These saponin mixtures are the adjuvants for the canine immunoprophylactic licensed [33–35] and experimental vaccines [36] against visceral leishmaniasis. The similarities in the potency of the humoral response induced by the QS21 and CP05–FML formulations are then probably also related to their similarities in composition and structure. The main difference found between the vaccines was that the QS21–FML vaccine induced a specific 1.5–2.1-fold increase in IgG, IgG2a and IgG3 over the CP05FML formulation. It has been proposed that the aldehyde group linked to C4 of the QS21 triterpene binds to the T cell receptor forming an imine that provides T cells with a B7-CD28 independent co-stimulatory signal, leading to T cell activation and TH1 immunity [1,37]. The aldehyde group then mimetizes the B7 ligand and potentializes the T cell response. Although the TH1 IgG2b subtype is enhanced by both saponins, the presence of the aldehyde group in QS21 but not in CP05 could then explain the increased induction of the typical TH1 IgG2a subtype. The position of the aldehyde group was shown to be determining the increase of a specific humoral response against FML. Indeed, the aldehyde in equatorial position was shown to be related to an effective humoral response induced by the sapogenin fraction of Riedel de Haen saponin [29].

In infections with intracellular obligatory parasites such as *Leishmania (L.) donovani*, only an effective cellular immune response might control the advance of the disease. A positive intradermal response to leishmanial antigen, in murine [15], canine [33,34,36] and human visceral leishmaniasis [38] reflects the reactive status of the immune system and is present at the beginning of the infection, suppressed with its progress and only recovered after repeated treatment. Human [39] and canine visceral leishmaniasis [40] provoke

a cellular immunosuppressive status. Asymptomatic or resistant dogs show a lymphoproliferative antigen-specific in vitro response and an in vivo cellular immune response expressed by a positive IDR to leishmanial antigens [41,42] indicating a degree of natural protection against the disease. In these dogs, high levels of IL2 and TNF were observed.

Conversely, susceptible or symptomatic dogs showed suppression of the cellular immune response in vivo or in vitro and a failure of IL2 and TNF production [40]. These results suggest a different kind of response of the TH1 or TH2 effector T lymphocytes (CD4), capable of determining resistance or susceptibility to the canine disease. Apparently, the absence of an in vitro response observed in the susceptible dogs is antigen-specific [40]. CD4 lymphocytes are predominant in Type 4 of delayed type hypersensitivity reactions. CD4 lymphocytes from the TH1 subset are involved in the intradermal response to *Leishmanial* antigens in the murine model [43].

A protective vaccine against visceral leishmaniasis should be formulated with an effective adjuvant that will enhance and maintain the specific IDR response in vaccinees [15,34,36]. In this investigation, the mean average of IDR was 0.76 mm for QS21 and 0.78 mm for CP05 with no significant differences. These values were higher than those previously described for QS21 purified from QuilA by Santos et al. (2002) [15] using the murine model (0.6 mm). Considering that the intradermal response to the leishmanial antigen in vaccinated mice is mediated by CD4 helper cells [43], the capability of the non-aldehyde containing saponin CP05 of inducing an effective IDR indicates that other moieties in the molecule stimulate CD4 helper cells as well. Vaccination with the sapogenin fraction of CP05 in the murine model should clarify this question. Our results disclosed the relevant adjuvant potential of the newly described CP05 saponin of *Calliandra pulcherrima* Benth pointing out its possible use in new vaccine formulations.

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