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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 [3β,16α,28[2*E*,6*S*[2*E*,6*S*(2*E*,6*S*)]]]-olean-12-en-28-oic acid 3-[[*O*-α-L-arabinopyranosyl-(1→2)-*O*-α-L-arabinopyranosyl-(1→6)-2-(acetylamino)-2-deoxy-β-D-glucopyranosyl]oxy]-16-hydroxy-*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)-6-*O*-[6-[[2-*O*-2,6-dimethyl-1-oxo-6-(β-D-xylopyranosyloxy)-2,7-octadienyl]-[(6-deoxy-β-D-glucopyranosyl)oxy]-2,6-dimethyl-1-oxo-2,7-octadienyl]-β-D-xylopyranosyl]oxy]-2,6-dimethyl-1-oxo-2,7-octadienyl]-β-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 μg/ml) than for CP05 (13 μg/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.

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 normonoterpenes 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 ligang (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 using 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_5 (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. ^{1}H NMR spectra were recorded at 200 MHz and ^{13}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_{254}, Merck) using the following solvent systems: (A) $CHCl_3$–MeOH–H_2O (55:45:5, v/v/v) for triterpenoidal saponin CP05; (B) $CHCl_3$–MeOH (95:5 (v/v)) for sapogenin and (C) *n*-BuOH–C_5D_5N–H_2O (60:40:30, v/v/v) for monosaccharides. Spray reagents were orcinol–sulfuric acid for triterpenoidal saponin CP05 and monosaccharides, and $CeSO_4$ 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 $CHCl_3$–MeOH–H_2O (55:45:5, v/v/v) afforded one homogeneous compound CP05 (285 mg), R_f 0.50 which gave a dark blue color with orcinol–H_2SO_4.

The QS21 saponin was isolated from Riedel De Haen, Saponin pure® (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_f 0.43 which gave a dark blue color with orcinol–H_2SO_4. 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 ^{1}H and ^{13}C NMR data with the literature [16].