JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Immunoadjuvant Activity, Toxicity Assays, and Determination by UPLC/Q-TOF-MS of Triterpenic Saponins from *Chenopodium quinoa* Seeds

Simone G. Verza,*[†] Fernando Silveira,[‡] Samuel Cibulski,[§,∥] Samuel Kaiser,[†] Fernando Ferreira,[‡] Grace Gosmann,[†] Paulo M. Roehe,[§,∥] and George G. Ortega[†]

[†]Programa de Pós-graduação em Ciências Farmacêuticas, Faculdade de Farmácia, Universidade Federal do Rio Grande do Sul, Porto Alegre, Av. Ipiranga 2752, 90610-000 Porto Alegre, RS, Brazil

[‡]Laboratorio de Carbohidratos y Glicoconjugados, Facultad de Química y Departamento de Desarrollo Biotecnológico, Facultad de Medicina, Instituto de Higiene, UdelaR., Av. Alfredo Navarro 3051, Montevideo, Uruguay

[§]Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil

[∥]Equipe de Virologia, FEPAGRO Saúde Animal, Instituto de Pesquisas Veterinárias Desidério Finamor, Eldorado do Sul, RS, Brazil

S *Supporting Information*

ABSTRACT: The adjuvant activity of *Chenopodium quinoa* (quinoa) saponins on the humoral and cellular immune responses of mice subcutaneously immunized with ovalbumin (OVA) was evaluated. Two quinoa saponin fractions were obtained, FQ70 and FQ90, and 10 saponins were determined by UPLC/Q-TOF-MS. Mice were immunized subcutaneously with OVA alone or adjuvanted with Quil A (adjuvant control), FQ70, or FQ90. FQ70 and FQ90 significantly enhanced the amount of anti-OVA-specific antibodies in serum (IgG, IgG1, and IgG2b) in immunized mice. The adjuvant effect of FQ70 was significantly greater than that of FQ90. However, delayed type hypersensitivity responses were higher in mice immunized with OVA adjuvanted with FQ90 than mice treated with FQ70. Concanavalin A (Con A)-, lipopolysaccharide-, and OVA-stimulated splenocyte proliferation were measured, and FQ90 significantly enhanced the Con A-induced splenocyte proliferation. The results suggested that the two quinoa saponin fractions enhanced significantly the production of humoral and cellular immune responses to OVA in mice.

KEYWORDS: *Chenopodium quinoa, saponins, UPLC/Q-TOF-MS, immunoadjuvant, OVA*

■ INTRODUCTION

The seeds of *Chenopodium quinoa* Willd (quinoa) have been harvested for centuries in American countries, specifically in the Andean region.[1,2] The quinoa seeds have a high nutritional value. They contain a high protein content, in particular essential amino acids (lysine, methionine, and cystine) and carbohydrates.[1−4] High levels of energy, calcium, manganese, iron, phosphorus, copper, and potassium were identified in quinoa seeds that were used to prepare breads, cakes, and drinks, too.[1] The bitterness in quinoa seeds has been attributed to saponins, triterpenoid glycosides, which have been studied for their antimicrobial, antifungal, antiviral,[4,5] and immunoadjuvant activities, to enchance immune responses and mucosal responses.[6,7]

Immunoadjuvants are intended to enhance or modulate the humoral and/or cellular immune responses to antigens.[8,9] Aluminum salts have been the most widely used immunoadjuvants in humans for its capacity to induce strong humoral (Th2-related) immune responses, although cell-mediated immune response mechanisms (Th1-related), such as delayed type hypersensitivity (DTH), are poorly stimulated.[10] Although aluminum salts are generally regarded as safe, several potential side effects, such as the formation of granulomas, eosinophilia, myofasciitis, allergenicity, and neurotoxicity, have been associated with the usage of this type of adjuvants.[8,9]

As a general rule, the more active the immunoadjuvant potential, the higher the expected toxicity, as observed with the Freund's complete adjuvant or lipopolysacharide (LPS), which elicit both Th1 and Th2 responses. Thus, lower toxicity and higher immunoadjuvant capacity are great challenges in the research and development of adjuvants for human use.[9−11]

Concerning immunoadjuvant activity, particular attention has been drawn to *Quillaja saponaria* saponins.[12−15] Among those, Quil A, a saponin fraction extracted from *Q. saponaria* bark, has been shown in a number of studies to be a potent Th1- and Th2-stimulating adjuvant. However, Quil A toxicity has restrained its inclusion in preparations for human use.[16]

Besides Quillaja saponins, other vegetable saponins have been investigated as immunoadjuvants,[16−18] including *C. quinoa* Willd,[6,7] for which the results obtained were controversial. In view of that, in the present study, an evaluation was made on the immunoadjuvant activity of quinoa saponins on humoral and cellular immune responses of mice immunized subcutaneously with OVA. These were accompanied by studies to determine its toxicity. The chemical structure of the main saponins present in *C. quinoa* seeds was

Received: December 6, 2011
Revised: March 6, 2012
Accepted: March 6, 2012
Published: March 6, 2012

 © 2012 American Chemical Society dx.doi.org/10.1021/jf205010c | *J. Agric. Food Chem.* 2012, 60, 3113−3118

determined by ultraperformance liquid chromatography/quadrupole time-of-flight−mass spectrometry (UPLC/Q-TOF-MS).

MATERIALS AND METHODS

Plant Material. Quinoa real (*C. quinoa*) seeds were harvested in Cochabamba, Bolívia. The raw material was identified and air-dried at 35 °C during 72 h previously (Memmert, Germany).

Preparation of the Freeze-Dried Extract and Quinoa Saponin Fractions. The dried quinoa seeds (100 g) were extracted with 1 L of 40% v/v hydroethanolic solution by moderate magnetic stirring (IKA, Staufen, Germany) for 1 h at 50 °C. The mixture was filtered through a Whatmann paper filter no. 2, concentrated, and freeze-dried. The freeze-dried powder was stored in light-protected glass until use. Such an extract was named as EXQ (94% yield). An aliquot of EXQ (700 mg) was dissolved in 20 mL of water and fractionated onto a 50 cm × 2.8 cm column containing 40 g of Diaion HP 20 (Supelco, Bellefonte, PA). The column flow rate was 5.5 mL/min, following a decreasing polarity gradient with methanol:water mixtures (30, 50, 70, and 90% of methanol) of 500 mL each. The intermediate and the less polar fractions obtained after elution were concentrated under vacuum at 40 °C separately, freeze-dried, and coded as FQ70 (15.6% yield) and FQ90 (5.0% yield).

UPLC/Q-TOF-MS Conditions for Saponin Analysis. The saponin separation was performed employing a Waters Acquity Ultra Performance LC system (Waters, Milford, MA) equipped with a binary solvent manager, sample manager, and a photodiode array detector (PDA), coupled with a Q-TOF mass spectrometer. The data were processed using the MassLynx V4.1 software package. High-purity nitrogen was used as a nebulizer and auxiliary gas. Argon was used as a collision gas. ESI capillary voltage was set at +3.0 kV for positive ion mode. Source and desolvation temperatures were set at 100 and 250 °C, respectively. The desolvation and cone gas flows were 380 and 50 L/h, respectively. The sample cone voltage was set at 33 V, and the collision energy (CE) was set at 4 eV. The mass scan range was from 200 to 1500 *m/z*. An UPLC column Hypersil Gold RP-18 100 mm × 2.1 mm i.d., 1.9 μm (Thermo Scientific, Bellefonte, PA) was used. The mobile phase consisted of 0.1% aqueous formic acid (solvent A) and acetonitrile (solvent B). The gradient elution program was as follows: 0−1.3 min, 25.0% B; 1.3−4.5 min, 25.0−30.0% B; 4.5−7.5 min, 30.0−35.0% B; 7.5−10.5 min, 35.0−40.0% B; 10.5−13 min, 40.0% B; 13−17 min, 40.0 to 25% B; and 17−22 min, 25% B. The flow rate was 0.3 mL/min, the injection volume was 5 μL, and the analyses were performed at 30 ± 1 °C. The concentration of the FQ70 and FQ90 solutions was 50 μg/mL.

Toxicity Assays with Quinoa Saponin Fractions. *Hemolytic Activity Assay.* The microhemolytic assay was performed with a 1% guinea pig red blood cell (RBC) suspension in saline solution (SS). One hundred eighty microliters of RBC was mixed with 20 μL of each of the preparations under test in U-bottom microplates (Thermo Scientific). Quinoa saponin fractions FQ70 and FQ90 were assayed over a concentration range of 10 μg/mL to 10 mg/mL. Quil A (Breentag Biosector, Denmark) dilutions (from 500 to 5 μg/mL in SS) were tested in parallel. SS was included as a negative control. Microplates were incubated for 30 min at 37 °C and centrifuged at 700*g* for 5 min. A fixed volume (75 μL) of each supernatant was transferred to a flat-bottom microplate, and the absorbance was measured at 415 nm in an Anthos 2020 enzyme-linked immunosorbent assay (ELISA) reader (Biochrom, Cambridge, United Kingdom). The hemolytic activity was expressed as the concentration capable of causing 50% of the maximum hemolysis (HD_{50}). Each sample was tested in triplicate. HD_{50} values were calculated from the sigmoidal curves using the OriginPro 8 software.

Acute Toxicity against Artemia salina. The toxicity of the quinoa saponin fractions was evaluated in the *A. salina* (brine shrimp) lethality test.[19] *A. salina* encysted eggs (10 mg) (Maramar, Rio de Janeiro, Brazil) were incubated in 100 mL of seawater under artificial light at 28 °C and pH 7−8. After incubation for 48 h, nauplii were collected with a Pasteur pipet.

FQ70 and FQ90 samples were dissolved in water separately. A 50 μL aliquot of seawater was placed in each well of a 96-well microtiter plate. Subsequently, 50 μL aliquots of each of the quinoa saponin fractions at a starting concentration of 4.0 mg/mL were placed in the plate and serially (2-fold) diluted. The last row was left with seawater only as a control. One hundred microliters of a suspension containing about 10 larvae was added to each well; plates were then incubated at room temperature and examined after 24 h. Dead larvae in each well were counted after microscopic examination, and lethality was expressed as LC_{50} index calculated using Minitab 14.

Acute Toxicity in Mice. Acute toxicity of quinoa saponin fractions was tested by subcutaneous inoculation of 200 μL of phosphate-buffered sterile saline (PBS) containing 300 μg of either FQ70 or FQ90. Both saponin fractions were inoculated at the dorsal region of five 6 weeks old male mice of the CF-1 (for each preparation), in two doses, 1 week apart. Mice were monitored daily for 14 days in search for signs of toxicity (lethality, local swelling, loss of hair, and piloerection). A positive control group of five mice was inoculated with a solution of Quil A (50 μg dissolved in 200 μL of sterile saline). Negative control mice were inoculated with SS only. The experimental procedures were approved by the University's Research Ethics Committee (Protocol no. 2008211).

Adjuvant Effect of *C. quinoa* Saponins. *Animals.* Six weeks old male mice of the CF-1 breed were purchased from the Fundação Estadual de Produção e Pesquisa em Saúde (Porto Alegre, Brazil). Mice were housed in polypropylene cages, acclimatized for 1 week prior to use, and maintained at 22 ± 3 °C and a 12 h light−dark cycle. Feed and water were supplied ad libitum. All of the experimental procedures fulfilled the Guide for the Care and Use of Laboratory Animals[20] and were approved by the University's Research Ethics Committee (Protocol no. 2008211).

Antigen-Adjuvant Preparation and Mice Immunization Protocol. Seven groups of six mice each were randomly divided and inoculated subcutaneously in the hind neck on days 1 and 14. Animals were immunized with 100 μg of ovalbumin (OVA) (Profos AG, Regensburg, Germany) alone or dissolved in saline containing two doses of FQ70 (133 and 266 μg), FQ90 (133 and 266 μg), or 50 μg of Quil A. All experimental vaccine were prepared, filtered through 0.22 μm filters, and kept at 4 °C until use. Blood samples were collected at day 28 after a first immunization for further assaying.

Immunoassays for Antibodies. Total concentrations of anti-OVA (IgG) and specific IgG1 and IgG2b were determined in each individual serum sample by ELISA, as follows: ELISA plates (Greiner Bio-One, Netherlands) were coated with OVA antigen at a previously determined antigen dilution (1:100 v/v) in acetate buffer (pH 5.0) and incubated overnight at 4 °C. Plates were then washed three times with PBS containing 0.05% Tween-20 (PBS-T) and blocked with bovine serum albumin solution (1% in PBS; Sigma, St. Louis, MO) at 37 °C for 1 h. One hundred microliters of each diluted serum sample (1:1000 and 1:2000) was added in duplicate to wells and incubated for 1 h at 37 °C. Subsequently, plates were washed three times with PBS-T, and 50 μL/well of an appropriate dilution of the following peroxidase conjugates was added: antimouse IgG (Sigma) diluted 1:10000 in PBS-T, IgG1 (Sigma) diluted 1:5000 in PBS-T, or IgG2b (Sigma) diluted 1:5000 in PBS-T. Plates were then incubated for 1 h at 37 °C. After another round of washes with PBS-T, 100 μL/well of *ortho*-phenylenediamine (Sigma) plus 0.003% H_2O_2 (Merck, Darmstadt, Germany) was added, and the plates were incubated for 15 min at 37 °C. The reaction was stopped by addition of 1 N HCl 50 μL/well (Merck). The absorbance was measured in an Anthos 2020 ELISA reader (Biochrom) at 492 nm. In each plate, a positive control serum sample (a pool of positive sera) was used to plot a standard curve. Antibody titers were expressed in arbitrary units (AU/mL) determined by comparison with the standard curve.

DTH Assay. DTH responses were assayed 28 days postpriming. Briefly, 10 μL of OVA was subcutaneously injected in the right footpad of the mice's hind limb, in three mice from each group. The thickness

of the injected footpads was measured 24 h later with a caliper. The average induration measured on the footpad of mice from the control group (injected with SS) was deduced from all readings.

Splenocyte Proliferation Assay. Splenocytes were collected 28 days after the second immunization under aseptic conditions, immersed in RPMI 1640 (Invitrogen, NY), minced, and mechanically dissociated to obtain a homogeneous cell suspension. Erythrocytes were lysed with ammonium chloride (0.8%, w/v). After centrifugation (380*g* at 4 °C for 10 min), pelleted cells were washed three times in RPMI 1640 and resuspended in the same medium supplemented with 0.05 mM 2-mercaptoethanol, 100 IU/mL penicillin, 100 μg/mL streptomycin, 2 mM L-glutamine, and 10% fetal bovine serum. By trypan blue dye exclusion, cell counting revealed >95% viability. Splenocytes were seeded at 2.5×10^5 cell/mL in 100 μL of complete medium into each well of a 96-well flat-bottom microtiter plate. Subsequently, 200 μL/well of concanavalin A solution (Con A) at 5 μg/mL (Biochrome, Berlin, Germany), OVA (10 μg/mL), lipopolysaccharide (LPS) at 10 μg/mL (Biochrome, Berlin, Germany), or medium only was added. Plates were then incubated at 37 °C in a humid atmosphere with 5% CO_2. After 44 h, 50 μL of MTT (Sigma) solution (2 mg/mL) was added to each well and incubated for 4 h. The plates were centrifuged at 1400*g* for 5 min, and the untransformed MTT was removed carefully by pipetting. Next, a dimethyl sulfoxide (DMSO) solution (192 μL of DMSO; 8 μL of 1 N HCl) was added to wells in volumes of 100 μL. After 15 min of incubation, the absorbance was measured in an ELISA reader at 550 nm with wavelength reference fixed at 620 nm. The stimulation index (SI) was calculated as the ratio A_1/A_2, where A_1 is the absorbance coefficient recorded for mitogen-stimulated cultures, and A_2 is the absorbance of non mitogen-stimulated cultures. Contaminant endotoxins were analyzed by a gel-clot Limulus amebocyte lysate assay, to avoid false positive of the immunoadjuvant activity of quinoa saponins.

Statistical Analysis. The data were expressed as means ± standard errors (SEs). One-way analysis of variance followed by Tukey's test of multiple comparisons was used to compare the parameters between. Values of $P < 0.05$ were considered significant.

■ RESULTS AND DISCUSSION

Determination of Quinoa Saponins in FQ70 and FQ90 by UPLC/Q-TOF-MS. In a previous work, the total saponin content in FQ70 and FQ90 was determined at 83.3 and 68.8 g %, respectively (data not shown), by LC method. The total ion current (TIC) chromatograms obtained by the UPLC/Q-TOF-MS analysis of FQ70 and FQ90 are shown in Figure 1.

The eight triterpenic saponins determined in FQ70 are bidesmosides and derived from five different aglycones moieties linked to different sugar side chains (Table 1). Apart from two less polar saponins observed in FQ90, derived from oleanolic acid and hederagenin, the saponin compositions of both FQ70 and FQ90 are rather similar (Figure 1 and Table 1). The structures of quinoa saponins were determined based on molecular weight, calculated from their respective pseudomolecular ions ($[M + H]^+$ or $[M + Na]^+$), their respective MS spectra, and comparison with literature data. The compound comprising an acidic sugar (compound **8**) Table 1) gave an additional signal at $[M + 2Na - H]^+$, which is probably arising from the sodium salt.[21]

All saponins identified in FQ70 and FQ90 had been reported previously in *C. quinoa* seeds.[22,23] In accordance with Madl et al.[21] and Gómez-Caravaca et al.,[2] we grouped the saponins by their aglycone moiety. Because of the different polarity of the phytolaccagenic acid, its derivatives are eluted first, hederagenin- and serjanic acid-derived saponins are eluted at intermediate retention times, and oleanolic acid-derived saponins are eluted at the end of the chromatographic run (Figure 1). The major peaks at 4.68 and 8.85 min in the quinoa

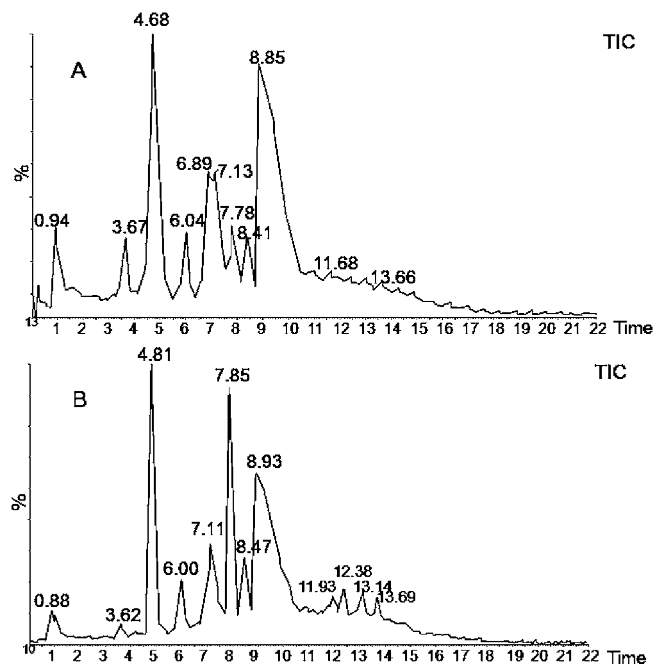


Figure 1. TIC chromatograms of FQ70 (A) and FQ90 (B).

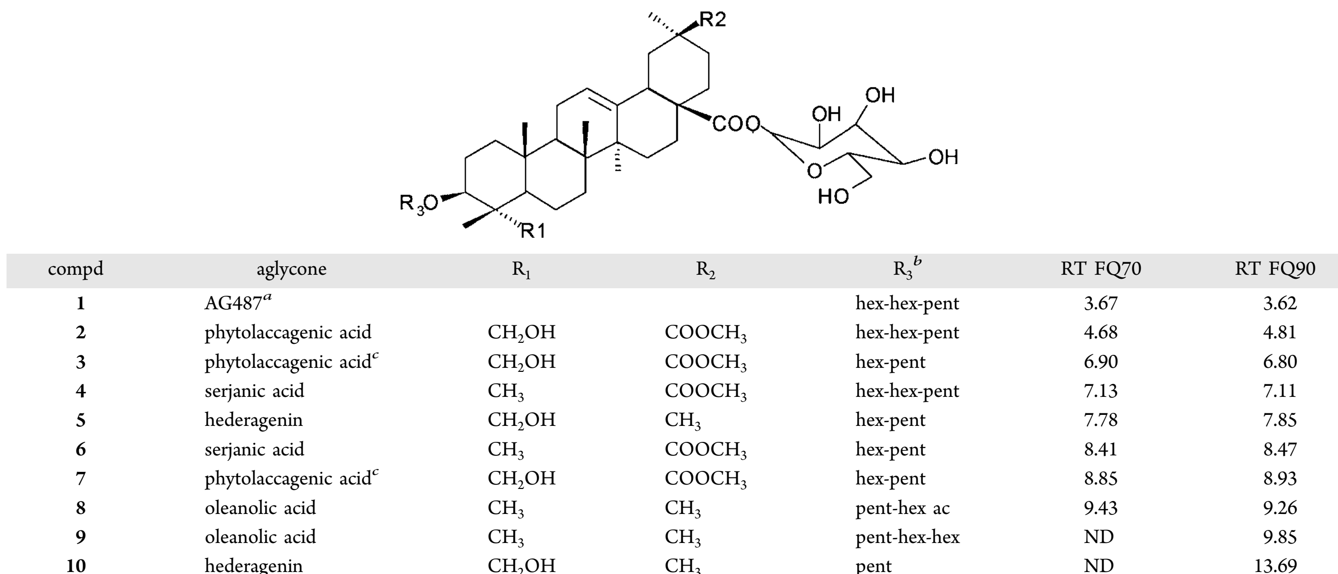
saponin fractions were both ascribed to phytolaccagenic acid saponins, in agreement with Madl et al.[21]

FQ90 contains four additional saponins (Figure 1) whose aglycone moieties were identified from the mass spectrometric data. Neither the anomeric configurations of their glycosidic linkages nor the identity of the monosaccharide residues could be defined because of limits of the MS. The peak with retention time of 11.93 min corresponded to saponin derived from oleanolic acid, while the peak at 12.38 min corresponded to a serjanic acid-derived saponin.

Hemolytic Activity and Acute Toxicity Assays. The hemolytic activity of quinoa saponin fractions was assayed and compared to Quil A. The HD_{50} values for FQ70, FQ90, and Quil A were 820 ± 2.9, 61.5 ± 3.9, and 14.1 ± 2.6 μg/mL, respectively. As compared to the high hemolytic activity displayed by Quil A, the two quinoa saponin fractions were significantly less hemolytic. A previous study on the hemolytic activity of quinoa saponins[4] revealed that monodesmosides derived from oleanolic acid were hemolytic, while bidesmosides were nonhemolytic. Here, similarly, the chemical structures of saponins in FQ70 and FQ90 were bidesmosides, as determined by UPLC/Q-TOF-MS. The exact mechanism responsible for the hemolytic activity of saponins is actually unknown. Nonetheless, the saponin's amphiphily and specific chemical features in their structures are often quoted for that effect.[16,24,25]

Toxicity is a critical issue in the development of new immunoadjuvants. The brine shrimp assay is expected to detect physiologically active and toxic compounds.[19] FQ70 and FQ90 fractions behaved differently in the brine shrimp toxicity assay. While FQ70 was almost atoxic for the shrimps ($P < 0.05$), for FQ90 the calculated LC_{50} value was 138.0 μg/mL. These findings are indicative that physiologically active compounds remain in the less hydrophilic fraction (FQ90) of the quinoa saponin preparations under study. Despite their similarity, FQ90 have additional saponins derived from hederagenin and oleanolic acid, which may be accountable for its higher toxicity to brine shrimps. Toxicity to *A. salina* has also been earlier

Table 1. Triterpenoid Saponins Determined in FQ70 and FQ90 Fractions of *C. quinoa* after UPLC/Q-TOF-MS Analysis



compd	aglycone	R_1	R_2	R_3[b]	RT FQ70	RT FQ90
1	AG487[a]			hex-hex-pent	3.67	3.62
2	phytolaccagenic acid	CH_2OH	$COOCH_3$	hex-hex-pent	4.68	4.81
3	phytolaccagenic acid[c]	CH_2OH	$COOCH_3$	hex-pent	6.90	6.80
4	serjanic acid	CH_3	$COOCH_3$	hex-hex-pent	7.13	7.11
5	hederagenin	CH_2OH	CH_3	hex-pent	7.78	7.85
6	serjanic acid	CH_3	$COOCH_3$	hex-pent	8.41	8.47
7	phytolaccagenic acid[c]	CH_2OH	$COOCH_3$	hex-pent	8.85	8.93
8	oleanolic acid	CH_3	CH_3	pent-hex ac	9.43	9.26
9	oleanolic acid	CH_3	CH_3	pent-hex-hex	ND	9.85
10	hederagenin	CH_2OH	CH_3	pent	ND	13.69

[a]The exact structure was not determined.[21] [b]Hex, hexose; Pent, pentose; and HexAc, hexuronic acid. [c]The compounds agree with the oligosaccharide sequence and aglycones, but the sugar anomeric configuration cannot be distinguished by MS; RT, retention time (min.); ND, not detected.

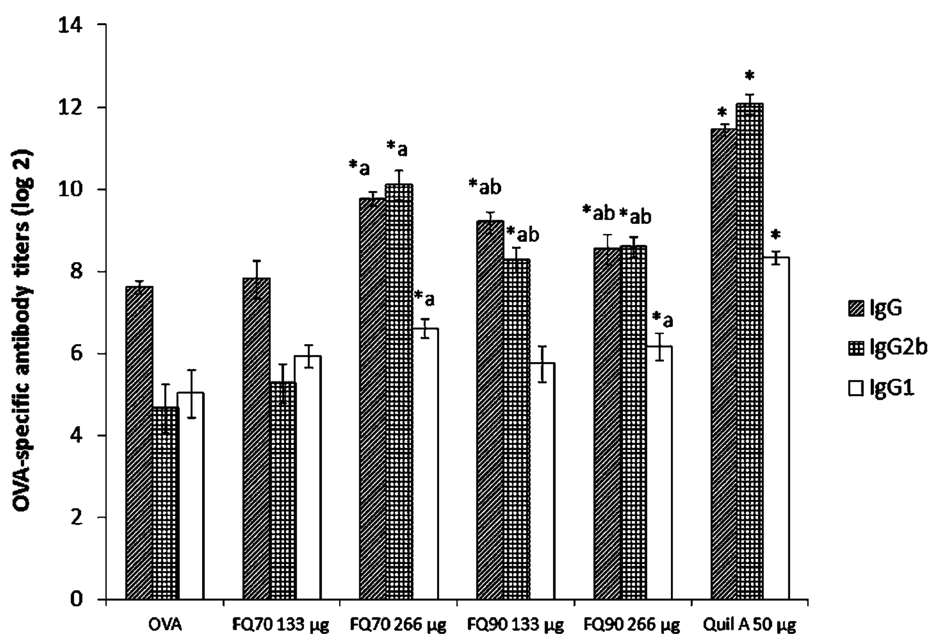


Figure 2. OVA-specific serum IgG, IgG1, and IgG2b antibodies in OVA-immunized mice. The values are presented as means ± SEs. Significant differences with the OVA group are indicated as $^*P < 0.05$; those with OVA/Quil A are indicated as $^a\ P < 0.05$.

demonstrated in saponins derived from hederagenin and oleanolic acid.[26]

Regarding acute toxicity, no lethality was detected after subcutaneous administration to mice of up to 300 μg of both FQ90 and FQ70. In addition, no signs of local toxicity (local swelling, loss of hair, and piloerection) were observed in mice inoculated with 300 mg of FQ70. On the other hand, in control mice inoculated with 50 μg of Quil A, no lethality was observed. However, local swelling and continuous piloerection were detected in all control mice. In view of these findings, it was decided to test the immunoadjuvant activity of FQ70 and FQ90 at a concentration below of 300 μg per dose of immunogen.

Immunoadjuvant Activity. The search of new immunoadjuvants based on plant-derived saponins has increased in recent times in search for safer and more effective vaccines for animals and humans.[9,12,15,16,24,25,27−32] In view of that, in the present study, the immunoadjuvant activity of quinoa saponins was evaluated on the humoral (Th2-related) and cellular immune responses (Th1-related) of mice immunized against OVA. The endotoxin level was less than 0.5 EU/mL in quinoa saponin fractions, indicative of no endotoxin contamination.

Effect of Quinoa Saponin Fractions on the OVA-Specific Serum Antibody Responses. The OVA-specific IgG, IgG1, and IgG2b antibody titers in mice sera were measured 28 days after the first immunization (Figure 2). Significant enhancement in

OVA-specific IgG, IgG2b, and IgG1 titers ($P < 0.05$) was observed in mice immunized with preparations adjuvanted with Quil A, FQ70 (266 μg), and FQ90, when compared to control mice inoculated with nonadjuvanted (OVA).

The IgG isotype responses of OVA-immunized mice were selectively stimulated, depending on the quinoa saponin fraction administered. There were no significant differences among the OVA-specific IgG isotypes levels in mice immunized with FQ70 at a dosage of 133 μg and mice immunized with OVA only. However, the IgG and IgG2b responses to FQ70 at 266 μg were more significantly stimulated than FQ90 (133 and 266 μg). Therefore, these findings indicate that quinoa saponins fractions, mainly FQ70, the less hemolytic ones, could significantly result in the increase of serum antibody production in mice immunized with OVA.

Recently, immunoadjuvant and hemolytic activities of saponins from several vegetable sources have been investigated, including plants such as *Achyranthes bidentata*, *Bupleurum chinense*, *Glycyrrhiza uralensis*, *Anemone radeana*, and *Platycodon grandiflorum*.[9,25,29,30] The saponins extracted from these five species were able to enhance immune responses with less side effects than Quil A, as estimated by its hemolytic activities.[9,25,29,30] Evidence accumulated in recent years suggests that the immunoadjuvant effect of saponin preparations does not necessarily correlate with its hemolytic activities.[9,25,29,30] Thus, saponins that possess significantly adjuvant effect with relatively lower hemolytic activity may be interesting candidates for future use as adjuvants as FQ70. In the present study, both saponin fractions, FQ70 and FQ90, were able to boost immune responses when compared to controls. However, as FQ70 was shown to be less hemolytic, this fraction seems more attractive for further studies on widening its application as a vaccine adjuvant. Future studies should concentrate on separation and evaluation of immunoadjuvant activity of individual saponins present in FQ70.

DTH Response. Statistical analysis revealed significant differences between groups injected with OVA alone and those treated with OVA adjuvanted with FQ90 (133 μg) and Quil A (50 μg) (Figure 3).

No significant differences were observed between the OVA group and the groups of FQ70 (133 and 266 μg) and FQ90 (266 μg). FQ70, which promoted a higher serum antibody response than FQ90 in mice immunized with OVA, conversely presents a low DTH response. These results suggest a

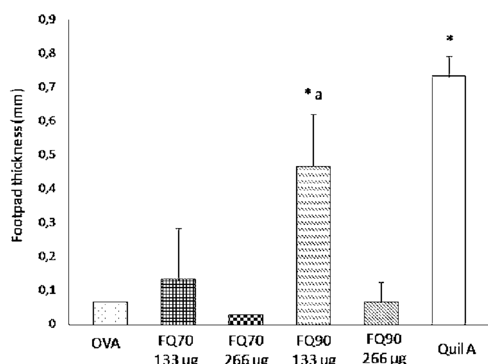


Figure 3. DTH responses in injected mice with OVA-antigen used in the preparation of the vaccines. The values are presented as means ± SEs. Significant differences with OVA groups are indicated as $^{*}P < 0.05$; those with the OVA/Quil A group are indicated as $^{a}P < 0.05$.

manifestation of cell-mediated immune response by FQ90, while FQ70 induces a humoral immune response.

Effect on Splenocyte Proliferation. The effects of the two quinoa saponin fractions on mitogen- and OVA-stimulated splenocyte proliferation assays were assessed in mice inoculated with the different antigen preparations (adjuvanted and nonadjuvanted) by the MTT colorimetric assay (Figure 4).

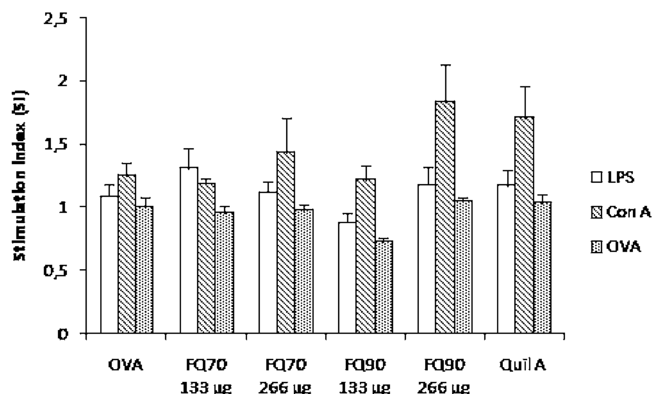


Figure 4. Effect of quinoa saponin fractions on mitogen- and OVA-stimulated splenocyte proliferation in OVA-immunized mice. The values are presented as means ± SEs. Significant differences with OVA groups are designated as $^{*}P > 0.05$.

Quil A and FQ90 (266 μg) significantly stimulated Con A-stimulated splenocyte proliferation in OVA-immunized mice ($P > 0.05$). However, no significant differences were observed in the Con A-, LPS-, and OVA-induced splenocyte proliferation among groups inoculated with OVA, OVA plus FQ70, and OVA plus FQ90 (133 μg). Furthermore, no significant differences were observed in the LPS- and OVA-induced splenocyte proliferation among OVA/Quil A and OVA groups. These findings suggested that FQ90 at a concentration of 266 μg exerted some stimulus on the splenocyte proliferation in mice immunized with OVA. FQ90 significantly enhanced the activation of T and B cells in mice immunized with OVA, therefore a substantial potential on the cellular immune responses.

The results obtained here suggest that FQ70 and FQ90 selectively modulate the type of immune response. FQ70 seems to exert its adjuvant effect only on Th2-related responses, whereas FQ90 seems to be able to stimulate both Th1 and Th2 responses, although the latter to a minor extent. FQ90 was more hemolytic and more toxic to brine shrimps than FQ70; moreover, it was less effective in stimulating humoral immune responses to OVA in mice than FQ70. These findings indicate that, indeed, the two quinoa saponin fractions examined here have distinct adjuvant properties, where modifications in the composition of saponins seem to influence its adjuvant activity. Thus, oleanolic acid derivatives may be involved in the Th1 responses induced by FQ90. These results are supported by other studies focusing the immunoadjuvant activity of oleanane type saponins from *G. uralensis*[25] and *A. bidentata*.[29] Similarly, the phytollacagenic acid-derived saponins, the predominant peaks in quinoa saponin fractions, seem to be correlated to the Th2 response induced above all by FQ70 rather than FQ90.

Further promising results with regard to the potential use of quinoa saponin as immunoadjuvant were achieved after intragastric and intranasal coadministration with either cholera toxin or OVA.[7] In such cases, IgG and IgA antibody titers were

potentiated, whereas IgG2a levels remained unaffected. The similarity between results reported by those researchers and those presented here with FQ70 and FQ90 will be further extended to provide a more complete picture on the immunoadjuvant activities of such saponins fractions.

■ ASSOCIATED CONTENT

Supporting Information

MS spectra of *C. quinoa* saponins present in FQ70 and FQ90. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Tel: +55 5133085278. Fax: + 55 5133085437. E-mail: simofar@gmail.com.

Funding

This study was financially supported by Conselho Nacional de Desenvolvimento Cientifico e Tecnológico (CNPq)—PROSUL project.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We are grateful to Centro de Desenvolvimento Tecnológico Farmacêutico (CDTF-UFRGS) for technical support.

■ REFERENCES

(1) Kuljanabhagavad, T.; Thongphasuk, P.; Chamulitrat, W.; Wink, M. Triterpene saponins from *Chenopodium quinoa* Willd. *Phytochemistry* **2008**, *69*, 1919−1926.

(2) Gómez-Caravaca, A. M.; Segura-Carretero, A.; Fernández-Gutiérrez, A.; Caboni, M. F. Simultaneous determination of phenolic compounds and saponins in quinoa (*Chenopodium quinoa* Willd) by a liquid chromatography-diode array detection-electrospray ionization-time-of-flight mass spectrometry methodology. *J. Agric. Food Chem.* **2011**, *59*, 10815−10825.

(3) Dini, I.; Schettino, O.; Simioli, T.; Dini, A. Studies on the constituents of *Chenopodium quinoa* seeds: isolation and characterization of new triterpene saponins. *J. Agric. Food Chem.* **2001**, *49*, 741−746.

(4) Woldemichael, G. M.; Wink, M. Identification and biological activities of triterpenoid saponins from *Chenopodium quinoa*. *J. Agric. Food Chem.* **2001**, *49*, 2327−2332.

(5) Stuardo, M.; San-Martín, R. Antifungal properties of quinoa (*Chenopodium quinoa* Willd) alkali treated saponins against *Botrytis cinerea*. *Ind. Crop. Prod.* **2008**, *27*, 296−302.

(6) Bomford, R.; Stapleton, M.; Winsor, S.; Beesley, J. E.; Jessup, E. A.; Price, K. R.; Fenwink, G. R. Adjuvanticity and ISCOM formation by structurally diverse saponins. *Vaccine* **1992**, *10*, 571−577.

(7) Estrada, A.; Li, B.; Laarveld, B. Adjuvant action of *Chenopodium quinoa* saponins on the induction of antibody responses to intragastric and intranasal administered antigens in mice. *Comp. Immunol. Microb.* **1999**, *21*, 225−236.

(8) Bruner, R.; Jensen-Jarolin, E.; Pali-Schöll, I. The ABC of clinical and experimental adjuvants—A brief of overview. *Immunol. Lett.* **2010**, *128*, 29−35.

(9) Sun, Y.; Mingquan, L.; Liu, J. Haemolytic activities and adjuvant effect of *Anemone raddeana* saponins (ARS) on the immune responses to ovalbumin in mice. *Int. Immunopharmacol.* **2008**, *8*, 1095−1102.

(10) Brewer, J. M. How do aluminium adjuvants work? *Immunol. Lett.* **2006**, *102*, 10−15.

(11) Aguilar, J. C.; Rodriguez, E. G. Vaccine adjuvants revisited. *Vaccine* **2007**, *25*, 3752−3762.

(12) Barr, I. G.; Sjölander, A.; Cox, J. C. ISCOMs and other saponin based adjuvants. *Adv. Drug Delivery Rev.* **1998**, *32*, 247−271.

(13) Kensil, C. R.; Patel, U.; Lennick, M.; Marciani, D. Separation and characterization of saponins with adjuvant activity from *Quillaja saponaria* Molina Cortez. *J. Immunol.* **1991**, *146*, 431−437.

(14) Pearse, M. J.; Drane, D. ISCOMATRIX® adjuvant for antigen delivery. *Adv. Drug Delivery Rev.* **2005**, *57*, 465−474.

(15) Skene, C. D.; Sutton, P. Saponin-adjuvanted particulate vaccines for clinical use. *Methods* **2006**, *40*, 53−59.

(16) Sun, H.-X.; Xie, Y.; Ye, Y.-P. Advances in saponin-based adjuvants. *Vaccine* **2009**, *27*, 1787−1796.

(17) Oda, K.; Matsuda, H.; Murakami, T.; Katayama, S.; Ohgitani, T.; Yoshikawa, M. Relationship between adjuvant activity and amphipathic structure of soyasaponins. *Vaccine* **2003**, *21*, 2145−2151.

(18) Sun, H.-X.; Xie, Y.; Ye, Y.-P. ISCOM and ISCOMATRIX. *Vaccine* **2009**, *27*, 4388−4401.

(19) Meyer, B. N.; Ferrigni, N. R.; Putnam, J. E.; Jacobsen, L. B.; Nichols, D. E.; McLaughlin, J. L. Brine shrimp: A convenient general bioassay for active plant constituents. *Planta Med.* **1982**, *45*, 31−34.

(20) Committee for the Update of the Guide for the Care and Use of Laboratory Animals. *Guide for the Care and Use of Laboratory Animals*; The National Academic Press: WA, 2010.

(21) Madl, T.; Sterk, H.; Mittelbach, M. Tandem mass spectrometric analysis of a complex triterpene saponin mixture of *Chenopodium quinoa*. *J. Am. Soc. Mass Spectrom.* **2006**, *17*, 795−806.

(22) Dini, I.; Tenore, G. C.; Schettino, O.; Dini, A. New oleanane saponins in *Chenopodium quinoa*. *J. Agric. Food Chem.* **2001**, *49*, 3976−3981.

(23) Zhu, N.; Sheng, S.; Sang, S.; Jhoo, J.-W.; Bai, N.; Karwe, M. V.; Rosen, R. T.; Ho, C.-T. Triterpene saponins from debittered quinoa (*Chenopodium quinoa*) seeds. *J. Agric. Food Chem.* **2002**, *50*, 865−867.

(24) Marciani, D. J.; Press, J. B.; Reynolds, R. C.; Pathak, A. K.; Pathak, V.; Gundy, L. E.; Farmer, J. T.; Koratich, M. S.; May, R. D. Development of semisynthetic triterpenoid saponin derivatives with immune stimulating activity. *Vaccine* **2000**, *18*, 3141−3151.

(25) Sun, H.-X.; Pan, H.-J. Immunological adjuvant effect of *Glycyrrhiza uralensis* saponins on the immune responses to ovalbumin in mice. *Vaccine* **2006**, *24*, 1914−1920.

(26) Ma, W. W.; Heinstein, P. F.; McLaughlin, J. L. Additional toxic bitter saponins from the seeds of *Chenopodium quinoa*. *J. Nat. Prod.* **1989**, *52*, 1132−1135.

(27) Rajput, Z. I.; Hu, S.; Xiao, C.; Arijo, A. G. Adjuvant effects of saponin on animal immune responses. *J. Zheijang Univ.-Sci. B* **2007**, *8*, 153−151.

(28) Song, X.; Hu, S. Adjuvant activities of saponins from traditional chinese medicinal herbs. *Vaccine* **2009**, *27*, 4883−4890.

(29) Sun, H.-X. Adjuvant effect of *Achyranthes bidentata* saponins on specific antibody and cellular response to ovalbumin in mice. *Vaccine* **2006**, *24*, 3432−3439.

(30) Sun, H.-X. Haemolytic activities and adjuvant effect of *Bupleurum chinense* saponins on the immune responses to ovalbumin in mice. *Vaccine* **2006**, *24*, 1324−1331.

(31) Xie, Y.; Pan, H.; Sun, H.; Li, D. A promising balanced Th1 and Th2 directing immunological adjuvant saponins from the root of *Platycodon grandiflorum*. *Vaccine* **2008**, *26*, 3937−3945.

(32) Yang, Z.-G.; Sun, H.-X.; Fang, W.-H. Haemolytic activities and adjuvant effect of *Astragalus membranaceus* saponins (AMS) on the immune responses to ovalbumin in mice. *Vaccine* **2005**, *23*, 5196−5203.