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Chemical properties and adjuvant activity of a galactoglucomannan from *Acrocomia aculeata*

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ABSTRACT

A galactoglucomannan (GGM) with an estimated weight-average molar mass of 3.5×10^5 was obtained from an aqueous extract of the mesocarp of fruits of *Acrocomia aculeata* (Jacq.) Lodd. by fractionation by Sephacryl S-300 HR and Sephadex G-25. Chemical and spectroscopic studies indicated that GGM has a main chain of (1 → 4)-linked β-D-mannopyranosyl residues attached to an initial chain of (1 → 3)-linked β-D-galactopyranosyl residues and a terminal chain of (1 → 4)-linked α-D-glucopyranosyl residues which comprised galactose, glucose and mannose in the molar ratio of 18:22:60. The adjuvant potential of the polysaccharide on the cellular immune response against ovalbumin antigen was investigated using *in vivo* assays.

1. Introduction

As part of a search for bioactive natural products, we have undertaken the examination of the mesocarp of fruits of *Acrocomia aculeata* (Jacq.) Lodd. (Arecaceae). This palm is native to dry hillsides and open forests from Central America to Southern Brazil (Braga, 1976). The coconut-flavored seed kernels of the fruits are eaten raw. The mesocarp is a food rich in fixed oil, protein and crude fiber (Belén-Camacho et al., 2005). Brazilians eat it as a strengthening tonic (Pott & Pott, 1994). Galactoglucomannans (GGMs) have been found in mosses (Geddes & Wilkie, 1972), ferns (Bremner & Wilkie, 1971), secondary walls of gymnosperms (Tenkanen, Makkonen, Perttula, Viikari, & Teleman, 1997) and angiosperms (Kubacková, Karácsonyi, & Bilisics, 1992) and primary walls of monocotyledons (Jakimow-Barras, 1973) and dicotyledons (Alam & Richards, 1971; Eda et al., 1984; Fischer, Wegryzn, Hallett, & Redgwell, 1996; Schröder et al., 2001). GGMs are characterized by a backbone of (1 → 4)-linked β-D-glucopyranosyl units and (1 → 4)-linked β-D-mannopyranosyl units, with the latter substituted at *O*-6 with D-galactopyranosyl units. One of the more recent detailed structural characterizations was of the GGM isolated from kiwifruit (*Actinidia deliciosa*) (Schröder et al., 2001). Hannuksela and du Penhoat (2004) have reported an *O*-acetylated GGM isolated from spruce thermomechanical pulp with mannopyranosyl units acetylated at C-2 and C-3 with a degree of acetylation around 0.28–0.37. One of the more complex GGM was isolated from cultures of *Nicotiana plumbaginifolia* (Sims, Craik, & Bacic, 1997). The GGM backbone was predominantly alternating (1 → 4)-linked β-D-glucopyranosyl units and (1 → 4)-linked β-D-mannopyranosyl units with the latter substituted at *O*-6 with either single α-D-galactopyranosyl or β-D-galactopyranosyl-(1 → 2)-α-D-galactopyranosyl residues. Small amounts of arabinose and xylose were shown to be covalent residues of the GGM, attached at *O*-6 of the (1 → 4)-linked β-D-mannopyranosyl units. The present paper is concerned with the isolation, chemical characterization and evaluation of the immunoadjuvant activity of a GGM from the mesocarp of fruits of *A. aculeata*.

2. Materials and methods

2.1. Plant material

Fruits of *A. aculeata* (Jacq.) Lodd. were collected from the Botanical Garden of the Federal University of Rio de Janeiro (Rio de Janeiro, Brazil) in February 2004.

2.2. Analytical techniques

Carbohydrate content was analyzed by colorimetric assays according to the procedure of Dubois, Gilles, Hamilton, Hebers, and Smith (1956), without previous hydrolysis of the sample, and by gas chromatography–electron impact mass spectrometry (GC–EIMS) of the alditol acetates (Sawardeker, Sloneker, & Jeanes,

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1965). Protein content was analyzed by the method of Bradford (1976). The experimental data were tested for statistical differences using the Student's *t*-test. The weight-average molar mass of galactoglucomannan (GGM) from *A. aculeata* was estimated from the calibration curve of elution using dextrans of known molecular weight as standards (2,000,000, 413,000, 282,000, 148,000, 68,000, 37,000, 19,500 and 9500; Sigma) on Sephacryl S-300 HR (5 × 85 cm; Pharmacia) (Tomoda et al., 1990).

Dialysis was carried out using tubing with an M_r cut-off 12,000. The optical rotations were measured on a Perkin Elmer 243B polarimeter. Vis and IR spectra were measured on a Shimadzu UV-1601 and on a Perkin Elmer FT-IR 1600 spectrometers, respectively.

^{1}H and ^{13}C NMR spectra were obtained on a Bruker DRX-600 NMR spectrometer operating at 600 MHz for δ_H and 150 MHz for δ_C, in D_2O containing sodium 2,2-dimethyl-2-silapentane-5-sulfonate as an internal standard. Gas chromatography (GC) was carried out with flame ionization detector (FID), using a glass capillary column (0.31 mm × 25 m) SE-30. GC–EIMS were taken on a VG Auto SpecQ spectrometer operating at 70 eV. Thin-layer chromatography (TLC) of monosaccharides were performed on silica gel coated plates (Merck) in *n*-BuOH–pyridine–H_2O (6:4:3), and sugars were detected by spraying with orcinol–H_2SO_4 (Barreto & Parente, 2006). Paper chromatography was carried out on Whatman No. 1 paper using the following solvent systems: (A) EtOAc–pyridine–H_2O (2:1:2) and (B) *n*-PrOH–EtOAc–H_2O (7:2:1). Chromatographic detection reagent was alkaline silver nitrate (Buchala & Meier, 1973).

2.3. Extraction

Fresh mesocarps of *A. aculeata* (2 kg), previously cut into small pieces, were extracted with hot water (6 l) at 80 °C under stirring for 1 h. The aqueous extract was filtered through Whatman filter paper (4 μm) and the filtrate centrifuged. By precipitation with two volumes of EtOH (12 h stirring and 24 h standing at 4 °C), a resulting precipitate was obtained following centrifugation and subsequent lyophilization (yield: 3.574 g, 0.18%). The amorphous powder (3.574 g) was dissolved in 0.01% sodium sulfate (800 ml) and added to 5% cetyltrimethylammonium bromide (160 ml). After centrifugation, the supernatant was poured into two volumes of EtOH and the precipitate obtained was dissolved in water (500 ml), dialyzed and lyophilized to yield crude polysaccharide (yield: 420 mg). It was extracted from mesocarps according to Pereira, da Silva, Pereira, and Parente (2000).

2.4. Fractionation

A sample of the crude polysaccharide (100 mg) was dissolved in 0.1 M Tris–HCl buffer (2 ml; pH 7.0), and applied to a Sephacryl S-300 HR column (5 × 85 cm; 1650 cm^3) with a flow rate of 1 ml/min. The carbohydrate content of each fraction was measured by spectrometry according to the colorimetric method reported by Dubois et al. (1956). Fractions of 5 ml corresponding to the peak galactoglucomannan (GGM) (1250–1650 ml) (Fig. 1) were pooled, dialyzed and freeze-dried. The obtained powder was dissolved in water (2 ml) and applied to a Sephadex G-25 column (1.5 × 50 cm; 15 g) with a flow rate of 1 ml/min and 5 ml fractions were collected (100–150 ml). The obtained eluate was concentrated and lyophilized to yield GGM (23 mg). The carbohydrate content of the fractions was measured. This procedure was repeated four times to obtain GGM (92 mg). It was obtained according to Pereira et al. (2000).

2.5. Molar carbohydrate composition and D,L configurations

The molar carbohydrate compositions of GGM (1 mg) and its six partial acid hydrolysis products (100 μg each one) were 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, Gerwig, Vliegenthart, & Clamp, 1975). The configurations of the glycosides were established by capillary GC and GC–EIMS of their trimethylsilylated (−)-2-butylglycosides (Gerwig, Kamerling, & Vliegenthart, 1978).

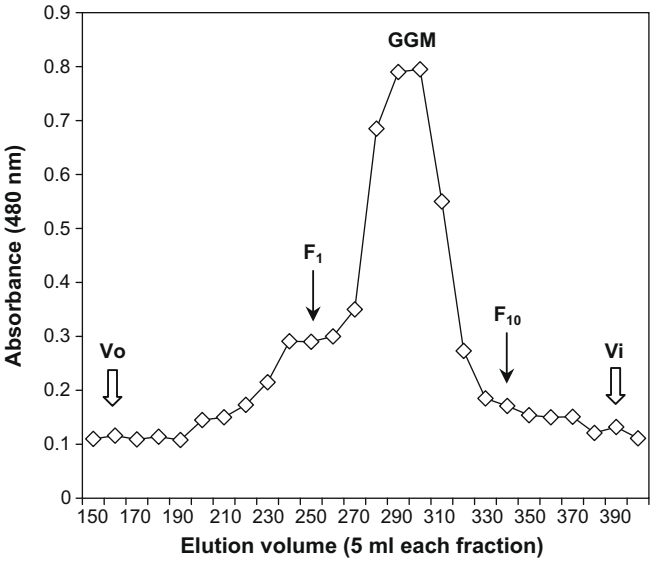

Fig. 1. Elution diagram of GGM polysaccharide from Sephacryl S-300 HR (0.1 M Tris–HCl). V_o, void volume; V_i, inner volume; F_1, initial fraction pooled; F_{10}, final fraction pooled.

2.6. Methylation analysis

GGM (1 mg) and its six partial acid hydrolysis products (100 μg each one) were dissolved in dimethylsulfoxide (200 μl) in a Teflon-lined screw-cap tube. Lithium methylsulfinyl carbanion (200 μl) was added to each solution under an inert atmosphere and the mixture was sonicated for 6 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). The chloroform phase was washed with (6 × 4 ml), dried (sodium sulfate), 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).

2.7. Periodate oxidation of GGM

GGM (25 mg) was dissolved in water (25 ml). After addition of 0.1 M sodium metaperiodate (25 ml) the reaction mixture was kept at 5 °C in the dark. The periodate consumption was measured by a spectrometric method (Dixon & Lipkin, 1954). The oxidation was completed after 5 days, then 2 ml of the solution were used for the measurement of formic acid liberation by titrating with 0.01 N sodium hydroxide after addition of one drop of ethylene glycol.

2.8. Smith degradation and analysis of products

The residue of the reaction mixture was successively treated with ethylene glycol (0.3 ml) and sodium borohydride (120 mg) at 5 °C for 16 h, then adjusted to pH 5 by addition of acetic acid.

The solution was concentrated and applied to a column (5.5 × 72 cm) of Sephadex G-15. Fractions were collected at 50 ml, and the eluates obtained from tubes 10 to 14 were combined, evaporated and lyophilized (Tomoda, Satoh, & Ohmori, 1978). The product (1 mg) was hydrolyzed with 1 N sulfuric acid at 100 °C for 6 h, the hydrolysates were derived to alditol acetates and determined by GC–EIMS as described by Sawardeker et al. (1965).

2.9. Partial acid hydrolysis of GGM

GGM (50 mg) was treated with 25 mM oxalic acid (20 ml) for 6 h at 100 °C. Only glucose and galactose were released. The insoluble residue was then heated with 0.1 M sulfuric acid for 6 h at 100 °C in a sealed tube. The neutralized hydrolysate was shaken with activated charcoal and filtered. The aqueous phase contained galactose, mannose and glucose and was discarded. The charcoal was then washed on a filter with 1%, 10% and 50% aqueous ethanol solutions. Thin-layer chromatography on silica gel coated plates showed that the first eluate contained mainly monosaccharides. Paper chromatography showed that the other two, which were combined, contained mainly oligosaccharides. These oligosaccharides were purified by paper chromatography using the solvent systems A and B to give six components: **1** (2.2 mg), **2** (2.7 mg), **3** (3.2 mg), **4** (1.8 mg), **5** (1.3 mg) and **6** (6.7 mg). They were identified by examination of the hydrolysis products of the oligosaccharides and methylation analyses. The partial acid hydrolysis of GGM was done according to Buchala and Meier (1973).

2.10. Immunization procedure

Male Swiss mice (three months old) were subcutaneously immunized twice with weekly interval with 100 μg ovalbumin (OVA) in 100 μl of saline (SAL) as the control group or with 100 μg of OVA mixed with 100 μg of galactoglucomannan (GGM) or Freund's Complete Adjuvant (FCA) (Sigma) or Freund's Incomplete Adjuvant (FIA) (Sigma), each one dissolved in 100 μl of saline as vehicle. Delayed type hypersensitivity (DTH) responses were assessed by measuring the increment in the right footpad thickness after subcutaneous challenge with 100 μg of OVA in 100 μl of SAL one week after the second immunization. The footpad thickness were 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 of SAL in the left hind footpad served as controls. The ovalbumin specific responses were obtained by subtracting the response to OVA challenge in unimmunized control mice (Mowat, Donachie, Reid, & Jarrett, 1991).

2.11. Toxicity assays

The toxicity of the polysaccharide was evaluated by subcutaneous administration on the back of mice with several dilutions (0.5, 1.0, 2.5, 5.0 mg) in 500 μl of saline solution. Injections were performed twice at weekly intervals, and the mice were monitored for 3 days. Saline solution treated animals were included as control group and the toxicity was assessed by lethality, local swelling and loss of hair at the site of injection (Sun & Liu, 2008).

3. Results and discussion

The crude polysaccharide was extracted with hot water from the fresh mesocarps of *A. aculeata,* previously cut into small pieces. Carbohydrate and protein contents of the crude polysaccharide were determined to be 89.94% and 10.06%, respectively. A sample of this fraction was fractionated by means of Sephacryl S-300 HR and desalted by means of Sephadex G-25 gel permeation chromatography, leading to the isolation of a neutral polysaccharide, protein free fraction GGM (Fig. 1). The fractionation procedure was monitored by carbohydrate content (Dubois et al., 1956). GGM was determined to be composed of galactose, mannose and glucose by the identification on TLC of the acid hydrolysates and by GC of the trimethylsilylated methyl glycoside derivatives prepared from the monosaccharides. Quantitative determination showed that the molar ratio of galactose, glucose and mannose was 18:22:60. The absolute configurations of the galactose, glucose and mannose were determined by GC and GC–EIMS of their trimethylsilylated (−)-2-butylgalactoside, (−)-2-butylglycoside, and (−)-2-butylmannoside. D-Galactopyranose, D-glucopyranose and D-mannopyranose were identified. GGM showed positive specific rotation, $[\alpha]_D^{20}$ +60° (*c* 1.0, H_2O).

The sequence of the sugar chain of GGM was established by methylation analysis (Parente et al., 1985), periodate oxidation and Smith degradation (Tomoda et al., 1978) and partial acid hydrolysis (Buchala & Meier, 1973). The permethylated GGM was hydrolyzed with acid, converted into the alditol acetates, and analyzed by GC and GC–EIMS. GGM afforded 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl mannitol, 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl glucitol, 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl mannitol, 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl glucitol and 1,3,5-tri-*O*-acetyl-2,-4,6-tri-*O*-methyl galactitol. GGM was oxidized with sodium metaperiodate. TLC and GC–EIMS analyses identified galactose, indicating (1 → 3)-linked galactosyl residues. Glycerol and erythritol were identified, indicating that (1 → 4)-linked glycosyl residues exist in GGM. This result is according to that obtained by methylation analysis. Controlled mild hydrolysis of GGM (Fig. 2) with acid gave galactose and glucose as the main products. Further hydrolysis of GGM gave the following oligosaccharides (Fig. 3): 3-*O*-β-D-galactopyranosyl-D-galactose (**1**), 4-*O*-β-D-galactopyranosyl-D-mannose (**2**), 4-*O*-β-D-mannopyranosyl-D-mannose (**3**), 4-*O*-β-D-mannopyranosyl-D-glucose (**4**), 4-*O*-α-D-glucopyranosyl-D-glucose (**5**) and *O*-β-D-mannopyranosyl-(1 → 4)-*O*-β-D-mannopyranosyl-(1 → 4)-D-mannose (**6**). The isolation of these oligosaccharides shows that there is a contiguous mannosyl residue.

Galactose and mannose were assigned the β-pyranose form from characteristic peaks of 812, 871 and 890 cm^{-1} in FT-IR spectrum (Barreto & Parente, 2006). A characteristic absorption at 840 cm^{-1} was detected due to an α-configuration (Pereira et al., 2000). The anomeric signals in the ^1H NMR spectrum of GGM at δ 4.65, 4.72 and 5.40 were assigned to (1 → 4)-linked β-D-Manp, (1 → 3)-linked β-D-Galp and (1 → 4)-linked α-D-Glcp, respectively (Hua, Zhang, Fu, Chen, & Chan, 2004; Pereira et al., 2000; Saulnier, Brillouet, & Moutounet, 1992; Tomoda, Shimizu, Shimada, & Suga, 1985). The ^{13}C NMR spectrum of GGM showed signals for anomeric carbons at δ 100.5, 102.3 and 105.6 attributed to (1 → 4)-linked β-D-Manp, (1 → 4)-linked α-D-Glcp and (1 → 3)-linked β-D-Galp, respectively. Others were present at δ 77.6 (*O*-substituted C-4 β-D-Manp), 78.9 (*O*-substituted C-4 α-D-Glcp) and 82.6 (*O*-substituted C-3 β-D-Galp), and 60.0, 61.4 and 62.0 (unsubstituted C-6) (Barreto & Parente, 2006; Pereira et al., 2000; Saulnier et al., 1992; Yamada, Kiyohara, & Otsuka, 1984). These data showed that GGM extracted from mesocarp of fruits of *A. aculeata* represents certain structural characteristics: it is composed of galactose, glucose and mannose in the molar ratio of 18:22:60 and has a main chain of (1 → 4)-linked β-D-mannopyranosyl residues attached to an initial chain of (1 → 3)-linked β-D-galactopyranosyl residues and a terminal chain of (1 → 4)-linked α-D-glucopyranosyl residues as shown in Fig. 2.

The formulation of effective vaccines generally requires an adjuvant to stimulate an appropriate immune response. An efficient adjuvant should have negligible toxicity and enhances the humoral or cellular immune response to a specific antigen (Marciani, 2003). Several studies have determined the immunopotentiating

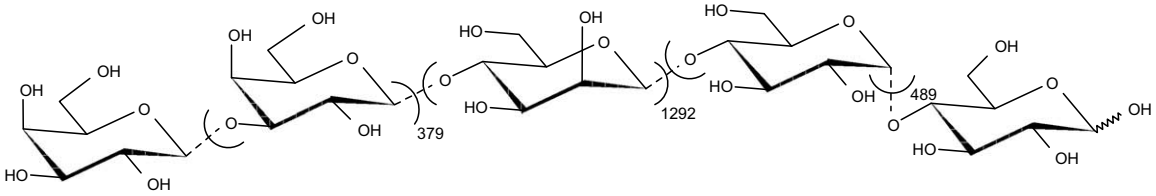

Fig. 2. Schematic representation of the galactoglucomannan (GGM), where the values 379, 489, and 1292 were calculated on the basis of the weight-average molar mass of GGM (3.5×10^5) and the molar composition of galactose:glucose:mannose (18:22:60).

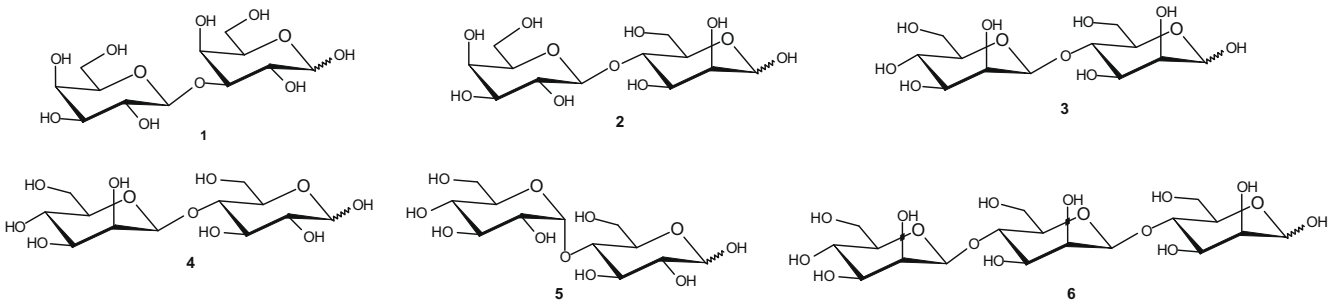

Fig. 3. The partial acid hydrolysis products **1**–**6** of GGM.

properties of polysaccharides from plant origin, including adjuvant effects (Ebringerová et al., 2008; Popov et al., 2006; Zhao, Kan, Li, & Chen, 2005). In the traditional medicine, complex mannoglycans isolated from different sources were shown to possess immunomodulatory activities, such as stimulation of lymphocyte proliferation (Ebringerová et al., 2008), promotion of antibody production (Sun & Liu, 2008) and enhancement of phagocytic activity (Barreto & Parente, 2006). In order to investigate the immunoadjuvant property of the isolated polysaccharide, its adjuvant potential on the cellular immune response against ovalbumin antigen was investigated (Fig. 4). Delayed type hypersensitivity (DTH) reaction was measured as an *in vivo* assay of cellular immune response. In this study, mice immunized with ovalbumin conjugated with galactoglucomannan (GGM) showed remarkable responses greater than those when the antigen was combined with commercial adjuvants. This response developed rapidly after immunization and persisted at high levels for at least 3 days (Mowat et al., 1991).

The structural similarities between the galactoglucomannan isolated from *Acrocomia aculeata* and other immunomodulatory complex mannoglycans may help to explain the potent adjuvant activity of the polysaccharide. For example, the main chain composed of (1 → 4)-linked β-D-mannose may be responsible for the activation of the cellular immune response, since this backbone is shared by the immunomodulating galactoglucomannan isolated from *Picea abies* (Ebringerová et al., 2008). In the galactoglucomannan isolated from *Dioscorea opposita* (Zhao et al., 2005), the stimulation of lymphocyte proliferation was abolished after the removal of terminal units of β-D-galactose, which suggested that these residues are important for the immunostimulatory activity. Finally, the terminal residues composed of (1 → 4)-linked α-D-glucose may be involved in macrophage stimulation through phagocytosis enhancement, as occurs in the immunomodulatory polysaccharides isolated from *Costus spicatus* (da Silva & Parente, 2003). Probably, the immunopotentiating activity of the polysaccharide may result from enhanced antigen presentation and stimulation of macrophage activity. Since targeting is the mechanism by which an adjuvant–antigen complex is delivered to antigen presenting cells for processing (Marciani, 2003), the pattern recognition receptors present in these cells can bind polymers containing the carbohydrate residues present in the polysaccharide (Popov et al., 2006). In order to discard the hypothesis of endotoxin contamination, the toxicity of the polysaccharide was evaluated. When the animals were immunized subcutaneously on the back, there is no lethality observed. Local swelling or loss of hair was not observed in mice inoculated with two doses ranging from 0.5 to 5.0 mg diluted in saline solution at weekly intervals (Sun & Liu, 2008). In conclusion, these results suggested that the galactoglucomannan (GGM) present in the fruits of *A. aculeata* is potentially applicable as dietary fiber, similarly as other polysaccharides of the same class (Ebringerová et al., 2008) and as component of nutritional

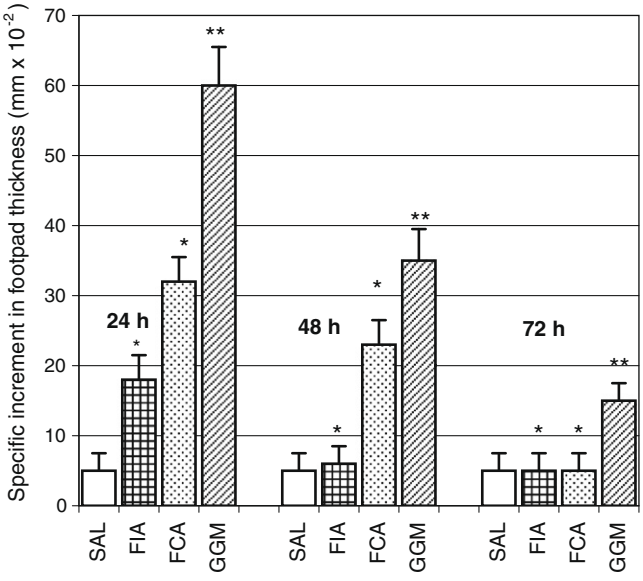

Fig. 4. Delayed type hypersensitivity responses after two subcutaneous immunizations with 100 μg of ovalbumin and 100 μg of each adjuvant. Results are mean ± *SEM* ($n = 5$); $^{*}p < 0.05$, $^{**}p < 0.01$ significantly different from the saline control. Student's *t*-test. SAL, saline solution; FCA, Freund's Complete Adjuvant; FIA, Freund's Incomplete Adjuvant, GGM, galactoglucomannan from *Acrocomia aculeata*.

supplements or pharmaceuticals with immunopotentiating properties, justifying the use of this plant as a food source and in the traditional medicine.

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