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Chemical properties and antiulcerogenic activity of a galactomannoglucan from *Syagrus oleracea*

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ABSTRACT

A galactomannoglucan (GMG) with an estimated weight–average molar mass of 415,000 g/mol was obtained from an aqueous extract of the mesocarp of fruits of *Syagrus oleracea* (Mart.) Becc. by fractionation by Sephacryl S-300 HR and Sephadex G-25. Chemical and spectroscopic studies indicated that GMG has a 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, mannose and glucose in the molar ratio of 30:33:37. Results of the present study indicated that the polysaccharide GMG of *S. oleracea* significantly inhibited gastric lesions induced by ethanol, showing a gastroprotective property.

1. Introduction

The genus *Syagrus* belongs to Arecaceae family which includes about 3000 to 3700 species distributed among 240 to 387 genera, out of which about 200 native and 200 exotic species of palms grow in Brazil (Lorenzi, 1992). *Syagrus oleracea* (Mart.) Becc., known in Brazil as catolé, is one of the native species which has its origin and habitat in the northeast and southeast regions of Brazil. Almost all of this palm is utilised by humans and animals. The nuts are an important component of animal nutrition, and kernels are used in the manufacture of sweets. Besides, kernel oil is used for edible purposes. Due to its multiple uses, and to the fact that this palm is easy to cultivate, its plantation becomes lucrative. In Brazil, the palmetto of *S. oleracea* is used for the treatment of stomach ulcers (Silva, 2009). As part of our ongoing efforts in discovering new naturally occurring polysaccharides, we describe the isolation, chemical characterisation and evaluation of the antiulcerogenic activity of a galactomannoglucan (GMG) from the mesocarp of fruits of *S. oleracea*.

2. Materials and methods

2.1. Plant material

The fruits of *S. oleracea* (Mart.) Becc. were collected from a catolé farm situated near Senador Pompeu, Ceará state, northeast region of Brazil in August 2002. The identity of the plant was confirmed by Prof. José Paz Parente, Federal University of Rio de Janeiro, using bibliographic data (Lorenzi, 1992). A voucher specimen (No. LQPM-50) is maintained in the Laboratory of Chemistry of Medicinal Plants at Federal University of Rio de Janeiro. The seeds were separated from the fruits manually using a stainless steel knife. The seeds were broken and the mesocarps were separated and cut into small pieces.

2.2. Analytical techniques

Carbohydrate content was analysed 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, 1965). Protein content was analysed 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 galactomannoglucan (GMG) from *S. oleracea* 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. Visible and IR spectra were measured on a Shimadzu UV-1601 and on a Perkin Elmer FT-IR 1600 spectrometer, respectively.

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^{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-sulphonate as an internal standard. Gas chromatography (GC) was carried out with flame ionisation detector (FID), using an SE-30 glass capillary column (0.31 mm × 25 m). Mass spectra were collected on a VG Auto SpecQ spectrometer operating at 70 eV. Thin-layer chromatography (TLC) of monosaccharides was performed on silica gel coated plates (Merck) in *n*-BuOH:pyridine:H_2O (6:4:3, v/v/v), 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, v/v/v) and (**B**) *n*-PrOH:EtOAc:H_2O (7:2:1, v/v/v). Chromatographic detection reagent was alkaline silver nitrate (Buchala & Meier, 1973).

2.3. Extraction

Fresh mesocarps of *S. oleracea* (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 lyophilisation (yield: 4.032 g, 0.20%). The amorphous powder (4.032 g) was dissolved in 0.01% sodium sulphate (900 ml) and added to 5% cetyltrimethyl ammonium bromide (180 ml). After centrifugation, the supernatant was poured into two volumes of EtOH and the precipitate obtained was dissolved in water (560 ml), dialysed and lyophilised to yield crude polysaccharide (yield: 405 mg). It was extracted according to Pereira, da Silva, Pereira, and Parente, 2000.

2.4. Purification of the polysaccharide

A sample of the crude polysaccharide (100 mg) was dissolved in the eluent 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 galactomannoglucan (GMG) (1100–1550 ml) (Fig. 1) were pooled, dialysed 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 lyophilised to yield GMG (23 mg). The carbohydrate content of the fractions was measured. This procedure was repeated 7× to obtain GMG (92 mg), using the method of Pereira et al. (2000).

2.5. Partial acid hydrolysis of GMG

GMG (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 sulphuric acid (5 ml) for 6 h at 100 °C in a sealed tube. The hydrolysate was neutralised with 0.1 M NaOH (10 ml), 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. These oligosaccharides were purified by paper chromatography using the solvent systems **A** and **B** to give six components: **1** (1.1 mg), **2** (1.7 mg), **3** (2.5 mg), **4** (2.7 mg), **5** (1.5 mg) and **6** (3.5 mg). They were identified by

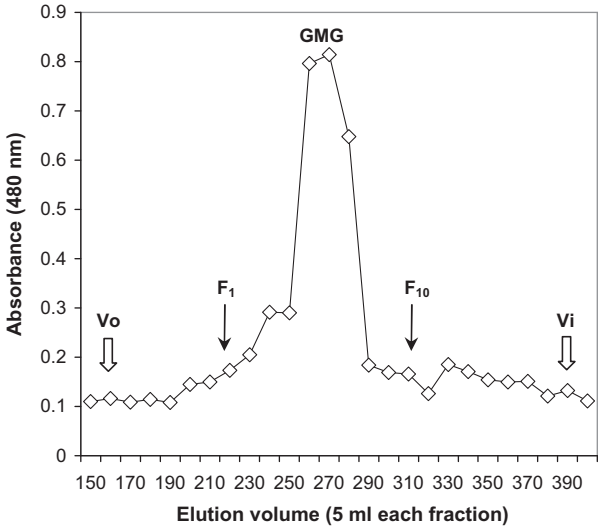

Fig. 1. Elution diagram of GMG polysaccharide from Sephacryl S-300 HR (0.1 M Tris–HCl). V_o, void volume; V_i, inner volume; F_1, initial fraction pooled; F_{10}, final fraction pooled.

examination of the hydrolysis products of the oligosaccharides and methylation analyses. The partial acid hydrolysis of GMG was done according to Buchala and Meier (1973).

2.6. Molar carbohydrate composition and D,L configurations

The molar carbohydrate compositions of GMG (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).

2.7. Methylation analysis

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

2.8. Periodate oxidation of GMG

GMG (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.

2.9. 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 glacial acetic acid. The reaction mixture was treated with repeating addition of ethanol followed by evaporation. The concentrated solution (2 ml) was applied to a column (5.5 × 72 cm) of Sephadex G-15. The column was eluted with water, and fractions of 50 ml were collected and analysed by the phenol–sulphuric method (Dubois et al., 1956). The eluates obtained from tubes 10 to 14 were combined, concentrated and lyophilised (Tomoda, Satoh, & Ohmori, 1978). The product (1 mg) was hydrolysed with 1 N sulphuric 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).

2.10. Animals

Male Swiss mice (three months old, 25–35 g) were obtained from the central animal care facilities, Health Sciences Centre, Federal University of Rio de Janeiro, Brazil. The mice were maintained under standard laboratory conditions (12 h light/dark cycle, at 22 ± 2 °C). Standard pellet food and water were available *ad libitum*. The animals were deprived of food 24 h prior to the experiment. The experimental protocol was performed according to the "Principles of Laboratory Animal Care" (NIH Publication 85–23, revised 1985).

2.11. Antiulcerogenic activity

Antiulcerogenic activity was evaluated by measuring acute gastric lesions induced by absolute ethanol (Yamada et al., 1991). Male Swiss mice in groups of five were fasted for 24 h before the experiment and administered orally with 1 ml of pure water as the negative control, or galactomannoglucan (100 mg/kg), or the reference compound cimetidine (100 mg/kg) dissolved in vehicle as a positive control. One hour after the treatments, all animals received orally 200 μl of absolute ethanol to induce gastric lesions. The animals were killed 1 h after treatment with the ulcerogenic agent and the stomachs removed, opened along the greater curvature and rinsed with physiological saline to determine the lesion damage. The degree of gastric mucosal damage was evaluated from digital pictures using a computerised image analysis system. The percentage of the total lesion area (haemorrhagic lesions) to the total surface area of the stomach was defined as the ulcer index (Hamauzu, Irie, Kondo, & Fujita, 2008).

3. Results and discussion

Carbohydrate and protein contents of the crude polysaccharide were determined to be 88.96% and 11.04%, respectively. The high protein content of the crude polysaccharide is probably due to the high content of α-(1 → 4)-linked D-glucopyranosyl residues in the polysaccharide (Pereira et al., 2000). The polysaccharide 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 glycosides derivatives prepared from the monosaccharides. Quantitative determination showed that the molar ratio of galactose, mannose and glucose was 30:33:37. The absolute configurations of the galactose, mannose and glucose were determined by GC and GC–EIMS of their trimethylsilylated (−)-2-butylgalactoside, (−)-2-butylmannoside and (−)-2-butylglucoside. D-Galactopyranose, D-mannopyranose and D-glucopyranose were identified. The galactomannoglucan (GMG) showed positive specific rotation, $[\alpha]_D^{20}$ +80° (c 1.0, H_2O). The weight–average molar mass of GMG was estimated to be 415,000 g/mol based on the calibration curve of elution volume of standard dextrans from gel filtration on Sephacryl S-300 HR.

The sequence of the sugar chain of GMG 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 GMG was hydrolysed with acid, converted into the alditol acetates, and analysed by GC and GC–EIMS. GMG 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. GMG was oxidised with sodium metaperiodate (Tomoda et al., 1978). 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 GMG. This result is according to that obtained by methylation analysis. Controlled mild hydrolysis of GMG (Fig. 2) with acid gave galactose and glucose as the main products. Further hydrolysis of GMG 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 are contiguous mannosyl residues.

Galactose and mannose were assigned the β-pyranose form from characteristic peaks of 811, 871 and 891 cm^{-1} in the FT-IR spectrum (Barreto & Parente, 2006). A characteristic absorption at 841 cm^{-1} was detected due to an α-configuration (Pereira et al., 2000). The anomeric signals in the ^{1}H-NMR spectrum of GMG at δ 4.64, 4.72 and 5.41 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 GMG showed signals for anomeric carbons at δ 100.4, 102.4 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 77.5 (*O*-substituted C-4 β-D-Manp), 78.8 (*O*-substituted C-4 α-D-Glcp) and 82.5 (*O*-substituted C-3 β-D-Galp), and 60.1, 61.5 and 62.1 (unsubstituted C-6) (Barreto & Parente, 2006; Pereira et al., 2000; Saulnier et al., 1992; Yamada, Kiyohara, & Otsuka, 1984). These data showed that GMG extracted from mesocarp of fruits of *S. oleracea* possesses certain structural characteristics: it is composed of galactose, mannose and glucose in the molar ratio of 30:33:37 and has a 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.

According to the literature, complex mannoglycans isolated from different sources have been shown to possess important biological activities, such as stimulation of lymphocyte proliferation, promotion of antibody production (Ebringerová et al., 2008), enhancement of phagocytosis and inhibition of capillary permeability (Barreto & Parente, 2006). In order to confirm the utilisation of *S. oleracea* in traditional medicine, the gastroprotective property of the polysaccharide was investigated. The antiulcerogenic activity was evaluated by measuring the inhibition of acute gastric lesions induced by absolute ethanol (Yamada et al., 1991). Ethanol tends to dissolve the components of the mucous membrane of the stomach, bringing gastric blood flow to a standstill. This contributes to the development of the haemorrhage and necrotic aspects of tissue injury (Guth, Paulsen, & Nagata, 1984). By macroscopic observations, in the control animals that received

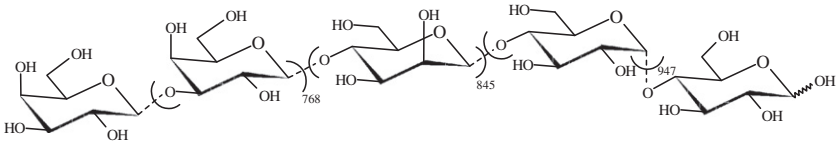

Fig. 2. Schematic representation of the galactomannoglucan (GMG), where the values 768, 845, and 947 were calculated on the basis of the weight–average molar mass of GMG (4.15×10^5) and the molar composition of galactose:mannose:glucose (30:33:37).

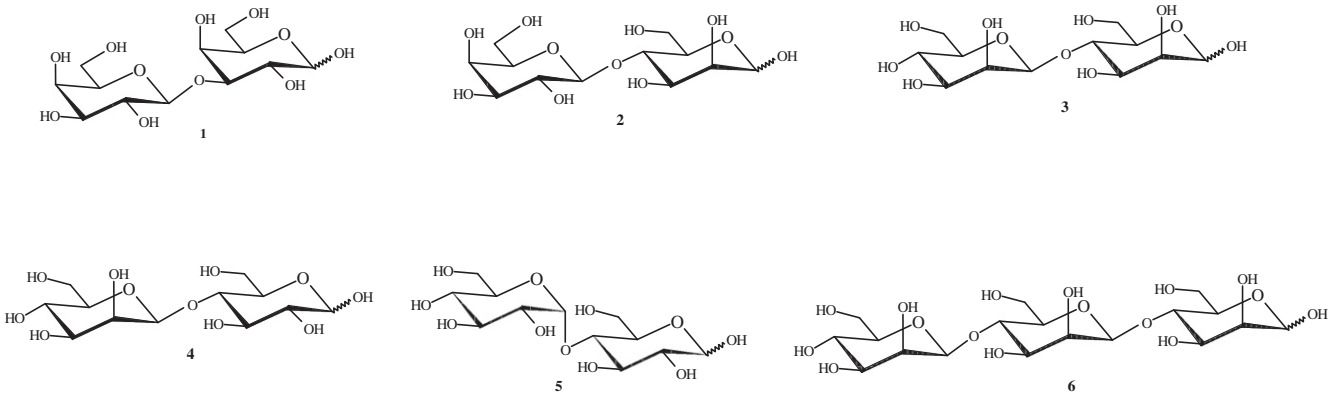

Fig. 3. The partial acid hydrolysis products **1**–**6** of GMG.

only water before absolute ethanol administration, intense and widespread gastric hyperaemia and thickened lesions were evident. In contrast, the stomachs of the animals which received the polysaccharide showed an aspect close to normality, with significant reduction in gastric hyperaemia and in number and severity of lesions. This protective action is closely related to the reference compound cimetidine at the same dosage (Singh et al., 2008). The intensity of gastric ulcers was quantified by the percentage of the injury area in relation to the control group (Fig. 4).

In the case of gastric ulcers induced by absolute ethanol, the polysaccharide probably interferes with the ulcerogenic mechanism, showing a cytoprotective property. The mechanisms which protect the gastric mucosa against acute attack by necrotic agents involve a variety of events (Galati, Monforte, Tripodo, d'Aquino & Mondello, 2001). Among these, a crucial role is played by mucus production, which is an important protective factor for the gastric mucosa and consists of a viscous, elastic, adherent and transparent gel formed by water and glycoproteins that covers the entire gastrointestinal mucosa. The protective properties of the mucus barrier depend not only on the gel structure but also on the amount or thickness of the layer covering the mucosal surface (Laine, Takeuchi, & Tarnawski, 2008).

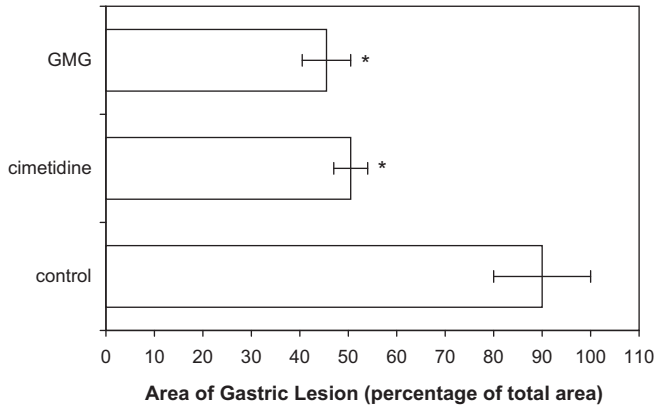

Fig. 4. Antiulcerogenic activity of galactomannoglucan (GMG, 100 mg/kg, p.o.) and the reference compound cimetidine (100 mg/kg, p.o.) against ethanol induced gastric lesions. Results are mean ± S.E.M. ($n = 5$); $^{*}p < 0.05$, $^{**}p < 0.01$, significantly different from the control group.

The use of this plant in traditional medicine against gastric disorders can be explained by the structural similarities between the galactomannoglucan and other antiulcerogenic polysaccharides. For example, the main chain composed of (1 → 4)-linked β-D-mannose may be responsible for the protection of the mucosa surface, forming a protective coating, since this backbone is shared by the bioactive polysaccharide isolated from *Aloe vera*, a medicinal plant used to treat gastric ulcers (Chow, Williamson, Yates, & Goux, 2005). Additionally, the initial chain of (1 → 3)-linked β-D-galactose and the terminal residues composed of (1 → 4)-linked α-D-glucose can also be involved in the antiulcerogenic activity, as occurs in the gastroprotective polysaccharide isolated from *Cochlospermum tinctorium*, which possesses these type of linkages (Nergard et al., 2005). Probably, these combined structural features synergistically contribute to the mode of action of this high molecular weight polysaccharide, protecting the mucosa by increasing the mucus synthesis, preventing the penetration of the necrotizing agent or interacting with the macromolecules of the gastric surface (Galati, Monforte, Tripodo, d'Aquino, & Mondello, 2001). In conclusion, these results suggested that the galactomannoglucan present in the fruits of *S. oleracea* is potentially applicable as dietary fibre, in a similar way to other polysaccharides of the same class (Ebringerová et al., 2008) and as component of nutritional supplements or pharmaceuticals with gastroprotective properties, justifying the use of this plant as a food source and in traditional medicine.

Acknowledgements

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