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Short communication

Chemically sulfated galactomannan from *Dimorphandra gardneriana* seed: Characterization and toxicity evaluation

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ABSTRACT

Dimorphandra gardneriana galactomannan (DG) was sulfated in pyridine:formamide using chlorosulfonic acid as the sulfation agent. The degree of substitution was 0.32, determined from the sulfur percentage. Confirmation of sulfation was obtained by FTIR spectroscopy through the presence of an asymmetrical S=O stretching vibration at 1259 cm^{-1}. NMR data showed that the sulfation occurred on primary hydroxyl groups. NMR and GPC data indicate degradation during reaction with elimination of galactose. At the maximum tested concentration of 1000 μg/mL, unmodified DG polysaccharide did not show a statistically significant cytotoxicity in Vero cells by the MTT method. Therefore, the CC_{50} > 1000 μg/mL obtained for the sulfated polysaccharides from *D. gardneriana* in Vero cells point to its lower cytotoxicity than the sulfated galactomannan from *Mimosa scabrella*.

1. Introduction

Sulfated polysaccharides (natural and chemically modified) possess physiological activities such as anti-inflammatory and anti-coagulation (Alban, Schauerte, & Franz, 2002; Dore et al., 2013;), antiviral (Chrestani et al., 2009; Ono et al., 2003; Pires, Gorin, Reicher, & Sierakowski, 2001), antioxidant (Dore et al., 2013; Wang, Guo, et al., 2010; Wang, Wang, et al., 2010) and antitumor (Gamal-Eldeen, Helmy, Talaat, & Ragab, 2007; Tao, Zhang, & Zhang, 2009; Zong et al., 2013), which depend on the structure and degree of sulfation (DS) (Alban et al., 2002), molar mass (Barbucci, Lamponi, Magnani, & Renier, 1998), chain conformation (Tao et al., 2009) and glycosidic linkages (Mulloy, Mourão, & Gray, 2000).

The aim of this work was the preparation and characterization of chemically sulfated galactomannan from *Dimorphandra gardneriana Tul.* (DG) seeds and the evaluation of the toxicity of modified and unmodified polysaccharide.

D. gardneriana Tul. and *Dimorphandra mollis Benth* pods are used to produce rutin, a flavonoid which is used to strengthen capillaries, inhibits some cancer cells and also possesses antioxidant and anti-inflammatory properties (Sánchez-Rodríguez, Ruiz, Ferreres, & Moreno, 2012). Brazil exported 403,167 kg of rutin in 2011 (Abiquifi, 2012) which represents US$ 13,774.446.00. In order to produce this amount of rutin, 5000 tons of pods were used. Taking into account the amount of seeds discharged from pods in the rutin production, the weight of the seed and yield of galactomannan from *D. gardneriana* (Cunha, Vieira, Arriaga, de Paula, & Feitosa, 2009), 75 tons of galactomannan could be have been produced in 2011.

Galactomannan from *D. gardneriana* shows *M*/*G* ratio closed to that of guar gum (*M*/*G* = 1.82) and a molar mass of 3.9×10^7 g/mol (Cunha et al., 2009). Formulations based on this galactomannan were evaluated as ophthalmic viscosurgical devices (OVD) (Pires et al., 2010). These authors reported that 3% galactomannan solution concentration showed potential to be used as effective OVD.

2. Experimental

2.1. Isolation and purification of galactomannan (*D. gardneriana*)

Mature pods of *D. gardneriana Tul.* were collected in Crato-Ceara-Brazil in August–September 2006. The seeds were separated from pods and the isolation and purification of the galactomannan from seeds were carried out as described by Cunha et al. (2009).

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2.2. Sulfation procedure

The sulfation procedure was based on the methodology described by O'Neill (1955) with modification proposed by Moura Neto, Maciel, Cunha, de Paula, and Feitosa (2011). Conditions used in this work are described in Table 1.

2.3. Characterization of sulfated galactomannan

The contents of nitrogen, carbon and sulfur were determined for the sulfated galactomannan (SDG) by elemental microanalysis using a Perkin-Elmer CHN 2400 instrument. Fourier transform infrared (FTIR) spectra were recorded with KBr pellets on a FT-IR Shimadzu 8300 spectrophotometer. 1D (^{1}H and ^{13}C broad band, BB) spectra of 3% (m/v) solutions in D_2O at 343 K were recorded on a Fourier transform Bruker Avance DRX 500 spectrometer. An inverse multinuclear gradient probe-head equipped with z-shielded gradient coils, and with Unix Silicon Graphics workstation was applied. Sodium 2,2-dimethylsilapentane-5-sulfonate (DSS) was used as the internal standard (0.00 ppm for ^{1}H). DEPT 135 spectrum was recorded in order to determine the hydrogenation of each carbon. The molar masses were analyzed by gel permeation chromatography using a Shimadzu LC-10AD chromatograph with a refractive index detector RID-6A at room temperature using an ultrahydrogel linear column (7.8 mm × 300 mm), flow rate of 0.5 mL min^{-1}, polysaccharide solution concentration of 0.1% (m/v), water as the solvent and 0.1 mol L^{-1} $NaNO_3$ as the eluent.

2.4. Cytotoxicity assay

Vero cells were cultured in 96-well microplates, and the monolayers were incubated for 72 h at 37 °C and 5% CO_2 with Dulbecco's modified Eagle's medium (DMEM) containing 5% fetal bovine serum (FBS), penicillin G (100 IU/ml), enrofloxacin (10 μg/mL) and amphotericin B (1.25 μg/mL) with 2-fold serial dilutions of the compounds at different concentrations, ranging from 1.9 to 1000 μg/mL. The *in vitro* toxicity of DG and SDG, which were sterilized by filtration through PVDF membranes (pore size 0.22 mm), was determined according to Denizot and Lang (1986), by quantifying the viable cells using 3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), which is converted into a purple formazan by mitochondrial dehydrogenases. Fifty percent cytotoxic concentration (CC_{50}) was defined as the polysaccharide concentration which could reduce by 50% the number of viable cells, when compared with a control without polysaccharide. Experiments were statistically expressed as mean ± standard deviation and analyzed by Student' *t*-test with $P < 0.01$ (Bolton, 1990). Variables exceeding the upper quantification limit were considered statistically significant.

3. Results and discussion

3.1. Degree of sulfation and yield

The degree of sulfation (DS) for the sulfated galactomannan (SDG) was determined by Eq. (1) (Melo, Feitosa, Freitas, & de Paula, 2002). This equation uses the % of sulfur and carbon obtained by microanalysis and takes into consideration the structure of the polysaccharide. DS is defined as the average number of $-SO_3Na$ groups inserted in each monosaccharide unit. Galactomannans are composed of hexose, so DS represents the number of $-SO_3Na$ groups per 6 carbons.

$$\mathrm{DS} = \frac{S\%/\text{atomic mass}}{C\%/\text{atomic mass} \times 6} = 2.25\left(\frac{S\%}{C\%}\right) \quad (1)$$

DS value obtained for sulfated galactomannan from *D. gardneriana* was smaller than those values observed for *Leucaena leucocephala*, *Mimosa scabrella*, and *Senna macranthera galactomannans* (Table 1).

The yield was calculated (Table 1) taking into account the increase of $-SO_3Na$ groups per monosaccharide unit as proposed by Moura Neto et al. (2011) for the sulfation of cashew gum (Eq. (2)).

$$\mathrm{Yield}\,(\%) = 100 \times \frac{m_f}{m_i(1 + ((\mathrm{DS} \times 102)/162))} \quad (2)$$

where m_f and m_i are the mass of sulfated galactomannan derivative and the initial galactomannan respectively. The value observed for SDG is similar to the one reported for the sulfation of *S. macranthera* galactomannan.

3.2. Characterization of sulfated galactomannan

Confirmation of sulfation was carried out using infrared spectroscopy. The SDG sample showed a new band at 1260 cm^{-1} (not observed in the galactomannan spectrum), attributed to asymmetrical stretching of S=O vibration (Grosev, Bozac, & Puppels, 2001; Mahner, Lechner, & Nordmeier, 2001; Mestechkina, Shcherbuking, & Shaskov, 2008; Ono et al., 2003; Pires et al., 2001).

^{13}C and ^{1}H NMR spectra of DG and SDG samples are shown in Fig. 1.The *M*/*G* ratio was obtained directly from relatives areas of the anomeric signals in ^{1}H NMR spectrum as described by Cunha et al. (2009). The *M*/*G* ratio of unmodified polysaccharide ($M/G = 1.93 \pm 0.02$) was close to the value obtained by Cunha et al. (2009) ($M/G = 1.82$). The SDG sample shows an *M*/*G* ratio of 2.85 ± 0.08. The high value observed for SDG suggests loss of galactose during the sulfation reaction. In the ^{13}C NMR spectrum of SDG, a new signal at 68.0 ppm is observed. Substitution of carbons with sulfate groups cause an increase in chemical shift in the order of 6–8 ppm (Chrestani et al., 2009; Mestechkina et al., 2008; Moura Neto et al., 2011; Wang, Guo, et al., 2010) which indicates a sulfate substitution in C-6 of monosaccharide units. The ^{13}C DEPT (Fig. 1F) experiment confirms this assignment as the new signal

Table 1
Comparison between the sulfation reaction conditions and the characteristics of the obtained galactomannan derivatives.

Species	Polysaccharide conc./(%m/v)	Sulfation reagent	SR/SU[a]	Temperature (°C)	Yield (g %)	DS	Ref.
D. gardneriana	0.7	CSA-py-FA	19.5	4	54[b]	0.32[c]	This Work
M. scabrella	0.5	CSA-py-FA	31.5	4	–	0.62	Ono et al. (2003)
L. leucocephala	0.5	CSA-py-FA	31.5	4	–	0.50	Ono et al. (2003)
S. macranthera	0.9	CSA-py-FA	29.2	8	56[b]	0.4	Pires et al. (2001)

CSA, chlorosulfonic acid; py, pyridine; FA, formamide.
[a] Molar ratio of sulfation reagent (SR) to sugar unit (SU).
[b] Calculated using Eq. (1).
[c] Calculated using Eq. (2).

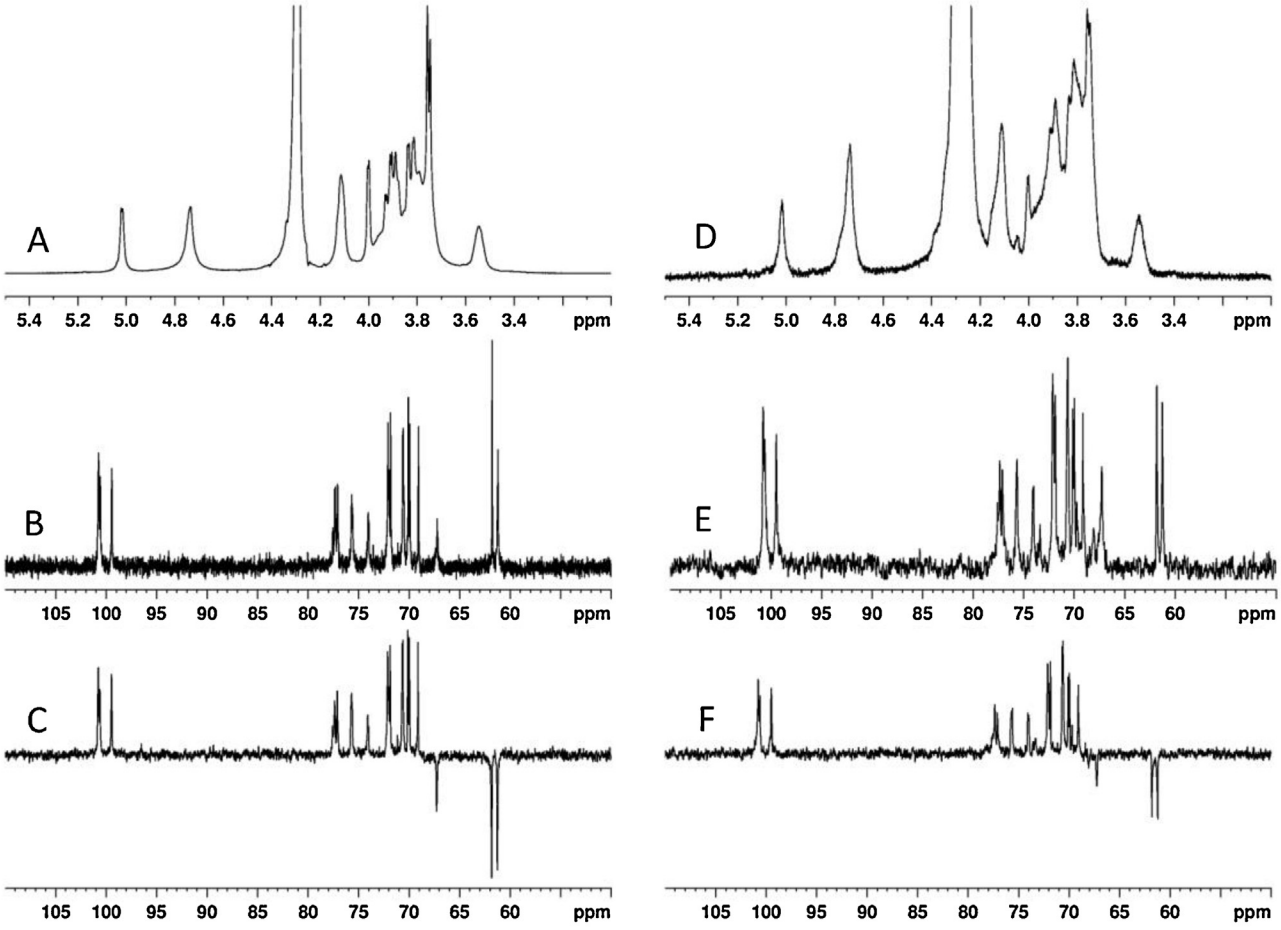

Fig. 1. (A) ^{1}H, (B) ^{13}C NMR and (C) DEPT spectra for unmodified *D. gardneriana* polysaccharide (DG); (D) ^{1}H, (E) ^{13}C NMR and (F) DEPT spectra sulfated *D. gardneriana* polysaccharide (SDG).

shows opposite amplitude to CH and CH_3 signals. The substitution is on primary CH_2 carbons.

Gel permeation chromatography (Fig. 2) was used to evaluate degradation during the sulfation reaction. As the absolute molar mass of charged polymer can only be estimated if the GPC is coupled with multiangle laser light scattering (MALLS) and this is not available in our equipment, the discussion on molar mass was based on comparison of the elution volume. If no degradation occurs, the insertion of $-SO_3Na$ groups in galactomannan chain would increase the hydrodynamic volume leading to a decrease in elution volume. In Fig. 2, SDG sample shows an increase in elution volume suggesting degradation, in agreement with the low yield and also corroborate with the NMR results. Degradation was also observed in sulfation of guar gum (Wang, Wang, et al., 2010).

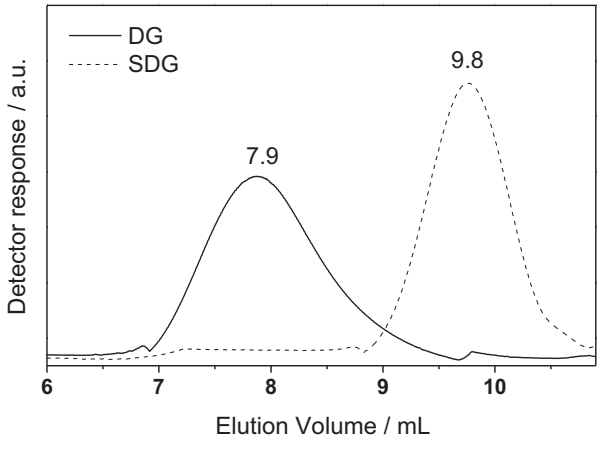

Fig. 2. GPC chromatogram of aqueous solutions of unmodified DG (continuous line) and sulfated DG (dashed line).

3.3. Cytotoxicity

At the maximum tested concentration of 1000 μg/mL, unmodified DG polysaccharide (Fig. 3A) did not show a statistically significant cytotoxicity in Vero cells by the MTT method. SDG (Fig. 3B) did not exhibit difference with the control without polysaccharide up to 500 μg/mL, but reduced by 30% the cells viability at 1000 μg/mL. According to these results, the DG and SDG polysaccharides presented $CC_{50} > 1000$ μg/mL.

In relation to the toxicity studies reported for *D. mollis*, the dry extract of its fruits, which is constituted mainly by a flavonoid called rutin, was described to be safe after 180 days of oral administration of 1000 mg/kg to rats, analyzing parameters like changes in body weight and hematocrit, in addition to others (Féres et al., 2006). They did not evaluate the toxicity of the galactomannan from the seeds of *D. mollis*. Cytotoxicity studies of neutral polysaccharides from leguminous seeds showed lack of cytotoxicity for the galactomannan from *M. scabrella* in Vero and MA-104 cells up to 645 μg/mL (maximum tested concentration) (Chrestani et al., 2009). This data is in accordance to the cytotoxicity results of DG sample.

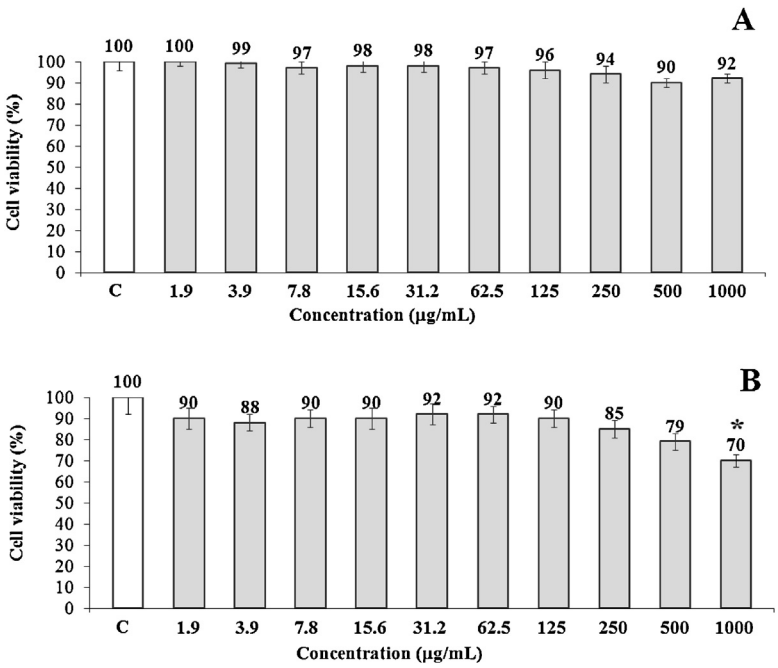

Fig. 3. Evaluation of cytotoxicity of unmodified (A) and sulfated (B) *D. gardneriana* polysaccharide in Vero cells after 72 h of incubation at 37 °C and 5% CO_2 by the MTT assay. Control: without polysaccharide treatment. Bars represents means, with vertical lines indicating standard deviations, $n = 3$, $^*P < 0.01$.

The cytotoxicity studies of the sulfated galactomannan from the seeds of *M. scabrella* presented a CC_{50} = 454 μg/mL in Vero cells, a CC_{50} > 625 μg/mL in MA-104 cells (Chrestani et al., 2009), and a CC_{50} = 1500 μg/mL in C6/36 cells (Ono et al., 2003). Therefore, the CC_{50} > 1000 μg/mL obtained for the SDG in Vero cells indicates lower cytotoxicity than the sulfated galactomannan from *M. scabrella*. This polysaccharide presents a higher degree of sulfation (DS) of 0.62, which could be related to the higher cytotoxicity.

The low cytotoxicity of SDG is important for the biological application of this polysaccharide, if activity comes to be proven in the future, or as matrix for drug delivery, for example.

4. Conclusions

Sulfated galactomannan produced from *D. gardneriana* seeds was obtained with DS of 0.32. Sulfation reaction leads to polymer chain degradation with release of galactosyl units as shown in GPC and NMR analysis. The new sulfate group was detected by FTIR and microanalysis. The sulfation occurs preferentially at the primary carbon atoms as observed in the NMR experiments. At the maximum tested concentration of 1000 μg/mL, unmodified DG polysaccharide did not show a statistically significant cytotoxicity in Vero cells by the MTT method. The CC_{50} > 1000 μg/mL obtained for the sulfated polysaccharides from *D. gardneriana* in Vero cells indicates lower cytotoxicity than the sulfated galactomannan from *M. scabrella*.

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