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Polysaccharide isolated from *Passiflora edulis*: Characterization and antitumor properties

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ABSTRACT

A hot water-extracted polysaccharide fraction (PFCM) of *Passiflora edulis* was characterized by microanalysis, infrared spectroscopy, NMR and high performance size-exclusion chromatography. The major component in PFCM is (1 → 4) linked galacturonic acid (esterified and unesterified). Neutral sugars such as arabinose, glucose, rhamnose, mannose, and fucose were also present. Traces of xylose and ribose were detected. The PFCM sample had a similar molar mass to that of pectin extracted from *P. edulis* under acidic conditions. Sarcoma 180 tumors treated with PFCM showed a growth inhibition ratio ranging from 40.59% to 48.73% depending on the dosage and type of PFCM administration (oral or intraperitoneal). Toxicological analysis shows that PFCM increases the cell types involved in primary defense mechanisms and no significant changes in the biochemical parameters and organs (e.g., kidney and liver) were observed. However, the use of PFCM treatment increased the spleen weight when compared with the use of 5-fluorouracil.

1. Introduction

Current therapies against cancer are still unsatisfactory mainly because of their side effects. In recent years, natural (or bio-) polysaccharides have been described as having antitumor activity (Leung, Liu, Koon, & Fung, 2006). These polysaccharides usually have low toxicity and few side effects, which make them appropriate for immunotherapy against cancer. The antitumor properties are generally related to macrophage activation and complement system modulation (Chihara, 1992; Wang et al., 1997).

P. edulis f. flavicarpa (passion fruit) is a plant from the *Passifloreaceae* family. This plant is economically important and very common in Brazil. Several parts of this plant are biologically active. The fruit extract reportedly exerts anxiolytic (Barbosa et al., 2008; Coleta et al., 2006), anti-inflammatory (Montanher, Zucolotto, Schenkel, & Fröde, 2007; Vargas et al., 2007) and antioxidant (Talcott, Percival, Pittet-Moore, & Celoria, 2003) activities.

Pectin from passion fruit has been extracted using diluted acid solution (Pinheiro et al., 2008; Yapo & Koffi, 2006). Pectins are complex polysaccharides consisting of (1 → 4)-linked galacturonic acid residues as the major component. Galacturonic acid is present as free acid (or salt) and in the methyl ester form. Neutral sugars such as rhamnose, arabinose, xylose, mannose, fucose and glucose have also been found in pectins from different sources (Duan, Zheng, Dong, & Fang, 2004; Mukhiddinov, Khalikov, Abdusamiev, & Avloev, 2000; Petersen, Meier, Duus, Clausen, 2008; Tamaki, Konishi, Fukuta, & Tako, 2008; Yapo & Koffi, 2006). Pectin has been extracted from passion fruit with diluted nitric acid and characterized by Yapo and Koffi (2006). The main component was found to be galacturonic acid, but neutral sugars such as rhamnose, arabinose, xylose, mannose, fucose and glucose were also present (Yapo & Koffi, 2006). The molar mass of pectin extracted from passion fruit ranges from 6.4×10^4 to 5.1×10^4 g/mol depending on the treatment used to obtain the polysaccharide (Yapo & Koffi, 2006).

In this study, the polysaccharide fraction of *P. edulis f. flavicarpa* (PFCM) was obtained without the use of acidic conditions in the extraction protocol. The aims of this study were to characterize the polysaccharide obtained and investigate its antitumor

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properties. Hematological, biochemical and histopathological analyses were performed in order to evaluate the toxicity associated with the PFCM.

2. Materials and methods

2.1. Reagents

5-Fluorouracil (5-FU) and *O*-phenylenidiamine dihydrochloride were purchased from Sigma–Aldrich (St. Louis, MO, USA). All other reagents were of analytical grade. The dried fruit (*P. edulis f. flavicarpa*) peel flour was obtained from Pectina do Brazil based at the technology development park PADETEC (*Parque de Desenvolvimento Tecnológico do Ceará*) in Fortaleza, Brazil.

2.2. Animals

Swiss mice (female, 25–30 g), obtained from the central animal house of the Federal University of Ceará (UFC), Brazil, were used. Animals were housed in cages with free access to food and water. All animals were kept under a 12:12 h light–dark cycle (lights on at 06:00). Animals were treated according to the ethical principles of animal experimentation of COBEA (*Colégio Brasileiro de Experimentação Animal*), Brazil. The Animal Studies Committee of UFC has approved the experimental protocol.

2.3. Cells

The cytotoxicity of the PFCM sample was tested against HL-60 (human leukemias), MDA-MB-435 (melanoma), SF-295 (brain), and HCT-8 (human colon) cell lines, all obtained from the National Cancer Institute, Bethesda, MD, USA. Cells were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 μg/mL streptomycin and 100 U/mL penicillin, and incubated at 37 °C in a 5% CO_2 atmosphere.

Sarcoma 180 tumor cells have been maintained in the peritoneal cavities of the Swiss mice at the Laboratory of Experimental Oncology at UFC since the mid 1980s.

2.4. Polysaccharide extraction

Flour samples (20 g) were dispersed in distilled water at 3% (w/v) and kept under continuous stirring for 2 h at a temperature of 90–100 °C. The solution pH was 6.0 and remained constant during the extraction. After filtration through fine cloth, the extract was centrifuged at 17,000 × *g* for 20 min at 20 °C. The supernatant was filtered through a G3 vacuum funnel and concentrated under reduced pressure. The pH of the resulting concentrate was adjusted to 7.0 with NaOH. The polysaccharide was precipitated by addition of ethanol (1:4 (v/v)), washed with ethanol/acetone, dried at 40 °C and stored (6.97 g). The isolated polysaccharide was referred to as PFCM.

2.5. Polysaccharide characterization

2.5.1. Monosaccharide composition

The monosaccharide composition of the polysaccharide was determined after hydrolysis with 4 M trifluoroacetic acid for 5 h at 96 °C. Methanol was added to the hydrolyzed material and TFA was eliminated by rotaevaporation of the methanol/TFA mixture. The released monosaccharides were converted into alditol acetate by successive reduction with $NaBH_4$ followed by acetylation with pyridine–Ac_2O. Gas–liquid chromatography (GLC) analysis was performed on a 3800 Varian Gas Chromatograph connected to a 2000 R-12 Varian Ion-Trap Mass Spectrometer, with He as the carrier gas (2.0 mL/min). A DB-23 capillary column was used with a heating program of 40 °C/min up to 220 °C (constant temperature). The resulting derivatives were identified by their typical electron impact breakdown profiles. The uronic acid content was determined using the meta-hydroxydiphenyl method (Blumenkrantz & Asboe-Hansen, 1973). A galacturonic acid standard curve was used.

2.5.2. Elemental microanalysis

The nitrogen content of the polysaccharide was determined by elemental microanalysis using a Perkin-Elmer CHN 2400 analyzer and related to the total protein content through a conversion factor of 6.25.

2.5.3. High-performance size-exclusion chromatography (HPSEC)

The peak molar mass of the PFCM sample was determined by HPSEC using a Shimadzu LC-10AD chromatograph with a refractive index (RID-6A) detector at room temperature on an Ultrahydrogel linear column (7.8 mm × 300 mm). A volume of 50 μL of the polysaccharide solution (0.1% (w/v)) was injected onto the column, and elution was carried out with 0.1 mol/L $NaNO_3$ at a flow rate of 0.5 mL/min. The average molar mass was estimated using pullulan standard of 5.9×10^3–7.9×10^5 g/mol molar range. The correlation between the pullulan standard molar mass and the elution volume is given by Eq. (1):

$$\log \quad M = 14.016 - 0.951\, V_e \quad R = 0.991 \tag{1}$$

where V_e is the elution volume.

2.5.4. Infrared spectroscopy

FTIR spectra were recorded with KBr pellets on an FTIR Shimadzu 8300 spectrophotometer with a resolution of 2 cm^{-1}. The spectra were obtained from 30 scans.

2.5.5. Nuclear magnetic resonance

^1H and ^{13}C broadband spectra of 3% (w/v) solutions in D_2O at 70 °C were recorded on a Fourier transform Bruker Avance DRX 500 spectrometer with an inverse multinuclear gradient probe-head equipped with z-shielded gradient coils, and with Silicon Graphics. Sodium 2,2-dimethylsilapentane-5-sulfonate (DSS) was used as the internal standard (0.00 ppm for ^1H).

2.6. Antitumor activity of PFCM

2.6.1. Determination of the effect of PFCM on tumor cells in culture

The tumor cell growth was quantified by the ability of living cells to reduce the yellow dye 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-*2H*-tetrazolium bromide (MTT) to a dark blue formazan product (Mosmann, 1983). For all experiments, cells were seeded in 96-well plates (10^5 cells $well^{-1}$ for adherent cells or 0.5×10^5 cells $well^{-1}$ for suspended cells in 100 μL of medium). After 24 h, PFCM (0.5–100.0 μg/mL), dissolved in distilled water (10 μL of PFCM plus 90 μL of medium) was added to each well (using the HTS – high-throughput screening – biomek 3000 – Beckman Coulter, Inc., Fullerton, CA, USA) and incubated for 72 h at 37 °C in a CO_2 incubator. 5-FU (5-fluorouracil) was used as the positive control. At the end of the incubation, the plates were centrifuged and the medium was replaced by fresh medium (150 μL) containing 0.5 mg/mL MTT. Three hours later, the formazan product was dissolved in 150 μL DMSO and the absorbance was measured using a multiplate reader (DTX 880 Multimode Detector, Beckman Coulter, Inc., Fullerton, CA, USA). The drug effect was quantified as the percentage of the control absorbance of the reduced dye at 595 nm.

2.6.2. Determination of the effect of PFCM on tumor growth in vivo

Ten-day-old Sarcoma 180 ascite tumor cells (2×10^6 cell/500 μL) were implanted subcutaneously into the left hind groin of the mice. One day after inoculation, the PFCM was dissolved in distilled water and administered for 7 days (10 or 25 mg/kg, intraperitoneally administered, i.p. and 50 or 100 mg/kg, orally administered, p.o.) in mice transplanted with the Sarcoma 180 tumor. 5-FU (25 mg/kg) was used as a positive control. The negative control was injected with 0.9% NaCl. On day 8, peripheral blood samples from the control and treated mice were collected from the retro-orbital plexus under light ether anesthesia and the animals were then sacrificed by cervical dislocation. The tumors, livers, spleens and kidneys were extirpated, weighed and fixed in 10% formaldehyde. The inhibition ratio (%) was calculated using the following formula: inhibition ratio (%) = $[(A - B)/A] \times 100$, where A is the average tumor weight of the negative control and B is that of the treated group. Body weights were measured at the start and on the last day of treatment. The blood samples were used for hematological and biochemical analyses.

2.7. Toxicological analysis

2.7.1. Determination of the effect of PFCM on biochemical parameters

Blood samples of the treated animals were collected from the retro-orbital plexus under light ether anesthesia. Biochemical analysis was performed on serum samples obtained after centrifugation of total blood without anticoagulants, at 2500 rpm for 15 min. Standardized diagnostic kits by LABTEST® spectrophotometer (Lagoa Santa, MG, Brazil) were used in the spectrophotometric determination of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and urea levels.

2.7.2. Determination of the effect of PFCM on hematological parameters

For the hematological analysis, one aliquot of blood per animal was placed in ethylenediaminetetraacetic acid (EDTA) and various hematological parameters (platelet count and total and differential leukocyte count) were carried out by standard manual procedures using light microscopy.

2.8. Histopathology and morphological analyses

After being fixed with formaldehyde, the tumors, livers, spleens, and kidneys were grossly examined for size or color changes and hemorrhaging. Portions of the tumor, liver, spleen and kidney were then cut into small pieces, followed by staining of the histological sections with hematoxylin and eosin. Histological analysis was performed by light microscopy. The recognition and the extension of liver, kidney or spleens lesions attributed to the drugs were recorded.

2.9. Statistical analysis

Data are presented as mean ± S.E.M. The differences between experimental groups were compared by ANOVA (analysis of variance) followed by Student–Newman–Keuls ($p < 0.05$) using the GRAPHPAD program (Intuitive Software for Science, San Diego, CA).

3. Results and discussion

3.1. Polysaccharide characterization

The extraction of polysaccharide from passion fruit flour was carried out with hot water without acidification. The yield of the polysaccharide in relation to the dry flour was 34.9%. The analytical data for the PFCM polysaccharide are shown in Table 1. The protein content (Table 1) of the PFCM sample is lower than that obtained for the passion fruit flour extracted with acetic acid (4.05%). This protein content may be due to the presence of a polysaccharide–protein complex as observed in other polysaccharides. Pectin extracted with nitric acid has a protein content ranging from 0.9% to 5.1% depending on the treatment used in the process (Yapo & Koffi, 2006).

Table 1
Analytical data for *Passiflora edulis* polysaccharide (PFCM).

Analytical data	Values (g/100 g of PFCM)
Moisture	14.3
Ash[a]	13.8
Protein (%N × 6.25)	3.0
Polysaccharide	82.7

[a] As ash is due to degradation reaction it is not account for polysaccharide composition.

Table 2
Sugar composition of polysaccharide extracted from *Passiflora edulis*.

Sugar	Values (g/100 g of polysaccharide)
Galacturonic acid	44.2
Arabinose	11.8
Rhamnose	10.6
Glucose	11.8
Mannose	9.0
Galactose	6.1
Xylose	3.6
Ribose	1.3
Fucose	1.6

The major component of PFCM is galacturonic acid (Table 2). The neutral sugars determined by GC/MS analysis were arabinose, glucose, rhamnose, and mannose. Small amounts of fucose, xylose and ribose were also detected. These neutral sugars, with the exception of ribose, have also been found in the wall material of yellow passion fruit flour (Yapo & Koffi, 2006). The data in Table 2 suggest that the PFCM has linear homogalacturonan (HG) and neutral sugar – branched rhamnogalacturonan-1 (RG-1) structures. The low galacturonic acid/rhamnose ratio indicates that RG-1 is present in a higher proportion than HG. This result is very different from those reported by Yapo and Koffi (2006) for pectin obtained by acid extraction from passion fruit where the structure mainly consisted of homogalacturonan.

HPSEC chromatograms of the PFCM using the refractive index detector are shown in Fig. 1. Only one broad peak with a maximum elution volume at 9.71 mL appears. The molar mass of the polysaccharide was estimated using Eq. (1) and the value was 6.0×10^4 g/mol. This value is in the range found by Yapo and Koffi (2006) for pectin extracted from yellow passion fruit (6.4×10^4–5.1×10^4 g/mol).

The FTIR spectrum of the PFCM polysaccharide (Fig. 2) shows bands at 1740 cm^{-1} and 1653 cm^{-1}, attributed, respectively, to the C=O stretching vibration of esterified and unesterified galacturonic acid (Iagher, Reicher, & Ganter, 2002; Marrique & Lajolo, 2002; Souza et al., 2009). The degree of esterification (DE) of PFCM was calculated using the approach proposed by Marrique and Lajolo (2002). In this calculation the area of the FTIR peak due to the stretching vibration of the carbonyl group of esterified uronic acid (1740 cm^{-1}) was related to that of the unesterified uronic acid (1653 cm^{-1}) through Eq. (2) (Marrique & Lajolo, 2002).

$$DE\,(mol\%) = 124.7R + 2.20 \quad (2)$$

where $R = A_{1740}/(A_{1740} + A_{1653})$. The low value of DE for FPCM (26.2 ± 0.8 mol%) is consistent with the results observed

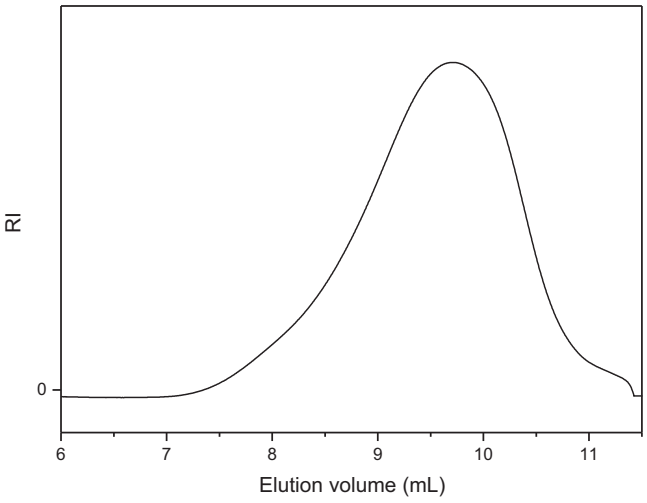

Fig. 1. HPSEC chromatogram of *P. edulis* polysaccharide (PFCM).

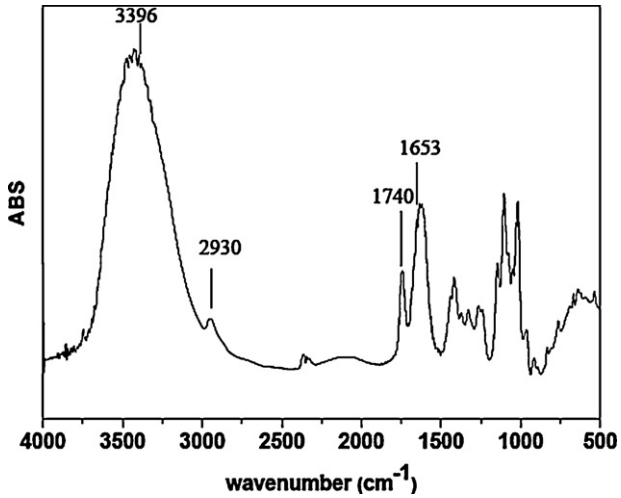

Fig. 2. FT-IR spectrum for KBr pellets of *P. edulis* polysaccharide (PFCM).

for the pectin obtained from passion fruit by acid extraction at low temperature (32.4–26.3 mol%) by Yapo and Koffi (2006).

The ^{1}H NMR spectrum of PFCM (Fig. 3) shows an intense signal at δ 3.80 due to the methyl groups of esterified galacturonic acid (GalA Me) (Cozzolino et al., 2006; Tamaki et al., 2008). Two signals at δ 2.17 and δ 2.06 were assigned to acetyl groups linked to 2-O and 3-O-galacturonic acid, respectively (Tamaki et al., 2008). The methyl group of L-rhamnose was observed at δ 1.25. The signals at δ 5.3 and 5.13 were attributed, respectively, to the H-1 of rhamnose and galacturonic acid. The H-5 of esterified galacturonic acid was detected at δ 4.9 while that for unesterified galacturonic acid was found in the range of δ 4.6–4.7 (Coenen, Bakx, Verhoef, Schols, & Voragen, 2007).

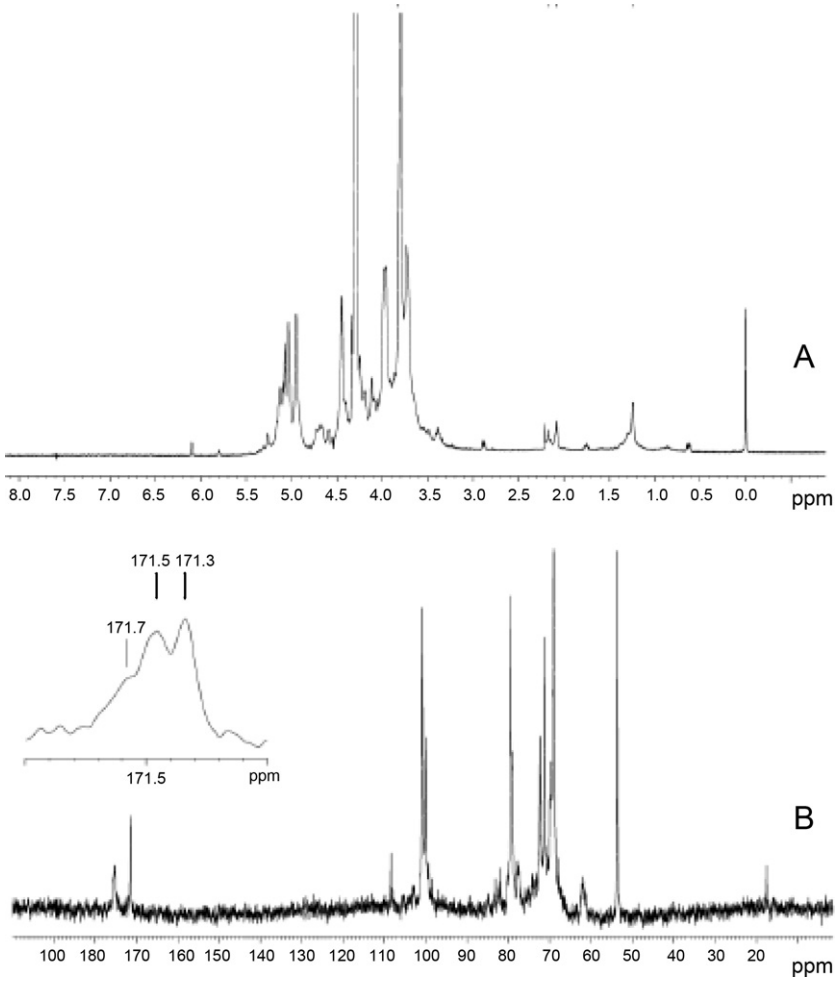

Fig. 3. NMR spectra for *P. edulis* polysaccharide (PFCM) in D_2O at 70 °C (A) ^{1}H NMR spectrum; (B) ^{13}C NMR spectrum.

The ^{13}C NMR spectrum of PFCM (Fig. 3) shows a resonance peak at δ 53.6 characteristic of the methyl ester of GalA Me. The signals at δ 101.1; 100.9 and 108.5 can be assigned to anomeric carbons of galacturonic acid (GalA), galacturonic acid methyl ester (GalA Me) and arabinofuranose (Ara), respectively. A low intense signal at δ 17.5 confirms the presence of rhamnose in the polysaccharide. In the carbonyl region of the spectrum the two signals at δ 171.3 and 175.3 correspond to C=O of GalA and GalA Me, respectively.

The distribution of methoxyl groups in acidic polysaccharide were investigated by Westerlund, Aman, Andersson, Andersson, and Rahman (1991) and Catoire, Goldberg, Pierron, Morvan, and Penhoat (1998) using the ^{13}C NMR pattern of the carbonyl region. The authors proposed that the carbon chemical shift of the carbonyl groups is influenced by the next-neighbor effects of the preceding and following residues. Triads of unesterified (U) and esterified (E) galacturonic acid such as UEU, EEE and UUU can be identified (where underscore denotes the observed residue) (Catoire et al., 1998; Westerlund et al., 1991). Taking into account the split of the carbonyl resonance, triads of the fully esterified acid have lower chemical shifts (EEE) than those where unesterified acid units are present in the triads (UEU). The triads with only unesterified groups are observed in the region of δ 175 and δ 177 (Catoire et al., 1998). Fig. 3B shows the amplification of the region related to the PFCM carbonyl groups. In the δ 171–172 region two sharp signals can be observed and a shoulder at δ 171.3; δ 171.5 and δ 171.7 assigned to the GalA Me in the triads EEE, UEE and UEU, respectively. A broad signal was observed between δ 173.0 and 175.7 due the resonances of the unesterified region. The EEE is more intense (43%) than UEE (35%) and UEU (22%) suggesting a high population of fully esterified triads (Catoire et al., 1998; Westerlund et al., 1991).

3.2. Antitumor activity of PFCM

The antitumor properties of PFCM were investigated using *in vitro* and *in vivo* models. As demonstrated for other polysaccharides (Gonzaga et al., 2009; Lins et al., 2009; Sousa et al., 2007), PFCM is not able to inhibit the proliferation of cancer cells in cultures at the tested concentrations (IC_{50} values greater than 100 μg/mL). 5-Fluorouracil, used as the positive control, showed IC_{50} values in the range of 0.36–12.59 μg/mL for HCT-8 and HL-60, respectively.

On the other hand, a significant reduction in the tumor weight was observed in PFCM-treated animals ($p < 0.05$). On day 8, the average tumor weight of the control mice was 1.37 ± 0.08 g. The intraperitoneal administration of PFCM (10 and 25 mg/kg) led to tumor inhibition of 70.40% and 72.89%, respectively. The oral administration (50 and 100 mg/kg) inhibited the tumor growth by 40.59% and 48.73% (Fig. 4). This inhibition is related to the occurrence of extensive areas of coagulative necrosis as observed through the histopathological analysis of the tumors extirpated from treated mice (data not shown).

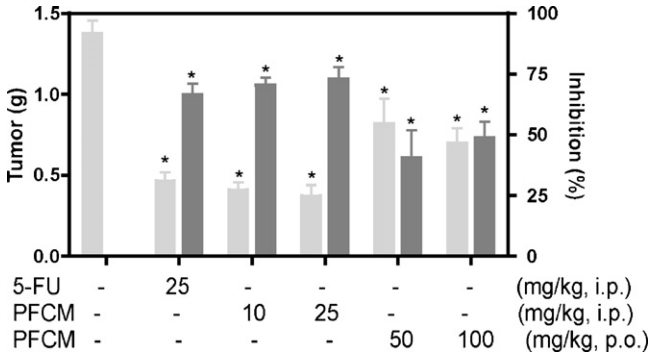

Fig. 4. Effect of the polysaccharide isolated from *P. edulis* (PFCM) and 5-FU on mice transplanted with Sarcoma 180 tumor. The graph shows the tumor growth inhibition (dark grey) and tumor weight (light gray). Data are presented as mean ± S.E.M. of eight animals. *, ($p < 0.05$) compared with saline by ANOVA followed by Student–Newman–Keuls test.

Table 4
Effect of the polysaccharide isolated from *Passiflora edulis* (PFCM) on the biochemical changes on peripheral blood. Mice were injected with Sarcoma 180 (2.0×10^6 cells/animal s.c.). The animals were treated, starting one day after tumor implant, for seven consecutive days.

Drug	Dose (mg/kg/day)	AST (UI/L)	ALT (UI/L)	Urea (mg/dL)
Healthy mice				
Saline	–	47.8 ± 12.5	14.1 ± 5.7	38.3 ± 4.1
S-180				
Saline	–	55.6 ± 10.5	17.3 ± 8.0	45 ± 6.1
5-FU	25	69.0 ± 14.1	21 ± 14.3	53 ± 10.3
PFCM	25	49.8 ± 13.8	19.5 ± 7.1	33.7 ± 8.4
PFCM	50	64.0 ± 12.5	21 ± 6.4	39.0 ± 5.8
PFCM	100	61.0 ± 15.2	22 ± 9.4	39.6 ± 7.8

Data are presented as mean ± S.E.M. of five animals.

This pattern of host-mediated antitumor activity has also been observed for other polysaccharides, such as alginates from *Sargassum vulgare*, sulfate polysaccharides from *Champia feldmannii* and a polysaccharide–protein complex from *Agaricus blazei* (Gonzaga et al., 2009; Lins et al., 2009; Sousa et al., 2007) using the same models. It has been suggested that this activity is related to the modulation of the immune system. Thus, the bioactive polysaccharides are considered to be biological response modifiers, acting through the direct or indirect activation of different components of the immune system such as macrophages, T-lymphocytes, B-lymphocytes and natural killer cells (Leung et al., 2006). The mechanisms involved in this process are not fully elucidated, but the observed responses in animal models include the inhibition

Table 3
Effects of the polysaccharide isolated from *Passiflora edulis* (PFCM) on organ weights. Mice were injected with Sarcoma 180 (2.0×10^6 cells/animal s.c.). The animals were treated, starting one day after tumor implant, for seven consecutive days.

Drug	Dose (mg/kg)	Liver (g/100 g body weight)	Spleen (g/100 g body weight)	Kidney (g/100 g body weight)
Healthy mice				
Saline	–	4.59 ± 0.190	0.18 ± 0.030	1.46 ± 0.050
Mice transplanted with tumor S180				
Saline	–	5.18 ± 0.188	0.63 ± 0.071[a]	1.17 ± 0.025
5-FU	25 (i.p.)	4.86 ± 0.191	0.46 ± 0.039[a,b]	1.19 ± 0.041
PFCM	10 (i.p.)	4.70 ± 0.190	0.66 ± 0.030[a,c]	1.14 ± 0.039
PFCM	25 (i.p.)	4.76 ± 0.328	0.73 ± 0.035[a,c]	1.06 ± 0.240
PFCM	50 (p.o.)	5.30 ± 0.160	0.69 ± 0.039[a,c]	1.08 ± 0.045
PFCM	100 (p.o.)	5.20 ± 0.140	0.60 ± 0.034[a,c]	1.16 ± 0.044

Data are presented as mean ± S.E.M. of eight animals.
[a] ($p < 0.05$) compared with healthy animals.
[b] ($p < 0.05$) compared to saline experimental group.
[c] ($p < 0.05$) compared to 5-FU by (ANOVA) followed by Student–Newman–Keuls test.

Table 5
Effect of the polysaccharide isolated from *Passiflora edulis* (PFCM) on the hematological changes in peripheral blood. Mice were injected with Sarcoma 180 (2.0×10^6 cells/animal s.c.). The animals were treated, starting one day after tumor implant, for seven consecutive days.

Drug	Dose (mg/kg/day)	Platelet (10^5 cells/μL)	Total leukocytes (10^3 cells/μL)	Differential leukocytes count (%)			
				Eosinophil	Lymphocyte	Neutrophil	Monocyte
Healthy mice							
Saline	–	7.52 ± 0.39	5.96 ± 0.27	0.40	66.8	19.60	12.80
S-180							
Saline	–	5.94 ± 0.52	2.8 ± 0.4[a]	0.6	55.2[a]	37.6[a]	7.0
5-FU	25	4.13 ± 0.21[a]	1.4 ± 0.20[a,b]	0.0	92.9[a,b]	3.8[a,b]	3.3[a,b]
PFCM	25	6.1 ± 0.1[c]	5.6 ± 1.1[b,c]	0.0	52.0[a,c]	37.4[a,c]	9.6[c]
PFCM	50	7.3 ± 1.1[c]	5.9 ± 1.3[b,c]	0.0	67.6[b,c]	27.4[a,b,c]	5.0[a,b]
PFCM	100	6.3 ± 1.0[c]	4.8 ± 0.6[b,c]	0.89	68.6[b,c]	26.2[a,c]	4.3[a,b]

Data are presented as mean ± S.E.M. of five animals.
[a] ($p < 0.05$) compared with healthy group by ANOVA followed by Student–Newman–Keuls test.
[b] ($p < 0.05$) compared with saline experimental group by ANOVA followed by Student–Newman–Keuls test.
[c] ($p < 0.05$) compared with 5-FU group by ANOVA followed by Student–Newman–Keuls test.

of cancer cell growth, angiogenesis and metastasis (Glinsky & Raz, 2009; Leung et al., 2006). It is important to note that the effect of PFCM is comparable with or, more commonly, greater than that observed with other polysaccharides using the Sarcoma 180 tumor model (Gan, Zhang, Yang, & Xu, 2004; Gonzaga et al., 2009; Lins et al., 2009; Liu et al., 2006; Sousa et al., 2007).

3.3. Toxicological analysis

One important aspect of this study is the toxicological profile of these polysaccharides. In general, the treatment is well tolerated and the observable side effects are potentially reversible (Gonzaga et al., 2009; Leung et al., 2006; Lins et al., 2009; Sousa et al., 2007, 2008). In a preliminary assessment of the toxicological effects of the PFCM treatment, morphological alterations of the liver, kidney and spleen were evaluated based on the weight and histopathological analysis. Also, basic metabolic alterations were monitored including urea, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels. Finally, a hematological evaluation was carried out.

No significant changes in the liver or kidney weights were observed in the PFCM treated animals (Table 3). However, after the treatment with PFCM, the spleen weights were significantly increased when compared with the 5-FU group ($p < 0.05$). No significant changes in the body weight gain were noted among the groups (data not shown).

Histopathological analysis of the liver samples did not show any alterations related to the PFCM treatment, suggesting that this organ was not affected by the treatment. Likewise, there were no differences related to the ALT and AST levels (Table 4). The kidneys removed from PFCM-treated animals, on the other hand, showed discrete hydropic change and some areas of focal necrosis in the proximal tubular epithelium (at 25 mg/kg), glomerular and tubular hemorrhage. The glomeruli structure was essentially preserved, which suggests that these alterations could be considered potentially reversible (Olsen & Solez, 1994). These results are in agreement with the findings presented by Sousa et al. (2007, 2008) and Lins et al. (2009), and the kidney seems to be the main toxicological target for this class of compounds (Sousa et al., 2008). However, no significant changes were observed in the urea level (Table 4). In fact, there is a considerable delay before blood urea levels are increased after renal alteration (Varley, Gowenlock, & Bell, 1980). In the spleen, PFCM-treated mice showed an increase in the white pulp and nest of megakaryocytes, which suggests an immunomodulatory activity.

The animals transplanted with Sarcoma 180 tumors showed a significant decrease ($p < 0.05$) in the total numbers of circulating peripheral leukocytes compared to healthy animals. In addition, 5-FU showed a considerable reduction in total leukocytes ($p < 0.05$). Moreover, there was a significant difference ($p < 0.05$) in the percentage of lymphocytes and neutrophils in the peripheral blood of animals transplanted with Sarcoma 180 tumors when compared to the normal group treated with saline (Table 5). Treatment with PFCM positively influenced the increased numbers of total leukocytes. Moreover, PFCM treatment normalized the percentage of lymphocytes and neutrophils in the peripheral blood. These results indicate that the PFCM increased the cell types involved in the primary defense mechanism.

4. Conclusions

In conclusion, PFCM has linear homogalacturonan (HG) and neutral sugar – branched rhamnogalacturonan-1 (RG-1) structures and low DE. The low galacturonic/rhamnose ratio indicates that RG-1 is present in a higher proportion than HG. PFCM exhibited *in vivo*, but not *in vitro*, antitumor effects with no expressive toxicity. This activity seems to be related to its immunostimulating properties.

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