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Structural characterization of blackberry wine polysaccharides and immunomodulatory effects on LPS-activated RAW 264.7 macrophages

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ABSTRACT

Three polysaccharide fractions were isolated from blackberry wine. The crude extract BWPs was obtained with ethanol precipitation and freeze-thawing process, it was then submitted to Fehling treatment, giving soluble BWPFs and insoluble BWPFp fractions. These fractions were characterized by Gas Chromatography-Mass Spectrometry (GC–MS) and Nuclear Magnetic Resonance (NMR). Major polysaccharides were identified for each fraction: mannan, type II arabinogalactan and type I rhamnogalacturonan for BWPs, a mannan formed by a major chain of α-Man*p*(1 → 6)-linked units, *O*-2 substituted with α-D-Man*p*(1 → 2)-linked side chains for BWPFp and a AG II formed by a major chain of *β*-D-Gal*p*(1 → 3)-linked, substituted at *O*-6 by side chains of the *β*-D-Gal*p* (1 → 6)-linked, which then are substituted at *O*-3 by non-reducing units of *α*-L-Ara*f* and a RG I, formed by [→4)-*α*-D-Gal*p*A-(1 → 2)-*α*-L-Rha*p*-(1→]$_n$ for BWPFs. Anti-inflammatory effects of polysaccharide fractions were evaluated in RAW 264.7 cells. Fractions markedly reduced nitric oxide (NO) and pro-inflammatory cytokine production (TNF-α and IL-1β) in LPS-treated cells.

1. Introduction

Vinification technologies are among the oldest known to humans. In recent years, nongrape fruit wines have caught the attention of alcoholic beverages consumers due to scientific evidence relating health benefits and habitual consumption of a wide variety of fruits and their products (Vasantha Rupasinghe, Joshi, Smith, & Parmar, 2017). These benefits have added value to these wines and consequently increased economic importance of this beverage segment. Emerging fruit wines include those of blueberries, cherries, strawberries, and blackberries. Blackberry wine is produced by yeast fermentation of natural sugars present in blackberry juice. It is recognized as a natural source of essential minerals and bioactive phytochemicals that can play an important role in health promotion and disease prevention (Petravić-Tominac, et al., 2013). Information regarding blackberry wines is very scarce. Previous studies investigate mainly the mineral composition, phenolic compounds and polyphenols (Amidzic Klaric, Klarić, Velić, & Vedrina Dragojevic, 2011; Amidzic Klaric, Klarić, & Mornar, 2011). In addition to promoting beneficial effects as antioxidants, phenolic compounds can act as inhibitors of carbohydrate-utilizing enzymes and as potential inhibitors of inflammation (Arozarena, et al., 2012; Johnson, Mejia, Fan, Lila, & Yousef, 2013). Health benefits and sensory properties attributed to fermented berry products are mainly attributed to high amounts of polyphenolic compounds present in this fruit (Lim, Hwang, & Shin, 2012). Also, phenolic compounds from the beverage had an impact to attenuate development of obesity and fasting blood glucose in C57BL/6J mice (Johnson, Wallig, Luna Vital, & Mejia, 2016). In addition to the compounds already described in the literature, blackberry wine has many other constituents that have not been structurally elucidated and can also be responsible for biological activities, such as polysaccharides.

Polysaccharides present in blackberry wine are derived from yeast cell walls used in the fermentation process or blackberry fruits (Boulet, Williams, & Doco, 2007). Polysaccharides from different sources have shown therapeutic effects including anti-tumor, anti-ulcer, anti-complementary, anti-coagulant, hypoglycemic agents and anti-inflammatory activities (Jin, Liu, Zhong, Sun, & Zhang, 2017; Leivas et al., 2016; Nergard, et al., 2005; Ochoa, Iacomini, Sassaki, & Cipriani, 2017; Wang et al., 2017). Macrophages are immune cells implicated in the initiation of inflammatory responses, secreting several pro-inflammatory mediators, including nitric oxide (NO) and pro-inflammatory cytokines, like tumor necrosis factor (TNF- α) and

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interleukin 1-β (IL-1β) (Lee & Park 2015). Therefore, this study reports fractionation and structural characterization of blackberry wine polysaccharides and evaluate their inhibitory effects on NO, IL-1β and TNF-α production in Lipopolysaccharide (LPS)-induced RAW 264.7 macrophages.

2. Materials and methods

2.1. Polysaccharide source

The blackberry wine (750 mL) was obtained after fermentation of mature blackberry fruits (*Rubus fruticosus*) in October of 2010 and it was produced by Adega Porto Brazos (Ponta Grossa, Paraná, Brazil).

2.2. Polysaccharide extraction and purification

After concentration under reduced pressure, blackberry polysaccharides were precipitated by addition of cold EtOH (3× vol.) and stand at 4 °C overnight. After centrifugation (8.000 rpm at 4 °C, 20 min) the precipitate was dissolved in water and dialyzed (6–8 kDa cut-off membrane) against distilled water for 72 h, originating polysaccharide fraction blackberry wine polysaccharide (BWP). This fraction was again solubilized in water (150 mL) and submitted to freeze-thawing process thrice (Gorin & Iacomini, 1984), which resulted in water soluble, blackberry wine polysaccharide-soluble (BWPs) and an insoluble, blackberry wine polysaccharide-insoluble (BWPi) fraction. The former was submitted to Fehling treatment, resulting in two fractions: supernatant, blackberry wine polysaccharide-Fehling supernatant (BWPFs) and precipitate, blackberry wine polysaccharide-Fehling precipitate (BWPFp).

2.3. Homogeneity and molecular weight analysis

BWPs, BWPFs and BWPFp samples homogeneity and isolated BWPFp sample average molar mass (Mw) were determined by high-performance size-exclusion chromatography (HPSEC) using refractive index and multi-angle laser light scattering detectors. Polysaccharide permeation was performed in serial array of columns with exclusion sizes of 7×10^6 (Ultrahydrogel 2000), 4×10^5 (Ultrahydrogel 500), 8×10^4 (Ultrahydrogel 250) and 5×10^3 (Ultrahydrogel 120) Daltons using 0.1 M aq. $NaNO_2$ containing 200 ppm aq. NaN_3 at 0.6 mL/min as eluent. Samples were filtered through a 0.22 μm membrane (Millipore) and injected (100 μL, loop) at a concentration of 1 mg/mL. Specific refractive index increment (dn/dc) was determined for polysaccharide purified BWPFp and data were analyzed and processed in ASTRA software (Wyatt Technologies). Final graphics were drawn using GraphPad Prism software version 5.01 for Windows (GraphPad Software, San Diego, CA, USA).

2.4. Monosaccharide analysis

Polysaccharides (~5 mg) were hydrolyzed using 2 M trifluoroacetic acid (TFA) (1 mL) at 100 °C for 12 h and then evaporated to dryness. Samples were then solubilized in 400 μL D_2O for NMR analysis (Sassaki et al., 2014).

After NMR analysis, the solution was reduced with $NaBH_4$ (2 mg) at room temperature overnight, neutralized after addition of acetic acid (500 μL) and evaporated to dryness. Resulting boric acid was removed as trimethyl borate by co-evaporation with MeOH in a nitrogen stream. Resulting alditols were converted to acetyl esters with Ac_2O-pyridine (1:1 v/v, 0.5 mL) at 100 °C for 1 h and extracted with $CHCl_3$. Organic phase was washed thrice with 5% $CuSO_4$, once with distilled water and dried at room temperature. Acetylated samples were analyzed by GC–MS (Varian Saturn 3800 Gas Chromatograph coupled to a Varian 4000 ion trap mass spectrometer) using a DB-225-MS capillary column (30 m × 0.25 mm) with injector at 250 °C and oven programed from 50 °C (initial) to 230 °C at 40 °C/min, He being the carrier gas (1 mL/min) and dissolved in acetone (Sassaki et al., 2008). Uronic acid contents of polysaccharides were determined by the colorimetric *m*-hydroxybiphenyl method (Filisetti-Cozzi & Carpita, 1991), using galacturonic acid standard.

2.5. Methylation analysis

Polysaccharides BWPs, BWPFs and BWPFp were methylated using NaOH/DMSO-MeI as described by Ciucanu and Kerek (1984). Per-*O*-methylated polysaccharides were submitted to methanolysis with 3% HCl-MeOH (80 °C, 2 h) followed by hydrolysis with formic acid (45%, 12 h) (Biermann, 1988). The latter was dried and reduced with $NaBD_4$ and acetylation was performed as described above. The partially *O*-methylated alditol acetates (PMAA) were examined by GC–MS (Varian Saturn 3800 Gas Chromatograph coupled to a Varian 4000 ion trap mass spectrometer), using a DB-225-MS capillary column (30 m × 0.25 mm), injector 250 °C, oven start at 50 °C (held 1 min) to 185 °C (40 °C/min) held 10 min and then to 210 °C (40 °C/min) held for 15 min, He being the carrier gas (1 mL/min). Chromatograms peaks were identified by their typical electron impact fragmentation profiles and retention times (Sassaki, Gorin, Souza, Czelusniak, & Iacomini, 2005). BWPFs was also submitted to carboxy-reduction according to Taylor and Conrad (1972), resulting BWPFs-CR sample.

2.6. NMR spectroscopy

Polysaccharides (10 mg) were dissolved in 0.5 mL D_2O with internal standard 10 μL 3-trimethylsilyl-$^2H_4$-propionic acid sodium salt (TMSP) 1%. Nuclear magnetic resonance analyses were performed using a Bruker Avance *III* NMR spectrometer operating at 14.1 Tesla (600.13 MHz for ^1H) equipped with an inverse 5 mm probe head (QXI) at 303 K. 1D ^1H and ^{13}C NMR were collected after 90° pulse calibration for each sample. ^1H and ^{13}C chemical shifts were determined by 2D NMR experiments. 2D ^1H-^{13}C multiplicity-edited HSQC was performed by correlation via double inept transfer with decoupling during acquisition using sensitivity improvement trim pulses as compiled in the pulse program hsqcedetgpsisp2.2 using 6993 Hz (^1H) and 24900 Hz (^{13}C) widths and a recycle delay of 1.080 s. 2D correlation maps were recorded using quadrature detection in the indirect dimension and 24 scans per series of 1024 × 320 W data points, with zero filling in F1 (2048), prior to Fourier transformation (Sassaki et al., 2014).

2.7. Cell culture and reagents

Murine macrophage cells (RAW 264.7) were grown in Dulbecco's modified Eagle's medium (DMEM) with 4.5 g L^{-1} glucose supplemented with 10% fetal bovine serum and 20 mM sodium bicarbonate (Cultilab, Campinas, SP, Brazil). All cultures were performed in culture plates (Falcon BD, San Jose, CA, USA). Lipopolysaccharide from *Escherichia coli* O55:B5 and the other reagents were purchased from Sigma (St Louis, MO, USA).

2.8. Cell viability assay

Cell viability was determined by MTT (3- (4,5-Dimethylthiazol-2-yl)-2,5-bromo diphenyltetrazolium) method (Mosmann, 1983), where reduction of crystal tetrazolium forming through mitochondrial enzymes is possible only in viable cells. Cells were plated in 24 well plates (4.8×10^5 cells/well) and treated with different concentrations of BWPs, BWPFs and BWPFp polysaccharides (0.1, 1.0, 10 and 100 μg/mL), dissolved in DMEM. After 24 h, 350 μL/well of the 5 mg/mL MTT solution was added. After 4 h of incubation, cell supernatant was removed and 500 μL of DMSO (Dimethylsufoxide) were added for cell lysis and crystal solubilization. Absorbance then was recorded at 570 nm wavelength.