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Polysaccharides from green and black teas and their protective effect against murine sepsis

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ABSTRACT

Polysaccharides from green and black teas were obtained via hot aqueous extraction, and after fractionation, chemical analysis showed that they consisted mainly of a rhamnogalacturonan containing a long sequence of →4)-6-*O*-Me-α-D-Gal*p*A-(1→, interrupted by α-L-Rha*p* residues, which were substituted by type II arabinogalactan chains. The polysaccharides were similar, except that black tea contained lesser galacturonic acid residues (35%), when compared with green tea (65%). Starch was present in both teas. The polysaccharides were tested, via oral administration in mice against induced-polymicrobial sepsis, at doses of 30, 50 and 100 mg/kg. Green and black tea polysaccharides were capable of reducing mortality rate by 40% and 25% respectively, compared to sham-controls. The influx of neutrophils was also affected, decreasing its accumulation in lungs and tissue injury. The difference between the green and black tea polysaccharides seems to result from the processing that the leaves have undergone and the different abilities to prevent mice death can be related to the different uronic acid contents.

1. Introduction

After water, teas from *Camellia sinensis* L. (mainly green and black teas) are considered to be the most consumed beverage worldwide. The main difference among the teas is based on the process of auto-oxidation catalyzed by the enzymes polyphenol oxidase (PPO) and peroxidase. Basically, oxidation is a process to which the leaves of *C. sinensis* interact with oxygen, after being processed (e.g. being minced, crushed or dried) that results in darkening. Those from *C. sinensis* are thus classified as white, green, oolong, black and Pu'erh teas which undergo unfermented, semi-fermented, and fermented processes (Engelhardt, 2010; Ho, Lin, & Shahidi, 2008).

Products of secondary metabolism, especially flavonoids from *C. sinensis*, have been widely studied for their chemical composition and biological effects (Scoparo et al., 2012; Sharangi, 2009), although the polysaccharides have received less attention. However, in infusions, many compound classes are extracted from the leaves, including polysaccharides which are then ingested. It is known that many plants used in folk medicine contain polysaccharides with a recognized variety of properties, including immunological, anti-radiation, anticoagulation, anti-cancer, anti-HIV, hypoglycemic and gastric protection activities (Cipriani et al., 2006; Wang, Wang, Li, & Zhao, 2001; Xie & Nie, 2006; Zhou, Xie, & Fu, 2001).

Recent studies have shown that the structures of polysaccharides are closely related to their biological activities (Cooper, Morré, & Morré, 2005; Harold & Graham, 1992), so elucidation of their structures is fundamental for understanding structure–activity relationships. Some structural studies have been carried out on tea polysaccharides, being described a polysaccharide composed of rhamnose, glucose, galactose, arabinose and xylose (Zhou, Xie, & Nie, 2004). Also, other polysaccharides were identified as neutral (1→4)-β-galactan and pectin-type polysaccharide formed by blocks of (1→4)-α-D-galactopyranosyluronic acids interconnected by (1→2)-linked rhamnose residues (Wang, Wei, & Jin, 2009a, 2009b).

Several pharmacological properties have been attributed to polysaccharides from different sources, although few investigations have correlated their structure with sepsis treatment. Sepsis is a considerable health problem and a leading cause of morbidity and mortality in many intensive care units. It represents a state of overproduction of pro-inflammatory mediators which frequently occurs after various noxious injuries, especially bacterial infection arising from abdominal surgery, appendicitis, perforated ulcers, or an ischemic bowel (Angus, Linde-Zwirble, Lidicker, & Clermont, 2001; Cohen, 2002). We now

Abbreviations: AcOH, acetic acid; Ac_2O, acetic anhydride; CLP, cecal ligation and puncture; EtOH, ethanol; $CHCL_3$, chloroform; HPSEC, high-performance size-exclusion chromatography; GC–MS, gas chromatography–mass spectrometry; MPO, myeloperoxidase; TMSP-d_4, 2,2,3,3-tetradeuterium-3-trimethylilsilylpropionate; GT, green tea; BT, black tea; GSP, green soluble polysaccharide; BSP, black soluble polysaccharide.

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evaluate differences in the chemical structure of the polysaccharides isolated from both green and black teas and their protection against sepsis, in a murine model of cecal ligation and puncture (CLP), and their effects on neutrophil migration.

2. Experimental methods

2.1. Plant material

Green and black teas were purchased in a local market (Curitiba, State of Paraná, Brazil), as commercial processed leaves.

2.2. Polysaccharide extraction and fractionation

Green and black teas (50 g of each) were submitted to aqueous extraction (100 °C, 500 mL, ×3). The extracts were combined and evaporated to 250 mL. High molecular weight components, mainly polysaccharides, were precipitated by addition of cold EtOH (3 vol.), and centrifuged (8,000 rpm at 4 °C, 20 min). The sediment was then dissolved in H_2O and dialyzed, in 12,000 Da cutoff membrane, against tap water for 24 h to remove the remaining low-molecular weight compounds, giving rise to crude polysaccharide fractions. These were frozen and then allowed to thaw at room temperature (Gorin & Iacomini, 1984), resulting in green (GSP) and black soluble polysaccharide (BSP) fractions and insoluble fractions which were separated by centrifugation as described above. The insoluble fractions were not analyzed.

2.3. Monosaccharide analysis

GSP and BSP (1 mg) were each hydrolyzed with 0.5 mL of 2 M TFA at 100 °C for 8 h, then the solution was evaporated, and the residue was dissolved in water (1 mL). The resulting monosaccharides were submitted to thin layer chromatography (TLC) on silica gel-60 (Merck), the eluant being ethyl acetate:acetic acid:*n*-propanol:water (4:2:2:1 v/v), followed by development with orcinol-sulfuric acid at 100 °C (Skipski, 1975). The monosaccharides were reduced with 2 mg $NaBH_4$ yielding alditols, which were acetylated in Ac_2O-pyridine (1:1 v/v, 0.5 mL) at room temperature for 12 h (Wolfrom & Thompson, 1963a, 1963b). The resulting alditol acetates were extracted with $CHCl_3$, and analyzed by GC–MS (Varian, Saturn 2000R-3800 gas chromatograph coupled to a Varian Ion-Trap 2000R mass spectrometer), using a DB-225-MS column (30 m × 0.25 mm × 0.25 μm) programmed from 50 to 220 °C at 40 °C/min, with He as carrier gas. Monosaccharides were identified by their relative retention times and typical electron ionization spectra (EI — 70 eV). The uronic acid contents of the soluble fractions were determined using the colorimetric *m*-hydroxybiphenyl method of Filisetti-Cozzi and Carpita (1991).

GSP and BSP (10 mg) were each submitted to carboxy-reduction through the carbodiimide method (Taylor & Conrad, 1972), using $NaBH_4$ as the reducing agent, giving the reduced products (GSP-CR, BSP-CR), and releasing their neutral equivalent monosaccharide.

2.4. Methylation analysis

The fractions GSP, BSP, GSP-CR and BSP-CR (5 mg) were each per-*O*-methylated in DMSO (0.5 mL), powdered NaOH (30 mg) and iodomethane (0.5 mL), adapted from the method of Ciucanu and Kerek (1984). The suspension was neutralized with acetic acid and dialyzed (12,000 Da cutoff) to eliminate reagents. The samples were lyophilized and alkylated polysaccharides were then hydrolyzed with 45% aq. v/v formic acid (1 mL) for 12 h at 100 °C, filtered and evaporated to dryness. The partially *O*-methylated monosaccharides were reduced with $NaBD_4$ and acetylated as described above, giving rise to a mixture of partially *O*-methylated alditol acetates, which were analyzed by GC–MS similar to what was described above, except that the temperature program was 50 to 210 °C in 30 min. Identification of the partially *O*-methylated alditol acetates was based on the *m*/*z* spectra (EI — 70 eV), and by comparison with the standard library (Sassaki, Gorin, Souza, & Czelusniak, 2005), the results being expressed as a relative percentage of each component.

2.5. Nuclear magnetic resonance (NMR) analysis

Mono- (^{13}C, ^{1}H and DEPT-135) and bi-dimensional $^{1}H/^{13}C$ (HSQC) NMR spectra were obtained by employing a Bruker Avance III 400 MHz spectrometer. Samples were dissolved in D_2O at ~40 mg in 400 μL and analysis was carried out at 70 °C. The chemical shifts were expressed in δ (ppm), standardized with TMSP-d_4 (sodium 2,2,3,3-tetradeuterio-3-trimethylsilyl-propionate) δ = 0 for ^{13}C and ^{1}H.

2.6. Animals

Male albino Swiss mice (3 months old, weighing 25–30 g), from the University of Paraná colony, were used for biological tests. They were maintained under standard laboratory conditions, with a constant 12 h light/dark cycle and controlled temperature (22 ± 2 °C), and standard pellet food (Nuvital®, Curitiba/PR, Brazil) and water were available ad libitum. All experimental procedures were previously approved by the Institutional Ethics Committee of the university (authorization number 430).

2.7. Sepsis induction by cecal ligation and puncture (CLP)

Mice were randomly grouped into five clusters of 10 mice: sham-operation, CLP plus vehicle (water p.o.), and CLP plus GSP or BSP (30, 50 and 100 mg/kg, p.o.). According to the body weight (~30 g), they were administered with 50 μL of each tea polysaccharide solution. Ketamine (80 mg/kg) and xylazine (20 mg/kg) were injected intraperitoneally to anesthetize the mice prior to surgical procedures. Polymicrobial sepsis was induced by CLP as previously described (Rittirsch, Huber-Lang, Flierl, & Ward, 2009). A midline incision of ~1.5 cm was performed on the abdomen and the cecum was carefully exposed and 50% of the distal portion was ligated. The cecum was then punctured three times with a sterile 16-gauge needle and squeezed to extrude fecal material from the wounds. The cecum was replaced and the abdomen was stitched surgically. Sham-control animals were treated identically, but no cecal ligation or puncture was carried out. Each mouse received subcutaneous sterile saline injection (1 mL) for fluid resuscitation after surgery. The mice were then kept on a heating pad (35 °C) until they recovered from the anesthesia. Food and water, ad libitum, were provided throughout the experiment. The survival rate was monitored for 7 days, 12 h each. During this period, vehicle (water) and treatments were orally administered daily.

In another set of experiment, 1 h prior to the surgery, mice were orally treated with vehicle, GSP, or BSP (30 and 50 mg/kg, p.o.). After 6 h post-operation, mice were sacrificed. Their lungs were collected and frozen for further use to determine the myeloperoxidase (MPO) activity.

2.8. Lung MPO activity

MPO activity was measured in order to determine neutrophil influx, according to established protocols (Bradley, Priebat, Christensen, & Rothstein, 1982). Briefly, the lung tissue was homogenized in 0.5 mL of 50 mM potassium buffer pH 6.0 with 0.5% hexadecyltrimethylammonium bromide, sonicated on ice, and then centrifuged at 14,000 rpm for 15 min at 4 °C. Supernatants were then assayed at a 1:20 dilution in reaction buffer (9.6 mM 3,3,5,5-tetramethylbenzine, 150 nM H_2O_2 in 50 mM potassium phosphate