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Anti-inflammatory and angiogenic activity of polysaccharide extract obtained from Tibetan kefir

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ABSTRACT

The search for new bioactive molecules is a driving force for research pharmaceutical industries, especially those molecules obtained from fermentation. The molecules possessing angiogenic and anti-inflammatory attributes have attracted attention and are the focus of this study. Angiogenic activity from kefir polysaccharide extract, via chorioallantoic membrane assay, exhibited a pro-angiogenic effect compared with vascular endothelial factor (pro-angiogenic) and hydrocortisone (anti-angiogenic) activity as standards with an EC50 of 192 ng/mL. In terms of anti-inflammatory activity determined via hyaluronidase enzyme assay, kefir polysaccharide extract inhibited the enzyme with a minimal activity of 2.08 mg/mL and a maximum activity of 2.57 mg/mL. For pharmaceutical purposes, kefir polysaccharide extract is considered to be safe because it does not inhibit VERO cells in cytotoxicity assays.

1. Introduction

Many studies have been conducted with different molecules, extracts and products obtained by fermentation in order to reveal and confirm their biological activity and use in the development of new pharmaceutical, cosmetic and nutraceutical products.

Kefir is a fermented milk product with an acidic taste and a creamy consistency that originated in the Balkans, Eastern Europe and the Caucasus (Fontán et al., 2006; Serafini et al., 2014). Kefir has beneficial effects on human health thanks to its antimicrobial (Anselmo et al., 2010), immunoregulatory (Hong et al., 2009), antiallergenic (Wei-Sheng et al., 2010), antitumoral (Gao et al., 2013), anti-inflammatory (Diniz et al., 2003), antidiabetic (Young-In et al., 2006) and antimutagenic (Guzel-Seydim et al., 2006) activities. The bioactivities of the kefir are attributed to the microorganisms and the substances within it, such as exopolysaccharides produced during the fermentation process of kefir grains.

Polysaccharides obtained from different sources, such as plants, animals and microorganisms, have been studied for possessing different biological activities: antibiotic, antioxidant, anticoagulant, anti-mutant, immuno-stimulation and anti-cancer (Yu et al., 2009; Wijesekara et al., 2011; Zong et al., 2012). However, polysaccharides derived from microorganisms—especially bacteria—are still being assessed for these activities and others (e.g., angiogenesis and anti-inflammatory).

The first studies on angiogenesis were proposed by Folkman (1971) who applied the concept of vascular bed control (i.e., stimulation or inhibition of new vessels) in an attempt to elucidate this process and also as an alternative treatment for various medical conditions (Folkman, 1971). In general, the endothelial cells lining the lumen of blood vessels are quiescent, and neovascularization is virtually absent. However, when these cells are stimulated they have early vascular neoformation, which can occur via two different mechanisms: vasculogenesis and angiogenesis (Lamalice et al., 2007; Adams and Alitalo, 2007).

Angiogenesis is the formation of a new network of vessels from preexisting vessels, a process that occurs via budding or intussusception. For angiogenesis to occur, tissue stimulation is necessary to induce and increase the expression of pro-angiogenic factors such as Vascular Endothelial Factor (VEGF) and Fibroblast Growth Factor (Folkman and Klagsbrun, 1987; Dias et al., 2002).

Angiogenesis is essential for the repair and development of tissues such as wound healing, the development of collateral circulation in ischemic tissues in corpus luteum, endometrium, placenta formation and hair growth. However, it can also be associated with pathological conditions such as cancer and eye changes (Adams and Alitalo, 2007; Folkman and Klagsbrun, 1987).

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The inflammatory process consists of a series of events that are related to tissue injury or infection. The inflammation, proliferation and remodeling of tissues are phases that occur in the synthesis and degradation of the extracellular matrix involving the activation and inhibition of the hyaluronidase enzyme (Bornstein and Sage, 2002).

Hyaluronidase is an enzyme that hydrolyses hyaluronic acid, an acid whose function is to ensure that cells remain adhered to one other. Fragmentation of this polymer significantly decreases intracellular viscosity, facilitating the proliferation of these cells from the tissues, leading to a consequent degradation of the extracellular matrix that promotes inflammation (Jeong et al., 2000). Hyaluronidase can be inhibited by chemicals or by immunological methods using natural inhibitors such as phenolic compounds, flavonoids and esters, phenolic aldehydes, alcohols, etc. (Jeong et al., 2000; Salmen, 2003).

This study aimed to evaluate the angiogenic and anti-inflammatory activity of polysaccharide extract obtained by fermentation of Tibetan kefir in whey.

2. Materials and methods

2.1. Obtaining the polysaccharide extract

We obtained the bioactive compound to basic exopolysaccharide extract (ExPP) via fermentation of Tibetan kefir in a medium composed of whey supplemented with 30% glucose (w/v), 2% bacteriological peptone (w/v), 4% potassium phosphate monobasic (w/v) and 4% sodium citrate dehydrate (w/v); the fermentation medium was pasteurized at 63 °C for 30 min. All of the reagents that we used were analytical grade. The inoculum rate was 6% (w/v) of the 10^8 CFU/g consists of the following bacteria *Lactobacillus kefiranofaciens*, *Lactobacillus helveticus*, *Lb. lactis*, *Lb. lactis* subsp *cremoris*, *Lb. lactis* subsp *lactis*, *Lb. casei*, *Lb. kefiri*, *Leuconostoc mesenteroides*, *Pseudomonas* sp., *Pseudomonas putida*, *Pseudomonas fluorecenses* and 10^8 CFU/g consists of the following yeast *Kazachstania unispora*, *Kazachstania exigua*, *Kluyveromyces lactis*, *Kluyveromyces marxianus*, *Kluyveromices siamensis*, *Saccharomices cerevisae*, *Saccharomices unisporus*, *Saccharomices martiniae*, *Candida humilis*, respectively, and fermentation time was conducted at 37 °C for 48 h.

At the end of 48 h of fermentation, the fermented medium was centrifuged and filtered with 20% trichloroacetic (1:1) at 4000 *g* for 30 min at 4 °C. The supernatant was added to absolute ethanol (1:3) at 4 °C and stored at 4 °C for 24 h to precipitate the ExPP. Next, the centrifugation was repeated under the same conditions, as was the ethanol precipitation for an additional 24 h. After the second round of precipitation, the ExPP sample was centrifuged again under the same conditions, and we dialyzed the precipitate in a dialysis membrane of 10–12 kDa pore size (Sigma-Aldrich, St. Louis, MO, USA) against ultra-pure water for 72 h. Next, the dialyzed sample was frozen and lyophilized (freeze-dryer, Modulyo©, Thermo Electron, USA).

2.2. Monosaccharide composition of the extract polysaccharide

Approximately 1 mg of lyophilized ExPP was subjected to total acid hydrolysis with 1 mL of 1 M trifluoroacetic acid for 4 h at 100 °C. Then, the lyophilized ExPP was reduced with sodium borohydride ($NaBH_4$, 20 mg) for 12 h. After reduction, the content was neutralized (pH 5–6) with 50% *v*/v acetic acid and freeze-dried. The dried product was co-distilled with methanol to remove boron salts. Next, an acetylation step was performed with 1 mL acetic anhydride for 1 h at 120 °C, and we analyzed the monosaccharides as alditol acetate derivatives using gas chromatography-mass spectrometry (GC-MS) in a Varian 3800 chromatography system using helium as a mobile phase (1.0 mL/min) in a DB-225MS capillary column (Durabond, 30 m × 0.25 mm; internal diameter 0.25 μm). The peak areas were determined via integration using the Varian software MS Data Review. All of the reagents were analytical grade unless otherwise specified.

2.3. Evaluation of angiogenic activity

2.3.1. Animals

We obtained fertilized eggs of *Gallus gallus* from a commercial poultry farm located in the Curitiba metropolitan region of Brazil. We maintained the eggs at ambient temperature (above 20 °C) until use. This project was approved by the Ethical Committee for Animal Experimentation of the Federal University of Paraná and registered under number 23,075.047773/2010-61.

2.3.2. Samples evaluated in biological models

We evaluated the biological activity of ExPP according to the pharmacological model for the dose-response curve. The polysaccharides were used in the ExPP in lyophilized (L), frozen (F), dialysate (D) and precipitate (Pt) forms, as necessary.

2.3.3. Evaluation of angiogenic activity

To evaluate the angiogenic activity of the polysaccharide extract, we used a modified chicken chorioallantoic membrane *ex ovo* assay (Boller et al., 2015). The study involved the evaluation of the ExPP (L) on a chorioallantoic membrane CAM capillary vessel in order to determine if the extract was angiogenic or antiangiogenic. In this assay, egg embryos were incubated at 37 °C and 60% relative humidity for 10 days. After that a 6 mm cellulose disc was laid over CAM with 10 μL of ExPP (L), VEGF (positive control) and hydrocortisone (HC – negative control) at 1, 3, 10, 30, 100, 300, 1000 and 3000 ng/mL. All analysis were made in triplicate and returned to the incubation chamber for a further 2 days.

The egg embryos were removed from incubation and a photomicrography was taken of each disc to count the total number of blood vessels surrounding the disc. This process was accomplished using a 30% milk powder solution injected inside the CAM sack and submitted to image analysis using the Image J program. The total blood vessels for each disc were plotted graphically (Graph Pad Prism, version 5.0) to obtain either 50% inhibition (IC50) or excitatory (EC50) concentrations.

2.4. Evaluation of anti-inflammatory activity in vitro

The polysaccharide (ExPP) was first tested in different presentation forms in order to review the best anti-inflammatory response by inhibiting the enzyme hyaluronidase. We studied the ExPP precipitated (ExPP (Pt)) with ethanol, ExPP lyophilized (ExPP (L)) and ExPP dialyzed (ExPP (D)) for 72 h against water and ExPP frozen (ExPP (F)) at the −80 °C and were tested at a concentration of 5 mg/mL. DMSO was used as positive control due to its ability to completely inhibit de hyaluronidase enzyme. Although not used as anti-inflammatory agent, it can simulate an effective anti-inflammatory substance. We also used a natural anti-inflammatory agent as positive control, propolis commercial extract, at same concentration (Reissing et al., 1955; Arossos and Davidson, 1967; Kuppusamy et al., 1990).

We determined the inhibition of hyaluronidase activity (Reissing et al., 1955; Arossos and Davidson, 1967; Kuppusamy et al., 1990). For the sample analysis, we placed 50 μL of ExPP (L) in different concentration (1, 2, 3, 5, 7 and 8 mg/mL) and 0.5 mL of potassium salt of hyaluronic acid (Sigma-Aldrich, St.Louis, MO, USA) (1.2 mg hyaluronic acid per mL of 0.1 M acetate buffer, pH 3.6, containing 0.15 M NaCl) in a reaction tube. The control tube consisted of the same reagent of test tubes without ExPP. All tube were incubated for 5 min at 37 °C and after that 50 μL of the enzyme hyaluronidase (350 units of the enzyme hyaluronidase type IV-S from bovine *testes*, Sigma-Aldrich, St.Louis, MO, USA - dissolved in the same buffer substrate at concentration 6.5 mg/mL) was added, and incubated at 37 °C for 40 min. The reaction was stopped by adding 10 L of 4 N sodium hydroxide solution and immediately placing 0.1 mL of 0.8 M potassium tetraborate into the reaction mixture and incubating it in a boiling bath for 3 min. After the incubation time, we added 3 mL of 4-dimethylaminobenzaldehyde