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Anti-diarrhoeal therapeutic potential and safety assessment of sulphated polysaccharide fraction from *Gracilaria intermedia* seaweed in mice

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ABSTRACT

Sulphated polysaccharides extracted from algae have been extensively studied for their diverse biological activities. Thus, the purpose of this study was to evaluate the chemical composition, the anti-diarrhoeal effect and acute toxicity of a sulphated polysaccharide fraction obtained from *Gracilaria intermedia* (SP-Gi). Initially, the FT-IR of SP-Gi revealed to be an agaran with sulphation at C-6 of the L-galactosyl residues. The anti-diarrhoeal activity of SP-Gi was evaluated in a castor oil-induced diarrhoea model. The effects of SP-Gi on enteropooling, Na^+-K^+-ATPase activity, gastrointestinal transit, and gastric emptying were then examined. Subsequently, the effect of SP-Gi on diarrhoea induced by cholera toxin (CT) and *Escherichia coli* was examined. In addition, an acute toxicity test was conducted in accordance with OECD guideline 423. Pre-treatment with SP-Gi reduces the total faeces, total diarrhoeal faeces, and enteropooling. SP-Gi (30 mg/kg *p.o.*) increased Na^+/K^+-ATPase activity and reduced gastrointestinal transit through anticholinergic mechanisms. ELISA demonstrated that SP-Gi can interact with GM1 receptors and CT. SP-Gi reduced diarrhoea induced by *E. coli* and prevented weight loss in the animals. Moreover, SP-Gi did not induce any toxicity signs. These results suggest that SP-Gi is a possible candidate for the treatment of diarrhoeal illnesses.

1. Introduction

Diarrhoea is a gastrointestinal disorder characterised by increased frequency of defaecation (three or more times per day), more fluidity of faeces, and/or the presence of blood and mucous, which can lead to electrolyte imbalance [1]. Despite improvements in public health and reduced mortality from diarrhoea in recent years, this disease continues to be one of leading causes of death among children less than five years of age worldwide [2].

In developing countries, infectious diseases, including enterotoxin-producing bacteria (*Vibrio cholerae* and *Escherichia coli*), enteroinvasive bacteria (*Shigella* and *Salmonella*), parasites (*Entamoeba histolytica* and *Cryptosporidium parvum*), and viruses (Rotavirus) are the major causes of diarrhoea [3]. Moreover, other factors may be responsible for diarrhoea, including drugs, anxiety, infectious agents and toxins, among others [4].

Oral rehydration solutions (ORS) are the first line of treatment for diarrhoea worldwide. However, ORS currently available are unable to reduce the duration and severity of diarrhoea [5]. Therefore, the growing demand for molecules with therapeutic potential has led to increased interest in research involving organisms of marine origin, due to their great biodiversity [6]. Of these molecules, the sulphated polysaccharides obtained from

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seaweeds have great therapeutic potential in the treatment of various diseases. Furthermore, several biological activities of sulphated polysaccharides obtained from red algae, such as antioxidant [7], anti-inflammatory and antinociceptive [8], anticoagulant [9], anticancer [10], antiviral [11], anti-microbial [12], gastroprotective [13], and anti-diarrhoeal activities [14,15] have been reported.

Sulphated polysaccharides from red algae of the *Gracilaria* genus (Gracilariaceae, Rhodophyta) are composed mainly of sulphated galactans formed by alternating 3-β-D-galactopyranosyl residues (A-units) and 4-α-L-galactopyranosyl (or 3,6-anhydrogalactopyranosyl) residues (B-units) [16,17]. These macromolecules usually have high molecular weight and possess high electronegativity due to sulphated esters in their structure, which allow electrostatic interaction with specific proteins contributing to different biological activities [18].

The genus *Gracilaria* is widespread throughout tropical and temperate regions [19]. In Brazil, several species of the genus including *Gracilaria intermedia* are found in abundance on the northeast coast. Currently, there are few studies involving this species, limiting phylogenetic analysis and ecological surveys [20,21]. Despite the low number of scientific studies on *Gracilaria intermedia* and sulphated polysaccharides, and considering the therapeutic potential of sulphated polysaccharides in diseases of the gastrointestinal tract, it is essential to conduct research in this area. The results obtained may allow the adoption of new therapies and prophylactic measures. Thus, the purpose of this study was to investigate the anti-diarrhoeal effect of a sulphated polysaccharide fraction extracted from *G. intermedia* (SP-Gi) in mice and to conduct a safety assessment thereof.

2. Materials and methods

2.1. Extraction of the sulphated polysaccharide fraction

The red seaweed *G. intermedia* was collected in April 2013 from the Atlantic coast northeast of Brazil (Taíba Beach, São Gonçalo, CE), and a voucher of this specimen has been deposited in the Prisco Bezerra Herbarium Phycological Herbarium of Sea Sciences Institute at Federal University of Ceará (nº 2386). After collection, the seaweeds were washed with distilled water, before being dried at 25 °C. Polysaccharide extraction was performed according to the method described by Farias et al. [22]. The dried tissue (5 g) was milled and suspended in 250 mL of 0.1 M sodium acetate buffer (pH 5.0) containing 30 mg/mL of papain, 5 mM EDTA, and 5 mM cysteine, and incubated at 60 °C for 40 min (papain is a proteolytic enzyme, which is able to digest protein contaminant that may interfere with the biological assays). After filtration with a nylon membrane, the polysaccharides were precipitated by the addition of 16 mL of 10% cetylpyridinium chloride. After 24 h at room temperature, the mixture was filtered once again through a nylon membrane. Then, the polysaccharides were washed with 500 mL of 0.05% cetylpyridinium chloride dissolved in 100 mL of a 2 M NaCl/ethanol (100:15, v/v) solution, and precipitated with 200 mL of absolute ethanol. After incubation at 4 °C for 24 h, the precipitate was filtered and washed thoroughly with 80% ethanol, followed by absolute ethanol, acetone and dried under hot air flow (60 °C). To calculate the yield obtained, the following formula was used:

$$\text{Polysaccharides}(\%) = \text{mass (g) of dried material obtained} / \text{mass (g) of dried microalgae used for the extraction process x 100.}$$

2.2. Chemical analysis

Protein contaminants in the polysaccharide fraction were measured as previously described by Bradford [23] using bovine serum albumin (BSA) as standard. The Fourier transform infrared spectra (FT-IR) of the polysaccharide fraction were recorded with a Shimadzu IR spectrophotometer (model 8300) between 400 and 4000 cm^{-1} in KBr pellets.

2.3. Animals

Mice (Swiss strain, 25–30 g) of both sexes were obtained from the Federal University of Piauí. All animals were housed in temperature-controlled rooms and received food and water *ad libitum*. The animals were deprived of food for 22–24 h before the experimentation, but had free access to water. The animals were divided into groups of 6–8 animals per group for all treatments.

2.4. Ethics statement

Experimental methods and protocols used in this study were approved and performed in accordance with the guidelines of Institutional Animal Ethics Committee (protocol N° 11/2013). All efforts were made in order to minimize animal suffering. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and the ARRIVE Guidelines [24].

2.5. Drugs and chemicals

Papain was obtained from Merck (Kenilwoth, NJ, USA). Xylazine hydrochloride and ketamine hydrochloride were from Syntec (Cotia, SP, Brazil). Castor oil, bethanechol, atropine, cholera toxin, monosialoganglioside-GM1, Splittgerber's reagent, and 3,3′,5,5′-tetramethylbenzidine were from Sigma (St. Louis, MO, USA). Loperamide hydrochloride and naloxone hydrochloride were obtained from Janssen-Cilag Pharmaceuticals LTDA (São Paulo, SP, Brazil) and Cristália Pharmaceutical Chemicals Ltda (Nova Itapira, SP, Brazil), respectively. Sulphated atropine was obtained from Isofarma Industrial Farmacêutica Ltda (Fortaleza, CE, Brazil). All drugs were dissolved in saline or phosphate-buffered saline (PBS).

2.6. Evaluation of anti-diarrhoeal activity of SP-Gi

2.6.1. Castor oil-induced diarrhoea

Castor oil was used to induce diarrhoea according to the method described by Awouters et al. [25] and modified by Costa et al. [14]. Initially, group 1 received saline orally while groups 2, 3, and 4 received SP-Gi (3, 10, and 30 mg/kg *p.o.*, respectively), group 5 received loperamide (5 mg/kg *p.o.*), and group 6 received vehicle only. After 1 h, castor oil (10 mL/kg *p.o.*) was administered to the experimental groups (groups 1–5) for induction of diarrhoea, and group 6 received saline only. After the induction of diarrhoea, was detect the characteristics of diarrhoeal stools, including evaluation of the total amount of stools (mg) and the total amount of diarrhoeal stools (mg) excreted [14]. A sample of the small intestine was weighed, frozen, and stored at −70 °C until assayed for Na^+/K^+-ATPase activity.

The incidence and gravity of the castor oil-induced diarrhoea were recorded based on stool consistency, according to the method of Dicarlo et al. [26].

2.6.2. Castor oil-induced intestinal fluid accumulation (enteropooling)

An assessment of castor oil-induced enteropooling was conducted using the method previously described by Robert et al. [27]