Research Paper

Sulfated-polysaccharide fraction extracted from red algae *Gracilaria birdiae* ameliorates trinitrobenzenesulfonic acid-induced colitis in rats

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Abstract

Objectives The aim of this study was to evaluate the protective effect of the sulfated-polysaccharide (PLS) fraction extracted from the seaweed *Gracilaria birdiae* in rats with trinitrobenzenesulfonic acid (TNBS)-induced colitis.

Methods In the experiments involving TNBS-induced colitis, rats were pretreated with polysaccharide extracted from *G. birdiae* (PLS: 30, 60 and 90 mg/kg, 500 μL p.o.) or dexamethasone (control group: 1 mg/kg) once daily for 3 days starting before TNBS instillation (day 1). The rats were killed on the third day, the portion of distal colon was excised and washed with 0.9% saline and pinned onto a wax block for the evaluation of macroscopic scores. Samples of the intestinal tissue were used for histological evaluation and assays for glutathione (GSH) levels, malonyldialdehyde (MDA) concentration, myeloperoxidase (MPO) activity, nitrate and nitrite (NO_3/NO_2) concentration and cytokines levels.

Key findings PLS treatment reduced the macroscopic and microscopic TNBS-induced intestinal damage. Additionally, it avoided the consumption of GSH, decreased pro-inflammatory cytokine levels, MDA and NO_3/NO_2 concentrations and diminished the MPO activity.

Conclusions Our results suggest that the PLS fraction has a protective effect against intestinal damage through mechanisms that involve the inhibition of inflammatory cell infiltration, cytokine releasing and lipid peroxidation.

Introduction

The study for natural products with pharmacological properties has significantly contributed to the discovery of compounds with important applications.[1,2] Recently, marine algae have attention as a source of bioactive substances for the development of new drugs.[3] In particular, algae are a very important and commercially valuable resource for the food industry; they also serve as soil conditioners and are used in traditional folk medicine due to their known health benefits.[4,5]

Red seaweeds produce an innumerous variety of sulfated galactans and are rich sources of sulfated polysaccharides (PLS). PLS from the *Gracilaria* genus are composed mainly of the alternating 3-linked-β-D-galactopyranose unit (Gal) and the 4-linked-3,6-anhydro-α-L-galactopyranose unit (AnGal). The Gal unit can be substituted for either methyl or sulfate ester radicals.[6]

Recent studies have shown that PLSs extracted from marine red algae demonstrated antioxidant and anti-inflammatory effects,[7,8] decreasing the production and releasing of free-radical scavengers and preventing oxidative damage in the living organism.[9] However, few studies have correlated the protective effect of PLSs from seaweeds to the intestinal damages associated with the colitis induced by trinitrobenzenesulfonic acid (TNBS).

The inflammatory bowel diseases (IBD) refer essentially to two different diseases: Crohn's disease and ulcerative colitis (UC). Although the aetiology of IBD remains unclear, there is evidence that it involves immune, genetic and environmental factors, which are related to the initiation and development of colitis.[10,11] In the IBD, there is an increasing number of inflammatory cells that are found in areas of the intestine with chronic inflammation, resulting in overproduction of a variety of pro-inflammatory mediators including eicosanoids, platelet-activating factor, releasing of pro-inflammatory cytokines and specimens reactive of oxygen and nitrogen metabolites.[10–13]

Currently, there is no effective therapy to cure the disease but the mainstream treatment depends on reduction of the abnormal inflammation in the colon lining, and thereby relieves the symptoms of disease. The treatment depends on the severity of the disease; therefore treatment is adjusted for each individual.[14] Most people with mild or moderate IBD are treated with corticosteroids to reduce inflammation and relieve symptoms.[15] Nearly 25% of patients with UC requiring steroids therapy become steroid dependent after 1 year, and virtually all develop steroid-related adverse events.[16]

Considering the data above, it is plausible that the red algae and their derivative, the PLS, demonstrated marked antioxidant and anti-inflammatory effect, decreasing the production of free-radical scavengers and preventing oxidative damage. Thus, this study aimed at evaluating the protective effect of PLS fraction extracted from red algae *G. birdiae* in the trinitrobenzenesulfonic acid (TNBS)-induced colitis in rats.

Methods

Extraction of the PLS fraction

The extraction of the polysaccharide of *G. birdiae* was accomplished at the Laboratory of Biochemistry of Sea Algae at the Department of Biochemistry and Molecular Biology of the Federal University of Ceará. The marine red algae *Gracilaria* was collected at Flecheiras beach, Trairí, Ceará, Brazil, in September 2006, geographical localization: 03°13′ 25′ S and 39°16′ 65″ W. A voucher specimen (No. 40781) was deposited in the Herbarium Prisco Bezerra in the Department of Biological Sciences, Federal University of Ceará, Brazil. The samples were cleaned of epiphytes, washed with distilled water and stored at −20°C. The extraction procedure of polysaccharide was performed according with method previously described.[17]

Chemical structure of PLS fraction

The chemical structure was previously described.[18,19] Total sugar content of each fraction was determined according to the method of Dubois.[20] Protein fractions were obtained by Bradford's method.[21] Sulfate content was determined by the barium chloride gelatine method,[22] and the monosaccharide contents of red seaweed galactans was obtained by reductive hydrolysis.[23]

Animals

Male Wistar rats (160–180 g) deriving from Federal University of Piauí (UFPI). The animals were housed at 25 ± 2°C under a 12/12 h light/dark cycle and were deprived of food for 12–16 h before the experiments, with free access to water. Experiments were conducted in accordance with current established principles for the care and use of research animals (National Institutes of Health guidelines) and were approved by Ethics Committee of the Federal University of Piauí (protocol n° 036/12).

Induction of colitis

Colitis was induced ($n = 6$ rats/group) by intracolonic single instillation of a solution of 20 mg of TNBS in 50% ethanol (EtOH), and the animals were previously anesthetized with ketamine (80 mg/kg; intramuscularly, i.m.) and xylazine (10 mg/kg, i.m.). Control groups received an equivalent volume of saline. A rubber catheter was inserted into the rectum, 8 cm distal to the anus, and the TNBS solution was introduced. Animals were placed head down in a vertical position for 30 s and then returned to their cages. Three days after the induction of colitis, the rats were killed and the experimental protocols executed as described below.

Treatment protocols

In the experiments involving TNBS-induced colitis, rats were treated with PLS (30, 60 and 90 mg/kg; orally, p.o.) or dexamethasone (1 mg/kg; subcutaneously, s.c.) once daily for 3 days before and after TNBS instillation (only on first day). On the third day after induction of TNBS-colitis, the rats were killed, and the abdomens were then opened, and after the identification of the intestine, the portion of distal colon was excised and washed with 0.9% saline. Then this segment, measuring 5 cm, were weighed to determinate the colon oedema. The results were expressed in increase in colon weight (g)/5 cm ratios, compared with a normal control group, without colitis. The evaluation of macroscopic scores of lesion was performed by modifying the criteria previously described (Table 1).[24] Additionally, samples of intestinal tissue were then removed for the measurement of glutathione (GSH) concentration,[25] malonyldialdehyde (MDA) level,[26] myeloperoxidase (MPO) activity,[27] nitrate and nitrite (NO_3/NO_2) concentra-

Table 1 Criteria for macroscopic scoring intestinal lesion

Scores	Criteria
0	No damage
1	Focal hyperaemia; no ulcers
2	Ulceration without hyperaemia or bowel wall thickening
3	Ulceration with inflammation at one site
4	≥2 sites of ulceration and inflammation
5	≥2 major sites of ulceration and inflammation or one site of ulceration extending >1 cm along the length of intestinal
6–10	If damage covered >2 cm along length of intestinal, score is increased by 1 for each additional centimetre of involvement

tion[28] and cytokine levels[8] in inflamed colon tissue. Samples were fixed in 10% formalin for histopathological analysis.

Histological evaluation

For histological evaluation, the intestinal tissue was fixed in formalin for 24 h. After that, the samples were stored in a solution of 70% alcohol. The samples were embedded in paraffin and sectioned, and the sections were deparaffinized, stained with haematoxylin and eosin, and then examined under a microscope (blind analysis). The laminas were analysed according to the criteria described previously,[29] in which scores are assigned to the following parameters: loss of mucosal architecture (score of 0–3), cellular infiltration (score of 0–3), muscle thickening (score of 0–3), crypt abscess (score of 0–1) and goblet cell depletion (score of 0–1).

Myeloperoxidase assay

This assay was performed to evaluate the neutrophil infiltration into the intestinal mucosa during TNBS-induced colon damage. Tissue samples were collected and homogenized (50–100 mg) in 50 mM 50 mM K2HPO4 buffer (pH 6.0) containing 13.72 mM hexadecyltrimethylammonium bromide. Then, homogenate was centrifuged (40 000 × g for 7 min at 4°C), and the supernatant assayed by spectrophotometry for MPO activity determination at 450 nm. The results were expressed as the MPO units per milligram of colon tissue.[27]

GSH levels

The GSH levels in the fragments of intestinal tissue were determined according to the method described previously with modifications.[25]

MDA concentration

The MDA concentration was measured using the method described previously with modifications.[26]

Measurement of nitric oxide (NO₃/NO₂) concentration

Homogenate of intestinal tissue of the animals was incubated in a microplate with nitrate reductase for 12 h to convert nitrate (NO_3) to nitrite (NO_2). Nitric oxide production was determined by measuring nitrite concentrations in an ELISA plate reader at 540 nm using the Griess method.[28] Results were expressed as micromoles of nitrite using the internal standard curve.

Cytokine measurements

Samples of intestinal tissue were collected homogenized in sterile saline. After that, the interleukin IL-1β and tumour necrosis factor (TNF)-α levels were measured using ELISA kits according to the manufacturer's recommendations.[8] The homogenates were centrifuged at 0.8 g at 4°C for 10 min, and supernatants were stored at −80°C until further analysis. The results were expressed as picograms per millilitre of homogenate (pg/ml).

Statistical analysis

The results were expressed as means ± SEM. Statistical significance of differences between the groups was determined by one-way analysis of variance (ANOVA) followed by the Student–Newman–Keuls test. For categorical variables, the Kruskal–Wallis test followed by Dunn's test was performed. $P < 0.05$ was defined as statistically significant.

Results

PLS structure

The PLS fraction isolated from the red algae *G. birdiae* was previously identified.[15,16] This galactan is an agar-type polysaccharide composed mainly by β-D-galactopyranose linked to 3,6-anhydro-α-L-galactose with low methyl substituted groups. The structure is formed by →4–3,6 anhydro-α-L-galp (1→3) β-D-galp 1→ segments, with the possibility of an α-L-galp unit substituted at the 6-position for a sulfate ester.[16] In addition, the molar mass distribution was found to be within 2.6×10^6 and 3.75×10^5 g/mol, while the soluble carbohydrate, protein and sulfate contents were 85.5%, 2.5% and 8.4%, respectively.[15,16]

PLS reduced TNBS-induced macroscopic lesion scores

In Figure 1, we can observe that the administration intracolon of TNBS induced a significant ($P < 0.05$) increase of colon macroscopic intestinal lesions (18.00 ± 2.2 scores of lesion) when compared with saline group (0.60 ± 0.04 scores of lesion). The treatment with PLS (30,

60 and 90 mg/kg, p.o.) reduced (30 mg/kg: 4.40 ± 1.03 scores of lesion; 60 mg/kg: 3.20 ± 1.02 scores of lesion; 90 mg/kg: 2.80 ± 0.42 scores of lesion) the damage scores of macroscopic lesions in the colon tissue, with the maximal effect observed at a dose of 90 mg/kg. Similar effects were produced by dexamethasone (5.50 ± 1.65 scores of lesion) administration, a drug of choice for treating IBD.

Histopathological evaluation

TNBS-induced colitis promotes change in histological findings characterized by severe intestinal damage, a massive
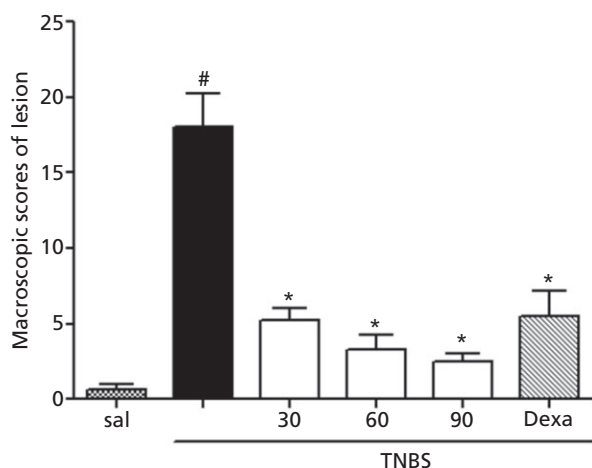

Figure 1 The polysaccharide fraction extracted from *Gracilaria birdiae* reduces TNBS-induced macroscopic intestinal damage. Rats were treated with saline (sal: control), PLS (30, 60 and 90 mg/kg, p.o.) or dexamethasone (1 mg/kg, s.c.) once daily for 3 days. On the third day, the rats were killed, and the abdomens were opened, and there was the identification of the intestine for the evaluation of macroscopic scores of lesion. The results are expressed as the mean ± SEM of the macroscopic scores of 5–7 animals per group. *$P < 0.05$ versus TNBS group; #$P < 0.05$ versus saline group (ANOVA followed by the Newman–Keuls post hoc test).

inflammatory cell infiltration, ulceration and muscle thickening. The histological examination of colon sections from those rats that received only TNBS into the colon revealed the presence of crypts showing extensive epithelial destruction (Figure 2 and Table 2). The histological evaluation of colons from rats treated with PLS 90 mg/kg revealed a pronounced reduction in the inflammatory response with moderate loss of epithelial cells and minimal inflammatory infiltration into the colonic tissue, resulting in a decreased microscopic damage score and showing a reduction of 45% in the total damage score, as compared with TNBS group.

PLS reduced TNBS-induced increase in wet weight of the colon

In Figure 3, we observed that TNBS induced a significant ($P < 0.05$) increase in the colon weight (1.03 ± 0.03 g; 75%) of the injured area in 5 cm of the bowel tissue, as compared with saline group (0.26 ± 0.01 g) or Dexa Group (0.68 ± 0.05). However, pretreatment with PLS 90 mg/kg significantly reversed (0.55 ± 0.05 g) the colon injury after TNBS colonic instillation.

PLS reduced TNBS-induced MPO activity

Figure 4 shows that the TNBS into the colon determined MPO activity in the concentration of 11.78 ± 2.393 units of MPO/mg of colon tissue, while the group treated with PLS 90 mg/kg decreased an activity of this enzyme at 2.578 ± 1.415 UMPO/mg of colon tissue, which was equivalent to a reduction of 78%.

PLS treatment interferes with TNBS-induced GSH and MDA levels in rat intestine

As showed in Figure 5A, the colitis produced a significant ($P < 0.05$) decrease in colonic GSH content (91.56 ± 14.96 mg/g of tissue) as compared with the saline group
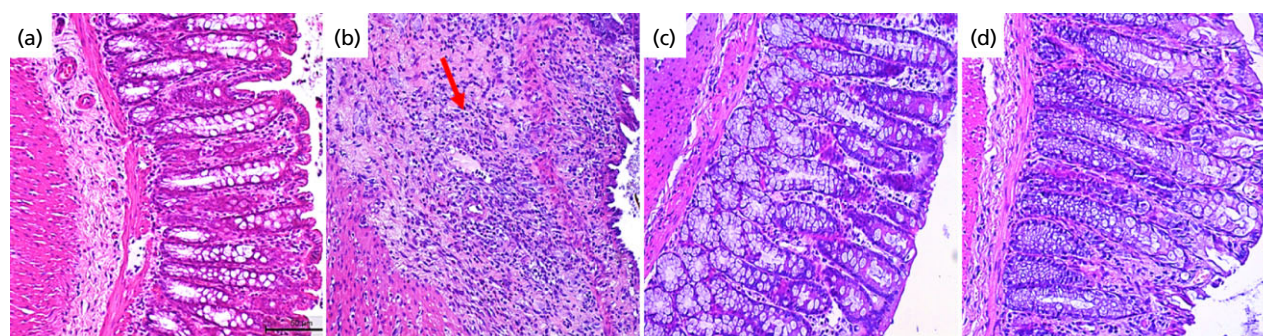

Figure 2 The polysaccharide fraction extracted from *Gracilaria birdiae* ameliorates the histopathological alterations in TNBS-induced colitis. Photomicrographs (200×, scale 50 μM) of the colon of mice control (a), TNBS (b), TNBS + PLS (90 mg/kg, p.o.) (c) and TNBS + dexamethasone (1 mg/kg, s.c.) (d), respectively. Note that TNBS-induced loss mucosal architecture, intense cellular infiltration (b, arrow). Treatment with *G. birdiae* was able reverted these alterations.

Table 2 Histological assessment of bowel damage

Criteria	Median score			
	Saline	TNBS	TNBS + PLS	TNBS + Dexa
Loss mucosal architecture	0	3 (1–3)[#]	1 (1–2)*	1 (1–3)*
Cellular infiltration	0	3 (1–3)[#]	2 (1–2)*	1.5 (1–3)*
Muscle thickening	0	3 (1–3)[#]	1 (1–2)*	1 (1–2)*
Crypt abscess	0	1 (1–1)	1 (1–1)	1 (0–1)
Goblet cell depletion	0	1 (0–1)	0 (0–1)	0 (0–0)
	Total damage score			
	0	11[#]	5*	4.5*

PLS, polysaccharide; TNBS, trinitrobenzenesulfonic acid. Score for histological damage expressed as mean ± SEM (*n* = 5). [#]$P < 0.05$ versus saline group; *$P < 0.05$ versus TNBS group (Kruskal–Wallis non-parametric test and Dunn's test were used for multiple comparisons of histological analyses).
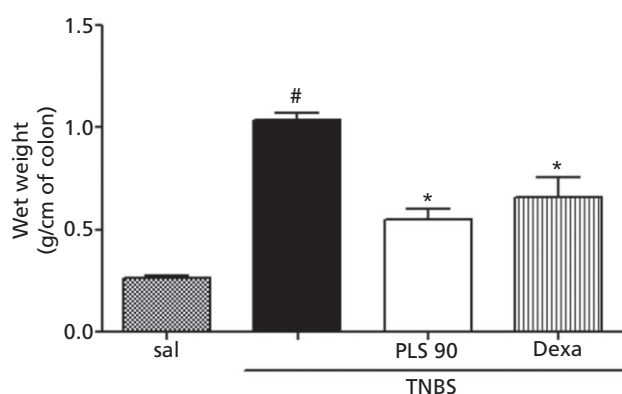

Figure 3 The PLS fraction extracted from *Gracilaria birdiae* reduces the wet weight of the colons of the rats treated with TNBS. Rats were treated with saline (sal: control), PLS (90 mg/kg, p.o.) or dexamethasone (1 mg/kg, s.c.) once daily for 3 days. On the third day, the rats were killed, and the abdomens were then opened. After identification of the intestine, samples of tissue (5 cm) were collected for the evaluation of wet weight of the colons. The results are expressed as the mean ± SEM of 5–7 animals per group. *$P < 0.05$ versus TNBS group; [#]$P < 0.05$ versus saline group (ANOVA followed by the Newman–Keuls post-hoc test).
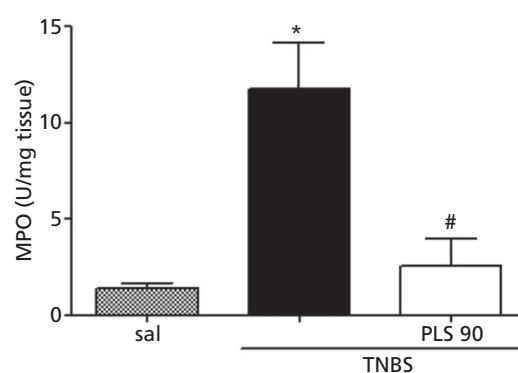

Figure 4 Effect of the polysaccharide fraction extracted from *Gracilaria birdiae* on the activity of intestinal tissue MPO in a rat model of TNBS-induced colitis. Animals were treated with saline (sal: control), PLS (90 mg/kg, *p.o.*) once daily for 3 days. On the third day, the rats were killed, the samples of colon were collected and MPO activity was evaluated. The results are expressed as the mean ± SEM of 5–7 animals per group. *$P < 0.05$ versus saline group; [#]$P < 0.05$ versus TNBS group (ANOVA followed by the Newman–Keuls post-hoc test).

(290.20 ± 16.74 mg/g of tissue). The treatment for 3 days with PLS in dose of 90 mg/kg prevent the consumption of GSH (286.30 ± 57.07 mg/g of tissue), which remained at a much higher concentration as compared with TNBS control group. On the another hand, Figure 5B shows that the oxidative stress in colonic mucosa induced by TNBS was evidenced by the significant increased of MDA (162.5 ± 25.63 nmol/g of tissue) concentration when compared with saline group (65.07 ± 17.93 nmol/g of tissue). PLS 90 mg/kg was able to inhibit the increase of MDA (23.95 ± 4.72 nmol/g of tissue) levels in inflamed mucosa tissue.

Nitric oxide (NO₃/NO₂) concentration

As shown in Figure 6, the TNBS group showed an increased level of NO_3/NO_2 in colon tissue (0.23 ± 0.03 μM) when this

group was compared with saline group (0.08 ± 0.01 μM). The treatment of PLS reduces the levels of nitrate and nitrite to (0.08 ± 0.00 μM) in the intestinal damage caused by TNBS instillation into the colon.

PLS decreased TNBS-induced IL-1β and TNF-α production

Figure 7 shows that the intracolonic administration of TNBS induced an increased of IL-1β (panel A; 6630 ± 561.5 pg/ml) and TNF-α (panel B; 106.8 ± 19.97 pg/ml) levels, comparing this group with saline group (IL-1β : 187.4 ± 31.65 pg/ml; TNF-α: 26.99 ± 7.771 pg/ml). Moreover, the treatment with PLS 90 mg/kg significantly reduced the concentrations of these cytokines (IL-1β : 2872.00 ± 267.20 pg/ml; TNF-α : 26.99 ± 2.67 pg/ml).
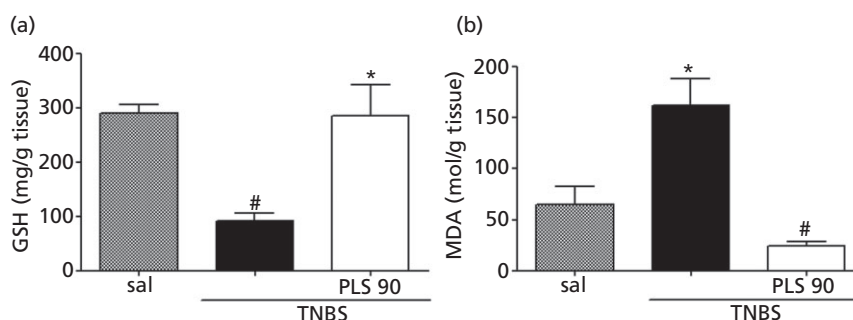
Figure 5 Effect of the PLS fraction extracted from *Gracilaria birdiae* on glutathione levels (a) and MDA concentration (b) in TNBS-induced colitis. Animals were treated with saline (sal: control), PLS (90 mg/kg p.o.) once daily for 3 days. On the third day, the rats were killed, the samples of colon were harvest, and glutathione level was evaluated. The results are expressed as the mean ± SEM of 5–7 animals per group. *$P < 0.05$ versus TNBS group; #$P < 0.05$ versus saline group (ANOVA followed by the Newman–Keuls post-hoc test).

Figure 6 Effect of the polysaccharide fraction extracted from *Gracilaria birdiae* on intestinal tissue concentration of nitric oxide (NO_3/NO_2). In the assay, animals were treated with saline (sal: control), PLS (90 mg/kg, p.o.) once daily for 3 days. On the third day, the rats were killed, the samples of colon were collected, and NO_3/NO_2 concentration was measured. The results are expressed as the mean ± SEM of 5–7 animals per group. *$P < 0.05$ versus TNBS group; #$P < 0.05$ versus saline group (ANOVA followed by the Newman–Keuls post hoc test).
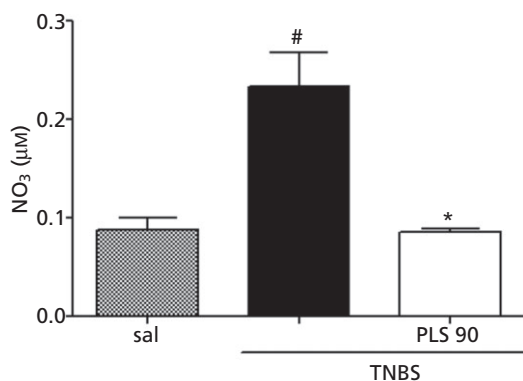
Discussion

The inflammatory bowel diseases (IBD) refer essentially to two different, yet closely related conditions: Crohn's disease and UC.[30] The development of inflammatory bowel disease (IBD) is characterized by frequent episodes of diarrhoea, abdominal pain, blood in the stool and weight loss over a period of months to years.[31] This leads to a discomfort and low quality of life for the person who bears this disease.

IBD is related to an abnormally exacerbated immune system to otherwise innocuous stimuli which are not properly abrogated by the feedback system normally downregulating the mucosal response towards luminal factors.[12] Early events of IBD occur long before symptoms appear. Thus, study in humans often needs to be retrospec-

tive, what can cause many errors. That has led to studies of animal models of IBD to determine early events in the disease process and to test new treatments.[32,33]

In this context, TNBS is a substance used as a valuable model to investigate the pathophysiological mechanism of IBD. Histological modifications occur in TNBS-induced colitis resemble that of IBD in terms of ulceration, inflammation and leucocyte infiltration. Although the acid property of TNBS is partly responsible for the mucosal damage, TNBS has been shown to elicit intestinal protein antigenicity by functioning as a hapten,[34] thereby developing an immune and inflammatory responses. The early acute phase of TNBS-induced inflammation is presumably essential to establish the continued progression of the colitis that is mediated by the T-cell dependent immunity.[35]

Many marine algae and their isolated constituents have shown beneficial therapeutic properties including analgesic, anti-inflammatory and gastroprotective actions.[30,36,37] Knowing that marine algae are an important therapeutic tool against gastrointestinal injury, this study aims to verify the effect of PLS fraction extracted from *G. birdiae* in TNBS-induced colitis in rats.

The results of this study demonstrated, for the first time, the effectiveness of the PLS action extracted from *G. birdiae* against TNBS-induced large intestine damage on the macroscopic and histological examinations in rats. These parameters were used as an indicator of disease-associated intestinal wall thickening and intensity of inflammation.[38] According to our results, we can infer that PLS fraction produce anti-inflammatory effect in TNBS-induced colon damage in rats.

Another parameter studied was the wet weight of the colon in animals with or without colitis. Our results demonstrated that the PLS 90 mg/kg decreased the wet weight of the colon. TNBS-induced colitis resembles that of IBD in terms of ulceration, inflammation and leucocyte infiltration,[39] causing an intense injury leading to an increase in
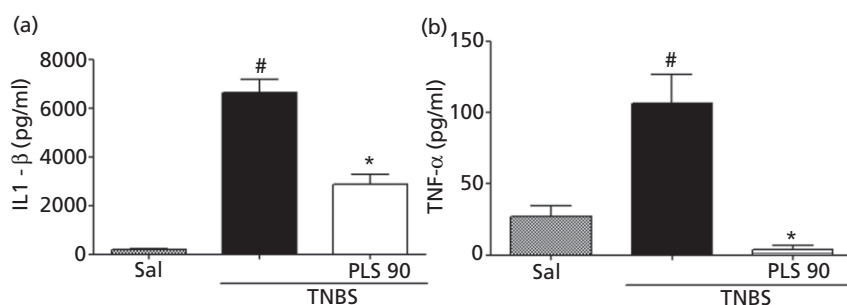
Figure 7 Effect of the polysaccharide fraction extracted from *Gracilaria birdiae* on intestinal levels of IL1-β and TNF-α in TNBS-induced colitis. Animals were treated with saline (sal: control), PLS (90 mg/kg, p.o.) once daily for 3 days. On the third day, the rats were killed, the samples of colon were collected, and cytokine concentration was evaluated. The results are expressed as the mean ± SEM of 5–7 animals per group. *$P < 0.05$ versus TNBS group; #$P < 0.05$ versus saline group (ANOVA followed by the Newman–Keuls post hoc test).

volume and weight of damaged intestine. Corroborating to the previous results, our substance decreased the inflammatory process in the colonic mucosa.

We can observe in our results the improvement of microscopic scores of lesion in the treatment with PLS (90 mg/kg). This substance also decreases the inflammatory response resulting and minimal inflammatory infiltration into the colonic tissue. Neutrophils were attributed as the cells responsible for disrupting epithelial integrity and causing colon injury in IBD.[40] Thus, the reduction of neutrophil infiltration in the animals treated with PLS contributes to the reduction in neutrophil-mediated colon tissue injury during colitis.

Corroborating with our results above, PLS fraction also decreased MPO activity. Neutrophil infiltration into the lamina propria is a common feature of colitis and probably accounts for significant non-specific injury from this disease.[40,41] During the migration, the neutrophils released MPO to penetrate into the injury tissue. MPO is an enzyme mainly found in azurophilic granules of neutrophils that produce the microbicidal molecule hypochlorite, a strong oxidant, upon the reaction with H_2O_2 and Cl^-. Moreover, it can serve as a good marker of inflammation, tissue injury and neutrophil infiltration in gastrointestinal tissues.[42,43] Thus, we can infer that the PLS decrease the mucosal damage by decreasing free radicals production derived of neutrophil infiltration during the inflammation in the colon.

Oxidative stress has been proposed to play an important role in the pathogenesis of inflammatory bowel disease and is related to neutrophil infiltration within the inflamed colon mucosa. The recruitment and activation of neutrophils during acute inflammation contribute to the overproduction of reactive oxygen and nitrogen species that overwhelm the tissue antioxidant protective mechanisms, resulting in oxidative stress, which perpetuates inflammation of the colon.[44] Therefore, this study investigated three oxidative stress markers: GSH and MDA and NO_3/NO_2 concentration.

Antioxidant compounds play an important role in various pathological conditions, including inflammation, neurodegenerative diseases and cancer.[45,46] It has been systematically reported in the literature that PLSs showing antioxidant activity, such as those extracted from marine algae, protect against cell death due to their ability to degrade excessive reactive oxygen species.[47] Nevertheless, few studies have correlated antioxidant potential with TNBS-associated injury of the intestinal tissue.

Our results demonstrated that the PLS fraction increased the levels of GSH in animals with TNBS-induced colitis. GSH, an endogenous antioxidant, protects the cells against oxidative damage, keeping the sulfhydryl groups (-SH) of proteins reduced and preventing them from reacting with free radicals.[48] Concentrations of endogenous antioxidants, such as GSH, are decreased significantly in patients with inflammatory bowel disease and in experimental models of colitis.[49,50] According this result, we can infer that PLS decreased the mucosa damage acting in the production and action of endogenous antioxidants.

Oxidative damage in colon tissue is a potential aetiological or triggering factor for IBD, because the detrimental effects of reactive oxygen molecules have been established in the inflammation process.[51] MDA is a product of lipoperoxidative processes that take place as a consequence of the colonic oxidative insult.[52] Our results demonstrated for the first time that the PLS reduced the MDA concentration in the colonic inflamed mucosa. Thus, we can infer that the action of PLS to reduce the inflammatory response in the colon is seemingly related to the decrease of oxidative stress.

The damage in intestinal mucosa induced by TNBS was accompanied with high levels of free radicals derivate of the degradation of NO (NO_3/NO_2 radicals).[53] Our result demonstrated that the PLS treatment decreased the

concentration of NO_3/NO_2 in the intestinal mucosa of the rats treated with TNBS. NO is a free radical with moderate reactivity. Its production in large quantities via the upregulation of inducible nitric oxide synthase (iNOS) can inhibit key enzymes in the mitochondrial electron transport chain.[53,54] In addition to this, high levels of nitric oxide from activated iNOS are toxic and can damage the tissue directly by the peroxynitrite formation after reaction with superoxide.[55] Thus, we infer the PLS has protective action against intestinal injury by decreasing the formation of peroxynitrite.

The inflammatory cells infiltration in the colitis is dependent of the overproduction of several pro-inflammatory mediators, for example cytokines.[13] Our results demonstrated that PLS 90 mg/kg decreased the concentration of these cytokines in inflamed intestinal mucosa. Cytokines, such as IL-1β and TNF-α, are increased in inflamed tissue, including the mucosa of IBD lesions.[56,57] These mediators are responsible for many of the features of inflammation; an important feature of UC is the recruitment of neutrophils and mononuclear cells. Leucocyte adherence and recruitment are increased in the microvessels in chronic disease,[58] mediated in part by the upregulation of adhesion molecules towards vascular endothelial cells by TNF-α and IL-1β. Moreover, increased levels of tissue-specific and inflammatory chemokines enhance leucocyte migration.[56] Thus, these results revealed that the PLS has an action to reduce the inflammatory response through inhibition of pro-inflammatory cytokines in the intestine injury resulted from TNBS-induced colitis in rats. A possible mechanism may involve the downregulation of the inflammatory response by inhibiting the synthesis and release of pro-inflammatory mediators.

Conclusions

In conclusion, our results suggest that PLS has a protective effect in the TNBS-induced colitis in rats, through mechanisms that involve the inhibition of inflammatory cell infiltration, reduction in the oxidative stress and of pro-inflammatory cytokines concentration. Thus, we suggest that PLSs may have potential applications in the development of novel therapeutic targets against the inflammatory bowel disease in humans.

Acknowledgements

References

1. Sousa FCF *et al*. Medicinal plants and their bioactive constituents: a scientific review of bioactivity and potential benefits in the anxiety disorders in animal models. *Rev Bras Farmacogn* 2008; 18: 642–654.

2. Correa MFP *et al*. Natural products from plant origin potentially useful in the asthma therapy. *Rev Bras Farmacogn* 2008; 18: 785–797.

3. Qi H *et al*. Antioxidant activity of different molecular weight sulfated polysaccharides from *Ulva pertusa* Kjellm (Chlorophyta). *J Appl Phycol* 2005; 17: 527–534.

4. Yang YF *et al*. Growth of *Gracilaria lemaneiformis* under different cultivation conditions and its effects on nutrient removal in Chinese coastal waters. *Aquaculture* 2006; 254: 248–255.

5. Jiao G *et al*. Chemical structures and bioactivities of sulfated polysaccharides from marine algae. *Mar Drugs* 2011; 9: 196–223.

6. Rees DA *et al*. Biogenesis of 3,6-anhydro-L-galactose. *Biochem J* 1961; 81: 347–352.

7. Silva RO *et al*. A sulfated-polysaccharide fraction from seaweed *Gracilaria birdiae* prevents naproxen-induced gastrointestinal damage in rats. *Mar Drugs* 2012; 10: 2618–2633.

8. Brito TV *et al*. Anti-inflammatory effect of a sulphated polysaccharide fraction extracted from the red algae *Hypnea musciformis* via the suppression of neutrophil migration by the nitric oxide signalling pathway. *J Pharm Pharmacol* 2013; 65: 724–733.

9. Souza MCR *et al*. Antioxidant activities of sulfated polysaccharides from brown and red seaweeds. *J Appl Phyco* 2007; 19: 153–160.

10. Podolsky DK *et al*. Pride and prejudice: inflammatory bowel disease models and drug development. *Curr Opin Gastroenterol* 2000; 16: 295–296.

11. Elson CO *et al*. Experimental models of inflammatory bowel disease. *Gastroenterology* 1995; 109: 1344–1367.

12. Fiocchi C. Inflammatory bowel disease: etiology and pathogenesis. *Gastroenterology* 1998; 115: 182–205.

13. Katz JA *et al*. Pathogenesis of inflammatory bowel disease. *Curr Opin Gastroenterol* 1999; 15: 291–297.

14. Botoman VA *et al*. Management of inflammatory bowel disease. *Am. Fam. Phys* 1998; 57: 57–68.

15. Hanauer SB *et al*. Postoperative maintenance of Crohn's disease remission with 6-mercaptopurine, Natural products in treatment of ulcerative colitis and peptic ulcer 119 mesalamine, or placebo: a 2-year trial. *Gastroenterology* 2004; 127: 723–729.

16. Faubion WA *et al*. The natural history of corticosteroid therapy for inflammatory bowel disease: a population-based study. *Gastroenterology* 2001; 121: 255–260.

17. Farias WR *et al*. Structure and anticoagulant activity of sulfated galactans. Isolation of a unique sulfated galactan from the red alga *Botryocladia occidentalis* and comparison of its anticoagulant action with that of

sulfated galactans from invertebrates. *J Biol Chem* 2000; 275: 29299–29307.

18. Souza BWS *et al.* Chemical characterization and antioxidant activity of sulfated polysaccharide from the red seaweed *Gracilaria birdiae*. *Food Hydrocoll* 2012; 27: 287–292.

19. Maciel JS *et al.* Structural characterization of cold extracted fraction of soluble sulfated polysaccharide from red seaweed *Gracilaria birdiae*. *Carbohydr Polym* 2008; 71: 559–565.

20. DuBois M *et al.* Colorimetric method for determination of sugars and related substances. *Anal Chem* 1956; 28: 350–356.

21. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976; 72: 248–254.

22. Lloyd AG *et al.* Infrared studies on sulphate esters. I. Polysaccharide sulphates. *Biochim Biophys Acta* 1961; 46: 108–115.

23. Stevenson TT, Furneaux RH. Chemical methods for the analysis of sulphated galactans from red algae. *Carbohydr Res* 1991; 210: 277–298.

24. Morris GP *et al.* Hapten-induced model of chronic inflammation and ulceration in the rat colon. *Gastroenterology* 1989; 96: 795–803.

25. Sedlak J, Lindsay RH. Estimation of total, protein-bound, and nonprotein sulfhydryl groups in tissue with Ellman's reagent. *Anal Biochem* 1968; 24: 1992–2005.

26. Mihara M, Uchiyama M. Determination of malonaldehyde precursor in tissues by thiobarbituric acid test. *Ana Biochem* 1978; 86: 271–278.

27. Bradley PP *et al.* Measurement of cutaneous inflammation: estimation of neutrophil content with an enzyme marker. *J Invest Dermatol* 1982; 78: 206–209.

28. Green LC *et al.* Analysis of nitrate, nitrite, and [15N]nitrate in biological fluids. *Anal Biochem* 1982; 126: 131–138.

29. Appleyard CB, Wallace JL. Reactivation of hapten-induced colitis and its prevention by anti-inflammatory drugs. *Am J Physiol* 1995; 269: 119–125.

30. Podolsky DK. Pride and prejudice: inflammatory bowel disease models and drug development. *Curr Opin Gastroenterol* 2000; 16: 295–296.

31. Farmer RG. Clinical features, laboratory findings and course of Crohn's disease. In: Kirsner JB, ed. *Inflammatory Bowel Disease*. Philadelphia, PA: WB Saunders, 1988: 175–184.

32. Strober W. Animal models of inflammatory bowel disease: an overview. *Dig Dis Sci* 1985; 30: 3–10.

33. Sartor RB *et al.* Granulomatous enterocolitis induced by purified bacterial cell wall fragments. *Gastroenterology* 1985; 89: 587–595.

34. Neurath M *et al.* TNBS-colitis. *Int Rev Immunol* 2000; 19: 51–62.

35. Zhang ZMD *et al.* Critical role of IL-17 receptor signaling in acute TNBS-induced colitis. *Inflamm Bowel Dis* 2006; 12: 382–388.

36. Chaves LS *et al.* Anti-inflammatory and antinociceptive effects in mice of a sulfated polysaccharide fraction extracted from the marine red algae *Gracilaria caudata*. *Immunopharmacol Immunotoxicol* 2013; 35: 93–100.

37. Damasceno SRB *et al.* Role of the NO/K ATP pathway in the protective effect of a sulfated-polysaccharide fraction from the algae *Hypnea musciformis* against ethanol-induced gastric damage in mice. *Rev Bras Farmacogn* 2013; 23: 320–328.

38. Lee JY *et al.* Inhibitory effects of Geijigajakyak-Tang on trinitrobenzene sulfonic acid-induced colitis. *J Ethnopharmacol* 2009; 126: 244–251.

39. Grisham MB, Yamada T. Neutrophils, nitrogen oxides, and inflammatory bowel disease. *Ann N Y Acad Sci* 1992; 664: 103–115.

40. Nosál'ová V *et al.* Effect of N-acetylcysteine on colitis induced by acetic acid in rats. *Gen Pharmacol* 2000; 35: 77–81.

41. Casagrande R *et al.* Protective effect of topical formulations containing quercetin against UVB-induced oxidative stress in hairless mice. *J Photochem Photobiol B* 2006; 84: 21–27.

42. Bamias G *et al.* Cytokines in the pathogenesis of ulcerative colitis. *Discov Med* 2011; 60: 459–467.

43. Martín AR *et al.* Resveratrol, a polyphenol found in grapes, suppresses oxidative damage and stimulates apoptosis during early colonic inflammation in rats. *Biochem Pharmacol* 2004; 67: 1399–1410.

44. Cuzzocrea S *et al.* Antioxidant therapy: a new pharmacological approach in shock, inflammation and ischemia/reperfusion injury. *Pharmacol Rev* 2001; 53: 135–159.

45. Zhao B. Natural antioxidants protect neurons in Alzheimer's disease and Parkinson's disease. *Neurochem Res* 2009; 34: 630–638.

46. Costa LS *et al.* Biological activities of sulfated polysaccharides from tropical seaweeds. *Biomed Pharmacother* 2010; 64: 21–28.

47. Urso ML, Clarkson PM. Oxidative stress, exercise, and antioxidant supplementation. *Toxicology* 2003; 189: 41–54.

48. Amirshahrokhi K *et al.* The effect of methylsulfonylmethane on the experimental colitis in the rat. *T Appl Pharmacol* 2011; 253: 197–202.

49. Tahan G *et al.* Melatonin expresses powerful anti-inflammatory and antioxidant activities resulting in complete improvement of acetic-acid-induced colitis in rats. *Dig Dis Sci* 2011; 56: 715–720.

50. Spitz DR *et al.* Metabolic oxidation/reduction reactions and cellular responses to ionizing radiation: a unifying concept in stress response biology. *Cancer Metastasis Rev* 2004; 23: 311–322.

51. Pavlick KP *et al.* Role of reactive metabolites of oxygen and nitrogen in inflammatory bowel disease. *Free Radic Biol Med* 2002; 33: 311–322.

52. Pacher P *et al.* Nitric oxide and peroxynitrite in health and disease. *Physiol Rev* 2007; 87: 315–424.

53. Kolios G *et al.* Nitric oxide in inflammatory bowel disease: a universal messenger in an unsolved puzzle. *Immunology* 2004; 113: 427–437.

54. Martínez-Flórez S *et al.* Quercetin attenuates nuclear factor-kappaB

activation and nitric oxide production in interleukin-1beta-activated rat hepatocytes. *J Nutr* 2005; 135: 1359–1365.

55. Kaulersch W *et al.* Polyclonal nature of the intestinal lymphocyte populations in inflammatory bowel disease: a molecular genetic evaluation of the immunoglobulin and T cell antigen

receptors. *Gastroenrerology* 1988; 95: 364–370.

56. Fiocchi C. Lymphokines and the intestinal immune response. Role in inflammatory bowel disease. *Immunol Invest* 1989; 18: 91–102.

57. Hatoum OA *et al.* The intestinal microvasculature as a therapeutic

target in inflammatory bowel disease. *Ann N Y Acad Sci* 2006; 1072: 78–97.

58. Mora JR, von Andrian UH. T-cell homing specificity and plasticity: new concepts and future challenges. *Trends Immunol* 2006; 27: 224–235.