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Original Article

Anti-inflammatory effects of methanolic extract of green algae *Caulerpa mexicana* in a murine model of ulcerative colitis

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ABSTRACT

Inflammatory bowel diseases, which include Crohn's disease and ulcerative colitis, are characterized by chronic and relapsed gut inflammation. *Caulerpa mexicana* is a type of green marine algae that can be found in tropical areas, such as the Brazilian Coastland. These macrophytes exhibit *in vitro* and *in vivo* anti-inflammatory properties such as the ability to reduce both cell migration to different sites and edema formation induced by chemical irritants. The aim of this study was to examine the effect of the *C. mexicana* methanolic extract on the treatment of colitis induced by dextran sodium sulfate. Acute experimental colitis was induced in BALB/c mice by treatment with 3% dextran sodium sulfate orally for 14 days. During this 14-day period, *C. mexicana* methanolic extract (2 mg/kg/day) was given intravenously on alternate days. Treatment with the methanolic extract significantly attenuated body weight loss and severe clinical symptoms. This was associated with a remarkable amelioration of colonic architecture disruption and a significant reduction in pro-inflammatory cytokine production. These results suggest that the anti-inflammatory action of *C. mexicana* methanolic extract on colorectal sites may be a useful therapeutic approach for inflammatory bowel diseases.

Introduction

Inflammatory bowel diseases (IBD) including Crohn's disease (CD) and ulcerative colitis (UC), are characterized by chronic and relapsed gut inflammation. UC is associated with intestinal inflammation and often results in weight loss, diarrhea accompanied by blood and mucus, fever, gastric dysmotility and colon shortening (Hendrickson et al., 2002; Byrne and Viney, 2006; Cho, 2008). Traditional therapeutic agents, such as 5-aminosalicylates (5-ASA) and corticosteroids, are still used to treat IBD. Some immunomodulators, such as azathioprine and 6-mercaptopurine, as well as antibiotics are becoming important in steroid resistant and steroid-dependent patients (Cho et al., 2011). Considering the serious side effects associated with the conventional treatment, natural products, including those from marine origin, have been studied to aid in the improvement of IBD clinical symptoms (D'Orazio et al., 2012).

Caulerpa mexicana is a green marine algae, from the Bryopsidales Order and the Cauleparceae Family, which can be found especially in tropical areas such as the Brazilian Coastland (Neto et al., 2008). These macrophytes have many bioactive compounds, such as polysaccharides, terpenes and flavonoids, which have different pharmacological activities with antitumor, antiprotozoal, antiviral, antioxidant, antinoceptive, anti-inflammatory and anticoagulant effects (Rocha et al., 2007; Souza et al., 2009a,b; Matta et al., 2011; Torres et al., 2014). Recently, we showed that aqueous and methanolic extracts of *C. mexicana* have *in vitro* and *in vivo* anti-inflammatory properties that reduce the pro-inflammatory cytokine levels in macrophage culture supernatants stimulated with LPS and decrease the cell migration to different sites that occurs following stimulation with zymosan. The extracts also reduce edema formation induced by chemical irritants (Bitencourt et al., 2011).

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The oral administration of dextran sulfate sodium salt (DSS) is widely used as a model for UC. This model is characterized by mucosal infiltration of inflammatory cells, epithelial injury and ulceration (Kim et al., 2010). Another study reported that mice with DSS-induced colitis exhibited phenotypic characteristics similar to human acute and chronic UC (Okayasu et al., 1990). We previously showed that *C. mexicana* extracts exhibit anti-inflammatory activity (Bitencourt et al., 2011). Therefore, the aim of this study was to examine the treatment effects of the *C. mexicana* methanolic extract on DSS-induced colitis.

Materials and methods

Extraction and isolation of the methanolic extract of C. mexicana

The green algae *Caulerpa mexicana* was collected in the coastal region of Bessa beach (7°03′52″ S/34°49′51″ W), Joao Pessoa, in the Paraiba State of Brazil in April 2008. The specimen was identified by Dr. George Emmanuel Cavalcanti de Miranda. Voucher specimens of *C. mexicana* (JPB 13985) have been deposited in the Lauro Pires Xavier Herbarium at the Federal University of Paraiba, Brazil. Fresh algae samples were washed, dried, lyophilized and exhaustively extracted with methanol in a Soxhlet apparatus, to obtain the methanolic extract.

Animals

Male BALB/c mice (6–8 weeks old) were used in the experiment. All mice were housed five per cage at a room temperature of 22 ± 2 °C and under a 12 h:12 h light/dark cycle. They had free access to food and water. Groups of five animals were used in each test group and control animals received saline only. All *in vivo* experiments were approved by "Ethics Committee on Animal Use, CEUA/UFRN," under protocol number 044/2009, which was in accordance with the guidelines of the Brazilian Committee for Animal Experimentation (COBEA).

Assessment of DSS-induced colitis

Previously we evaluate the dose-response of *C. mexicana* methanolic extract in peritonitis, ear swelling and air pouch models, and the dose that showed better anti-inflammatory effect was 2 mg/kg, and this is chosen to be used in colitis model (Bitencourt et al., 2011). Our colitis model was developed following the methodology described by Kim et al. (2010) and Melgar et al. (2006) with the aim of evaluate colitis during its acute phase. Therefore, experimental colitis was induced by giving mice drinking water *ad libitum* containing DSS 3% (w/v) for 14 days. Mice were monitored carefully every day to confirm that they consumed approximately equal volumes of DSS-containing water. For the experiment, the mice were divided into three experimental groups. The first group was kept as vehicle-treated control and treated with saline in the same route as the methanolic extract, and the second group was given drinking water with DSS only throughout the experimental period. The other group consisted of mice receiving 3% DSS and administered methanolic extract (2 mg/kg/day) by intravenous route on alternate days for 14 days. The mice treatment with the methanolic extract began on the same day that DSS administration in drinking water.

Evaluation of disease activity index (DAI)

The mice were checked daily for colitis development by monitoring body weight, gross rectal bleeding and stool consistency. The overall disease severity was assessed by a clinical scoring system on a scale of 0–4. Briefly, the scoring was as follows: 0, no weight loss, no occult blood in the stools and normal stool consistency; 1, weight loss of 1–5%, no occult blood and normal stool consistency; 2, 5–10% weight loss, positive for fecal occult blood and loose stools; 3, 10–20% weight loss, positive for fecal occult blood and loose stools; and 4, greater than 20% weight loss, gross rectal bleeding and diarrhea.

Colon tissue culture

Intestine tissue samples corresponding to the transverse colon were exhaustively washed with RPMI-1640 medium containing 2% fetal bovine serum and gentamicin before being cut into smaller pieces. Then, approximately 0.5 cm of tissue was placed in 1 ml of 10% FBS containing RPMI-1460 medium in 24-well tissue culture plates and incubated for 24 h at 37 °C in 5% CO_2. Thereafter, the supernatants were collected for determination of IL-6, IL-12, TNF-α, IFN-γ and IL-17 by ELISA.

ELISA for cytokines

The IFN-γ, IL-17, IL-12, TNF-α and IL-6 concentrations in the cell-free culture supernatants of the colon tissues were measured in triplicate, utilizing a set enzyme-linked immunosorbent assay kit (eBioscience, San Diego, CA, USA) according to manufacturer's instructions.

Histopathology

After the period of induction of colitis by DSS, animals were euthanized; fragments of 0.5 cm of ascendant colon were obtained, fixed in a solution of 4% formalin and used for histological analysis by conventional tissue preparation methods. Tissue samples were viewed under the light microscope (40×) after hematoxylin and eosin (H&E) staining.

Results

Treatment of mice with C. mexicana *methanolic extract decreased the DAI after colitis induction*

The parameter used to evaluate the development of the disease was the DAI, which is associated with observed clinical symptoms such as weight loss, stool consistency, fecal blood and diarrhea. In our model the DAI was evaluated individually during the 14 days of the study. The highest DAI score was observed in DSS group (Fig. 1D, closed squares), which exhibited intense weight loss from the 11th day, as shown in Fig. 1C (closed squares), as well as persistent and severe diarrhea (Fig. 1A) and fecal blood (Fig. 1B). On the other hand, the group treated with the methanolic extract had weight development similar to the group that received normal water (Fig. 1C, closed cycles), low disease development rate (Fig. 1D, closed triangles), with no blood in the stool (Fig. 1B, closed triangles) and little diarrhea (Fig. 1A, closed triangles) compared with the group receiving DSS. Overall, the DAI score of the *C. mexicana*'s methanolic extract-treated group was significantly lower than that of the DSS control group from day four following administration of DSS.

The treatment of mice with C. mexicana *methanolic extract ameliorated the tissue damage in the colon after colitis induction*

To evaluate the tissue damage after colitis induction in mice, histological analysis was conducted on colon sections. For the colon histological analyses, the slides were stained with hematoxylin and eosin and then evaluated on the light microscope. Photomicrographs were subsequently obtained at 40, 100 and 200× magnifications. Fig. 2 shows that the animals receiving

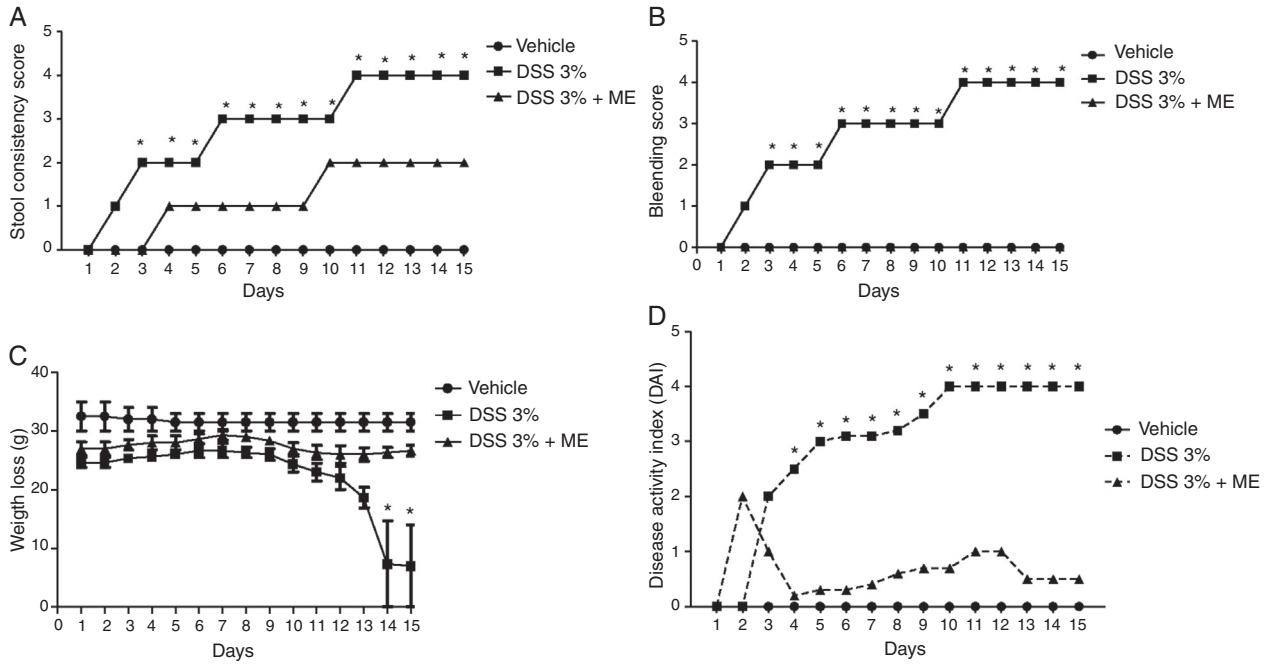

Fig. 1. The disease activity index (DAI) in mice treated with *Caulerpa mexicana* methanolic extract in the colitis model. (A) Stool consistency score, (B) bleeding score, (C) weight loss score and (D) total DAI score. The mice were administered 3% DSS in their drinking water (*ad libitum*) for 14 days with or without methanolic extract (ME) treatment (2 mg/kg/day *i.v.* up to 14 days); saline was used as a negative control. Changes in DAI level were evaluated daily throughout the 15-day experimental period. $^{*}p < 0.05$ in the vehicle-treated control group *vs.* the DSS-induced colitis group; significances between treated groups were determined by ANOVA.

water without DSS presented normal colon structures with crypts and other morphological structures preserved (Fig. 2A and B). On the other hand, colon samples from the mice that received DSS showed typical inflammatory changes in the colon architecture and a diffuse inflammatory infiltrate, composed mainly by mononuclear cells. At certain points the inflammation was more intense, promoting small foci of glandular destruction and moderate ulceration, crypt dilation and goblet cell depletion as shown in Fig. 2C and D. Although they had a slight inflammatory reaction decrease, the group treated with the methanolic extract had an acute inflammatory infiltrate observed on the entire length of the mucosa that was intense in some areas, leading to glandular destruction and ulceration points. In some areas, abscesses with exudate were present, although these were less intense and more focal when compared with the group that received only DSS (Fig. 2E and F).

The treatment of mice with methanolic extract of C. mexicana *decreased Th1 and Th17 cytokine production in the colon culture supernatant*

Because cytokines are important mediators of inflammation in colitis models, the next step was to evaluate the Th1 and Th17 cytokine levels in the *in vitro* colon tissue culture supernatant. The results presented in Fig. 3 show an intense production of IFN-γ (Fig. 3A), IL-6 (Fig. 3B), IL-12 (Fig. 3C), IL-17A (Fig. 3D) and TNF-α (Fig. 3E) in the colon culture supernatants of mice that received DSS in their drinking water. However, the mice treated with the methanolic extract of *C. mexicana* exhibited a significant decrease in the levels of these cytokines compared with the group that received DSS in their drinking water.

Discussion

UC, which is major form of IBD, is a nonspecific inflammatory disease of the large intestine. In addition, it is a lifelong illness with profound emotional and social impacts, it causes serious intestinal tract damage and its progression to chronic UC can lead to colon cancer (Xavier and Podolsky, 2007; Shanahan and Bernstein, 2009). The UC induced in mice by drinking water containing DSS leads to clinical symptoms such as weight loss, diarrhea, fecal blood, mucosal ulceration and colonic shortening (Kim et al., 2010). In this study, we evaluated the development of the disease for 14 days (Melgar et al., 2006). The highest DAI score was observed in the DSS group that developed intense weight loss, fecal bleeding and persistent and severe diarrhea. On the other hand, the animals treated with the methanolic extract did not show the clinical symptoms of colitis or weight loss.

Because UC is characterized by an intense inflammatory process, the ability of the methanolic extract to reduce the colitis clinical symptoms demonstrates the extract's anti-inflammatory activity. In addition, the colon histological analysis shows that the animals that received water with DSS presented typical inflammatory changes in their colon architecture that was slightly attenuated when the animals were treated with the methanolic extract. These effects on clinical symptoms and on histological parameters could be due to the presence of antioxidant compounds, such as β-carotene and α-tocopherol that have been isolated from *C. mexicana* (Sousa et al., 2008). The inhibition of free radicals by these compounds could be decisive in decreasing the oxidative stress that causes damage to the intestinal epithelial barrier (Zhu and Li, 2012; Zheng et al., 2012). Furthermore, we reported previously that the *C. mexicana* methanolic extract inhibits cell migration in a model of peritonitis (Bitencourt et al., 2011) and we believe that this inhibition could also be happening in the colitis model presented here, contributing to the attenuation of the clinical symptoms as well as leading to the slight improvement on the histological analysis observed by the inflammatory infiltrate reduction in the colons of *C. mexicana* treated animals.

The DSS induced-colitis animal model has a number of advantages over other models. These advantages include simple experimental methods, reproducibility of the development time-course as well as colitis severity among individual mice, and relative uniformity of the induced lesions. Therefore, this experimental

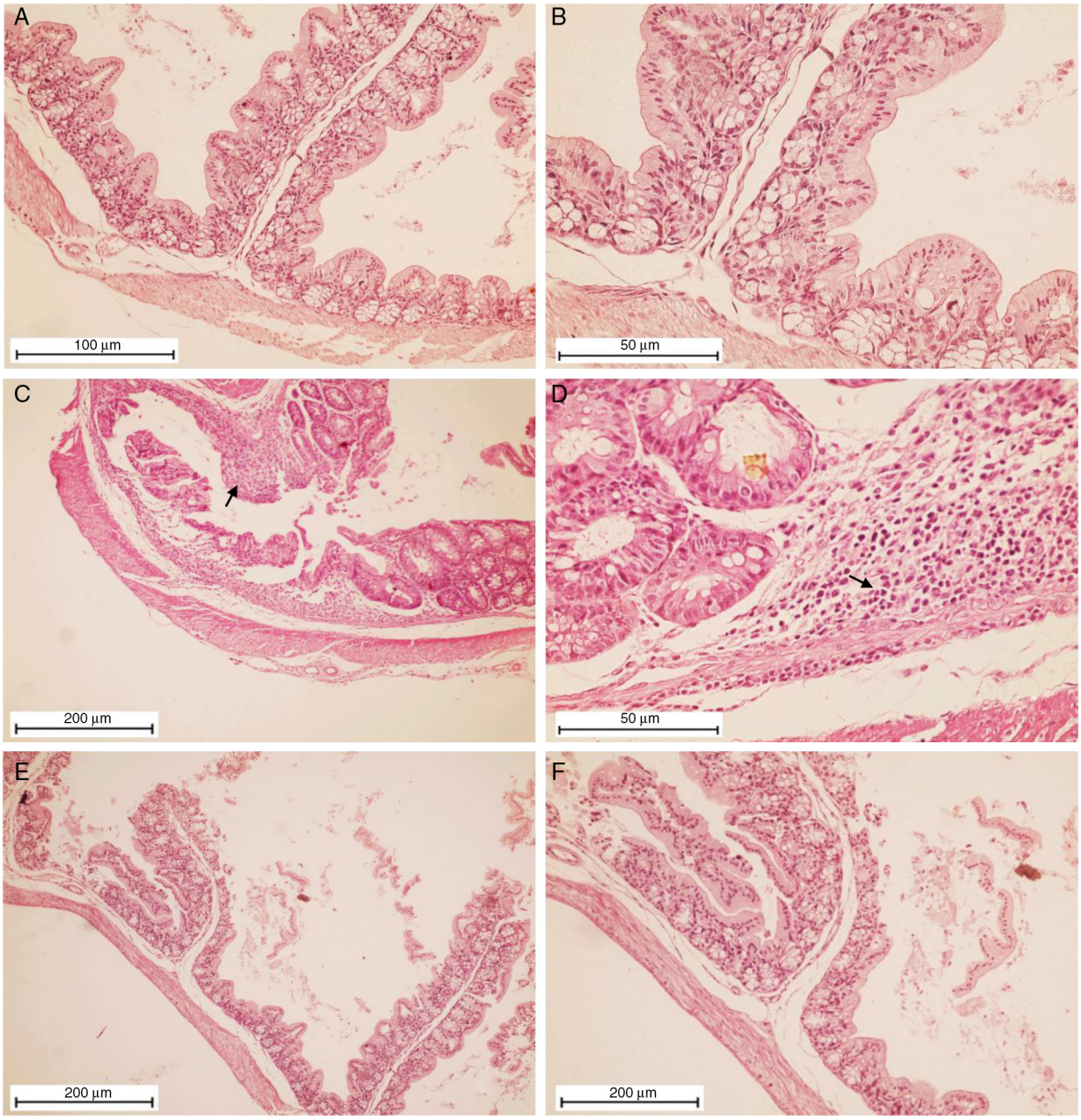

Fig. 2. Tissue damage in the colon of mice with DSS-induced colitis treated with methanolic extract of *Caulerpa mexicana*. Saline was used as a negative control (A and B). Representative sections of colon tissue from mice that received in their drinking water 3% DSS which were treated with methanolic extract of *C. mexicana* (2 mg/kg, *i.v.*, up to 14 days), showing an improvement in inflammatory processes and restoration of mucosal tissue (E and F) and colon sections from animals that were not treated with the extract under study (C and D), showing the destruction of the mucosal tissue which has been replaced by inflammatory granulation tissue (arrow). Histological changes were determined by H&E staining. Original magnification in A and F was 100×, in B and D was 200× and in C and E was 40×.

model is reliable for studying the pathogenesis of UC and for testing drugs or phytochemicals for treatment (Cunha et al., 2013; Lucero et al., 1996). The literature shows that there is an imbalance between pro-inflammatory and anti-inflammatory cytokines in UC (Papadakis, 2004). Pro-inflammatory cytokines are responsible for determining the nature of the immune response in UC and can stimulate the rapid synthesis and secretion of inflammatory mediators, such as reactive oxygen and nitrogen species, leukotrienes, platelet activating factor and prostaglandins (Neuman, 2007). The hyperactivation of immune cells is another important factor in IBD progression and is associated with high levels of pro-inflammatory cytokines from Th1 and Th17 pattern of immune response, such as TNF-α, IL-6 and IFN-γ, which are known to be involved in colon damage (Gálvez, 2014). Cytokines are important mediators of inflammation and elevated levels of pro-inflammatory cytokines are observed not only in the inflamed gut of IBD patients but also in animals with DSS-induced colitis (Neuman, 2007). Because of this it is interesting to evaluate if the pattern of immune response in colitis model induced by DSS could be changed by treatment with the methanolic extract of *C. mexicana* and this pattern can be evaluated by the type of Th1 and Th17 cytokines produced at the site of injury.

Our findings showed that the levels of all cytokines analyzed, including IFN-γ, IL-12, TNF-α (Th1 pattern), IL-6, and IL-17A (Th17 pattern), were higher in the colon tissue of DSS-treated mice compared with those not treated with DSS; however, these levels were reduced by treatment with the methanolic extract obtained from the green seaweed *C. mexicana*. These results indicate that the

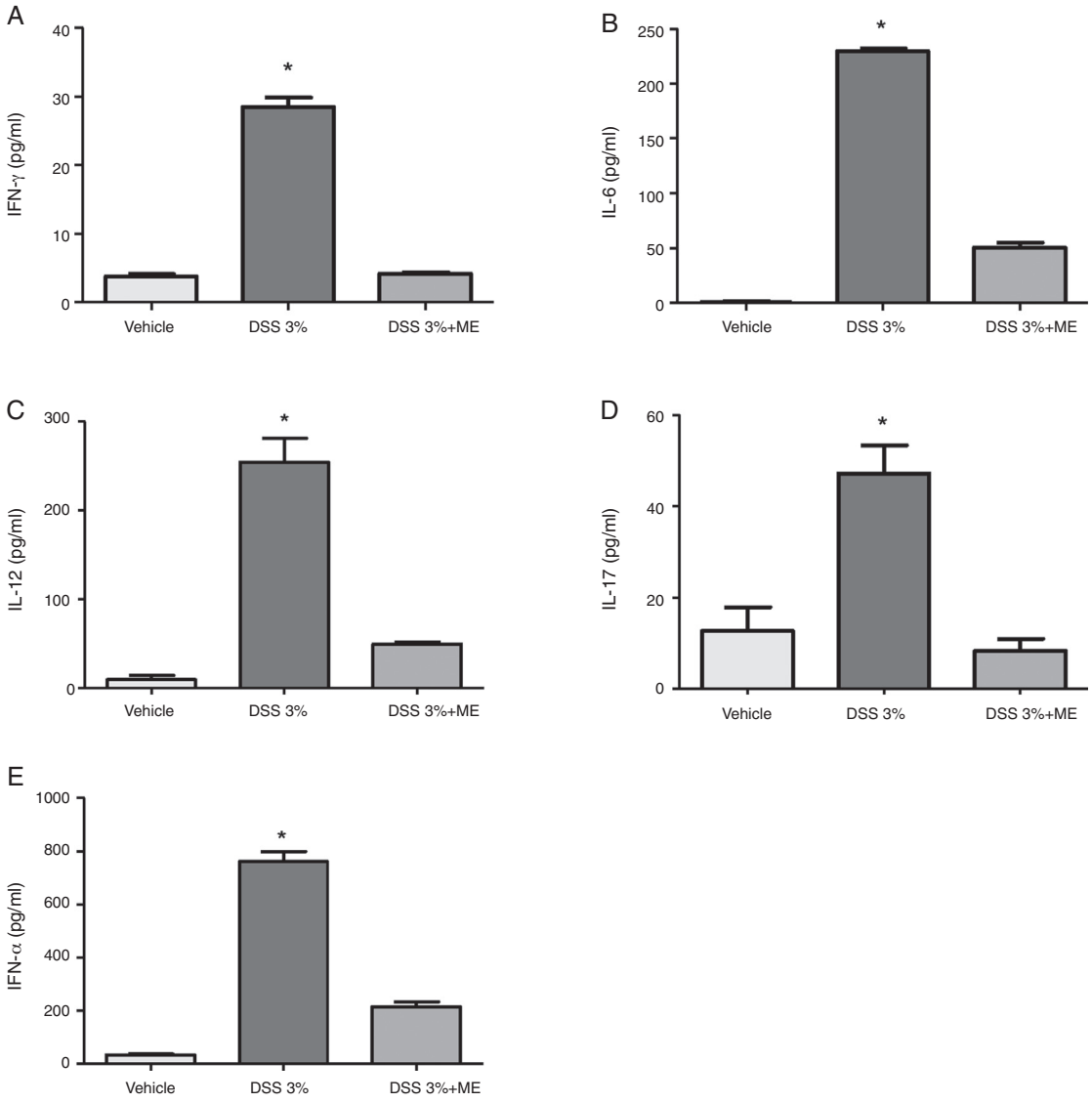

Fig. 3. Levels of Th1 and Th17 cytokines observed in cultured colonic tissue of mice with DSS-induced colitis treated with *Caulerpa mexicana* methanolic extract. Mice were administered 3% DSS in their drinking water (*ad libitum*) for 14 days with or without *C. mexicana* methanolic extract (2 mg/kg/day *i.v.* up to 14 days); saline was used as a negative control. The production of (A) IFN-γ, (B) IL-6, (C) IL-12, (D) IL-17A and (E) TNF-α were determined as described in Material and Methods section. Values represent mean ± SD ($n = 5$) $^*p < 0.05$ in the vehicle-treated control group *vs.* the DSS-induced colitis group; significance between the treated groups was determined by ANOVA.

methanolic extract exerts an anti-inflammatory effect in UC by negatively regulating the pro-inflammatory cytokine levels. Reduction of the levels of these inflammatory mediators may have occurred by two possible mechanisms. The first is associated with the capacity of the *C. mexicana* methanolic extract to directly inhibit cytokine secretion from innate immune cells, as previously demonstrated (Bitencourt et al., 2011), and the latter is related to the reduction of cell migration to the colonic mucosa as an indirect mechanism to decrease cytokine levels.

Currently some studies have shown the efficacy of natural products in the treatment of experimental IBD. These studies demonstrated that curcumin–piperine mixtures in self-microemulsifying (CUR-PIP-SMEDDS), ellargic acid, mangiferin and olipein ameliorate the DSS-induced colitis in mice through the reduction in DAI and histopathological lesion, and downregulating inflammatory mediators such as MPO activity, IFN-γ, TNF-α and IL-6 levels, COX-2 and iNOS expression and interfering with the signaling pathways of p38 MAPK, NF-κB, and STAT3 (Dou et al., 2014; Li et al., 2015; Giner et al., 2011; Marín et al., 2013).

The green seaweed *C. mexicana* is composed of numerous compounds endowed with potential biological activities (Carrillo-Dominguez et al., 2002). Some of them could contribute to the negative effects mediated by the methanolic extract on cytokine secretion at the colonic mucosa and on inflammatory cell migration to the colon. In fact, compounds like caulerpin and other purified indolic alkaloids from *Caulerpa* species such as *Caulerpa racemosa*, have proven anti-inflammatory activity reducing cell migration in peritonitis models (Bamias and Cominelli, 2007) and sulphated polysaccharides isolated from *C. mexicana* has anti-inflammatory and antinociception activity (Carneiro et al., 2014).

Therefore, new studies are needed to isolate the compounds from the extract and evaluate their mechanisms of action. Furthermore, considering the great reduction in the clinical symptoms and cytokine secretion, but only slight amelioration of histological damage, it is possible that the association of the *C. mexicana* methanolic extract with conventional therapies or new approaches could also guarantee mucosal healing alongside improved clinical symptoms.

Conclusions

In summary, our data show that the treatment of mice with methanolic extract of *C. mexicana* significantly ameliorate the clinical signs observed in UC and decrease the levels of cytokine from Th1 (IFN-g, IL-12 and TNF-a) and Th17 (IL-6 and IL-17) immune response pattern and this decrease could be associated with reduction of tissue damage observed in the colon of the animals that received DSS. Therefore this extract appears promising for research on metabolites that may be effective in the treatment of UC.

Conflicts of interest

The authors declare no conflicts of interest.

Authors' contributions

MAOB and HMDS (MSc students) contributed to biological studies, running the laboratory work, analysis of the data and drafted the paper. GMFA (MSc student) contribute to *C. mexicana* extract production. GECM contributed in collecting plant sample and identification and herbarium confection. AMAM contribute with the mice production. JXAJ contributed to critical reading of the manuscript. EJDS contribute to histopathology analysis. JTS and BVOS designed the study, supervised the laboratory work and contributed to critical reading of the manuscript. All the authors have read the final manuscript and approved the submission.

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