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Beneficial role of *Pistacia lentiscus* aqueous extract in experimental colitis: Anti-inflammatory and potential therapeutic effects

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Abstract

Background: *Pistacia lentiscus L. (PL)* is a flowering plant traditionally used in the treatment of gastrointestinal disorders. The extracts of this plant are endowed with strong pharmacological activities. The aim of our current study was to investigate the anti-inflammatory and potential therapeutic effects of *PL* leaves aqueous extract (PLAE) against Dextran Sulfate Sodium (DSS)-induced acute colitis.

Materials and methods: The therapeutic effect of PLAE was evaluated after orally administration of 3% DSS alone or concomitantly with PLAE (50, 100 or 200 mg/Kg). Mucosal lesions were assessed by macroscopic and histopathological examination. In this context, hemorrhage, diarrhea, weight loss, and disease activity index (DAI) were determined daily throughout the experiment. In the same way, hematoxylin-eosin and Alcian blue staining of colonic mucosal were used to evaluate respectively mucosal damages and mucus production. Furthermore, the levels of nitric oxide (NO), and pro-inflammatory cytokines [tumor necrosis factor- α (TNF- α) and interleukin-6 (IL-6)] were measured in plasma, as well as in colonic explants and peritoneal macrophages cultures supernatants.

Results: Administration of DSS+PLAE indicated a significant reduction in clinical score of acute colitis DAI compared to DSS alone administration. Interestingly, histological analysis of the mucosa showed that DSS+PLAE-treated groups exhibited almost normal histology evidenced by an intact epithelium structure and less inflammatory cell infiltration in the mucosa. Alcian bleu staining revealed that DSS+PLAE-treated groups displayed almost normal mucus production. Importantly, a significant decrease in pro-inflammatory mediators (NO, IL-6 and TNF- α) levels in dose dependent manner was reported in plasma, and culture supernatants of colonic explants and peritoneal macrophages from DSS+PLAE-treated mice compared to the DSS group.

Conclusion: Our results showed that the systemic and local anti-inflammatory activities of aqueous leaves extract of *PL* improve the clinical signs of acute colitis. Our data suggest that PLAE has beneficial effects and could constitute a promising approach against acute ulcerative colitis by targeting the deregulated immune response.

Highlights

Administration of PLAE (*Pistacia lentiscus* aqueous extract) during acute DSS induced-colitis:

- Induces reduction in clinical score of acute colitis.
- Prevents colon histological lesions induced by DSS.
- Inhibits mucus production by Goblet cells in acute colitis.
- Induces a significant decrease in pro-inflammatory mediators (NO, IL-6 and TNF- α) levels in plasma, and culture supernatants of colonic explants and peritoneal macrophages.

1. Introduction

Inflammatory bowel disease (IBD) constitute a group of chronic and relapsing idiopathic inflammatory disorder of the intestine that include Crohn's disease (CD) and ulcerative colitis (UC) (Caruso et al. 2020, Geboes et al. 2018). The exact factors that trigger the chronic and relapsing intestinal inflammation remain unknown. Multiple lines of evidence suggest that the disease is caused by a confluence of genetic and environmental factors that alter gut homeostasis. It is also thought that IBD results from an aberrant and continuing immune response against endogenous flora and luminal antigens (Zhang and Li, 2014, Soufli et al. 2016, Toumi et al. 2021). Although the pathogenesis of both Crohn's disease and ulcerative colitis involves intestinal inflammation, these two disorders differ in several features, including the association with specific susceptibility loci and the type of immune response and pathology associated with disease (McGovern et al. 2015). CD has long been considered to be driven by Th1 and Th17 responses, whereas UC has been associated with a non-conventional Th2 response (Duché, 1992; Dray and Marteau, 2007). Immunological studies have recently focused on the mucosal innate immune responses, such as epithelial barrier integrity, innate microbial sensing, autophagy and the unfolded protein response. During the innate response, the intestine of patients with IBD is massively infiltrated by inflammatory cells, mostly macrophages and neutrophils that release large amounts of pro-inflammatory cytokines, such as TNF- α , IL-6, IL-17, IL-12, and interferon- γ (Abraham and Medzhitov 2011, Soufli et al. 2016, Toumi et al. 2021). The pro-inflammatory cytokines are involved in the up-regulation of inducible nitric oxide synthase (iNOS) expression as well as nitric oxide (NO) production. In fact, during IBD, the balance between pro-inflammatory cytokines, anti-inflammatory cytokines (IL-4 and IL-13), and immunoregulatory cytokines (IL-10 and transforming growth factors- β) is disturbed (Rafa et al. 2013).

The most commonly used treatments for IBD are 5-aminosalicylates (5-ASA), corticosteroids, immune-suppressors and immunomodulatory therapies that involve the use of humanized monoclonal antibodies mainly against TNF- α . However, treatments targeting the immune system have only an immunosuppressive effect (Taylor and Irving, 2011), and most of them have limitations in side effects and safety, such as drug dependence, adverse reaction, decline of immune function, and risk of cancer (Danese et al. 2012).

In this sense, it appears important to research and develop an affordable, cost-effective and side-effect-free therapeutic strategy. Therefore, highlighting a new therapeutic approach that targets the deregulated immune response is of great interest. There is growing evidence and focus that herbal remedies can help cure gastrointestinal illness and prevent relapse (Ukwe et al. 2010; Bozorgi et al. 2013; Zahouani et al. 2020). Among those medicines figures *Pistacia lentiscus* L. (PL), a flowering plant growing in the Mediterranean region. In fact, most members of the *Pistacia* have chemical and therapeutic similarities. Results from several studies have revealed potent pharmacological activities of various parts of this genus plants (Paterniti et al. 2017). The fruits, nuts, resin and leaves of *Pistacia lentiscus* are used for the treatment of eczema, asthma, kidney stones, throat infections, stomach and diarrhea ache, with astringent, antipyretic, antibacterial, anti-inflammatory and antiviral activities (Bozorgi et al. 2013; Paterniti et al. 2017).

The aim of our present study was to evaluate the anti-inflammatory and potential therapeutic effects of aqueous extract of *Pistacia lentiscus* leaves (PLAE) in the experimental model of Dextran Sulfate Sodium (DSS)-induced acute colitis.

2. Materials And Methods

2.1 *Pistacia lentiscus*

Pistacia lentiscus L. leaves were collected in East Algeria in December 2017. A specimen of *Pisactia lentiscus* L. has been identified as previously described (Boutemine et al. 2018).

2.2 Preparation of aqueous extract of PL (PLAE)

PL leaves were first washed with water, dried in the shade, and then powdered with a blender. The powder obtained was then dissolved in bi-distilled water. The mixture was subjected to continuous stirring for a period of 24 hours and then filtered and centrifuged. The supernatant obtained was lyophilised and the extract has been stored.

2.3 Acute cell toxicity of PLAE on hepa1c1c7 cell line

a- hepa1c1c7 cells line culture

Mouse hepatoma hepa1c1c7 (ATCC[®] CRL-2026) cell line was cultured in DMEM (Dulbecco's Modified Eagle's Medium, Hyclone Laboratories, Logan, Utah, USA) supplemented with 10% of fetal bovine serum (FBS), 100 unit/mL of penicillin and 100 g/mL of streptomycin (Gibco[®], Life Technologies, Carlsbad, CA, USA). Briefly, cells at exponential phase were seeded into 96-well plates at 10^5 cells/mL per well. Then, different concentrations of PLAE were added (0.304, 0.914, 2.74, 8.13, 24, 74, 220 and 666 µg/mL). Each treatment was tested in triplicate and the control group was given only culture medium without PLAE. The above plate were placed in a 5% CO₂ humidified-atmosphere incubator at 37 °C for 24h.

b- MTT assay

The cytotoxic effect of PLAE on hepa1c1c7 cell line was determined by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-difenylnitroimidazolium (MTT) assay. In this sense, at the end of culture time, 20 µL MTT (5 g/L) was added to each well and the plate was incubated at 37 °C for 4 h. The plate was then shaken for 15 min. Finally, the absorbance of each well was measured by microplate reader at 492 nm. The proliferation rate was calculated as A_{492} of treated/ A_{492} of untreated (control) cells \times 100% (Tankiewicz-Kwedlo et al. 2010).

2.4 Induction of acute colitis by sodium dextran sulfate (DSS)

Female BALB/c mice (18-22g) were allowed free access to food and water and were kept under normal conditions with a 12 h dark/light cycle. All our experiments were approved by Ethics Committee of the

Thematic Research Agency in Health Sciences (N°58/DFPR/ATRSS).

Induction of colitis was performed according to the Okayasu protocol (Okayasu et al. 1990, Chassaing et al. 2015). Mice received *ad libitum* 3% (w/v) of Dextran Sulfate Sodium (DSS) (Salt, Reagent Grade, MP Biomedicals, M.W 36-50 kDa, Ref: 160110) dissolved in drinking water for 7 days.

The mice were randomly divided into 6 groups as follows (Fig.1):

- 1) DSS group: mice received DSS dissolved in drinking water, plus 200 µl of distilled water by gavage each day (n = 8).
- 2) DSS+PLAE 1 group: mice received DSS dissolved in drinking water, plus 200 µl of aqueous extract dissolved in distilled water at 50 mg/Kg by gavage each day (n = 8).
- 3) DSS+PLAE 2 group: mice received DSS dissolved in drinking water, plus 200 µl of aqueous extract dissolved in distilled water at 100 mg/Kg by gavage each day (n = 8).
- 4) DSS+PLAE 3 group: mice received DSS dissolved in drinking water, plus 200 µl of aqueous extract dissolved in distilled water at 200 mg/Kg by gavage each day (n = 8).
- 5) PLAE group: mice received drinking water, plus 200 µl of aqueous extract dissolved in distilled water at 200 mg/Kg by gavage each day (n = 4).
- 6) Control (Ctrl) Group: mice received drinking water, plus 200 µl of distilled water by gavage each day (n = 8).

2.4.1 Effect of PLAE on clinical manifestations of acute colitis

In order to assess the severity of colitis, body weight, stool consistency, and blood in the stool were determined daily throughout the experiment, using the following classification system (Azuma et al. 2010b):

- Weight loss was noted as follows: score 0: none; score 1: 1 to 5%; score 2: 5 to 10%; score 3: 10-20%; score 4: > 20% weight loss.
- Diarrhea was scored as follows: score 0: normal; score 2: loose stools; score 4: watery diarrhea.
- Blood in the stool was scored as follows: score 0: normal, score 2: slight bleeding; score 4: rectal hemorrhage.

The sum of the three scores allowed us to calculate the disease activity index (DAI), to assess the severity of the inflammation throughout the experiment (Azuma et al., 2010b). The total DAI score ranged from 0 to 12.

On day 7, the mice were euthanized, the blood was collected by cardiac puncture and the plasma was separated and stored at -20°C for subsequent analysis. Next, an intraperitoneal washing was performed

for the recovery of peritoneal macrophages. After dissection, the colon and cecum of each mouse were isolated by separating them from the small intestine at the ileocecal junction and from the anus at the distal end of rectum. Next, colon was washed using cold PBS to remove faeces and measured. Raw pictures from all groups of mice were taken. Then, each colon was dichotomized as follows:

- A fragment was immersed in 4% formaldehyde for histological analysis.
- Small fragments were placed in DMEM supplemented with 10% FCS, 100 unit/mL of penicillin and 100 g/mL of streptomycin for explant culture.

2.4.1. Histological evaluation of colonic lesions

a- Histological Staining of colonic mucosal with hematoxylin-eosin (H&E)

Colon mucosa of mice were fixed in 4% buffered formaldehyde and embedded in paraffin. Tissue sections 2 µm thick were deparaffinized using xylene and rehydrated through a series of increasingly concentrated ethanol baths. Sections were stained with hematoxylin and eosin. Briefly, the tissue sections were first immersed in hematoxylin for 3-5 min. After rinsing, they were stained with eosin for 2 min. The histological criteria were based on the degree of tissue structure changes, as well as inflammatory cells infiltration.

Colonic inflammation scores were determined in the basis of previously described system for DSS-induced colitis (Azuma et al. 2010b, Soufli et al. 2015). The histological criteria were noted as follows:

- For the epithelium: score 0: normal morphology; score 1: Goblet cell loss; score 2: loss of crypt and presence of epithelium; score 3: loss of crypt and epithelium; score 4: loss of crypt and epithelium (large area).
- For infiltration: score 0: occasional inflammatory cells in the lamina propria; score 1: increased infiltration in the lamina propria mainly at the base of the crypts; score 2: confluence of the inflammatory infiltrate extending to the mucosa; score 3: confluence of the inflammatory infiltrate extending to the submucosa; score 4: transmural extension of the infiltrate (extensive).

b- Histological staining of colonic mucosal with Alcian Blue

Each piece of colon was stained with Alcian Blue to assess mucus production. This staining will color the contents of the cells pink and the mucus blue. For this, after *deparaffinization and rehydration*, colon sections of 2 µm thick were placed in the Alcian Blue dye for 15 min and then in nuclear red for 2 to 3 min. The intensity of the blue color in the crypts reflects a significant production of mucus.

Interpretation of all results (H&E and Alcian blue staining) was performed double-blind in the presence of a pathologist. The images were captured with a digital camera (Casio) on an optical microscope (Motic).

2.4.3 Culture of mice peritoneal macrophages

Peritoneal macrophages were obtained by washing the peritoneal cavity of mice using 5 mL of cold PBS (pH = 7.4). The cells suspensions were washed thrice with PBS by centrifugation at 2800 rpm for 5 min. Pellets recovered were re-suspended in DMEM, supplemented with 10% FCS. Cells were allowed to adhere at a density of 10^5 macrophages/mL in a 48-well microplate. After 2 hours, the wells were washed with PBS to remove non-adherent cells. Then, all macrophages were incubated in complete DMEM at 37°C and 5% CO₂. After 24 hours, the culture supernatants were recovered and then stored at -20°C for the subsequent determination of NO and cytokines levels.

2.4.4 *Ex vivo* Culture of colonic explants

Ex vivo culture of two pieces of the colon will provide key information regarding the severity of inflammation. In this context, the colonic sections (approximately 0.5 cm), cut longitudinally, were washed thrice serially in mixture of antibiotics (100 U/mL of penicillin and 0.1 mg/mL of streptomycin). The washed colons explants was then placed in a well (24 well plate) containing a drop of complete DMEM with 1% antibiotics (penicillin & streptomycin), supplemented with 10% FCS. After 2 hours, 500 µL of DMEM were added and the explants were incubated at 37°C and 5% CO₂ for 20h. Then, supernatants were collected and centrifuged for 10 min at 4°C and store at -20°C until determination of NO and cytokines levels.

2.4.5 Nitrite concentrations measurement

The Griess reaction was used to determine nitrite levels as an indicator of NO production, in plasma and culture supernatants of Ctrl, DSS, DSS+PLAE 2 and DSS+PLAE 3 groups, as described by Touil-Boukoffa et al. (1997). Briefly, samples were mixed with Griess reagent and distilled water. After incubation, the absorbance was determined at 543 nm by a spectrophotometer. The concentration of nitrites was determined using a standard curve with sodium nitrite.

2.4.6 Measurement of IL-6 and TNF- α levels

The levels of IL-6 and TNF- α were measured in the plasma and the culture supernatants of colonic explants, and peritoneal macrophages of Ctrl, DSS, DSS+PLAE 2 and DSS+PLAE 3 groups, using specific enzyme immunoassay kits (ELISA), according to the manufacturer's specifications (Invitrogen ThermoFisher scientific). Briefly, capture antibodies were fixed on 96-well plates. After 24h, the samples were added followed by detection antibodies conjugated with biotin. Then Avidin-HRP followed by tetramethylbenzidine (TMB) was added to reveal the reaction. Finally, the absorbance was measured at 450 nm with an ELISA reader. A standard curve was performed for each assay using recombinant cytokines (Analytical sensitivity: 3.7 pg/mL for TNF- α and < 3 pg/mL for IL-6).

2.5 Statistical analysis

Statistical analysis was performed using GraphPad Prism. All data were expressed as mean \pm SEM. The statistical significance was assessed using Student's t-test, with $p < 0.05$ being considered significant.

3. Results

3.1 Effect of PLAE on cell proliferation and viability

The Acute cell toxicity of PLAE on hepa1c1c7cell line (Fig. 2) was determined by 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay. We observed that exposure of cells to up 220µg/mL PLAE (equivalent to raw medicinal material) for 24 h did not reduce the cellular proliferation and viability (Fig. 2) compared to the control group (0µg/mL of PLAE). In addition, our results indicated that exposure of cells to 666µg/mL PLAE (three times more concentrated than raw medicinal material) for 24 h did not alter the cellular proliferation and viability. Our results clearly showed that PLAE has no cytotoxic activity on hepa1c1c7cell line at concentrations used in our experiments.

3.2 PLAE decreases the clinical manifestations of DSS-induced acute colitis

We determined daily throughout the experiment hemorrhage, diarrhea and weight loss. These parameters were used to determine the clinical score of acute disease activity index (DAI) (Fig. 3). Our results suggest that concomitant administration of PLAE at 200 mg/Kg with DSS significantly reduced the clinical manifestations and clinical score of acute colitis DAI in comparison to DSS administration alone (Fig. 3). These symptoms and signs of morbidity were more noticeable from day 5 to the end of the experimental period. Administration of PLAE at 100 mg/Kg highly reduced the clinical manifestations and clinical scores of DAI but did not improve mice weight loss induced by DSS (Fig. 3). However, we observed that the concentration of 50 mg/Kg did not improve the DSS-induced clinical manifestations (Fig. 3). Interestingly, the administration of the aqueous extract alone in absence of DSS had no effect on animals and the results were comparable to those obtained for the control group (Fig. 3).

3.3 PLAE prevents shortening of the colon during acute DSS-induced colitis

The colon shortening is a marker of inflammation in DSS-induced acute colitis model (Okayasu et al. 1990; Chassaing et al. 2015). Indeed, we noticed that in DSS group, the colon length (5.38 ± 0.18 cm) was significantly shortened as compared to control group (10.63 ± 0.36 cm, $p < 0.0001$). Administration of DSS+ PLAE significantly prevented the colon shortening in dose-dependent manner compared to DSS group (DSS+ PLAE 2: 7.66 ± 0.44 cm; DSS+ PLAE 3: 8.25 ± 0.61 cm vs DSS group: 5.38 ± 0.18 cm, $p < 0.001$) (Fig. 4). However, the administration of PLAE at 50mg/Kg (DSS+ PLAE 1 group) has no effect on DSS induced-colon shortening (DSS: 5.38 ± 0.18 cm vs DSS+ PLAE 1: 5.52 ± 0.26 cm, $p > 0.5$). In addition, the administration of aqueous extract alone (PLAE group) had no effect on colon size and the results were comparable to those obtained for control group (Fig. 4).

3.4 Effect of PLAE on colon histological lesions induced by DSS

The observed bleeding and changes in colon size in DSS group suggest significant tissue alterations. In order to confirm these observations, we performed histological analysis. Colon tissue sections of control mice revealed a well-differentiated, normal-polarity epithelium and well-preserved crypts, as well as a complete absence of inflammatory infiltrate (score 0) (Fig. 5a). In contrast, DSS-treated mice colon

displayed significant tissue damage as compared to control group (Fig. 5b). These damages are characterized by crypt deletion, complete goblet cell depletion, ulceration, mucosal erosion and replacement of epithelial cells by granulous tissue (score 4). Importantly, an intense infiltration of inflammatory cells into the mucosa and submucosa with a strongly increased size of mucosa-associated lymphoid tissue was observed, as well as the occasional presence of pseudo-polyps (score 4) (Fig. 5b).

Interestingly, we observed that treatment with DSS+PLAE 3 significantly reduced these tissue alterations (Fig. 5e). In fact, normal crypts were observed, with almost an intact epithelium structure, evidenced by a focal decrease in the height of the epithelium and less cellular infiltration in the mucosa. We noted in some cases the presence of some regenerative ulceration corresponding to remissions (score 1) (Fig. 5e). In DSS+ PLAE 2 group, a locally altered epithelium was observed accompanied by a focal deletion of crypts, and less inflammatory infiltrate compared to DSS group (score 2) (Fig. 5d). However, treatment with PLAE at 50 mg/Kg concomitantly with DSS has no effect on DSS-induced histopathological lesions (score 4) (Fig. 5c). In addition, PLAE group (Fig. 5f) presented histological structure comparable to that of control group (score 0).

3.5 PLAE inhibits mucus production by Goblet cells in acute colitis

In order to test the effect of PLAE on mucus production, we stained the colonic tissue sections with the Alcian blue stain. Mucosa of control mice had a normal structure and crypts composed of several well-differentiated Goblet cells stained blue, reflecting normal mucus production (Fig. 6a). On the other hand, the colonic mucosa of the DSS group was characterized by a hypocrine effect due to the disappearance of Goblet cells and their secretory vesicles. A considerable reduction in blue coloration was observed reflecting a decrease of mucus production (Fig. 6b). Interestingly, colonic mucosa in DSS+PLAE 2 and 3-treated mice (Fig 6 d, e) exhibited almost normal mucus production with some degree of focal loss of crypts in the DSS+ PLAE 2 group mucosa (Fig 6d). In addition, the PLAE group showed similar mucus production to the control group (Fig. 6f). Nevertheless, the DSS + PLAE 1 group was characterized by a hypocrine effect and disappearance of Goblet cells comparable to that observed in the DSS group (Fig. 6c).

Our macroscopic and histopathological evaluation revealed clearly that the dose 50 mg/Kg is too low and have no effect on DSS-induced colitis. Hence, the doses 100 mg/Kg and 200 mg/Kg were chosen for additional investigations.

3.6 Effect of PLAE on systemic, local and peritoneal macrophages productions of NO in acute colitis

It is well known that DSS-induced colitis is associated with prolonged activation of iNOS and increased NO production at systemic and local levels (Soufli et al. 2015, Soufli et al. 2016). In order to evaluate the effect of PLAE on systemic and local production of NO, we measured nitrites in plasma and culture supernatants of colon explants. Our results showed that the plasma levels of nitrites in DSS group were significantly higher compared to control group (22.04 ± 3.5 vs 4.70 ± 1.95 μ M, $P < 0.05$) (Fig. 7a). In contrast, mice treated with DSS+PLAE showed a dose-dependent decrease in systemic levels of nitrites

(DSS+PLAE 2: $11.65 \pm 6.76 \mu\text{M}$, DSS+PLAE 3: $5.38 \pm 1.99 \mu\text{M}$). In addition, DSS+PLAE 3 group indicated a significantly reduced NO level compared to DSS group ($5.38 \pm 1.99 \mu\text{M}$ vs 22.04 ± 3.5 , $p < 0.05$).

In addition, our data corroborated those measured in culture supernatants of colon explants. Indeed, local NO levels in DSS group were significantly higher compared to control group (0.6467 ± 0.1270 vs $0.1698 \pm 0.0763 \mu\text{M} / \text{mg}$ of explant) (Fig 7b). On the other hand, treatment with DSS+PLAE significantly reduced the level of nitrite in culture supernatants of colonic explants in dose-dependent manner (DSS+PLAE 2: $0.5884 \pm 0.1441 \mu\text{M} / \text{mg}$ of explant; DSS+PLAE 3: $0.1263 \pm 0.0465 \mu\text{M} / \text{mg}$ of explant). Furthermore, when compared to DSS group, local nitrite levels in DSS+PLAE 3 group decrease significantly compared to DSS group (0.1263 ± 0.046 vs $0.6467 \pm 0.1270 \mu\text{M} / \text{mg}$ of explant, $p < 0.01$) (Fig. 7b).

We also measured the nitrite levels produced by peritoneal macrophages cultured *in vitro*. Our results showed that nitrite levels in the culture supernatants of peritoneal macrophages obtained from DSS group was significantly increased as compared to those from control group (13.64 ± 2.78 vs $6.2 \pm 0.32 \mu\text{M} / 10^5 \text{ cells}$, $p < 0.05$). Interestingly, NO production by peritoneal macrophages from DSS+PLAE-treated mice was significantly reduced compared to DSS group in a dose-dependent manner (DSS+PLAE 2: $6.53 \pm 1.69 \mu\text{M} / 10^5 \text{ cells}$; DSS+PLAE 3: $4.45 \pm 1.98 \mu\text{M} / 10^5 \text{ cells}$) (Fig. 7c). In addition, we observed a significant decrease of nitrite production in DSS+PLAE 3 treated-group compared to DSS group (4.45 ± 1.98 vs $13.64 \pm 2.78 \mu\text{M} / 10^5 \text{ cells}$, $p < 0.05$) (Fig. 7c).

3.7 Effect of PLAE on systemic and local production of pro-inflammatory cytokines in acute colitis

DSS causes systemic and local inflammation characterized by increased production of pro-inflammatory cytokines (Sanchez-Fidalgo et al. 2010, Shi et al. 2011). Therefore, in order to evaluate the effect of PLAE on systemic production of pro-inflammatory cytokines, we measured plasma levels of IL-6 and TNF- α . Our results show that the plasma levels of both cytokines in DSS group were significantly higher compared to those of control group (IL-6: 31.55 ± 9.95 vs $8.18 \pm 1.2 \text{ pg/mL}$; $p < 0.01$, TNF- α : 85.20 ± 9.27 vs $42.5 \pm 9.27 \text{ pg/mL}$; $p < 0.05$) (Figure 8 a and b). In contrast, we observed a dose-dependent decrease in systemic levels of IL-6 and TNF- α in DSS+PLAE-treated mice [DSS+PLAE 2: IL-6: 39.26 ± 13.55 , TNF- α : $18.85 \pm 15.0 \text{ pg/mL}$; DSS+PLAE 3: IL-6: $11.20 \pm 1.68 \text{ pg/mL}$, TNF- α : $36.71 \pm 0.39 \text{ pg/mL}$] (Figure 8 a, and b). Furthermore, we observed a significant decrease of both pro-inflammatory cytokines in DSS+PLAE 3 treated-group compared to DSS group (IL-6: 11.20 ± 1.68 vs $31.55 \pm 9.95 \text{ pg/mL}$; $p < 0.01$; TNF- α : 36.71 ± 0.39 vs $85.20 \pm 9.27 \text{ pg/mL}$; $p < 0.05$).

Local production of IL-6 and TNF- α in colonic explant culture were strongly inhibited by PLAE. Levels of both cytokines secreted by colon explants of DSS group (IL-6: 13.54 ± 2.19 , TNF- α : $2.28 \pm 0.68 \text{ pg/mL/mg}$ of explant) were significantly higher in comparison to those of control group (IL-6: 3.09 ± 1.2 , TNF- α : $0.39 \pm 0.083 \text{ pg/mL/mg}$ of explant) (Figure 8 c and d). In contrast, treatment with PLAE significantly reduced IL-6 and TNF- α levels in culture of colonic explants in a dose-dependent manner (DSS+PLA 2: IL-6: 10.31 ± 4.34 , TNF- α : $1.17 \pm 1.1 \text{ pg/mL/mg}$ of explant; DSS+PLAE 3: IL-6: 6.30 ± 1.46 , TNF- α : $0.89 \pm 0.21 \text{ pg/mL/mg}$ of explant) (Fig. 8 c and d). Interestingly and in comparison to DSS group,

DSS+PLAE 3 group shows a significant decrease in local production of IL-6 and TNF- α (IL-6: 13.54 ± 2.19 vs 6.30 ± 1.46 pg/mL/mg of explant, $p < 0.001$, TNF- α : 2.28 ± 0.68 vs 0.89 ± 0.21 pg/mL/mg of explants; $p < 0.05$).

3.8 Effect of PLAE on pro-inflammatory cytokines production by peritoneal macrophages in acute colitis

We then measured the rate of pro-inflammatory cytokines IL-6 and TNF- α secreted by peritoneal macrophages cultured without any form of stimulation. The results showed that IL-6 and TNF- α levels in culture of peritoneal macrophages obtained from DSS group were higher than those from control group (IL-6: 15.60 ± 6.08 vs 70.43 ± 23.98 pg/mL/ 10^5 macrophages, $p < 0.05$; TNF- α : 58.11 ± 38.2 vs 32.7 ± 2.8 pg/mL/ 10^5 macrophages). In contrast, the production of IL-6 by peritoneal macrophages from DSS+PLAE 2 and 3-treated mice was significantly reduced as compared to DSS group (Fig. 9 a). In addition, we observed that TNF- α levels in DSS+PLAE 3 treated group was highly reduced compared to DSS group (58.11 ± 38.2 vs 39.28 ± 12.42 pg/mL/ 10^5 macrophages) (Fig.9 b).

4. Discussion

Due to the side effects of the drugs currently available, the use of medicinal plants for treatment of acute and chronic intestinal inflammatory diseases is attracting increasing interest. In our current study, we demonstrated for the first time the anti-inflammatory and therapeutic activities of the aqueous extract of *Pistacia lentiscus* leaves (PLAE) on DSS-induced acute colitis.

Crohn's disease and ulcerative colitis are the major forms of intestinal bowel diseases (IBD) characterized by bowel inflammation and abdominal pain. In recent decades, many animal models have been developed to characterize the complexity of IBD pathogenesis, defining the underlying molecular mechanisms and evaluating potential therapeutics for humans (Chassaing et al. 2015). In the present study, we chose to use experimental colitis model based on administration of dextran sodium sulphate (DSS) in mice. This model has been recognized for its speed, simplicity and reproducibility.

Our results showed that after DSS administration, all animals developed the symptoms of acute colitis characterized by progressive weight loss, diarrhea and rectal bleeding. In fact, we observed first clinical signs of colitis on day 3 after starting DSS administration, whereas on day 5 most animals started to present heavy bleeding. At macroscopic scale, we observed hemorrhagic erosion zones and ulcerations scattered throughout the colon, the cecum, and the rectum accompanied by colon size shortening. From histological point of view, we observed that DSS causes erosions and thinning of the epithelium surface, edematous zones, crypts dilation, as well as the infiltration of immune cells, mainly polynuclear neutrophils (PNN) and macrophages. The deterioration of the lower intestinal tract was accompanied by a depletion of Goblet cells and a decrease in mucus secretion. These results are consistent with those reported by several authors who speculated that DSS is a chemical colitogen with anti-coagulant properties capable of causing severe murine colitis which closely resembles human ulcerative colitis (Okayasu et al. 1990, Abdelouhab et al. 2012, Chassaing et al. 2015). The mechanism by which DSS

induces intestinal inflammation has not yet been fully described. However, some authors suggest that DSS carries a highly negative charge provided by sulfate groups, toxic to the colon epithelia monolayer. It thus induces erosions that compromise the barrier integrity leading to increased colonic epithelial permeability and allow to the dissemination of the pro-inflammatory intestinal contents in the underlying tissues. Poritz et al. (2007) observed that the administration of DSS induces changes in tight junction protein expression as early as one day after treatment and an inflammatory response characterized by leukocyte recruitment and increased production of pro-inflammatory cytokines (Toumi et al. 2013). These initial effects are followed by increasingly severe symptoms with severe diarrhea, bleeding and acute anemia. In addition, DSS anticoagulant property aggravates intestinal bleeding (Chassaing et al. 2014). All of these reactions cause narrowing of the colon size, progressive weight loss and death of the animals.

Importantly, our study showed that the oral administration of PLAE concomitantly with DSS significantly reduced the clinical, macroscopic and histopathological colitis scores in comparison to the administration of DSS alone. Our results are in line with those of several studies. Zahouani et al. (2020) revealed that pre-treatment with PLAE significantly reduced the lesion areas in acetic acid-induced acute colitis. In addition, Dellai et al. (2013) suggested that pre-treatment with PLAE at 200mg/Kg decreased the intensity of gastric mucosal damages at 74.9% compared with ethanol-HCl group in an experimental model of gastric ulcer. Furthermore, several other studies showed the benefit of *Pistacia lentiscus* (PL) extracts on ulcerative colitis. Recently, Ostovan et al. (2020) demonstrated that Mastic Oil of PL significantly reduced the total colitis index, including inflammation severity, inflammation extent, and crypt damage scores. Razi et al. (2000) suggested that supplementation with oleogum resin from PL delayed the onset and progression of the disease and helped prevent weight loss in the DSS model of colitis. In addition, Kim et al. (2009) showed that PL mastic decreased the macroscopic and histological damage in TNBS-induced colitis in rats. Our results are also in concordance with our previous study establishing that PL fatty oil pre-treatment or treatment significantly reduced ulcerated and hemorrhagic areas in ethanol-induced gastric ulcer (Boutemine et al. 2018).

Otherwise, our results revealed that DSS-administration induces a significant increase in systemic, local, and peritoneal macrophages production of NO compared to control group. This result suggests that NO is implicated in the intestinal inflammation. Our results are in agreement with previous studies conducted in our laboratory demonstrating that the administration of 3% DSS induced a significant decreases in NO production in plasma, colon and macrophages cultures compared to control group (Abdelouhab et al. 2012, Toumi et al. 2013, Toumi et al. 2014). Other authors demonstrated that the administration of 3% or 5% DSS increases iNOS expression and NO level in rat colon (Arafa et al. 2009, Larrosa et al. 2009). In addition, it was shown that inhibition of iNOS expression blunted DSS colitis in mice (Rahimi et al. 2010)

In the second part of our experiments, we aimed to determine the systemic, local, and macrophage production of the pro-inflammatory cytokines IL-6, and TNF- α . In agreement with previous studies, our results suggest that DSS administration significantly increase pro-inflammatory cytokines production (Zi-cong et al. 2020; Toumi et al. 2014). The infiltrated inflammatory cells observed in the mucosa and

submucosa after treatment by DSS may secrete pro-inflammatory cytokines (TNF- α , IL-1 β , IFN- γ , IL-12, IL-6). These cytokines lead with endotoxin (LPS) and many bacterial components present in the colon to activation of the transcription factor NF- κ B, and then the expression of iNOS (Toumi et al. 2013, Soufli et al. 2016). NO produced in large quantities by this enzyme, joins the superoxide anion ($O_2^{\cdot-}$) to generate peroxynitrite ion ($ONOO^-$) known for its deleterious effects on tissues via the nitration of proteins and lipid peroxidation (Korhonen et al. 2005). The latter mechanisms could be involved in the DSS-induced mucosal injuries. In addition, TNF- α can also activate NF- κ B and stimulate the production of pro-inflammatory cytokines (such as IFN- γ) that amplifies its own production (Zhou et al. 2006). IL-6 is another important pro-inflammatory cytokine that plays a central role in acute inflammation (Kishimoto et al. 2005). Thus, IL-6 can activate neutrophils, monocytes/macrophages, and lymphocytes at the site of inflammation. More importantly, IFN- γ , TNF- α , or IL-6 can disrupt the epithelial barrier function by apoptosis-independent mechanisms (Suzuki et al. 2011). Furthermore, these cytokines production upregulates the Cyclooxygenase-2 (COX 2) expression. This enzyme is responsible for producing prostaglandins associated with the mediation of inflammation (Vane et al. 1998). Kim et al. (2015) showed that the COX-2 and prostaglandins-2 levels were increased in the colon tissue concomitantly with TNF- α and IL-6 levels increases in DSS-treated mice as compared to those of control mice.

Interestingly, we observed that systemic, local, and peritoneal macrophages production of NO, IL-6 and TNF- α were significantly down-regulated when the DSS is administered concomitantly with PLAE. Our results are in agreement with previous studies. Zahouani et al. (2020) underlined the anti-inflammatory effect of PLAE oral pre-treatment in acetic acid-induced ulcerative colitis, by reducing IL-6 production. Ostovan et al. (2020) showed that *PL* mastic oil administered intra-rectally significantly decreased TNF- α level after 7 days of treatment in acetic acid-induced colitis. Our present findings are also in line with our previous results on ethanol-induced gastric ulcer model showing that pre-treatment or treatment with *PL* fatty oil significantly reduced iNOS expression in the gastric mucosa. Systemic and local production of NO was also reduced. Furthermore, a significant decrease in IL-6 and TNF- α level was observed in gastric explants culture (Boutemine et al. 2018).

The therapeutic and anti-inflammatory activities of PLAE observed during our study could be due to the presence of many bioactive phyto-chemical compounds. Interestingly, recent phytochemical analyses of *PL* leaves aqueous extract allowed the identification of numerous phenolic compounds such as flavonoids (isoquercetin and luteolin), phenolic acids (ellagic and dicaffeoylquinic), flavonols (catechin, rutin, and kaempferol) and tannins (Zahouani et al., 2020). Another study conducted on leaves powder, by Arab et al. (2014) revealed the presence of very high alkaloid and polyphenol content, particularly flavonoids (isoquercetin and luteolin), leucoanthocyanins and total tannins (mainly gallic tannins). In addition, Bozorgi et al. (2013) showed that *PL* leaves are rich in monoterpenes (α -pinene, limonene, sabinene and myrcene), and in sesquiterpenes (β -caryophyllene). In addition, *PL* leaves also contain sterols and triterpenes, saponosides as well as reducing compounds (oses, holosides and mucilage).

Polyphenols limit the immune response during intestinal inflammation by limiting tissue colonization by PNN. *In vitro* and *in vivo* studies have indicated that polyphenols inhibited phospholipase A2,

lipoygenase and COX 2 involved in the production of inflammatory mediators such as prostaglandins (Calder et al. 2006). These enzymes act also on NO production by modulating the activity of iNOS (Sanchez-Fidalgo et al. 2010). In addition, during intestinal diseases, polyphenols have a therapeutic effect similar to that of prebiotics. In fact, most polyphenols pass through the small intestine without being absorbed (Scalbert et al. 2000). Thus, polyphenols are biotransformed to their metabolites by the gut microbiota, resulting in their bioavailability increases. Moreover, polyphenols modulate the composition of the gut microbial community mainly by inhibiting pathogenic bacteria and stimulating beneficial bacteria (Lee et al. 2006, Ozdal et al. 2016).

Otherwise, flavonoids (such as quercetin) treatment has shown beneficial effects by the amelioration of colonic damage and the reduction of the DAI in the DSS model (Oz et al. 2013). The flavonoid anti-diarrheal effects have been related to their capacity to inhibit muscle contractility, enhance intestinal motility and reduce fluid intraluminal accumulation in the gut lumen (Di Carlo et al. 1993, Vezza et al. 2016). Besides, flavonoids significantly reduced neutrophil infiltration into the damaged colonic tissue in experimental colitis models (Seibel et al. 2009, Vezza et al. 2016). Flavonoids may inhibit pro-inflammatory cytokines production by blocking the binding of NF- κ B to DNA, as well as inhibiting mitogen-activated protein kinase (MAPK) and STAT activation (Salaritabar et al. 2017). In fact, Camuesco et al. (2004) and Vezza et al. (2016) proposed that the anti-inflammatory effects of quercitrin might be related to a decrease in iNOS expression through down-regulation of NF- κ B in colonic tissue. In addition, the administration of quercetin remarkably decreased the levels of different cytokines in the inflamed colon induced by DSS (Miller et al, 1999, Westphal et al. 2012, Oz et al. 2013). In another study, Marques et al. (2018) suggest that terpenes (monoterpenes and sesquiterpenes) reduce the pro-inflammatory cytokines TNF- α and IL-1 α levels and increase the production of IL-10. In addition, terpenes significantly inhibit the production of NO by macrophages *in vitro* and alter the activity of NF- κ B (Marques et al., 2018).

Finally, the study of Andersen and Markham reveal that the total absence of cyanogenic heterosides greatly reduces the toxicological risks associated with the use of *Pistacia lentiscus* L. These findings are in agreement with our results suggesting that PLAE has no cytotoxic activity on hepa1c1c7 cell line.

In summary, our study and the data on the effects of bioactives components present in *PL* leaves provides several hypothesis to explain the mechanism of PLAE action. PLAE can act as a therapeutic tool either by i) forming a protective membrane along the colon layers, ii) stimulating secretion of mucus by Goblet cells, iii) interacting directly with the sulfate groups of DSS, thus inhibiting their interaction with the colonic wall, and iv) eliminating pathogenic bacteria (such as *Helicobacter pylori*) which is involved in the aggravation of DSS-induced colitis. In addition, PLAE may act as an anti-inflammatory agent either at i) cellular level by inhibiting the activation and recruitment of immune cells, ii) membrane level by blocking pro-inflammatory cytokines receptors, or iii) intra-cellular level by reducing the expression of NF- κ B, and iNOS, and the production of pro-inflammatory cytokines.

According to our finding we conclude that PLAE is not efficient at 50mg/kKg, this dose was considered too low for therapeutic effects. However, 100mg/Kg PLAE could be prescript to mild colitis patient. Moreover, 200mg/Kg PLAE could treat the sever forms of colitis.

5. Conclusion

Collectively, our results suggest that the aqueous extract of the *Pistacia lentiscus* leaves have a systemic and local anti-inflammatory properties resulting in a therapeutic effect against DSS-induced acute colitis in mice. Thus, the *Pistacia lentiscus* aqueous extract offers new therapeutic possibilities for the treatment of human IBD.

Abbreviations

A: absorbance

CD: crohn disease

DAI: disease activity index

DMEM: Dulbecco's Modified Eagle's Medium

DSS: Dextran Sulfate Sodium

FBS: Fetal bovine serum

IBD: Inflammatory bowel disease

IL-6: interleukin-6

iNOS: inducible nitric oxide synthase

H&E: hematoxylin-eosin

LPS: [Lipopolysaccharide](#)

MTT: 3-(4, 5-dimethylthiazol-2-yl)-2, 5-difenyltetrazolium

NF-κB: Nuclear Factor-kappa B

NO: nitric oxide

OONO⁻ : peroxynitrite ion

O⁻² : superoxide anion

PL : *Pistacia lentiscus*

PLAE: *PL* leaves aqueous extract

PNN: polynuclear neutrophils

TNBS: Trinitrobenzenesulfonic

TNF- α : tumor necrosis factor- α

UC: ulcerative colitis

Declarations

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Compliance with ethical standards

Conflict of interest: The authors declare that they have no conflict of interest

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Figures

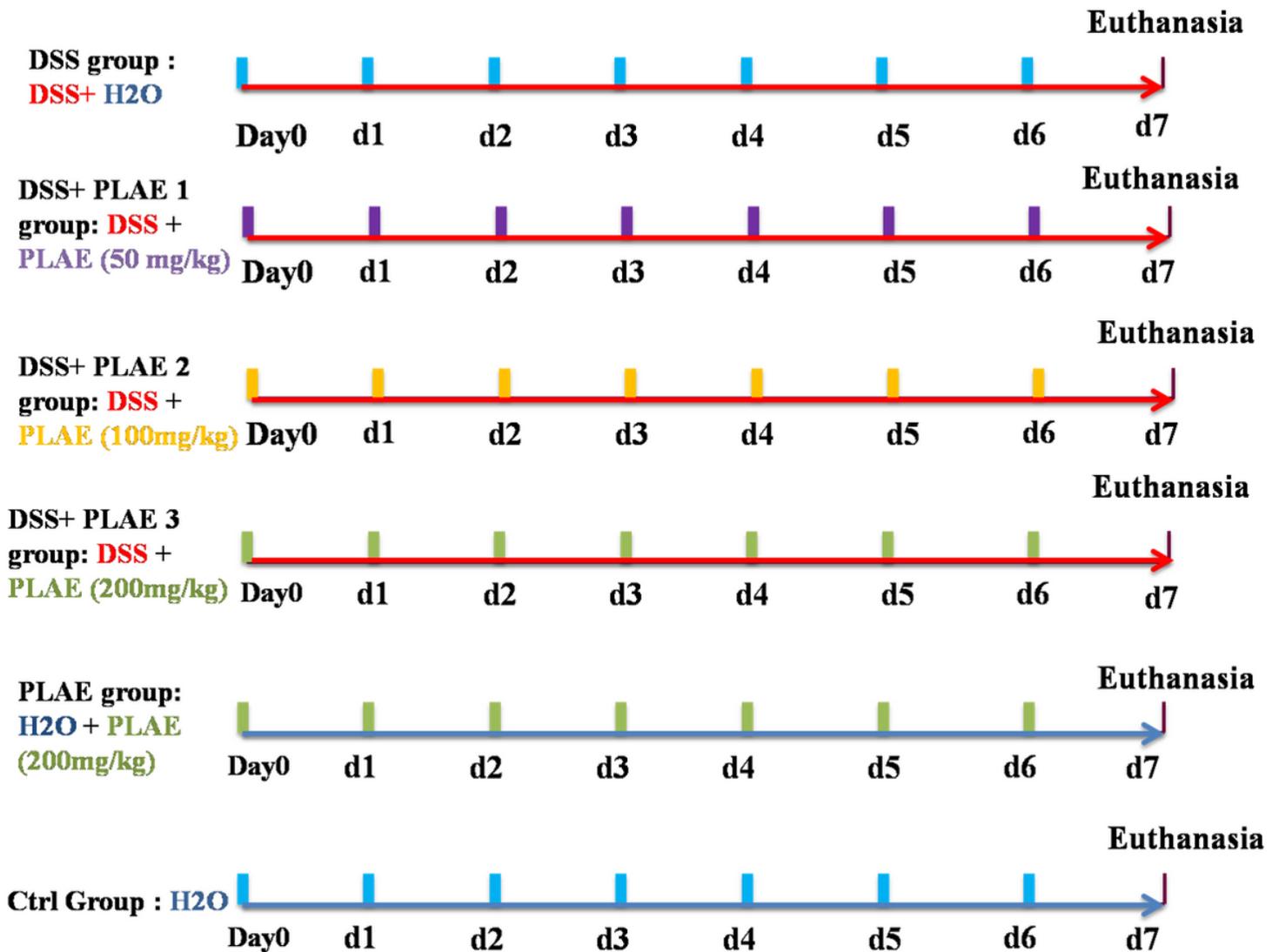


Figure 1

Design of acute colitis induction by DSS. (d: day, DSS: Dextran Sulfate Sodium , PLAE: Pistacia lentiscus leaves aqueous extract, H₂O: water, Ctrl: control) (n = 8 for all groups and n=4 for PLAE group).

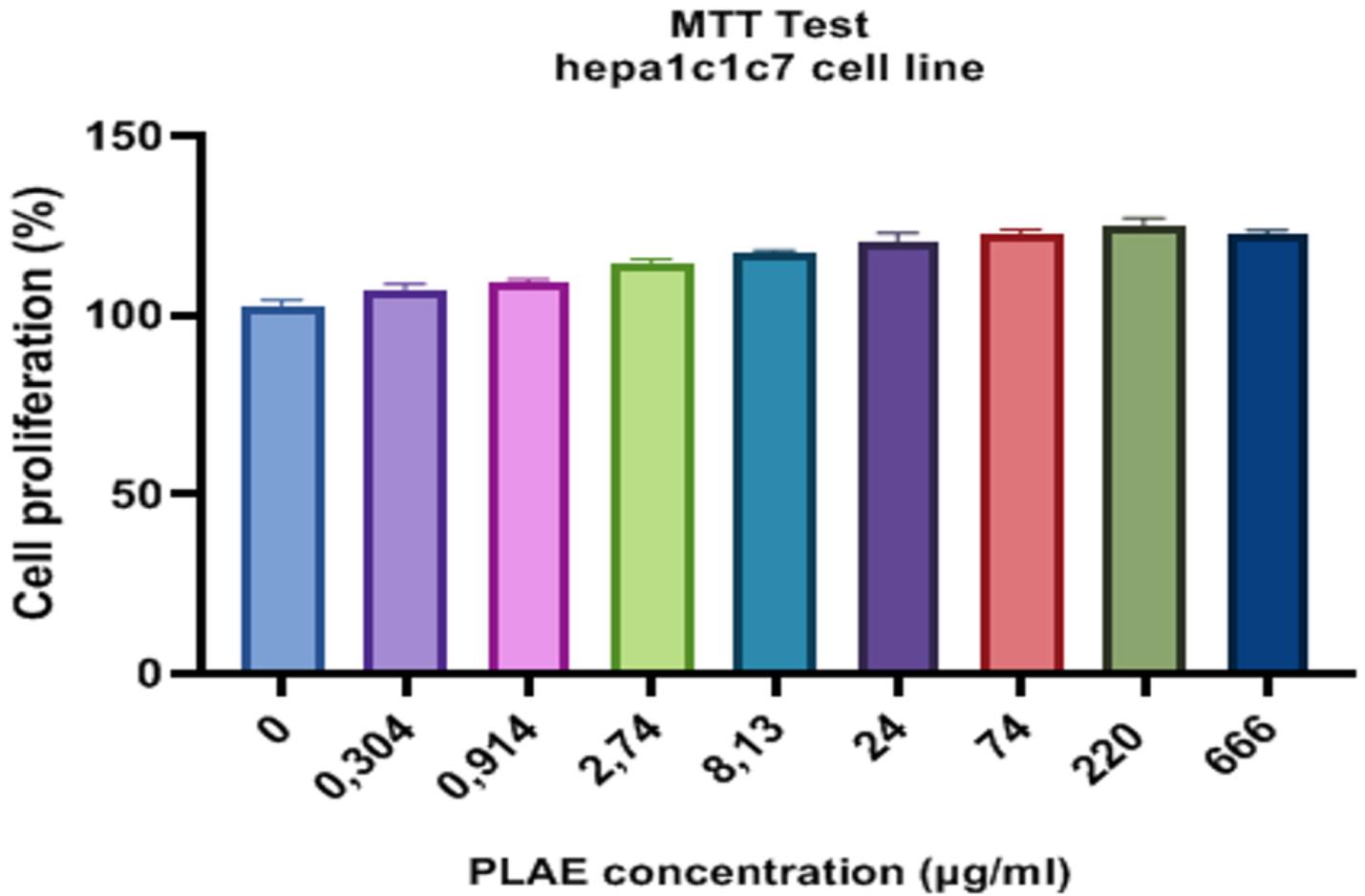


Figure 2

Effect of PLAE on hepa1c1c7 cell line proliferation. Acute cell toxicity was determined by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium (MTT) assay. Values are means \pm SEM of experiments carried out in triplicate. PLAE: Pistacia lentiscus leaves aqueous extract.

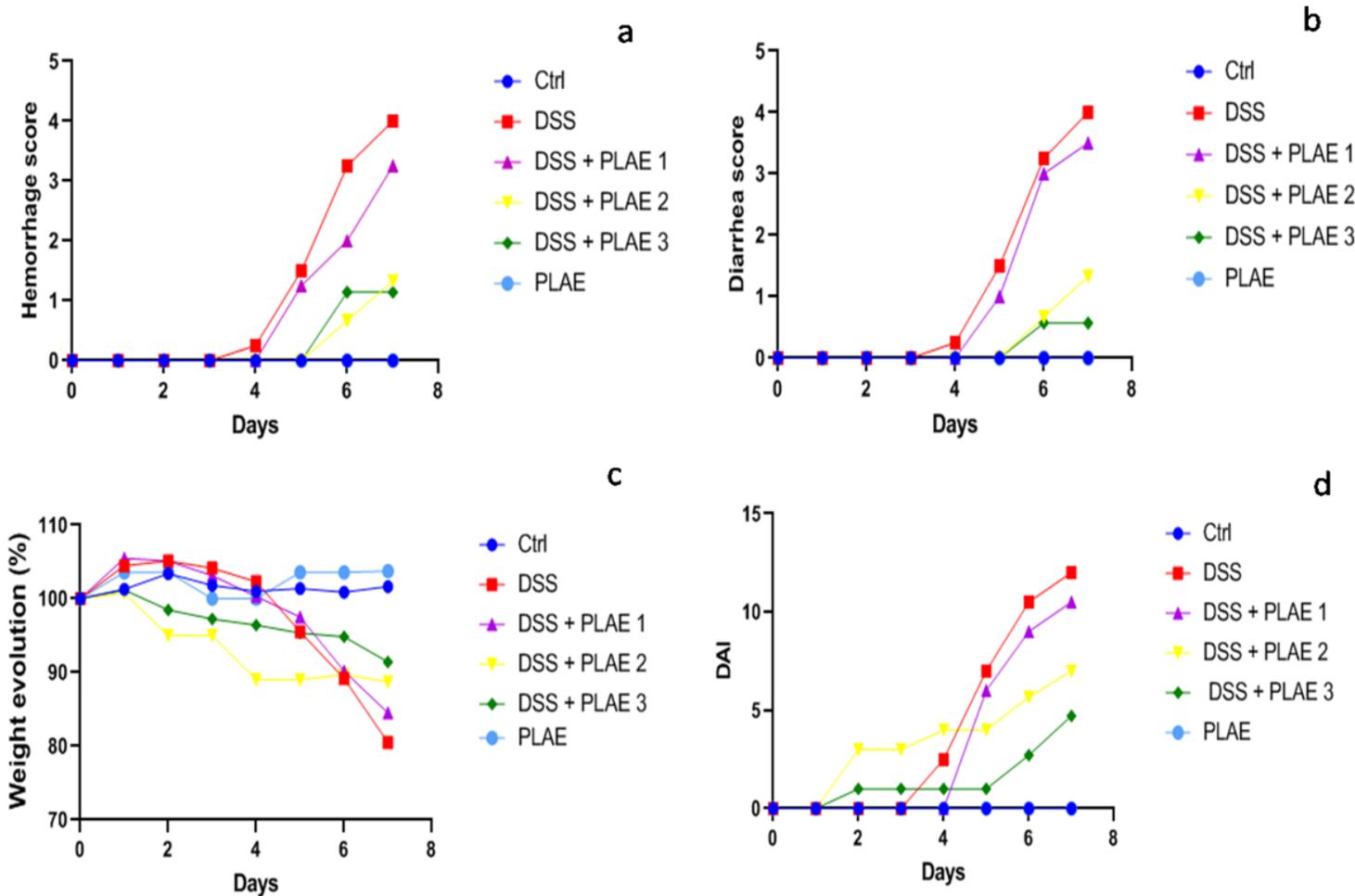


Figure 3

Effect of PLAE on clinical manifestations and clinical score of acute colitis DAI (disease activity index). All parameters were determined in mice (n=8) treated with DSS, in the absence or presence of PLAE after 7 days of treatment as described in material and methods (Ctrl: controls; DSS: Dextran Sulfate Sodium, PLAE: Pistacia lentiscus leaves aqueous extract, DSS+ PLAE 1: DSS + PLAE at 50 mg/Kg, DSS+PLAE 2: DSS+ PLAE at 100 mg/Kg, DSS+PLAE 3: DSS+ PLAE at 200 mg/Kg, PLAE: PLAE at 200mg/Kg alone).

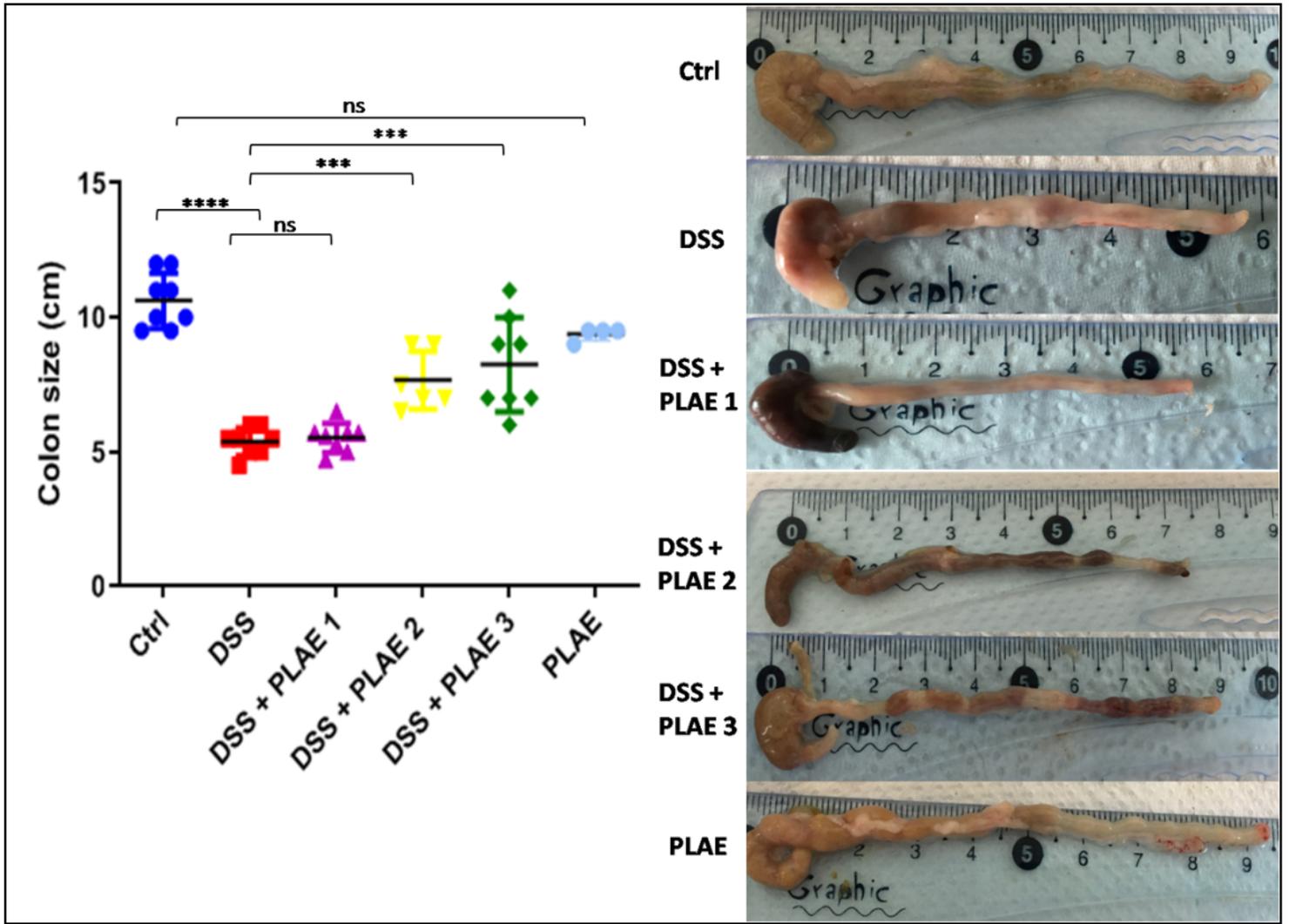


Figure 4

PLAE prevents shortening of the colon during acute DSS-induced colitis. Colon size was determined in mice (n=8) treated with DSS, in the absence or presence of PLAE after 07 days of treatment. Legends as in Fig. 3, **** p < 0.0001; *** p < 0.001, ns: not significant.

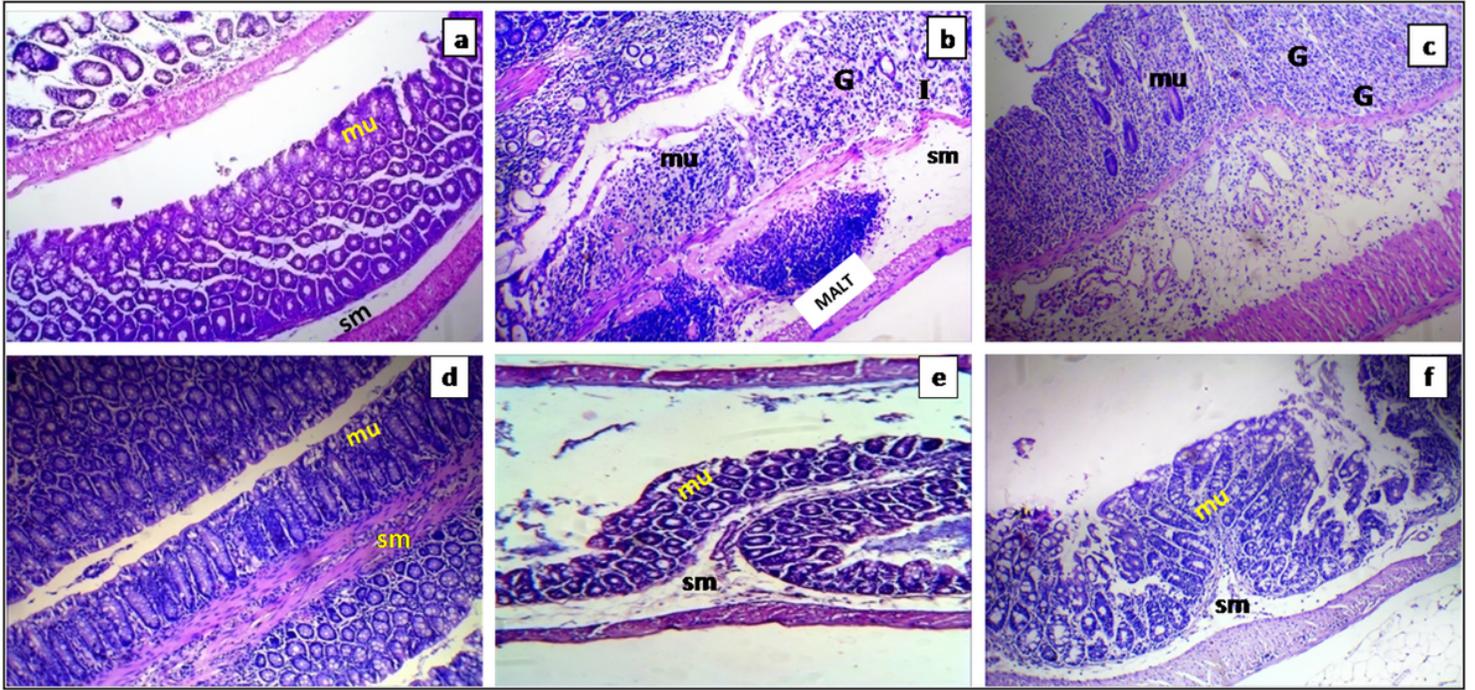


Figure 5

PLAE prevents colon lesions induced by DSS in acute colitis Histological analysis carried on colon tissue of mice by H&E stain (n=8) treated with DSS, in the absence or presence of PLAE after 07 days of treatment, Gx10. (a) control group, (b) DSS group, (c) DSS +PLAE1 group: DSS + PLAE at 50mg/Kg, (d) DSS +PLAE 2 group: DSS + PLAE at 100mg/Kg, (e) DSS +PLAE 3 group: DSS + PLAE at 200mg/Kg, (f) PLAE group: PLAE alone. [G: granulation tissue, I: inflammatory infiltrate, MALT: lymphoid tissue associated with mucous membranes, m: mucus, mu: mucosa, sm: submucosa].

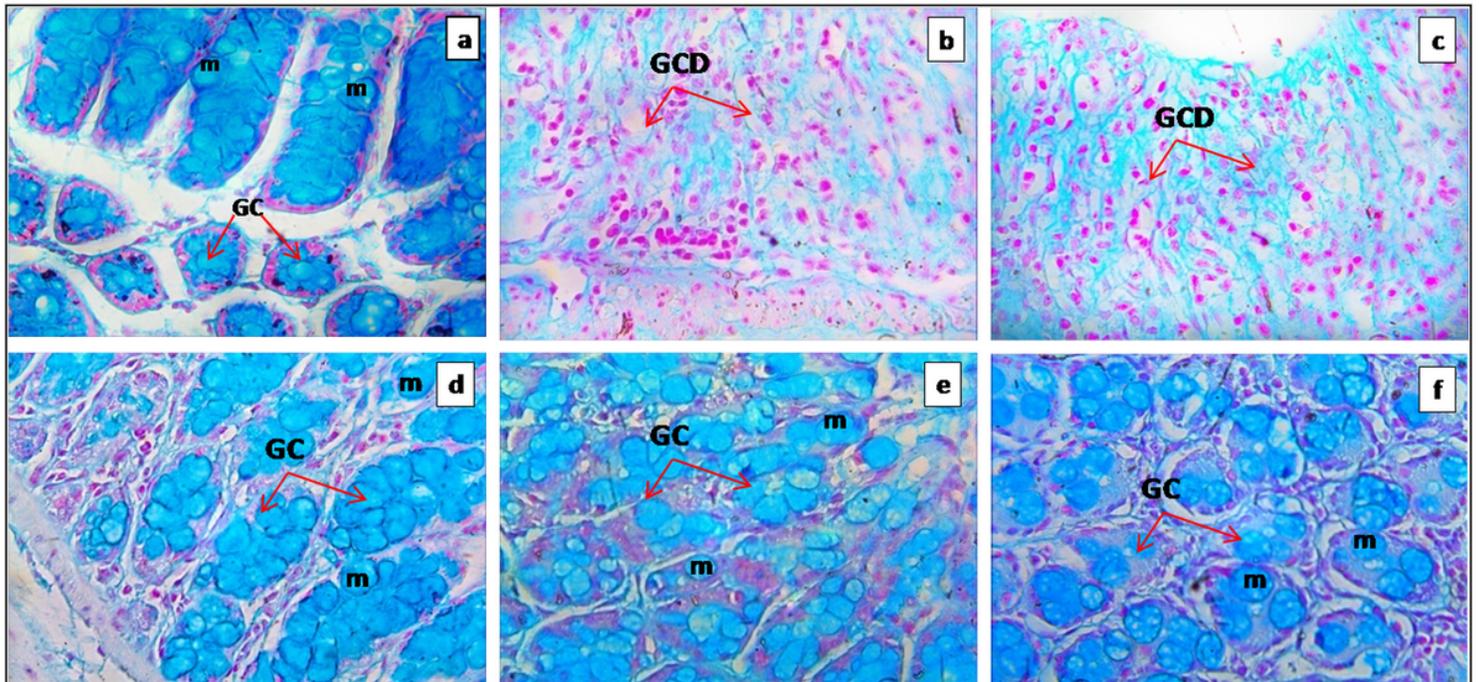


Figure 6

PLAE induces mucus production by Goblet cells in acute colitis Mucus production by Goblet cells determined in colon tissue of mice (n=8) treated with DSS, in the absence or presence of PLAE after 07 days of treatment, Gx100. (a) control group, (b) DSS group, (c) DSS +PLAE1 group: DSS + PLAE at 50mg/Kg, (d) DSS +PLAE 2 group: DSS + PLAE at 100mg/Kg, (e) DSS +PLAE 3 group: DSS + PLAE at 200mg/Kg, (f) PLAE group: PLAE alone. [GC: Goblet cells, GCD: Goblet cells depletion].

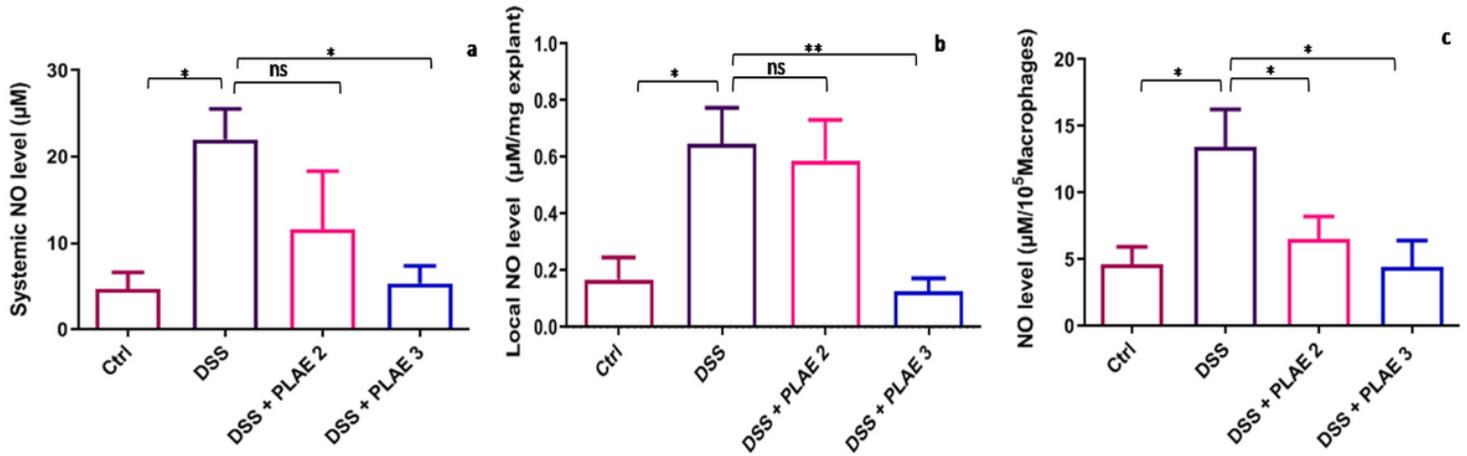


Figure 7

PLAE inhibits systemic, local and peritoneal macrophages production of NO in acute colitis. NO production was determined in plasma (a), culture supernatants of colonic explants (b) and in culture supernatants of peritoneal macrophages from mice (n=8) treated with DSS, in the absence or presence of PLAE after 07 days of treatment. Legends as in Figure 3, (*p<0.05, **p<0.01, ns: not significant).

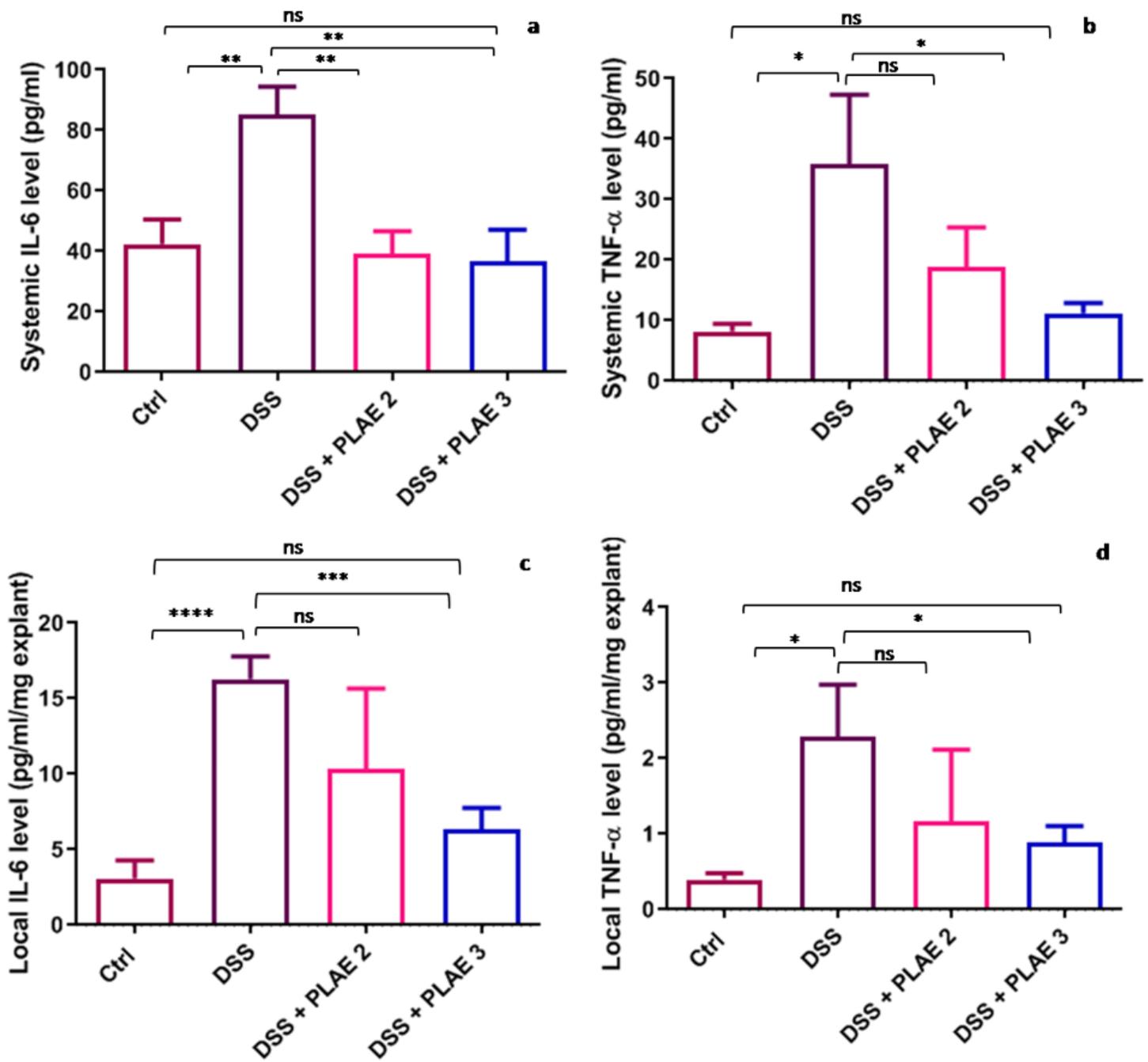


Figure 8

PLAE inhibits the production of pro-inflammatory cytokines in acute colitis. Production levels of (a) systemic IL-6, (b) systemic TNF- α , (c) local IL-6, (d) local TNF- α . Systemic levels were determined in plasma samples and local levels were determined in supernatants culture of colonic explants from mice (n=8) treated with DSS, in the absence or presence of PLAE after 07 days of treatment. Legends as in Figure 3 (**** p<0.0001, *** p<0.001, ** p<0.01, * p<0.05, ns: not significant).

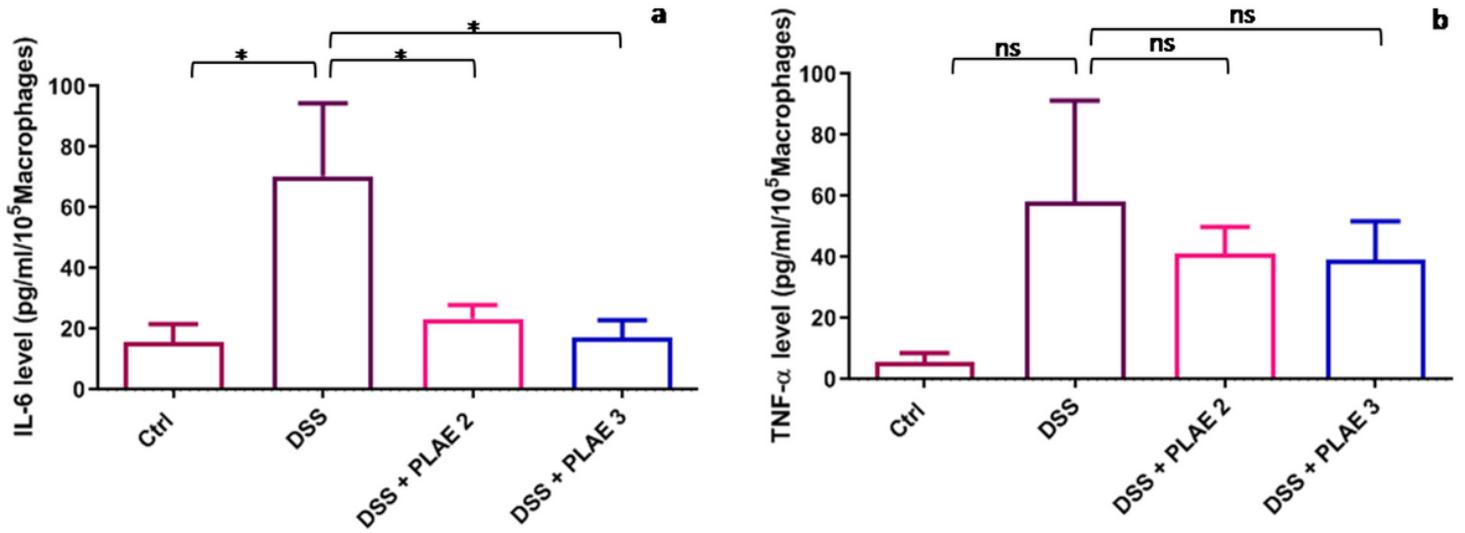


Figure 9

PLAE inhibits the production of pro-inflammatory cytokines by peritoneal macrophages in acute colitis. Production levels of (a) IL-6 and (b) TNF- α were determined in culture supernatants of peritoneal macrophages obtained from mice (n=8) treated with DSS, in the absence or presence of PLAE after 07 days of treatment. Legends as in Figure 3. (*p < 0.05, ns: not significant).

Supplementary Files

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