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ORIGINAL ARTICLE

Potential usefulness of methyl gallate in the treatment of experimental colitis

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Abstract Methyl gallate is a gallotannin widely distributed in nature. Previous studies have demonstrated its antioxidant, anti-inflammatory, antimicrobial and anti-tumor activities. In the present study, the activity of methyl gallate on experimental models of inflammatory bowel disease has been investigated. Experimental colitis was induced in Sprague–Dawley rats through the intracolonic instillation of an acetic acid solution (2 mL, 4% v/v). Methyl gallate (100 and 300 mg/kg, p.o.) and the reference drug mesalazine (100 mg/kg, p.o.) were tested. Methyl gallate induced a significant reduction in the colon weight/length ratio and macroscopic lesion score. Besides, the malondialdehyde content and the GSSG/GSH ratio were remarkably decreased. Furthermore, the administration of methyl gallate reduced the expression of COX_2, IL-6, TNFα and the severity of microscopic tissue damage induced by acetic acid, while the mean goblet cell density was significantly higher in both the group treated with methyl gallate and the one treated with mesalazine, in comparison with untreated animals. The Na^+K^+ATPase pump activity was recovered in treated groups (control: 827.2 ± 59.6, colitis: 311.6 ± 54.8, methyl gallate 100 mg/kg: 642.2 ± 175.0, methyl gallate 300 mg/kg: 809.7 ± 100.6, mesalazine: 525.3 ± 81.7). Methyl gallate was also found to induce a significant reduction in the castor oil-induced intestinal motility in Swiss mice, decreasing the peristalsis by 74.5 and 58.82% at 100 and 300 mg/kg p.o., respectively. This compound also antagonized the jejunum contractions induced by Ach and $CaCl_2$. This study demonstrates that methyl gallate exerts beneficial effects in a preclinical model of intestinal disorders.

Keywords Methyl gallate · Experimental colitis · Intestinal motility · Antispasmodic effect

Introduction

Progressive inflammatory diseases of the gastrointestinal tract result in long-term morbidity. Specifically, the incidence and prevalence of inflammatory bowel disease (IBD) are increasing in diverse regions around the world, indicating its emergence as a global disease. IBD is an idiopathic and chronic inflammation of the gastrointestinal tract characterized by mucosal ulceration that comprises two major conditions: ulcerative colitis (UC) and Crohn's disease (CD) (Garrido-Mesa et al. 2011). Its pathogenesis is still not completely understood; patients suffer chronic diarrhea, weight loss, abdominal pain, fever, and fatigue. IBD's treatment is focused on the induction of clinical remission, maintains adequate nutrition, and prevents relapses or reduces their duration. To date, no complete response has been achieved with conventional therapies (aminosalicylates, corticosteroids, and antibiotics); therefore, the development of new therapies that combine efficacy and lower side effects is an important goal in the IBD therapy (Algieri et al. 2013).

It has been described beneficial effects of polyphenol-rich plants or their isolated compounds, on reducing the

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severity and progression of IBD (Kaulmann and Bohn 2016). Furthermore, polyphenols, terpenoids, flavonoids and alkaloids, modulating the immune response, represent hopeful candidates for IBD therapy (Somani et al. 2015). Particularly, methyl gallate (MG), methyl 3,4,5 trihydroxy-benzoate, is a gallotannin and it is widely distributed in medicinal and food plants. It was described that MG possesses antioxidant, anti-inflammatory and anti-allergic properties (Cavalher-Machado et al. 2008; Gorzalczany et al. 2011; Wang et al. 2014). It was reported that MG inhibited the activity of cyclooxygenase-2 (COX_2) and 5-lipoxygenase in bone marrow-derived mast cells (Kim et al. 2006). Furthermore, MG modified the production of various inflammatory mediators, such as IL-6 (interleukin-6), IL-1β (interleukin-1β), as well as leukocyte activation and migration (Correa et al. 2016).

Although medicinal plants containing MG have shown beneficial properties for ulcers, gastritis and diarrhea (Akiko Hiruma-Lima et al. 2006; Calvo et al. 2007; Chena et al. 2006), there is no scientific evaluation of MG on gastrointestinal tract. Therefore, on the basis of these considerations, the present study was aimed to study the effect of MG on the gastrointestinal system in experimental models.

Materials and methods

Materials

Mesalazine, loperamide, atropine, castor oil, acetylcholine, Trizma-HCl, sodium deoxycholate, EGTA, NaF, phenylmethanesulfonylfluoride, sodium pervanadate and $CaCl_2$ were purchased from Sigma Chemical Co., St. Louis, MO., USA. Acetic acid was purchased from Merck (Darmstadt, Germany). Protease inhibitor cocktail 4% was purchased from Roche Hertfordshire, UK. Rabbit anti-COX_2 was purchased in Cell Signaling Technology, Danvers, MA, USA. Goat anti-TNFα, goat anti-IL6 and Secondary antibody were purchased in Santa Cruz Biotech, Inc. Dallas, TX, USA. All reagents were of analytical grade.

Test compound

Methyl 3,4,5-trihydroxy-benzoate (purity: 98%) was purchased from Sigma Chemical (Fig. 1).

Animals

The experiments were carried out on female Swiss mice weighing 25–30 g and female Sprague–Dawley rats (250–270 g), following international and local guiding principles and regulations concerning the care and use of laboratory animals for biomedical research. Such experiments were approved by the local Ethics Committee (ExpFyB: 0738657/11). The animals were kept in room maintained at 22 ± 2 °C with a 12-h light/dark cycle and had free access to a standard commercial diet and water ad libitum.

Ulcerative colitis model

Experimental design

Rats were divided into 5 groups of 5 animals each. One of them did not receive any treatment (control). The colitis was induced in the other groups. A reference group was treated with mesalazine (100 mg/kg, p.o.). Other groups received 100 and 300 mg/kg p.o. of MG. Animals were treated during 5 consecutive days. On the fourth day, the colitis was induced. All animals were killed on the fifth day.

Induction of colonic inflammation in rats

Colitis was induced in rats (fasted for 36 h, with access to water ad libitum) by administration of 2 mL of a 4% v/v acetic acid solution. A polyethylene tube was inserted into the rectum and along the colon up to a distance of 8 cm (Paiva et al. 2003). Animals were euthanized 24 h later.

Macroscopic inflammation scoring

Scores are assigned using an arbitrary scale based on clinical features of the colon and ranging from 0 to 4 as follows: 0 (no macroscopic changes), 1 (mucosal erythema), 2 (mild mucosal oedema, slight bleeding or small erosions), 3 (moderate oedema, slight bleeding ulcers or erosions) and 4 (severe ulceration, oedema and tissue necrosis) (Kannan and Guruvayoorappan 2013). In order to remove faecal residues, colon samples were cut longitudinally and cleansed with saline solution.

Histological evaluation

Samples of colon were fixed in 10% buffered formalin and embedded in paraffin wax. Sections were cut in a microtome at 5 μm thickness and stained with hematoxylin and eosin or Alcian blue for examination under a light microscope. Alcian blue was used in order to quantify Goblet cell, which was counted using ten crypts per colon tissue section.

Immunoblot analysis

Colons were homogenized in an ice-cold homogenization buffer (150 mM NaCl, 50 mM Trizma-HCl, 1% v/v sodium deoxycholate, 1 mM EGTA, 1 mM NaF, 1 mM phenylmethanesulfonylfluoride, 1 mM sodium pervanadate and a protease inhibitor cocktail 4%, pH 8.0) and centrifuged at 10,000 rpm for 10 min at 4 °C. The protein in supernatant was quantified by the Lowry method and resuspended in 6χ solution of SDS-sample buffer (62.5 mM Tris–HCl buffer, pH 6.8 containing 6% w/v SDS, 25% w/v glycerol, 5% v/v β-mercaptoethanol and 0.01% w/v bromophenol blue) and heated at 95 °C for 5 min. Equal amount of proteins (100 µg) was loaded onto 10% SDS-PAGE and transferred to PVDF membranes. After blocking for 1 h in 3% w/v nonfat milk in PBS buffer, membranes were incubated overnight at 4 °C with the corresponding primary antibodies: Rabbit anti-COX_2 (dilution 1:1000), Goat anti-TNFα (dilution 1:1000), Goat anti-IL6 (dilution 1:5000) and Rabbit anti-β tubuline (dilution 1:1000) and Rabbit anti-GAPDH (dilution 1:1500) as loading controls. For signal amplification, an antibody coupled to horseradish peroxidase (dilution 1:2000; Santa Cruz Biotech, Inc. Dallas, TX, USA) was used. Complexes were visualized by chemiluminescence detection (Pierce ECL Western blotting substrate). Densitometry analysis of the bands was performed using Image J (National Institute of Health, Bethesda, Maryland, USA). Protein band densities were normalized to the β-tubulin content (Lowry et al. 1951; Zancheta et al. 2015; Zgheel et al. 2014).

Thiobarbituric acid reactive substances (TBARs)

Malondialdehyde (MDA) formed during lipid peroxidation in colon samples was quantified spectrophotometrically at 635 nm (Mariotto et al. 2008). Colon tissues were homogenized in phosphate buffer (pH 7.4, 10%), 200 µL of this homogenate was mixed with 200 µL of 8.0% w/v SDS and 600 µL of water. Next, 3 mL of 0.4% TBA in 10% acetic acid was added, boiled for 1 h and cooled at room temperature. Finally, the concentration of MDA was measured and the results were expressed as nmol MDA/mg protein. The protein content was determined (Lowry et al. 1951).

Measurement of glutathione status

A reversed-phased liquid chromatography method coupled with electrochemical detection was carried out for quantification of GSH and GSSG, involving minor modifications of previous reported techniques (Rodriguez-Ariza et al. 1994). Standard solutions (GSH, GSSG) were prepared in mobile phase buffer at a concentration range of 0.3–20 µg/mL. Each colon sample (200 mg) was homogenized with 1.0 mL 1.0 M perchloric acid solution containing 2.0 mM EDTA and centrifuged at 15,000 rpm for 20 min at 4 °C. The lipid layer was discarded, and the limpid supernatant was filtered through a 0.45 µm cellulose ester membrane. Samples were frozen at −80 °C until use for GSH and GSSG determination. The analytical column used was a Gemini NX-SM C18 (150 × 4.6 ID, particle size: 5 µm, pore size: 110Å) and the mobile phase, 20 mM sodium phosphate buffer, pH 2.7. Chromatographic separation was performed isocratically at 25 °C and a flow rate of 1.0 mL/min. The potential settings of the detector were: guard cell, + 0.900 V; detector 1, + 0.450 V; and detector 2, + 0.900 V.

ATPase activity

Colon was homogenized twice in 0.32 M sucrose (pH 7.0) and centrifuged at 900*g* for 10 min; supernatants were spun down at 100,000*g* for 30 min in ultracentrifuge. Colon membranes were 10 min preincubated in 0.20 M Tris–HCl buffer (pH 7.4) followed by 30-min incubation period in a buffer containing 100 mM NaCl, 20 mM KCl, 3 mM $MgCl_2$, 0.20 M Tris–HCl buffer (pH 7.4) and 4 mM ATP. For determination of ouabain-sensitive ATPase (Na^+–K + ATPase), NaCl and KCl were omitted, and 100 µL of ouabain (1 mmol/L) or vehicle (water) was added to the assay. After incubation at 37 °C for 15 min, both reactions were stopped with 30% (w/v) trichloroacetic acid solution and then ATPase activity was monitored by colorimetric determination of orthophosphate released. Na^+–K + ATPase activity was determined as the difference between total and ouabain-insensitive ATPase (Magro et al. 2005). Besides, tubes containing enzyme preparations and assay media maintained at 0 °C throughout during the incubation period were used as blanks. Protein content in synaptosomal membrane fractions was determined.

Castor oil-induced diarrhea

Animals were divided into four groups. Two groups were treated with MG (100 and 300 mg/kg, p.o.), another group with loperamide hydrochloride (3 mg/kg, p.o.), and a fourth group with distilled water (0.1 mL/10 g, p.o.) 30 min before the administration of castor oil (0.3 mL/mouse). Mice were housed individually in cages containing a filter paper and observed for a period of 4 h (Uddin et al. 2005). The following parameters were observed: first watery defecation time, total number of solid, semi-solid and watery stool, and the number of watery stools.

Castor oil-induced intestinal motility

The effect of MG on castor oil-induced intestinal motility in mice was tested using the charcoal method (Mazzolin et al. 2013). Mice were fasted for 6 h and randomly assigned to four groups ($n = 6$) that received: water 0.1 mL/10 g p.o., 100 and 300 mg/kg p.o. of MG, or atropine 0.1 mg/kg i.p. Every 30 min, castor oil (0.3 mL/animal, p.o.) and then 10% activated charcoal (0.3 mL/mouse, p.o.) were administered to each mouse. Intestinal motility was expressed as the distance travelled by charcoal in 30 min as percentage of total small intestine length (from the pylorus to the ileal terminus).

Spasmolytic activity

Rats were fasted for 24 h and killed without any anesthetic agent, in order to avoid any influence on the tissue relaxation response. Jejunum samples of approximately 1 cm long were mounted in 10 mL organ baths containing Tyrode's solution, at 36 ± 1 °C and oxygenated with 95% O_2, 5% CO_2. Preparations were equilibrated for at least 30 min under 1 g resting tension, and isometric force was measured (Gorzalczany et al. 2013). The cumulative concentration–response curves for acetylcholine (Ach) were obtained in absence and presence of different concentrations of MG (0.03, 0.1, 0.3, and 1 mg/mL). For concentration–response curves of $CaCl_2$ (10^{-4}–3×10^{-2} M) Tyrode's solution was replaced by a calcium-free and high potassium concentration medium (K^+ 75 mM). Different concentrations of MG (0.03, 0.1, 0.3, and 1 mg/mL) were tested in these conditions. The effect of KCl (25 and 80 mM) and serotonin (10^{-4} M) was evaluated in absence and presence of different concentrations of MG.

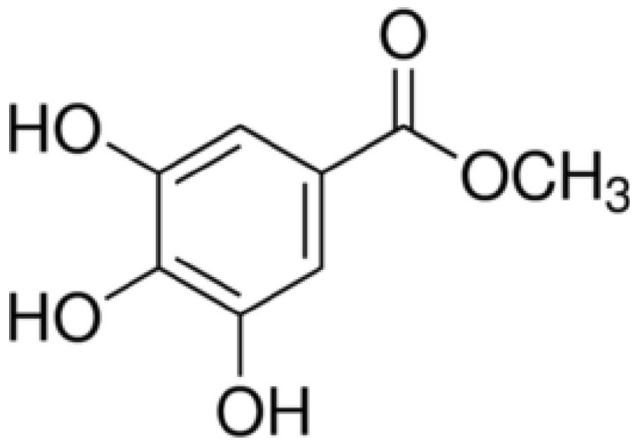

Fig. 1 Chemical structure of methyl gallate

Data analyses

Data are presented as the mean ± standard error of the mean (SEM). The statistical significance of differences between groups was assessed by means of analysis of variance (ANOVA) followed by Dunnett's test. p values < 0.05 were considered significant. The statistical analysis was carried out using the Instant statistical package (Graph Pad software, Inc., version 5 USA).

Results

Ulcerative colitis model

During the experiment, no mortality was observed in any rat. The macroscopic inflammation score showed a reduction in those animals who were treated with MG, as well as in the reference group (Fig. 2a). Moreover, acetic acid caused an increase in the wet weight/length ratio but the treatment with MG (300 mg/kg) and mesalazine, administered by the oral route, decreased the ratio significantly

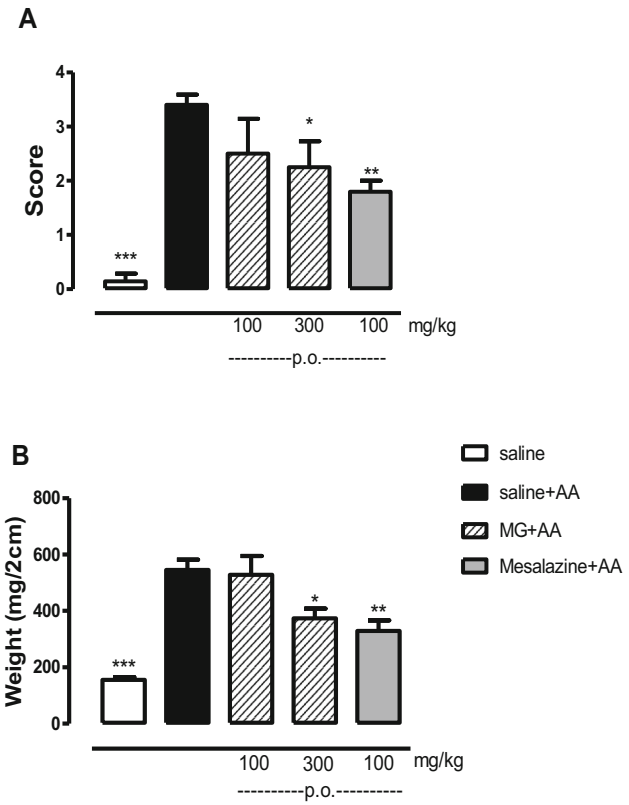

Fig. 2 Acetic acid-induced colitis model. Effect of MG on **a** score based on macroscopic colon lesions, **b** colon weight (mg tissue/2 cm tissue). Data are expressed as mean ± SEM, $n = 5$ rats. *$p < 0.05$, **$p < 0.01$ compared with acetic group. One way ANOVA, followed by Dunnett's test

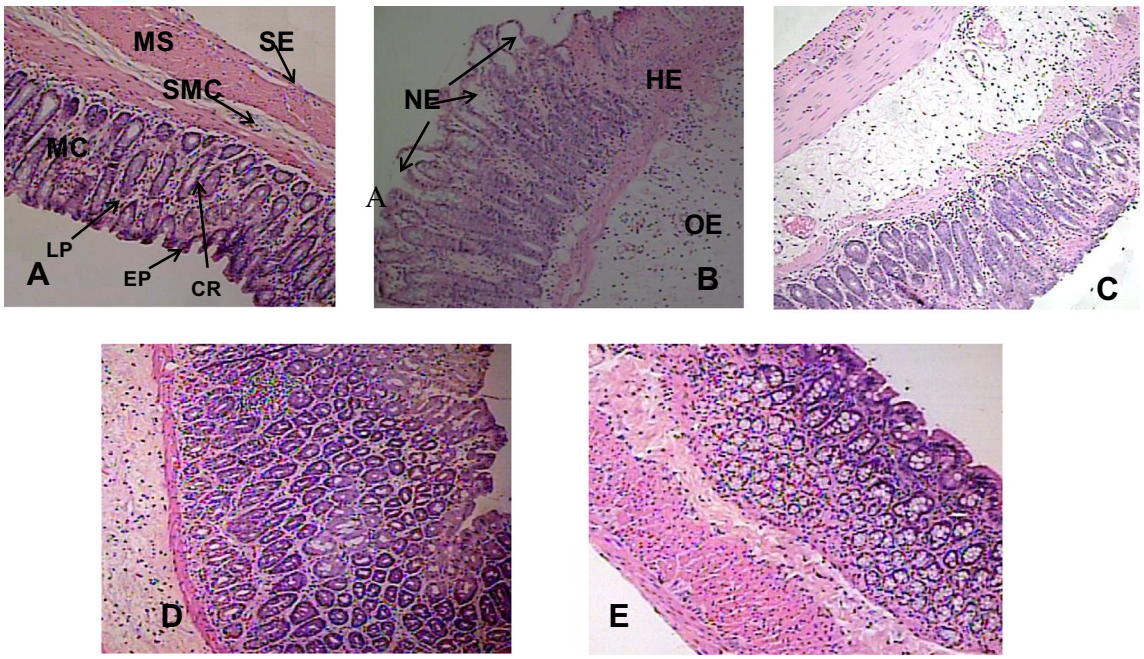

Fig. 3 Histological sections of colonic mucosa from **a** normal colon, **b** acetic acid (AA), **c** AA + mesalazine, **d** AA + MG (100 mg/kg, p.o.), **e** AA + MG (300 mg/kg, p.o.) stained with hematoxylin and eosin (10×). *MC* mucosa, *SMC* submucosa, *MS* muscle, *SE* serosa, *LP* lamina propia, *CR* cryptas, *EP* epithelio, *NE* necrosis, *OE* oedema, *HE* hemorrhage

(Fig. 2b). Both parameters are considered indicators of the severity and extent of the inflammatory response.

Furthermore, histological examination of the colonic sections showed a normal colon structure without neither inflammation nor necrosis foci in control animals (Fig. 3a). Nevertheless, acetic acid caused severe injury to the colon mucosa. It was observed degeneration and necrosis of epithelial cells, crypt distortion, diffuse polymorphonuclear leukocytes infiltration in the submucosa and lamina propia and evidence of massive hemorrhage with a significant accumulation of erythrocytes in the lamina propia (Fig. 3b). But, the treatment with MG (100 and 300 mg/kg p.o.) and mesalazine proved to reduce the severity of tissue damage induced by acetic acid (Fig. 3c–e).

Considering the mucosa protective role of goblet cells and taking into account that a reduction of them can be observed in ulcerative colitis (Kim and Ho 2010), the Alcian blue staining was performed in colon samples. Number of goblet cells, the storage of mucin and the mucus layer overlying the epithelium were diminished by the administration of acetic acid. These effects were reduced by MG and mesalazine treatments, showing a higher number of goblet cells in those experimental groups (Fig. 4).

In response to tissue damage induced by acid acetic, some inflammatory mediators were studied. The expression of COX_2 was up-regulated in experimental colitis, but this effect was reduced by MG and mesalazine (Fig. 5a). In the same sense, IL-6, a cytokine with central role in immune regulation and inflammation, showed the similar pattern (Fig. 5b). Furthermore, beneficial effects of MG in colitis groups were associated with suppression of the production of colonic TNF-α, a potent pro-inflammatory cytokine that is considered to play a key role in inflammatory intestinal condition (Fig. 5c).

On the one hand, acetic acid induced an increase in GSSG and a reduction of GSH in colon samples from colitis group, raising the ratio GSSG/GSH. But, both doses of MG and mesalazine recovered this relationship at normal level (control group) (Fig. 6a). On the other hand, the reduction in high level of TBARS in colitis model induced by 300 mg/kg of MG (Fig. 6b) could confirm the antioxidant activity of MG in our experimental condition.

An increase in electrolyte secretion and a decrease in electrolyte absorption have been reported in IBD as well as elevated levels of pro-inflammatory cytokines and ROS (Rezaie et al. 2007; Priyamvada et al. 2015). Na^+/K^+ ATPase maintains proper Na and K concentrations and contributes to normal cell volume regulation and solute absorption. Being the fundamental driving force of transepithelial ionic transport, it is likely that its activity may have an important influence in the intestinal tract water balance. Therefore, the Na^+/K^+ ATPase activity assay was carried out. MG reverted significantly the decrease in the Na^+/K^+ ATPase activity produced by acetic acid (Control: 827.2 ± 59.6; Colitis: 311.6 ± 54.8;

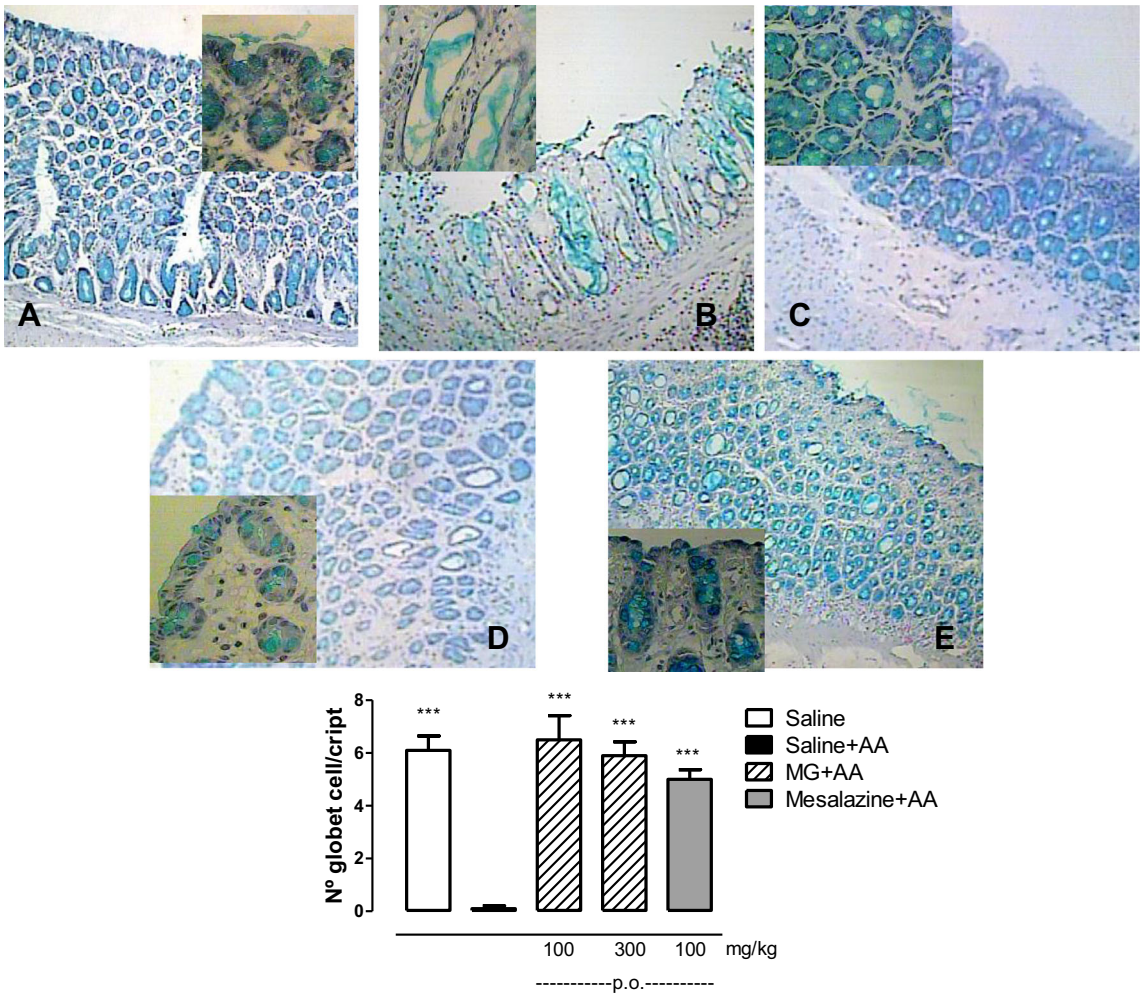

Fig. 4 Histological sections of colonic mucosa from **a** normal group, **b** acetic group, **c** AA + mesalazine, **d** AA + MG (100 mg/kg, p.o.), and **e** AA + MG (300 mg/kg, p.o.) stained with alcian blue (10× and 40×). The graph shows the number of goblet cells/crypt. Data are expressed as mean ± SEM, $n = 10$ crypts. ***$p < 0.001$ compared with acetic group. One way ANOVA, followed by Dunnett's test

MG 100 mg/kg: 642.2 ± 175.0; MG 300 mg/kg: 809.7 ± 100.6; mesalazine 100 mg/kg: 525.3 ± 81.7).

Effect of MG on castor oil-treated mice

No significant differences were found on the total number of watery stools between control and test groups (Fig. 7a). Both doses of MG (100 and 300 mg/kg) caused a significant increase in the time elapsed between the administration of the castor oil and the excretion of the first diarrhea feces as compared to the diarrhea control group (Fig. 7b). In the loperamide treated animals, a significant decrease in number of watery stools and a significant increase in latency time until the development of diarrhea were observed.

Additionally, in the castor oil-induced intestinal motility test in mice, MG (100 and 300 mg/kg, p.o.) significantly reduced the intestinal motility, decreasing the peristalsis 74.5 and 58.82%, respectively, compared to the control group. Similar results were obtained with the reference drug, atropine (72.1%) (Fig. 7c).

Spasmolytic activity

MG antagonized the jejunum contractions induced by Ach (1×10^{-9} to 1×10^{-5} M). Higher concentration significantly reduced the maximal response of the agonist in a concentration-dependent manner (E_{max} 0.03 mg/mL: 77.74%; E_{max} 0.1 mg/mL: 72.93%; E_{max} 0.3 mg/mL: 57.47%; E_{max} 1 mg/mL: 8.03) (Fig. 8a) suggesting as spasmolytic effect of the MG (IC_{50}: 0.78 mg/mL). Pretreatment with atropine (3×10^{-8} M), a classical muscarinic receptor antagonist, reduced the response of Ach, whereas at higher concentration (10^{-5} M), the effect was totally antagonized (data not shown).

Furthermore, serotonin receptors located in the enteric nervous system which resides within the intestinal wall are involved in the gastrointestinal motor and secretory

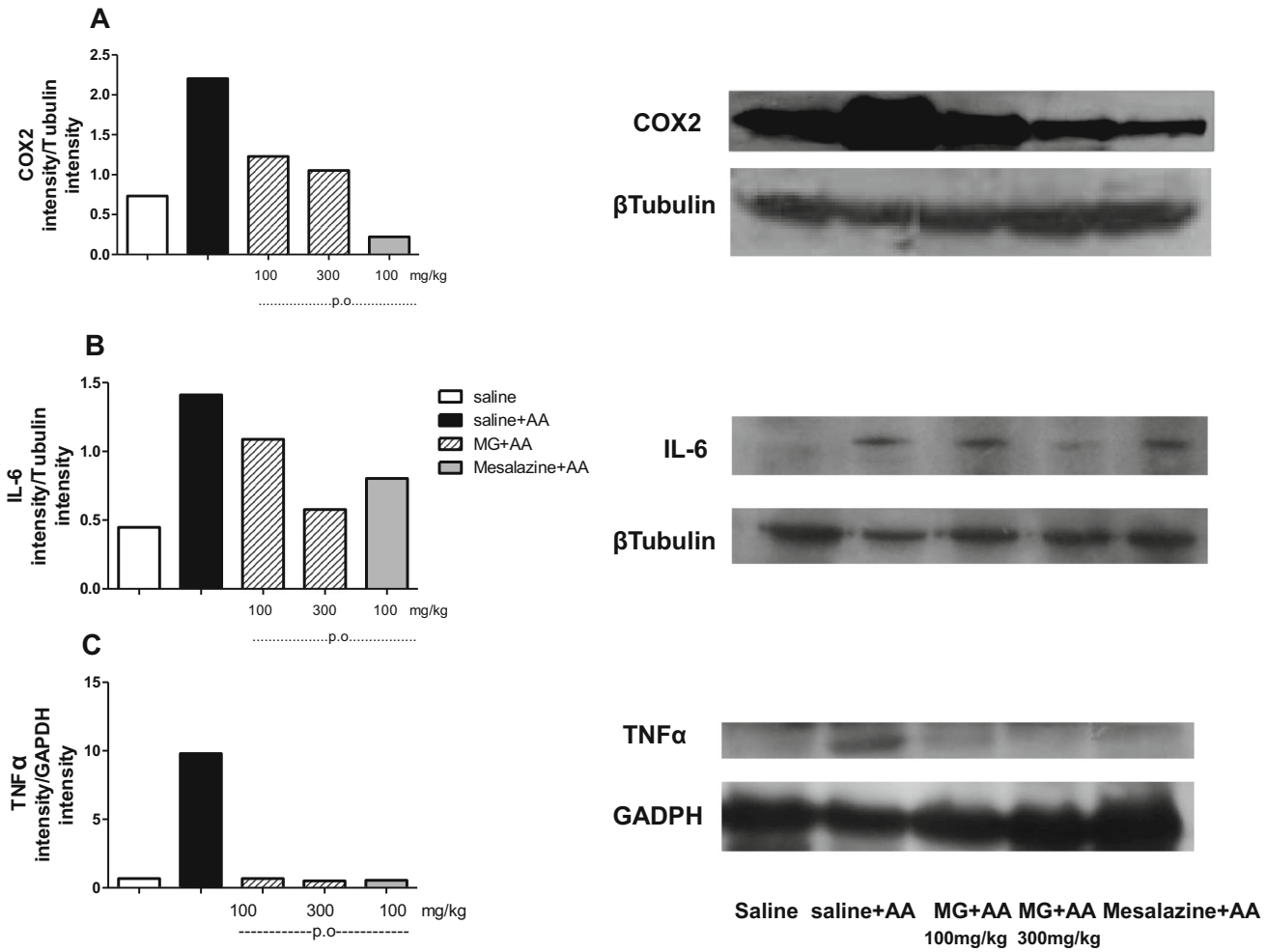

Fig. 5 Western Blot analysis showing the effect of MG on COX_2 (**a**), IL-6 (**b**) and TNF-α (**c**) in experimental groups

function. It is an important intracellular signaling molecule in gastrointestinal tract, and it has a key role in the pathogenesis of functional gastrointestinal disorder, such as IBD (Sikander et al. 2009). So, the effect of MG on jejunum contraction induced by serotonin was investigated. MG antagonized the effect of the neurotransmitter in a concentration-dependent manner (higher concentration reduced 90.67% the maximal response, IC_{50}: 0.07 mg/mL, Fig. 8d).

Besides neural factors, spasmolytic compounds can act through calcium channel, so in order to investigate if the observed effect was due to the blockade of calcium channels, the effect of MG on the muscle contractions induced by $CaCl_2$ (1×10^{-4} to 3×10^{-1} M) was analyzed. MG non-competitively inhibited the response-concentration induced by $CaCl_2$ (IC_{50}: 0.07 mg/mL) and higher concentrations reduced the maximal response in a 100% (Fig. 8b). Verapamil (10^{-6} M), a calcium channel blocker, was used as positive control (data not shown). Also, MG produced a relaxant effect on the tonic contractures obtained by 25 mM KCl (IC_{50}: 0.027 mg/mL) and 80 mM KCl (IC_{50}: 0.26 mg/mL) (Fig. 8c). The use of low K^+ (25 mM) and high K^+ (80 mM)-induced depolarization in the tissues can distinguish between K^+ channel opening and Ca^{2+} channel blocking. Both can cause smooth muscle relaxation by decreasing intracellular free calcium. But K^+ channel openers increase in K^+ efflux and Ca^{2+} antagonists inhibit calcium entry (Mehmood et al. 2015). The activity induced by MG in low and high K^+ conditions strongly suggests that MG could be considered a calcium influx antagonist (Fig. 8c).

Discussion

Experimental models of IBD constitute an approach to study the pathogenesis of human IBD. These models contribute to the understanding of the histopathological and morphological changes occurring in the intestinal tract and play a crucial role in the development of novel drugs to

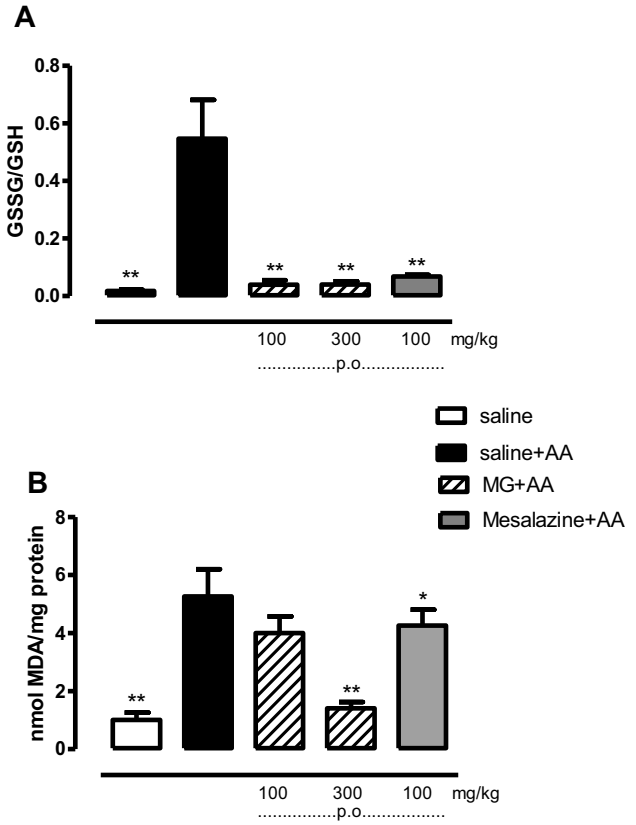

Fig. 6 Effect of MG on the GSSG/GSH ratio (**a**) and on lipid peroxidation (**b**) in colon tissue. Results are expressed as mean ± -SEM, $n = 5$ rats. *$p < 0.05$, **$p < 0.01$ compared with acetic group, One way ANOVA, followed by Dunnett's test

treat and control different diseases (Randhawa et al. 2014). In this sense, the intracolonic administration of diluted acetic acid causes lesions that bear a close resemblance to human ulcerative colitis in terms of pathogenesis, histopathological features and inflammatory mediators profile, thus representing a reproducible model for the screening of drugs to treat colitis (Minaiyan et al. 2014). This study represents the first demonstration that MG may exert beneficial effects on experimental intestinal disorders.

Acetic acid is known to induce colitis featured by the presence of colonic epithelial lesions and necrosis with inflammatory cell infiltrates. In the present study, the administration of 4% acetic acid caused a severe colonic inflammation characterized by crypt destruction, tissue necrosis with inflammatory cell infiltrates as well as depletion of colonic mucus. The oral administration of MG reduced the macroscopic and histopathologic damages induced by acetic acid. It is known that goblet cells secrete mucin, which is made up of glycoproteins that form a barrier that protects the bowel. Since mucin represents the first line of defense against physical and chemical injuries, the goblet cell hyperplasia induced by MG would be responsible, at least in part, for the beneficial properties of this compound. Thus, the administration of MG would represent a useful strategy to protect the integrity of the intestinal mucosa. An important aspect to be considered is that inflammation and oxidative stress are related processes mediating most of the chronic diseases, including IBD. Under physiological conditions, a certain level of reactive oxygen species (ROS) can be reduced by antioxidant activity. However, an excessive oxidant load resulting from increased ROS generation or decreased reduction reactions can overwhelmingly enhance membrane permeability, alter the inflammatory response, and result in lipid and protein modifications and DNA damage, thus contributing to the initiation and/or propagation of IBD (Rezaie et al. 2007). In experimental and clinical studies of IBD, it has been demonstrated that colonic GSH levels are decreased while lipid peroxidation (TBARS levels) is increased (Moura et al. 2015). In our experimental model, we also detected high levels of lipid peroxidation products as well as an increased GSSG/GSH ratio. Interestingly, the administration of MG reduced the levels of oxidative stress biomarkers, thus improving the intestinal redox status.

Not only is the enhanced production of ROS associated with chronic intestinal inflammation, but also it can disturb the colonic epithelial integrity. Alterations in the epithelial barrier function allowing the translocation of luminal antigens into the bowel wall as well as aberrant and excessive cytokine responses to such environmental stimuli may cause acute mucosal inflammation and exacerbate the signs and symptoms of IBD. The expression of COX_2 and pro-inflammatory cytokines, as indicator of the disease severity, was then analyzed. High levels of COX_2, TNF-α and IL-6 levels were found in animals treated with acetic acid. Although the cytokine network within the inflamed mucosa is complex, novel agents that target cytokines or cytokines signaling cascades are currently being tested in clinical trials, suggesting that the cytokine blockade is an interesting strategy in IBD therapy (Neurath 2014). Among these agents, MG seems to be a valuable candidate to reduce the deleterious effects of inflammation within the intestine.

In the colitis model used in this work, the increased levels of cytokines and oxidative stress biomarkers were accompanied by a reduction in the Na^+/K^+ ATPase activity. The latter finding would explain the increase in the wet weight/length ratio. These alterations in the pump functionality could therefore induce a modification in the electrolytic gradient across the epithelium, leading to water retention in the colon. An inverse relationship between the colonic Na^+K^+ ATPase activity and the degree of mucosal inflammation was observed. This decreased pump activity has also been observed along the large intestine of pediatric and adult IBD patients (Allgayer et al. 1988). In summary, the protective properties of MG on experimental colitis

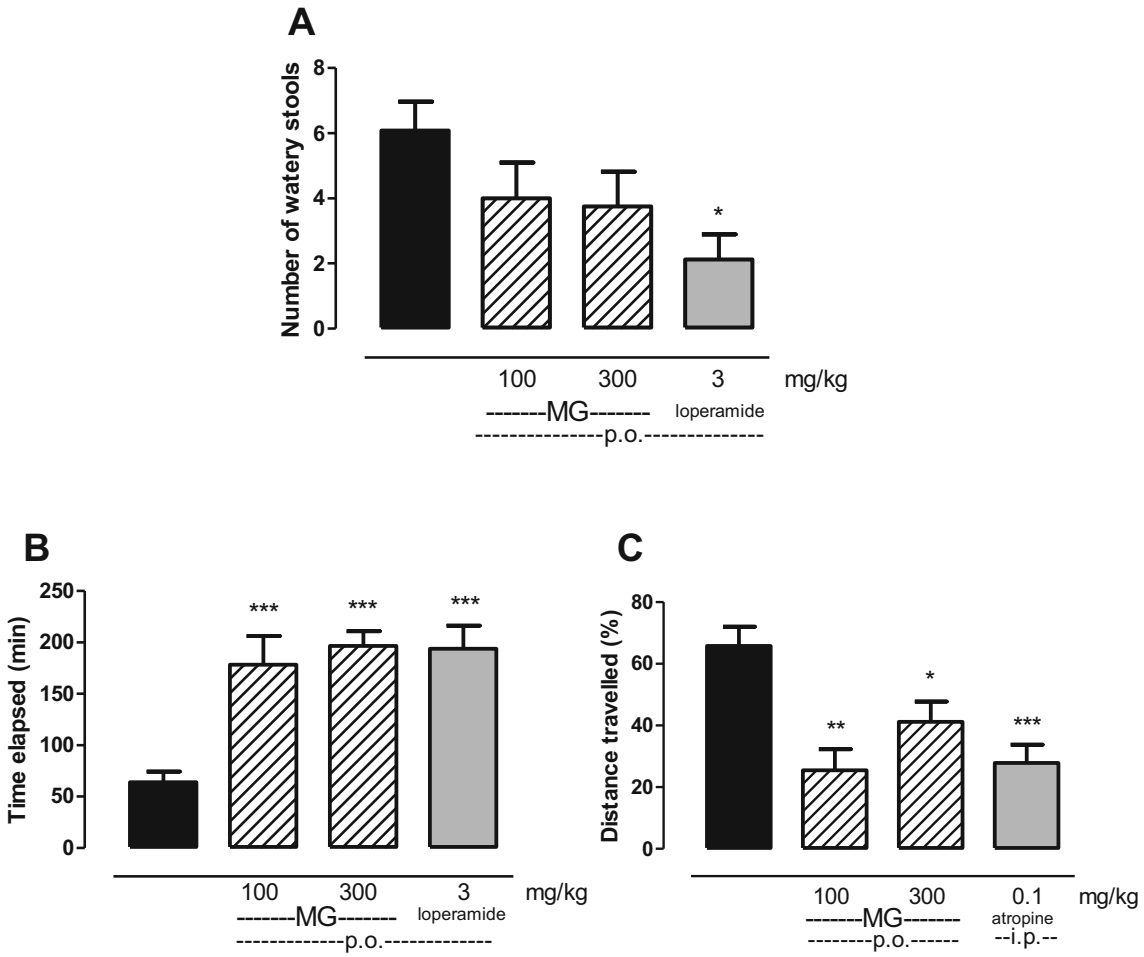

Fig. 7 Castor oil-induced diarrhoea and intestinal motility model in mice. **a** total number of watery stools, **b** time elapsed between the administration of the castor oil and the excretion of the first diarrhea feces, and **c** Distance travelled by charcoal marker expressed as % of total length of small intestine. Results are expressed as mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$. One way ANOVA, followed by Dunnett's test

may be attributed to its ability to decrease the oxidative stress biomarker levels and inflammation as well as to its capacity to recover the Na^{+}/K^{+} ATPase pump activity.

Intestinal inflammation also results in the disturbance of intestinal motility, which may reflect changes in smooth muscle function. Thus, diarrhea continues to be the most prevalent symptom in patients with IBD, probably caused by changes in the colonic motility and by the epithelial barrier dysfunction. Therefore, the development of strategies aimed at modifying the altered motility induced by inflammation and oxidative stress is desired. The castor oil is an effective laxative, since its active principle, ricinoleic acid, alters the permeability to electrolytes in the intestine and causes alterations in the intestinal motility (Uddin et al. 2005). MG would exert a beneficial effect by extending the time of onset of diarrhea and by reducing the intestinal motility, since a reduction in the distance travelled by charcoal in treated animals was observed. MG decreased the intestinal motility, suggesting that, in addition to its effect on experimental diarrhea, MG would also have an antispasmodic effect. It is well known that some spasmolytic agents have a potential therapeutic use in diarrhea, since they induce relaxation of the smooth muscle of the gut and help prevent an excessive loss of fluids. Besides, IBD is characterized by abdominal cramps and pain due to an increased colonic motility. Since cytokines sensitize primary afferent neurons in the intestinal lumen, intestinal spams are a frequent manifestation of inflammation. Such IBD symptoms could be treated by the administration of an antispasmodic agent. The gastrointestinal motor tone is regulated through multiple physiological mediators, such as acetylcholine and 5-hydroxytryptamine, and these agonists cause a stimulatory effect mediated through an increase in the cytosolic calcium levels; therefore, the ability of MG to block any of the above pathways or to antagonize the calcium influx could help to improve the complex signs of IBD on intestinal smooth muscle.

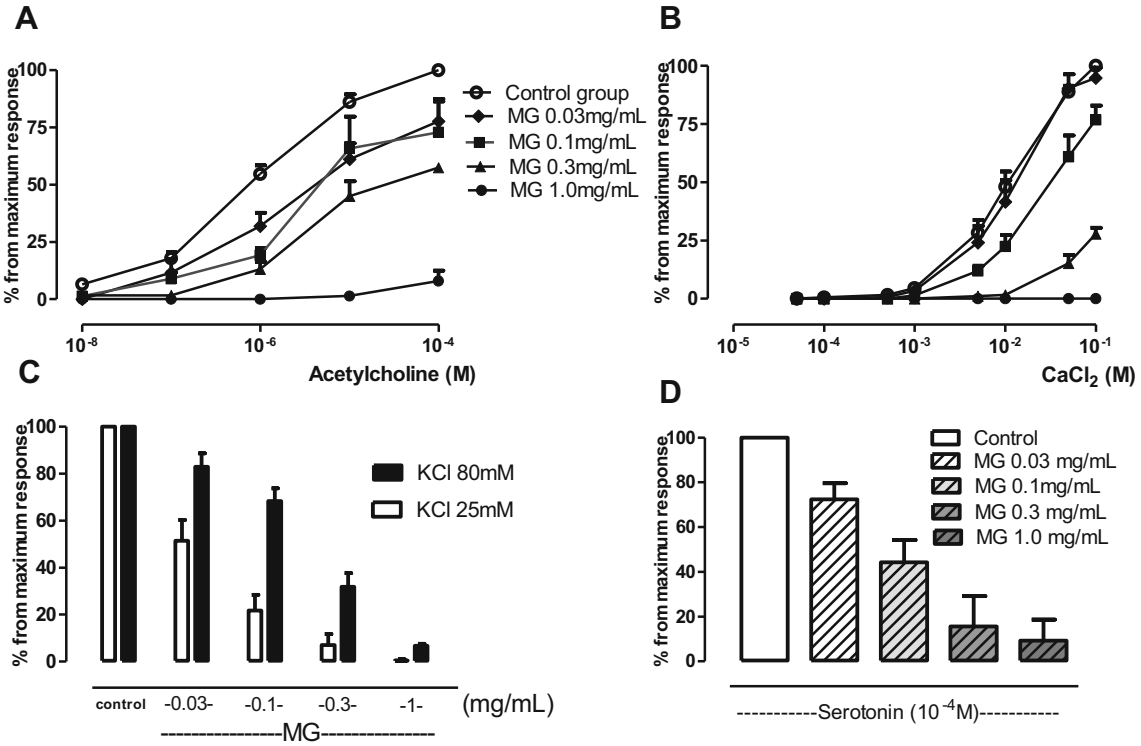

Fig. 8 Effect of MG on **a** cumulative log concentration–response curves for Ach, **b** cumulative log concentration–response curves for $CaCl_2$, **c** the maximum response induced by KCl and Serotonin (**d**)

By means of a multitarget approach, this study demonstrates, for the first time, that MG has antidiarrheal, spasmolytic, anti-inflammatory, and antioxidant effects in preclinical models of intestinal inflammation, suggesting that the administration of this compound could represent a promising strategy to target the multifactorial pathophysiology of IBD. Only part of the mechanisms responsible for beneficial effects induced by MG were studied herein; therefore, future research needs to fully evaluate the clinical potential of MG.

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