

Suppression of TNBS-induced colitis in rats by 4-methylesculetin, a natural coumarin: Comparison with prednisolone and sulphasalazine

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ABSTRACT

The aim of the present study was to compare the effects of the 4-methylesculetin with those produced by prednisolone and sulphasalazine and to elucidate the mechanisms involved in its action. Colitis was induced in rat by instillation of trinitrobenzenesulphonic acid (TNBS). The colon damage was evaluated using macroscopic, microscopic and biochemical analysis. In addition, *in vitro* studies were performed to evaluate cytokine production in cell cultures using the murine macrophage cell line RAW264.7, mouse splenocytes and the human colonic epithelial cell line Caco-2. 4-Methylesculetin produced a reduction of the macroscopic damage score and the recovery of the intestinal cytoarchitecture. These effects were associated with a prevention of the GSH depletion and an inhibition in AP activity. After colitis relapse, 4-methylesculetin improved the colonic inflammatory status as evidenced by histological findings, with a reduction in apoptosis, as well as biochemically by inhibition of colonic myeloperoxidase, alkaline phosphatase and metalloproteinase 9 activities. Paired with this inhibitive activity, there was a decrease in malondialdehyde content and in IL-1 β levels. *In vitro* assays revealed that 4-methylesculetin promoted an inhibition in IL-1 β , IL-8, IL-2 and IFN- γ production in cell cultures. In conclusion, 4-methylesculetin showed similar efficacy to that obtained with either prednisolone or sulphasalazine, both in the acute phase of colitis as well as following a curative protocol. The intestinal anti-inflammatory activity by 4-methylesculetin is likely related to its ability in reduce colonic oxidative stress and inhibit pro-inflammatory cytokine production.

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1. Introduction

Inflammatory bowel disease (IBD) refers to a group of conditions characterised by inflammation in the intestinal tract. Crohn's disease (CD) and ulcerative colitis (UC) account for the majority of these conditions [1]. These diseases are characterised by the uncontrolled response of the intestinal immune system against the normal enteric microflora, leading to abdominal pain and chronic diarrhoea for most of the patient's life [2]. The aetiopathogenesis of these diseases has not been fully elucidated but is currently presumed to result from a complex interplay among genetic, environmental, microbial and immune factors. Patients with IBD frequently experience relapse, and medical treatments are not always able to keep patients in remission in the long term.

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Current therapies involve a combination of aminosalicylates, corticosteroids and immunosuppressants such as azathioprine, methotrexate, cyclosporine and recently monoclonal antibodies against tumour necrosis factor α (TNF- α). These drugs have shown beneficial effects in the treatment of IBD, but they might impose risk to the patients, and not all patients respond to these treatments. They do not represent a cure for these diseases, and dose-dependent side effects are frequently associated with all of these treatments [3]. For this reason, research of new agents that combine pharmacologic efficacy and the absence of undesirable adverse effects is critical.

It has been proposed that reactive oxygen species (ROS) are involved in the development of tissue injury in patients with IBD. Indeed, the anti-inflammatory effects of corticosteroids in the treatment of IBD have been ascribed, in part, to their ability to reduce neutrophil infiltration, thus ameliorating oxidative insult [4]. Moreover, sulphasalazine has been suggested to produce beneficial effects by inhibiting the formation of ROS from neutrophils [5].

Previous studies have shown the beneficial effects of different natural antioxidant compounds in experimental models of rat colitis, including flavonoids like quercitrin [6,7], rutoside [8], morin [9], diosmin [10], hesperidin [10], isocoumarin [11] and coumarin derivatives [12,13]. Our preliminary studies showed that esculetin and 4-methylesculetin treatments prevent colonic damage induced by TNBS in rats [13]. This study showed that the presence of methyl group at C-4 in the esculetin molecules improves its antioxidant and intestinal anti-inflammatory activity [13]. This encouraged us to continue research with 4-methylesculetin to elucidate the mechanism of action and to compare its effects with those produced by prednisolone and sulphasalazine, two of the main drugs used for the treatment of IBD in humans.

2. Materials and methods

2.1. Materials

All chemicals, including 4-methylesculetin, prednisolone and sulphasalazine were provided by Sigma, unless otherwise stated. The test substances were dissolved in methylcellulose (1% w/v) and prepared fresh daily for administration to the animals.

2.2. Animals

Male Wistar rats (weighing 180–200 g) obtained from the Central Animal House of the São Paulo State University (UNESP), Botucatu, São Paulo (Brazil), were housed in standard environmental conditions (21 °C, 60–70% humidity) with 12 h light/dark cycles and air filtration. Animals had free access to water and food (Bio-base). Experimental protocols met the “Guidelines of Animal Experimentation” approved by the Commission of Ethics in Animal Experimentation (Protocol number 042/04-CEEA), Institute of Biosciences, São Paulo State University (UNESP).

2.3. Induction of colitis and assessment of the inflammatory process

Colitis was induced using the method originally described by Morris [14]. Briefly, animals were fasted overnight and then anaesthetised with halothane. Under anaesthesia, they were administered 10 mg of trinitrobenzenesulphonic acid (TNBS) dissolved in 0.25 ml 50% ethanol (v/v) by means of a Teflon cannula inserted 8 cm into the anus. During and after TNBS administration, the rats remained in a head-down position until they recovered from the anaesthesia. Rats from the non-colitic (normal) group received 0.25 ml of saline instead of TNBS. Two different protocols were followed.

2.3.1. Acute colitis

Rats received 5 mg/kg of 4-methylesculetin, 2 mg/kg of prednisolone or 50 mg/kg of sulphasalazine orally at 96, 72, 48, 24 and 2 hours before colitis induction. The dose of 5 mg/kg of 4-methylesculetin was chosen based in dose-response study previously performed in our laboratory [13]. The drugs were administered by means of an oesophageal catheter (volume: 5 ml/kg). Two additional groups were included for reference: a non-colitic group that received saline intracolically and the oral vehicle and a colitic group that received only the first dose of TNBS (TNBS-control without relapse) and the vehicle (5 ml/kg methylcellulose) orally. The animal body weights, the occurrence of diarrhoea (as detected by perianal fur soiling) and the total food intakes for each group were recorded daily. Animals from all groups ($n = 7$) were killed 48 h after colitis induction with an overdose of halothane.

2.3.2. Colitis relapse

In this protocol, colitis was initially induced with 10 mg of TNBS in 50% ethanol, as described previously. After 14 days, the animals were given a second dose of 10 mg of TNBS in an attempt to mimic the common relapses experienced in humans with IBD. The animals were divided into three groups, which were treated daily with the same drugs and doses used in acute colitis. The non-colitic and TNBS-control groups were similar to the acute colitis group. Treatments started 2 h after the first administration of TNBS and continued until the day before the animals were killed. Animals from each group were sacrificed after 1, 2 or 3 weeks of colitis induction, whereas all animals from the colitic control group without relapse were sacrificed after 3 weeks. The animals were killed with an overdose of halothane.

The colonic segments were obtained after laparotomy, and the occurrence of adhesions between the colon and adjacent organs was noted. The colonic segments were placed on an ice-cold plate, cleaned of fat and mesentery, and blotted on filter paper. The colon was weighed, and its length was measured under a constant load (2 g). The colon was opened longitudinally and scored for macroscopically visible damage on a 0–10 scale by two observers blinded to the treatments, according to the criteria described by Bell [15]. The colon was subsequently divided longitudinally into different pieces to be used for the following biochemical assays: myeloperoxidase (MPO) activity, alkaline phosphatase (AP) activity, total glutathione (GSH) content, malondialdehyde content (MDA), matrix metalloproteinase (MMP-9 and MMP-2) activity, and TNF- α and interleukin 1 β (IL-1 β) levels.

2.4. Biochemical assays in colonic specimens

2.4.1. Myeloperoxidase (MPO) activity

MPO activity was measured according to the technique described by Krawisz [16]. Samples were suspended in 1 ml of 50 mM phosphate buffer incorporating 0.5% hexadecyltrimethylammonium bromide (pH 6.0) and minced with scissors for 15 s on an ice-cold plate. The resultant suspension was subsequently diluted to a final 1:20 w/v ratio, homogenised for 1 min with an automatic Heidolph homogeniser, sonicated for 10 s and subjected to three freeze-thaw cycles. The homogenates were then centrifuged at 7000g and 4 °C for 10 min, and the supernatants were assayed for MPO activity. The results are expressed as MPO units per g of wet tissue.

2.4.2. Alkaline phosphatase (AP) activity

AP activity was measured spectrophotometrically using disodium nitrophenyl phosphate (5.5 mM) as a substrate in a 50 mM glycine buffer with 0.5 mM MgCl₂, pH 10.5 [17]. The results are expressed as mU per mg of protein.

2.4.3. Glutathione (GSH) content

Total GSH content was quantified with the recycling assay described by Anderson [18]. Samples were thawed, minced, diluted to a concentration of 1:20 (w/v) in ice-cold 5% (w/v) trichloroacetic acid and homogenised. The homogenates were centrifuged at 7000g for 15 min at 4 °C, and the supernatants were used to quantify glutathione content. The results are expressed as nmol per g of wet tissue.

2.4.4. TNF- α and IL-1 β levels

Colonic samples for the determination of TNF- α and IL-1 β levels were weighed, homogenised, minced on an ice-cold plate and suspended in a centrifugation tube with 10 mmol/L sodium phosphate buffer (pH 7.4) (1:5 w/v). The tubes were placed in a shaker submerged in a water bath (37 °C) for 20 min and then centrifuged at 9000g for 30 s at 4 °C; the supernatants were frozen at -80 °C until

assayed. TNF- α and IL-1 β levels were quantified by a DuoSet ELISA Kit to measure natural and recombinant rat enzyme (R&D Systems, Inc. USA). The results were expressed as pg per mg of protein.

2.4.5. Production of malondialdehyde (MDA)

Tissue concentrations of MDA were determined as an index of lipid peroxidation by the methodology described by Zingarelli [19]. The tissue samples were homogenised in 1.15% KCl solution. An aliquot (400 μ l) of the homogenate was added to a reaction mixture containing 200 μ l of 8.1% sodium dodecyl sulphate (SDS), 1500 μ l of 20% acetic acid (pH 3.5), 1500 μ l of 0.8% thiobarbituric acid, and 400 μ l distilled water. The samples were then boiled for one hour at 95 °C and centrifuged at 3000g for 10 minutes. The absorbance of the supernatant was measured by spectrophotometry at 532 nm and compared to a standard curve obtained using 1,1,3,3-tetramethoxypropane. The data were expressed as nmol per g of wet tissue.

2.4.6. Gelatine zymography for MMP-2 and MMP-9

Zymographic analyses were used to evaluate the gelatinolytic activity of MMP-2 and -9 in colon rats. Frozen samples of rat colons from all experimental animals were mechanically homogenised in a solution of 50 mM Tris-HCl, pH 7.5, with 0.25% of triton X-100 and 10 mM CaCl₂ portioned for 0.1 ml for every 30 mg of colonic tissue. Aliquots of 20 μ g of protein were subjected to electrophoresis under non-reducing conditions in an 8% polyacrylamide gel copolymerised with 0.1% gelatine. After electrophoresis, the gels were washed twice (for 15 min) in a solution of 2.5% Triton X-100 to remove the sodium dodecyl sulphate (Bio-Rad, CA, USA), followed by two washes in a buffer solution of 50 mM Tris-HCl, pH 8.0, for 5 min. Then the gels were incubated overnight in Tris-HCl buffer containing 5 mM CaCl₂ at 37 °C. Finally, the gels were stained with Coomassie Brilliant Blue R-250 [20]. The bands were analysed in an Image Master VDS 3.0 (Pharmacia Biotech). The values are expressed as integrated optical densities.

2.5. Histological findings

Representative whole gut specimens were taken from a region of the inflamed colon corresponding to the segment adjacent to the gross macroscopic damage and were fixed in 4% buffered formaldehyde. Cross-sections were selected and embedded in paraffin. Equivalent colonic segments were also obtained from the non-colitic group. Full-thickness sections of 6 μ m were obtained at different levels and stained with haematoxylin and eosin. After staining, images were subjected to analysis and photomicrography with a Leica microscope utilising Leica Qwin Plus version 3.3 e 3.40 for the evaluation of the histological damage score by methodology described by Stucchi [21].

The Feulgen reaction technique [22] was also applied to another histological section to analyse the degree of compaction of the nuclear chromatin in epithelial cells of the crypts that indicated the apoptosis of these cells. The results were expressed as the number of apoptotic cells divided by the number of total crypts.

2.6. In vitro effects of 4-methylesculetin on cytokine production

2.6.1. Effects on IL-1 β production in RAW264.7 cells

The murine macrophage cell line RAW264.7 was obtained from the Cell Culture Unit of the University of Granada (Granada, Spain) and cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FBS and 2 mM L-glutamine in a humidified 5% CO₂ atmosphere at 37 °C. These cells were seeded onto 24-well plates at a density of 5 \times 10⁵ cells per well and grown until confluence. Then they were incubated for 30 min with different concentrations of 4-methylesculetin (1–100 μ M). Afterwards, these cells

were stimulated with LPS (50 pg/ml) for 20 h, and the supernatants were collected, centrifuged at 10,000g for 5 min, and frozen until ELISA analysis of IL-1 β concentration.

2.6.2. Effects on IL-8 production in Caco-2 cells

The human colon adenocarcinoma cell line Caco-2, obtained from the Cell Culture Unit of the University of Granada (Granada, Spain), was used to test the ability of the 4-methylesculetin to inhibit the production/release of IL-8. Cells were grown in DMEM supplemented with 10% FBS and 2 mM L-glutamine in a humidified 5% CO₂ atmosphere at 37 °C. Caco-2 cells were seeded onto 24-well plates at a density of 5 \times 10⁵ cells per well and grown until formation of a monolayer. Then they were incubated for 30 min with different concentrations of 4-methylesculetin (1–100 μ M). After this, the cells were stimulated with IL-1 β (1 ng/ml) for 20 h, and the supernatants were collected, centrifuged at 10,000g for 5 min, and frozen until ELISA analysis of IL-8 concentration.

2.6.3. Effects on IFN- γ and IL-2 production on mouse splenocytes

Splenocytes were obtained from male 8-week-old Balb/c mice provided by the Laboratory Animal Service of the University of Granada (Granada, Spain). After sacrificing the mice with an overdose of halothane, the spleens were removed and homogenised in DMEM with 1% penicillin/streptomycin. After centrifugation (1500g, 5 min), the erythrocytes were lysed with a buffer (NH₄Cl 1.7 mol/L, KHCO₃ 0.12 mol/L, ethylenediamine-tetra-acetic 9 mmol/L) for 30 min at 4 °C. Resting cells were counted with a haemocytometer and cultured to perform proliferation and stimulation assays in culture medium containing DMEM and 10% FBS. Cells were incubated at 37 °C in a humidified 5% CO₂ atmosphere. These cells were incubated for 1 hour with different concentrations of 4-methylesculetin (1, 10, 50 or 100 μ M). After this time, the cells were stimulated with concanavalin A (5 μ g/ml) for 48 h, and the supernatants were collected, centrifuged at 10,000g for 5 min and frozen at -80 °C until ELISA analysis of IFN- γ and IL-2 concentrations.

2.6.4. Cell viability assays

The viability of the spleen-derived lymphocytes was quantified using the WST-1 Reagent (Roche, Germany) following the protocol previously described [23] and also by viable cell counting by conventional trypan blue staining using a haemocytometer.

Caco-2 and RAW264.7 cell viability was determined by crystal violet staining. Briefly, the cells were stained with crystal violet (0.2%) in ethanol for 30 minutes at room temperature. The plates were then washed 4 times with PBS. The cells were solubilised with 1% sodium dodecyl sulphate solution for 30 minutes. The cells were then centrifuged at 3000g for 5 minutes, and dye uptake was measured at 540 nm using a microplate reader.

2.7. Statistics

All results are expressed as the mean \pm S.E.M., and the differences between means were tested for statistical significance using one-way analysis of variance (ANOVA). Nonparametric data (score) are expressed as the median (range) and were analysed with the Kruskal-Wallis test. Differences between proportions were analysed with the χ^2 test. Statistical significance was set at $p < 0.05$.

3. Results

3.1. Acute colitis

The administration of TNBS resulted in colonic inflammation, which was associated with a significant reduction in body weight

after 48 hours in comparison with non-colitic rats (data not shown) and was likely related to a reduction in food intake (data not shown) and the presence of diarrhoea in this group (Table 1). The macroscopic analysis of the colonic specimens in the colitic rats revealed the existence of severe necrosis and inflammation of the mucosa, typically extending 5.0 cm along the colon (Table 1). This inflammatory process was associated with an increase in the colonic weight/length ratio. Biochemically, the colonic damage was characterised by an increase in colonic MPO, AP (Table 2) and metalloproteinase-9 activities (Fig. 1), as well as in TNF- α , IL-1 β (Fig. 1) and MDA contents (Table 2). Furthermore, significant colonic GSH depletion took place in the inflamed colon (Table 2). Histological assessment of colonic samples from the TNBS control group revealed severe transmural disruption of the normal architecture of the colon, with extensive ulceration and inflammation involving all the intestinal layers of the colon, with a median score of 16.5 (Fig. 2). These samples were also characterised by severe oedema, interstitial micro-haemorrhages and diffuse leucocyte infiltration, mainly composed of neutrophils and, to a lesser extent, lymphocytes and histiocytes (Fig. 2).

The compound 4-methylesculetin showed an intestinal anti-inflammatory preventative effect in colitic rats, similar to the effects obtained with prednisolone and sulphasalazine, as demonstrated by a decrease in the macroscopic damage scores, in the percentage of diarrhoea and in the incidence of adhesions between the colon and adjacent organs (Table 1). This benefit was also assessed biochemically by the increase in GSH content and the inhibition in AP activity (Table 2). Additionally, only prednisolone significantly reduced TNF- α levels (Fig. 1). In contrast, 4-methylesculetin and prednisolone reduced the microscopic damage score in comparison with the non-treated colitic group (Fig. 2). Thus, the histological studies showed that treatment with 4-methylesculetin and prednisolone promoted the recovery of the intestinal cytoarchitecture and a reduction in ulceration, oedema, mucus depletion and in the infiltration of inflammatory cells in the sub-mucosal layers (Fig. 2). In addition, 4-methylesculetin also reduced this infiltration into the lamina propria layer (Fig. 2). Finally, the number of apoptotic epithelial cells increased two-fold in the TNBS control group compared to the non-colitic group. However, none of the tested drugs was able to significantly reduce the number of apoptotic epithelial cells (Table 1).

3.2. Colitis relapse

The histological evaluation of the colonic damage in the chronic phase of the TNBS-colitis revealed that the damage was maximal seven days after TNBS instillation and decreased gradually during the following two weeks. At this time, the colonic segments still appeared macroscopically and microscopically ulcerated and inflamed (Table 3). The microscopic analysis also revealed leucocyte migration accompanied by mucus depletion and oedema. The inflammatory process was also demonstrated by a similar progres-

sion of the biochemical inflammatory parameters studied (Tables 4 and 5).

The administration of 4-methylesculetin, prednisolone or sulphasalazine once the colonic damage had been induced facilitated the recovery of the inflammatory process during the following two weeks. This was demonstrated both histologically and biochemically. Thus, at the first week, 4-methylesculetin significantly reduced the macroscopic damage score in comparison to the TNBS-control and apoptosis. These effects were similar to that observed with prednisolone and sulphasalazine treatments (Table 4). Microscopically, all treatments also reduced leucocyte infiltration and oedema in the mucosa compared with TNBS-control rats.

Biochemically, these beneficial effects were associated with a significant inhibition of MPO, AP and MMP-9 activities (Tables 4 and 5). In addition, 4-methylesculetin and prednisolone also decreased the MDA content (Table 4) and IL-1 β colonic levels (Table 5).

Colitis relapse induced by a second dose of TNBS was characterised by a reactivation of the colonic inflammatory process, and a significant increase in the microscopic and macroscopic damage score and in the weight/length ratio (Table 3) were observed when compared to the normal evolution of colonic damage in the animals that did not receive the second dose of TNBS (TNBS-control without relapse). Microscopically, the reactivation of the inflammatory process was characterised by an increase of leucocyte infiltration in the lamina propria and mucosa. Thus, these effects were associated with colonic oxidative damage, which caused a reduction in the GSH content and an increase in the others biochemical parameters evaluated in comparison with colitic animals without relapse (Tables 4 and 5).

After the colitis relapse, the treatment with 4-methylesculetin promoted a reduction in microscopic damage score and apoptosis (Table 3). These effects were biochemically accompanied by inhibition in MPO and AP activities (Table 4). The treatments with prednisolone and sulphasalazine were not able to significantly modify any of the microscopic or macroscopic parameters evaluated. Biochemically, both drugs were only able to counteract GSH content, whereas sulphasalazine also inhibited colonic AP activity (Tables 4 and 5).

3.3. Inhibition of cytokine production in cell cultures

The results obtained in these assays showed that 4-methylesculetin dose-dependently reduced the production of the different cytokines studied (Fig. 3). In LPS-stimulated RAW264.7 macrophages, the coumarin derivative inhibited IL-1 β release significantly at a concentration of 10 μ M and achieved a complete inhibition at a concentration of 50 μ M. Similarly, the incubation of 4-methylesculetin with IL-1 β -stimulated Caco-2 cells reduced the production of IL-8 at a concentration of 10 μ M and obtained a maximal inhibition of 70% at a concentration of 50 μ M. Finally, in concanavalin A-stimulated splenocytes, this compound

Table 1

Effects of 4-methylesculetin (5 mg/Kg), prednisolone (2 mg/Kg) and sulphasalazine (50 mg/Kg) treatment on macroscopic damage score, colonic weight, incidence of diarrhoea, adhesions and apoptosis in acute TNBS colitis.

Group	Macroscopic damage score (0–10) ¹	Colonic weight (mg/cm) ²	Diarrhoea (%) ³	Adherence (%) ³	Apoptosis/per crypt epithelial cells ²
Non-colitic	0 ^a	80.2 \pm 12.17 ^a	0 ^a	0 ^a	0.062 \pm 0.004 ^a
TNBS-control	8.0 (7–10) ^b	144.6 \pm 14.04 ^b	100 ^b	83 ^b	0.136 \pm 0.015 ^b
4-Methylesculetin	6.0 (3–7) ^c	138.0 \pm 22.17 ^{bc}	33 ^a	33 ^b	0.111 \pm 0.019 ^b
Prednisolone	6.0 (1–8) ^c	138.9 \pm 23.98 ^{bc}	33 ^a	0 ^a	0.087 \pm 0.012 ^a
Sulphasalazine	6.0 (3–7) ^c	132.8 \pm 23.52 ^{bc}	67 ^{bc}	33 ^a	0.089 \pm 0.013 ^a

¹ Score data are expressed as the median (range).

² Exclusion extension of lesion, Colonic weight and apoptosis data are expressed as the mean \pm S.E.M.

³ Diarrhoea and adherence are expressed in percentage. Value without a common letter differ $p < 0.05$.

Table 2
Effects of 4-methylesculetin (5 mg/Kg), prednisolone (2 mg/Kg) and sulphasalazine (50 mg/Kg) treatment on glutathione (GSH) content, myeloperoxidase (MPO) and alkaline phosphatase (AP) activities and malondialdehyde content (MDA) in acute TNBS colitis.

Group	GSH (nmol/g tissue)	MPO (U/g tissue)	AP (mU/mg protein)	MDA (nmol/g tissue)
Non-colitic	1768 ± 90.63 ^a	201 ± 26.20 ^a	5.44 ± 0.45 ^a	12.99 ± 1.83 ^a
TNBS-control	1046 ± 51.92 ^b	2246 ± 216.97 ^b	16.62 ± 1.43 ^b	20.07 ± 1.29 ^b
4-Methylesculetin	1638 ± 443.41 ^a	1590 ± 135.53 ^c	11.41 ± 1.55 ^c	18.00 ± 2.29 ^{ab}
Prednisolone	1698 ± 84.31 ^a	1441 ± 218.92 ^c	9.96 ± 1.39 ^a	15.58 ± 2.32 ^{ab}
Sulphasalazine	1695 ± 98.90 ^a	1550 ± 88.56 ^c	10.91 ± 1.20 ^c	16.57 ± 1.48 ^{ab}

Data are expressed as mean ± S.E.M. Means without a common letter differ, $p < 0.05$.

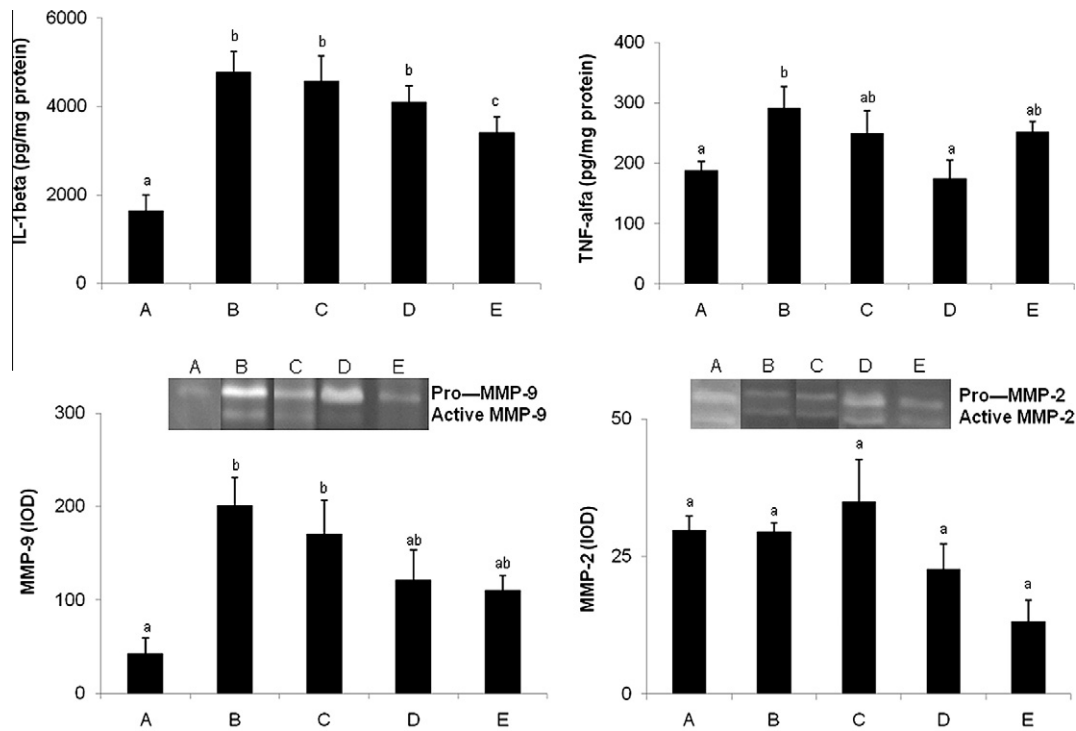


Fig. 1. Effects of different treatments in TNBS rat colitis in biochemical parameters (acute protocol). This figure showed tumour necrosis factor alpha (TNF-alfa) and interleucin 1-beta (IL-1beta) levels, matrix metalloproteinases 2 (MMP-2) and 9 (MMP-9) activities. (A) non-colitic; (B) TNBS-control; (C) 4-methylesculetin (5 mg/Kg), (D) prednisolone (2 mg/Kg) and (E) sulphasalazine (50 mg/Kg). Data are expressed as mean ± S.E.M. MMP activity is expressed as densitometry as integrated optical density (IOD). Value without a common letter differ $p < 0.05$.

inhibited either IL-2 or IFN- γ production at a concentration of 50 μ M, resulting 65% and 100% inhibition, respectively, at the highest concentration assayed. The viability assays revealed that most of the concentrations used were devoid of any significant cytotoxic effect, with the exception of RAW264.7, in which the concentration of 100 μ M of 4-methylesculetin showed an incidence of cytotoxic effects of approximately 25%.

4. Discussion

This study confirms the intestinal anti-inflammatory activity of 4-methylesculetin, at a dose of 5 mg/kg, in the TNBS model of rat colitis. This natural compound showed similar efficacy to that obtained with either prednisolone (2 mg/kg) or sulphasalazine (50 mg/kg), two of the main drugs currently used for the treatment of human IBD. This beneficial effect was observed following the preventative dosing protocol used in the acute phase of experimental colitis, as well as after a curative protocol in the chronic phase.

In the chronic colitis with relapse model, the colonic damage was initially characterised by a time-dependent recuperation of inflammatory status, similar to that described previously in [11].

Two weeks after the initial insult, the colonic segments still appeared macroscopically ulcerated and inflamed. As expected, the second administration of TNBS resulted in a reactivation of the colonic damage in comparison with that observed in the control colitic group without relapse.

The treatment with 4-methylesculetin, prednisolone or sulphasalazine showed an improvement of the intestinal inflammatory process in all time points studied, either before or after the second TNBS instillation. This was exhibited by a significant decrease in the macroscopic damage score and by an inhibition in colonic MPO activity, as this enzyme activity is a marker of neutrophil infiltration, which can be considered an index of inflammation damage [24]. Colonic inflammation was also characterised by an increase in AP activity, which has been attributed mainly to leucocyte activity [25]. AP enzyme activity has been considered a sensitive biochemical marker of intestinal inflammation [6,26,7] and its inhibition can be interpreted as a result of the anti-inflammatory effect of a given compound. In fact, 4-methylesculetin, prednisolone and sulphasalazine inhibited AP activity in the first week after TNBS instillation, while 4-methylesculetin and sulphasalazine also showed this activity after the colitis relapse.

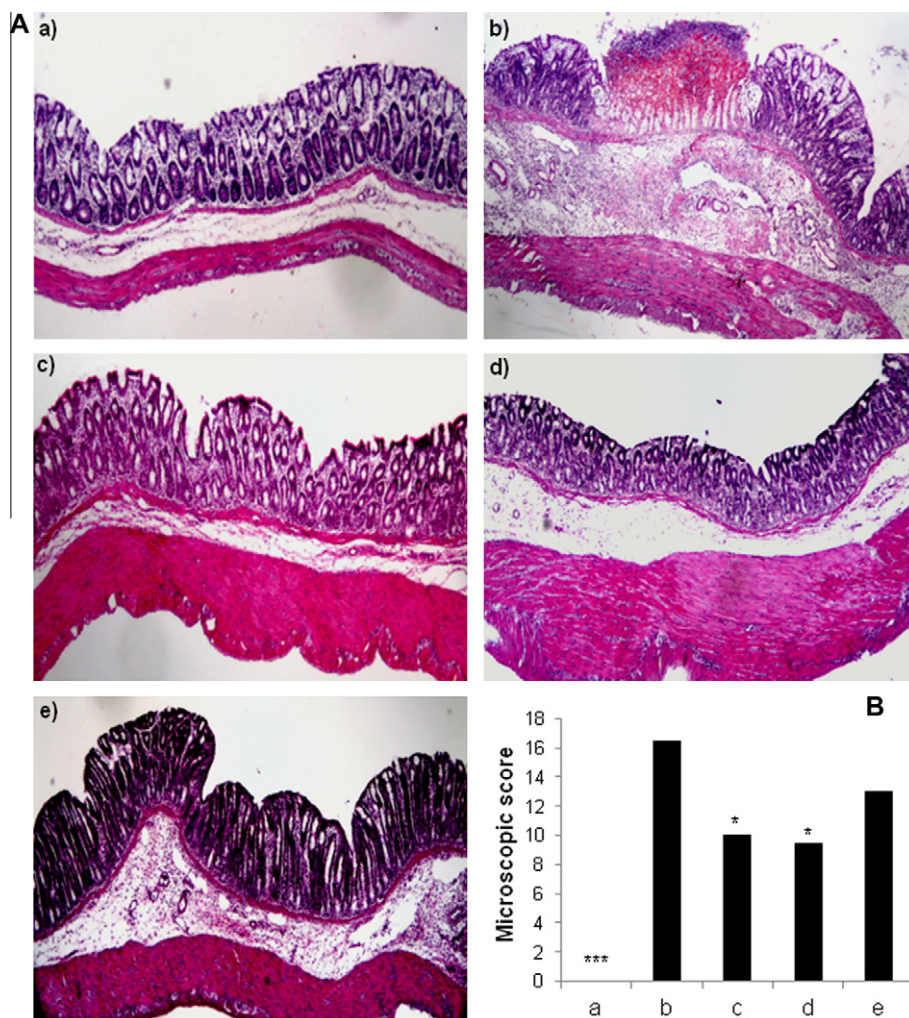


Fig. 2. Effects of different treatments in TNBS rat colitis (acute protocol) in histological analyses. (A) Histological sections of colonic mucosa stained with haematoxylin and eosin: (a) non-colitic; (b) TNBS-control; (c) 4-methylscutletin (5 mg/Kg), (d) prednisolone (2 mg/Kg) and (e) sulphasalazine (50 mg/Kg). (B) Colonic microscopic damage score. Data are expressed as means \pm SEM. * $p < 0.05$; *** $p < 0.01$ vs. TNBS control group.

IBD is characterised by a complex inflammatory cascade responsible for the tissue destruction. In this event, matrix metalloproteinases (MMPs), a group of zinc-dependent endopeptidases, participate in extracellular matrix degradation and remodelling. Their enzymatic activity is regulated by tissue inhibitors of the MMPs (TIMPs); however, under pathological situations, the increased amount of active MMPs cannot be controlled by TIMPs, resulting in extracellular matrix breakdown and tissue injury [27]. As a result, drugs that are able to block the tissue damage-related MMPs or are able to increase the tissue protection-related MMPs could be considered as good candidates for the treatment of this disease. In IBD, the MMP gelatinases MMP-2 and MMP-9 has been described to display antagonistic functions [28]. Epithelial-derived MMP-9 is an important mediator of tissue injury in colitis in human, whereas MMP-2 protects the colon against tissue damage and maintains gut barrier function [29]. It has been suggested that the inhibition of intestinal MMP-9 activity may be beneficial in IBD [28]. In the present study, and in comparison with non colitic rats, colonic MMP-9 activity was significantly increased in control colitic rats both in the acute and chronic phases of experimental TNBS colitis, but not one week after colitic relapse. However, colonic MMP-2 activity was only significantly inhibited one week after the first TNBS instillation. The administration of 4-methylscutletin, prednisolone or sulphasalazine to colitic rats once

colonic damage had been induced resulted in the inhibition of colonic MMP-9 activity seven days after TNBS administration and simultaneously maintained the protective activity of colonic MMP-2.

TNF- α and IL-1 β are clearly involved in the intestinal inflammatory process by inducing the expression of different pro-inflammatory proteins, including MMPs [30]. It is possible that the effect of 4-methylscutletin or prednisolone on MMP-9 activity is related, at least in part, to the ability of these compounds to reduce IL-1 β and TNF- α colonic levels.

Because oxidative damage is involved in the pathogenesis of IBD, antioxidant compounds have been studied for the treatment of these intestinal conditions [31]. 4-methylscutletin, similar to other coumarin derivatives, has been described to possess antioxidant and anti-inflammatory effects [32]. In a previous study, we reported the beneficial effect of 4-methylscutletin in TNBS-induced colitis in rats. We concluded that its activity is likely related to its antioxidant properties [13]. The results obtained in the present study confirm this observation because 4-methylscutletin improved the biochemical markers of colonic oxidative stress, as analysed by glutathione. Glutathione is a natural tripeptide that displays antioxidant activity, and depletion of glutathione is associated with experimental colitis [33] and MDA, the major end-product of the oxidation of polyunsaturated fatty acids.

Table 3
Effects of 4-methylsulculetin (5 mg/Kg), prednisolone (2 mg/Kg) and sulphasalazine (50 mg/Kg) treatment on macroscopic damage score, extension of lesion, microscopic damage score and apoptosis in chronic with relapse of TNBS colitis.

Group	Macroscopic damage score (0–10) ¹	Colonic weight (mg/cm) ²	Microscopic damage score (0–27) ²	Apoptosis/per crypt epithelial cells
<i>1 week</i>				
Non-colitic	0 ^a	89.0 ± 7.42 ^a	0 ^a	0.079 ± 0.014 ^a
TNBS-control	5.5 (5–7) ^b	147.5 ± 8.02 ^b	13.0 (10–23) ^b	0.198 ± 0.021 ^b
4-Methylsulculetin	3.5 (3–5) ^c	138.3 ± 4.15 ^b	10.5 (7–15) ^b	0.094 ± 0.02 ^a
Prednisolone	2.0 (0–5) ^a	137.7 ± 8.39 ^b	10.5 (7–14) ^b	0.109 ± 0.016 ^a
Sulphasalazine	3.0 (2–6) ^c	139.1 ± 10.75 ^b	12.0 (9–19) ^b	0.122 ± 0.016 ^a
<i>2 week</i>				
Non-colitic	0 ^a	102.0 ± 3.89 ^a	0 ^a	0.070 ± 0.007 ^a
TNBS-control	3.0 (2–4) ^b	146.3 ± 9.59 ^b	14.0 (10–17) ^b	0.273 ± 0.023 ^b
4-Methylsulculetin	2.0 (2–3) ^a	120.1 ± 5.56 ^a	11.5 (6–18) ^b	0.132 ± 0.018 ^c
Prednisolone	2.5 (0–3) ^a	134.9 ± 6.14 ^b	11.0 (9–12) ^b	0.140 ± 0.017 ^c
Sulphasalazine	3.0 (2–6) ^c	135.1 ± 2.72 ^b	8.0 (4–13) ^b	0.161 ± 0.02 ^c
<i>3 week</i>				
Non-colitic	0 ^a	97.9 ± 4.13 ^a	0 ^a	0.103 ± 0.009 ^a
TNBS-control with relapse	4.0 (2–4) ^b	138.1 ± 13.83 ^b	14.0 (12–18) ^b	0.236 ± 0.021 ^b
TNBS-control without relapse	2.0 (1–3) ^c	144.5 ± 3.30 ^b	12.0 (9–17) ^b	0.117 ± 0.013 ^a
4-Methylsulculetin	2.5 (1–3) ^c	151.8 ± 11.30 ^b	7.5 (4–9) ^b	0.080 ± 0.008 ^a
Prednisolone	2.5 (2–3) ^c	171.5 ± 10.21 ^b	13.0 (8–18) ^b	0.117 ± 0.01 ^a
Sulphasalazine	3.0 (2–5) ^c	171.0 ± 24.71 ^b	12.0 (6–13) ^b	0.099 ± 0.011 ^a

¹ Score data are expressed as the median (range).

² Colonic weight data and apoptosis are expressed as the mean ± S.E.M. Value without a common letter differ, $p < 0.05$.

Table 4
Effects of 4-methylsulculetin (5 mg/Kg), prednisolone (2 mg/Kg) and sulphasalazine (50 mg/Kg) treatment on glutathione (GSH) content, myeloperoxidase (MPO) and alkaline phosphatase (AP) activities and malondialdehyde content (MDA) in chronic with relapse of TNBS colitis.

Group	GSH (nmol/g tissue)	MPO (U/g tissue)	AP (mU/mg protein)	MDA (nmol/g tissue)
<i>1 week</i>				
Non-colitic	1500 ± 76.46 ^a	109.7 ± 6.77 ^a	7.23 ± 0.76 ^a	12.18 ± 0.93 ^a
TNBS-control	917 ± 26.21 ^b	318.6 ± 51.67 ^b	18.23 ± 4.18 ^b	17.08 ± 1.11 ^b
4-Methylsulculetin	1016 ± 58.79 ^c	177.5 ± 34.00 ^a	9.96 ± 1.53 ^a	12.99 ± 0.59 ^a
Prednisolone	1223 ± 77.02 ^c	128.4 ± 22.05 ^a	6.38 ± 0.89 ^a	12.56 ± 0.71 ^a
Sulphasalazine	1268 ± 65.64 ^a	154.6 ± 37.92 ^a	7.28 ± 1.18 ^a	17.47 ± 1.44 ^{bc}
<i>2 week</i>				
Non-colitic	2146 ± 78.71 ^a	85.0 ± 7.70 ^a	6.44 ± 0.95 ^a	7.96 ± 0.55 ^a
TNBS-control	1568 ± 123.04 ^b	160.1 ± 24.96 ^b	15.34 ± 2.12 ^b	13.61 ± 1.97 ^b
4-Methylsulculetin	1715 ± 91.67 ^b	121.7 ± 24.33 ^{ab}	7.60 ± 0.44 ^a	10.39 ± 1.13 ^{ab}
Prednisolone	1875 ± 128.04 ^b	114.7 ± 36.64 ^{ab}	10.30 ± 1.20 ^a	12.55 ± 0.98 ^{ab}
Sulphasalazine	1841 ± 216.08 ^b	100.9 ± 15.71 ^{ab}	8.99 ± 1.01 ^a	12.12 ± 1.22 ^{ab}
<i>3 week</i>				
Non-colitic	1741 ± 79.29 ^a	95.0 ± 11.74 ^a	6.81 ± 0.89 ^a	7.53 ± 0.81 ^a
TNBS-control with relapse	1252 ± 74.08 ^b	200.3 ± 25.54 ^b	11.92 ± 1.69 ^b	10.28 ± 0.79 ^b
TNBS-control without relapse	1436 ± 76.84 ^{ab}	154.4 ± 17.53 ^{ac}	7.84 ± 0.82 ^a	10.48 ± 1.07 ^{ab}
4-Methylsulculetin	1471 ± 11.83 ^{ab}	164.0 ± 9.16 ^c	6.60 ± 0.69 ^a	9.51 ± 0.46 ^{ab}
Prednisolone	1693 ± 111.90 ^{ac}	167.6 ± 4.62 ^{bc}	8.28 ± 0.83 ^{ab}	12.45 ± 1.37 ^b
Sulphasalazine	1676 ± 122.03 ^{ac}	175.9 ± 46.47 ^{bc}	7.58 ± 1.36 ^a	11.92 ± 1.51 ^b

Data are expressed as mean ± S.E.M. Means without a common letter differ, $p < 0.05$.

Glutathione is frequently measured as indicator of the “*in vivo*” lipid peroxidation and oxidative stress. In addition, the reduction in neutrophil infiltration, as detected by microscopic and biochemical analyses, can also contribute to these protective effects on the altered oxidative status in the inflamed colonic tissue.

It has been reported that the impairment in the epithelial barrier function can account for the pathophysiology of IBD, leading to a higher access of luminal agents to the intestinal tissue. Furthermore, this impairment is also associated with a functional loss of normal absorptive capacity and inappropriate secretion of intestinal fluid and electrolytes, thus promoting diarrhoea, abdominal cramps, excessive gas, and bloating [34]. One of the processes that has been proposed to be involved in the altered epithelial integrity is the increase in intestinal epithelial cell apoptosis [35]. Inhibition of cell apoptosis has been considered a symptomatic treatment op-

tion for ill patients with severe diarrhoea [34]. This inhibitory effect on epithelial cell apoptosis may reduce villus atrophy and epithelial destruction [36]. The present study reveals that 4-methylsulculetin, prednisolone and sulphasalazine reduced the number of intestinal epithelial cell apoptotic events across all time points studied during the chronic colitis with relapse. Different mechanisms can be involved in this effect, and one of them could be ascribed to the beneficial effects exerted by these compounds acting on the colonic oxidative status. Previously, it has been reported that the excessive oxidative stress to which the intestinal mucosa is subjected under inflammatory conditions might enhance epithelial apoptosis [37]. Therefore, it has been suggested that IFN- γ and TNF- α , the production of which is clearly increased by the infiltrating immune-competent cells in these intestinal conditions, contribute to epithelial cell apoptosis in the gut [34]. In the

Table 5

Effects of 4-methylesculetin (5 mg/Kg), prednisolone (2 mg/Kg) and sulphasalazine (50 mg/Kg) treatment on matrix metalloproteinase activity (MMP-9 and MMP-2), tumour necrosis factor α (TNF- α) and interleukin 1 β levels in chronic with relapse of TNBS colitis.

Group	MMP-9 (IOD)	MMP-2 (IOD)	TNF- α (pg/mg protein)	IL-1 β (pg/mg protein)
<i>1 week</i>				
Non-colitic	78.08 \pm 18.54 ^a	43.43 \pm 15.82 ^a	187.81 \pm 14.53 ^a	1370 \pm 138.55 ^a
TNBS-control	155.37 \pm 31.41 ^b	14.94 \pm 5.40 ^b	275.17 \pm 21.18 ^b	4412 \pm 800.83 ^b
4-Methylesculetin	60.25 \pm 10.67 ^a	33.82 \pm 4.16 ^{ab}	284.57 \pm 15.54 ^b	2055 \pm 326.80 ^a
Prednisolone	23.53 \pm 3.37 ^a	24.79 \pm 4.38 ^{ab}	170.82 \pm 13.38 ^a	1536 \pm 291.58 ^a
Sulphasalazine	70.17 \pm 12.89 ^a	34.03 \pm 8.81 ^{ab}	254.42 \pm 17.80 ^b	2289 \pm 309.30 ^a
<i>2 week</i>				
Non-colitic	51.09 \pm 3.46 ^a	39.29 \pm 5.11 ^a	141.19 \pm 16.60 ^a	594 \pm 117.60 ^a
TNBS-control	104.29 \pm 39.44 ^b	78.63 \pm 20.46 ^a	174.81 \pm 17.43 ^a	1257 \pm 225.45 ^b
4-Methylesculetin	138.08 \pm 33.13 ^b	68.86 \pm 35.01 ^a	136.55 \pm 22.59 ^a	942 \pm 126.73 ^{ab}
Prednisolone	94.02 \pm 26.99 ^{ab}	41.58 \pm 15.40 ^a	141.55 \pm 23.85 ^a	1562 \pm 244.74 ^b
Sulphasalazine	124.96 \pm 20.12 ^b	79.46 \pm 28.78 ^a	153.45 \pm 30.48 ^a	1546 \pm 226.46 ^b
<i>3 week</i>				
Non-colitic	70.06 \pm 19.26 ^a	37.66 \pm 8.64 ^a	65.28 \pm 12.53 ^{ab}	650 \pm 85.93 ^a
TNBS-control with relapse	102.09 \pm 17.69 ^a	32.95 \pm 10.14 ^a	89.73 \pm 13.27 ^{ab}	1045 \pm 89.60 ^{bd}
TNBS-control without relapse	103.58 \pm 37.32 ^a	41.92 \pm 4.39 ^a	59.00 \pm 5.96 ^a	570 \pm 118.36 ^{ab}
4-Methylesculetin	83.18 \pm 12.76 ^a	37.08 \pm 7.12 ^a	115.41 \pm 25.70 ^b	788 \pm 149.21 ^{ab}
Prednisolone	55.62 \pm 6.98 ^a	37.26 \pm 5.97 ^a	97.14 \pm 10.41 ^{ab}	2274 \pm 133.66 ^c
Sulphasalazine	84.21 \pm 28.08 ^a	47.99 \pm 20.29 ^a	116.24 \pm 11.38 ^b	1512 \pm 164.80 ^d

Data are expressed as mean \pm S.E.M. MMP activity are expressed in densitometry as Integrated Optical Density (IOD).

Means without a common letter differ, $p < 0.05$.

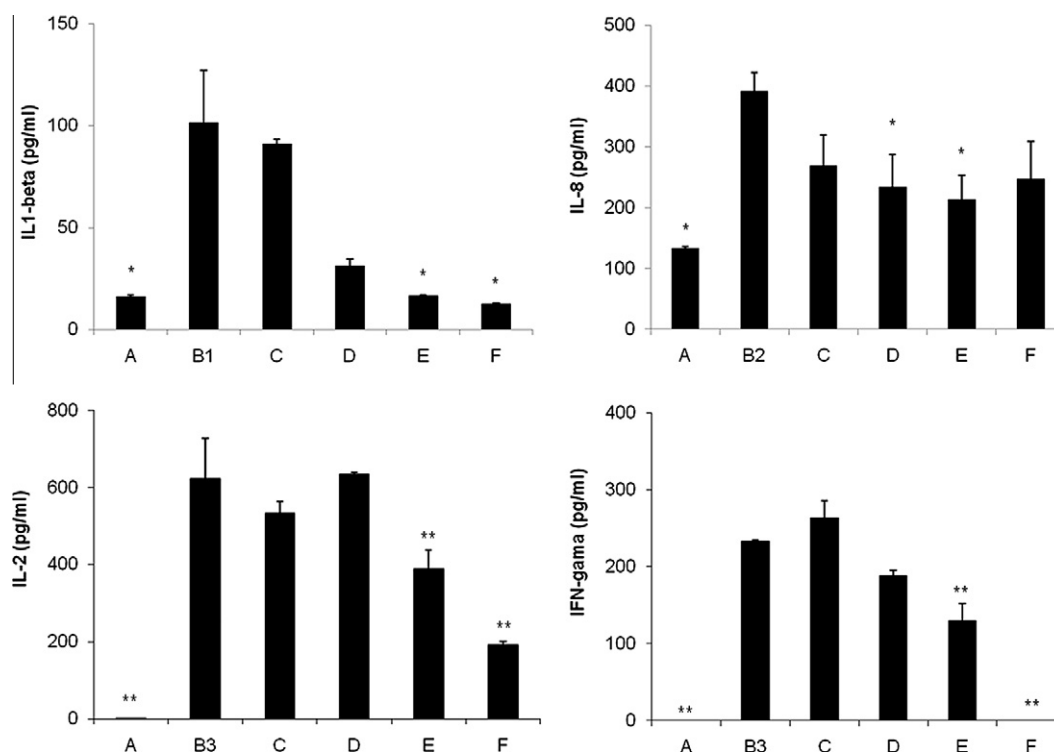


Fig. 3. In vitro effects of 4-methylesculetin on cytokine production in different cell cultures. IL-1beta in RAW264.7 cells, IL-8 in Caco-2 cells and IL-2 and IFN- γ in spleen cells. (A) non stimulated cells; (B1) LPS + DMSO; (B2) IL-1 + DMSO; (B3) Concanavalin + DMSO; (C) 4-methylesculetin 1 μ M; (D) 4-methylesculetin 10 μ M; (E) 4-methylesculetin 50 μ M and (F) 4-methylesculetin 100 μ M. Results are expressed as mean \pm S.E.M. * $p < 0.05$, ** $p < 0.01$ vs respective controls (B).

deregulated intestinal epithelial cells, the presence of TNF- α and INF- γ can promote caspase-8 activation, resulting in oxidative stress, the loss of membrane permeability and the occurrence of an apoptotic effect [36,38]. For this reason, the effects that these compounds can exert on cytokine production and release can also account to their beneficial effects in these intestinal conditions. The ability of both prednisolone [39] and sulphasalazine [40] to reduce excess cytokine production is regulated through inhibition of

NF- κ B activity. Of note, NF- κ B is a redox-sensitive transcription factor and is activated by oxidant stress in the inflamed intestinal mucosa [41].

Since 4-methyl-esculetin was also able to inhibit cytokine production, as well as prednisolone and sulphasalazine, it is possible that its anti-inflammatory effect was also related to inhibition of the NF- κ B activation, but new studies are necessary to elucidate this mechanism of action.

Finally, the results obtained in the “in vitro” assays showed that 4-methylscutletin was also able to inhibit the production of IL-8, IL-1 β , IL-2 and IFN- γ in a dose-dependent manner in different cell types involved in the intestinal immune response, i.e., epithelial cells (Caco2), macrophages (RAW264.7) and lymphocytes (mouse splenocytes). This immunomodulatory effect can account for its beneficial effect during intestinal inflammation. Considering that inhibition of IL-8, IL-1 β , IL-2 and IFN- γ production contributes with *in vivo* anti-inflammatory effects produced by 4-methylscutletin, it is plausible to suggest that effect was derived from absorption of 4-methylscutletin. In fact, several studies shown that 4-methylscutletin structurally-related compounds as coumarin and coumarin derivatives are promptly absorbed in small intestine, rapidly and widely distributed into tissues to produce its pharmacological effects [42–45].

5. Conclusion

The intestinal anti-inflammatory activity shown by 4-methylscutletin in the TNBS model of colitis can be ascribed to its ability to reduce colonic oxidative stress and inhibit the production of pro-inflammatory cytokines. Moreover, 4-methylscutletin also shows a similar efficacy to that demonstrated by prednisolone or sulphasalazine, two drugs currently used in the pharmacological treatment of IBD in humans. Additionally, it is interesting to note that the administration of 4-methylscutletin exerts both curative and preventive effects, showing the latter effect both when the mucosa is intact and when it is damaged and in the process of recovery.

Conflict of interest

The author declares that there are no conflicts of interest.

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