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Myrtle berries seeds aqueous extract inhibits in vitro human neutrophils myeloperoxidase and attenuates acetic acid-induced ulcerative colitis in rat

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Running title: MBSAE protection on ulcerative colitis in rat.

Abstract

We aimed in the present study to investigate the protective effect of myrtle (*Myrtus communis* L.) berries seeds aqueous extract (MBSAE) on acetic acid (AA)-induced colitis in rat as well as the mechanism implicated in this coli-protection. The use of LC/MS technique allowed us to identify 18 phenolic compounds in MBSAE. Secondly, we found that MBSAE inhibited luminol-amplified chemiluminescence of resting neutrophils and N-formyl-methionylleucyl-phenylalanine (fMLF) or phorbolmyristate acetate (PMA) stimulated neutrophils, in a dose-dependent manner. MBSAE had no effect on superoxide anion, but it inhibited H_2O_2 production in cell free system stimulated with horseradish peroxydase (HRPO) and MPO release from neutrophils. *In vivo*, the pre-treatment of rats with sulfasalazine (100 mg/kg) and MBSAE (25, 50, and 100 mg/kg) significantly reduced the AA-induced colonic mucosa lesion as well as histopathology changes. MBSAE counteracted AA-induced lipid peroxidation and ant antioxidant enzymes activities depletion such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx). We also found that myrtle extract inhibited the increase of plasma scavenging activity (PSA) and preserved non-enzymatic antioxidants content such a sulfhydryl groups (-SH) and reduced glutathione (GSH). More importantly, acetic acid administration increased colonic hydrogen peroxide (H_2O_2), free iron and calcium levels, while the MBSAE pre-treatment reversed all intracellular mediators perturbation.

In conclusion, our data suggests that MBSAE exerted a potential protective effect against AA-induced injury and oxidative stress in rat colon. This coli-protection might be related to in part, to its antioxidant and ROS scavenging activities or by negatively regulating Fenton reaction components such as H_2O_2 and free iron, which are known to lead to cytotoxicity mediated by intracellular calcium deregulation.

Key words: *Myrtus communis*, neutrophils, colitis, MPO, oxidative stress, Fenton reaction.

Abbreviations:

AA, acetic acid ; CAT, catalase ; DPPH, 2,2-diphenyl-1-picrylhydrazyl ; GPx, glutathione peroxidase ; fMLF, N-formyl-methionylleucyl-phenylalanine ; GSH, reduced glutathione; H_2O_2, hydrogen peroxide;MBSAE, myrtle berries seeds aqueous extract ; MDA, malondialdehyde ; MPO, myeloperoxidase ; PMA, phorbolmyristate acetate ; PSA, plasma scavenging activity ; ROS, Reactive oxygen species ; ROS, Reactive oxygen species ; -SH, sulfhydril groups ; SOD, superoxide dismutase.

1. Introduction

Inflammatory bowel disease (IBD), including ulcerative colitis and Crohn disease, are a chronically relapsing disorders of the intestine and they are characterized by aberrant immune responses to luminal bacteria in genetically susceptible subjects.[1,2] The active phase of ulcerative colitis (UC) is characterized by inflammation and injury in the colon.[3]The inflammation in ulcerative colitis is limited to the mucosal layer. It normally begins in the rectum where usually the highest activity of inflammation is found. In contrast, in Crohn's disease, the second major form on inflammatory bowel disease, the inflammation is transmural affecting all layers of the gut wall.[4] One of the main mechanisms involved in ulcerative colitis is oxidative stress induced via the generation of reactive oxygen species (ROS).[5] Oxidative stress arises from an imbalance between the generation of ROS and antioxidant in favour of the former. The ROS such as hydrogen peroxide H_2O_2, superoxide $O^{2\bullet-}$, and hydroxyl $OH^{\bullet}$ free radicals are toxic molecules capable of causing oxidative damages to lipids, proteins and DNA. However, under certain circumstances, their rate of production is dramatically elevated, affecting in turn the cellular mechanisms. [6] Lipids in general and polyunsaturated fatty acids in particular are the primary targets of ROS and elevated levels of lipid peroxidation products as well as impairment of membrane functions mediated through modifications of membrane lipids have been reported. [7] To mitigate and repair oxidative-induced damages and prevent ulcerative colitis, the use of plant-based remedies with antioxidant properties is highly desirable.

Myrtle (*Myrtus communis* L.), an endemic species from the Mediterranean area is widely used for its culinary and medicinal properties. Increasing body of evidences showed that Myrtle extracts were endowed with antioxidant, [8,9] anti-inflammatory,[10] antimicrobial,[11] antidiabetic[12] and anti-ulcer, among others.[13] These intriguing biological properties were attributed to a

plethora of bioactive components including organic acids, flavonoids and anthocyanes.[8] Despite that anti-ulcer effect of myrtle extract has been documented[13], its proptective effects against AA-induced colonic lesions has not received much attention.

Therefore, we aimed in the present study to evaluate the antioxidant properties of MBSAE as well as its protective effect against AA-induced colonic lesions in rats. The implication of some intracellular mediators in such coliprotection was also investigated.

2. Materials and methods

2.1. Reagents and Antibodies

PMA, fMLF, protease inhibitors were from Sigma–Aldrich (St Quentin Fallavier, France). Anti MPO antibody was from Abcam. Acztic acid, 5,5-dithio bis(2-nitrobenzoic acid) (DTNB), trichloroacetic acid (TCA), acetylcholine iodide, S-butyrylcholine, butylhydroxytoluene (BHT), 2,2-diphenyl-1-picrylhydrazyl (DPPH), methanol, ether, bovine serum albumin (BSA), orthophosphoric acid and NaCl were purchased from Sigma-Aldrich Co. (Germany).

2.2. Plant collection

Myrtle berries (*Myrtus communis* L.) seeds were collected in October, 2013 from the locality of Nefza (Northwestern Tunisia) and identified by the laboratory of taxonomy in the Faculty of Sciences of Tunis (FST)-Tunisia. The voucher specimens (No. MY01) has been deposited in the herbarium of the Higher Institute of Biotechnology of Beja and in the Department of Biological Sciences, Faculty of Science of Bizerte, Tunisia.

2.3. Plant extract preparation

The myrtle berries seeds were dried in an incubator at 50°C during 72 hours and powdered in an electric blender (MOULINEX Ovatio 2, France). Thereafter, seed powder was dissolved in double distilled water and incubated at room temperature for 24 h in a shaking bath. The sample was centrifuged at 10 000xg for 10 min and the supernatant was recovered, lyophilized and stored at -80°C until use.

2.4. Characterisation of phenolic compounds by HPLC-PDAESI-MS/MS

The lyophilized extract was dissolved (1 mg mL^{-1}) in methanol[14]and analyzed by LC-MS/MS using an Agilent Series 1100 LC system (Agilent Technologies, Palo Alto, Ca, USA) equipped with a photodiode array detector (PDA) and a triple quadrupole mass spectrometer type Micromass Autospec UltimaPt (Kelso, UK) interfaced with an ESI ion source. Separation was achieved using a Superspher® 100 (12.5 cm × 2 mm i.d., 4 mm, Agilent Technologies, Rising Sun, MD) at 45 °C.

Samples (20 µL) were eluted through the column with a gradient mobile phase consisting of A (0.1% acetic acid) and B (acetonitrile) with a flow rate of 0.25 mL min^{-1}. The following multistep linear solvent gradient was employed: 0–5 min, 2% B, 5–75 min, 88% B, 75.1–90 min, 2% B.

PDA was detected in the 200–700 nm wavelength range and the mass spectra were recorded in negative ion mode under the following operating conditions: capillary voltage, 3.2 kV; cone voltage, 115 V; probe temperature, 350 °C; ion source temperature, 110 °C. The spectra were acquired in an m/z range of 150–750 amu.

The identification of phenolic compounds was based on cochromatography with authentic standards, when available. PDA and mass spectra were used to affirm the identity of compounds previously reported in the literature.[15,16]

2.5. Ethics

Neutrophils were isolated from venous blood of healthy volunteers managed in the hematology and immunology department of Bichat Hospital, Paris, France. The investigations were approved by the local ethics committee and samples were obtained with the volunteers' and patients' written informed consent. All experiments were approved by the Institut

National de la Santé et de Recherche Médicale (INSERM) institutional review board and ethics committee. Data collection and analyses were performed anonymously.

2.6. Isolation and preparation of human neutrophils

Venous blood was collected from healthy adult volunteers and neutrophils were isolated by Dextran sedimentation and density gradient centrifugation as previously described by El-Benna and Dang.[17] Erythrocytes were removed by hypotonic lysis. Following isolation, the cells were resuspended in Hank's balanced salt solution (HBSS). The cells were counted and their viability was determined with the trypan blue exclusion method.

2.7. Measurement of ROS production by chemiluminescence

Isolated cells were resuspended in HBSS at a concentration of 1 million per mL. Cell suspensions (5×10^5) in 0.5 mL of HBSS containing 10 µM luminol in the presence or absence of MBSAE were preheated to 37°C in the thermostatted chamber of a luminometer (Berthold-Biolumat LB937) and allowed to stabilize. After a baseline reading, cells were stimulated with 0.1 µM fMLF or 100 ng/mL PMA. Changes in chemiluminescence were measured over a 30-min period.

2.8. Measurement of superoxide anion production

Isolated cells were also resuspended in HBSS at a concentration of 1 million per mL. Cell suspensions in 1 mL of HBSS containing 1 mg/mL cytochrome c in the presence or absence of MBSAE were preheated to 37°C in the thermostatted chamber of a spectrophotometer (Uvikon) and allowed to stabilize. After a baseline reading, cells were stimulated with 0.1 µM fMLF or 100 ng/mL PMA. Changes in absorbance were measured at 550 nm over a 15-min period.

2.9. Measurement of H_2O_2 inhibition by chemiluminescence

The effect of MBSAE on H_2O_2 was tested in a cell free system using horseradish peroxydase (HRPO). The reaction mixture contained 10 µM luminol in the presence or absence of MBSAE. The reaction was started by addition of 2.5 U/mL horseradish peroxydase (HRPO), and lucigeninchemiluminescence was measured at 37°C for 30 min in a luminometer (Berthold-Biolumat LB937).

2.10. Measurement of Released MPO from Neutrophils

Immunoblotting of MPO released by PMA stimulated neutrophils was done with primary rabbit antibodies against MPO (dilution: 1/10.000), and with a horseradish peroxidase (HRP)-labeled-goat anti-rabbit antibody (dilution: 1/10.000). The reaction was measured by using enhanced chemiluminescence (ECL) reagents.

2.11. Animals and treatments

Adult male *Wistar* rats (weighing 220–240 g, 15 weeks old and housed five per cage) were purchased from Pasteur Institute of Tunis and used in accordance with the local ethics committee of Tunis University for the use and care of animals and in accordance with the NIH recommendations.[18] They were provided with standard food (ALMES, TN) and water *ad libitum*, and maintained in animal house at controlled temperature (22 ± 2 °C) with a 12-12 h light–dark cycle. Rats were divided into six groups of 10 animals each. Group 1 and 2 served as controls and received bidistilled water (5 mL/kg, *b.w., p.o.*) during seven days. Groups 3, 4, and 5 were pre-treated with various doses of MBSAE (25, 50 and 100 mg/kg, *b.w. p.o.*), while group 6 was pre-treated with sulfasalazine (100 mg/kg, *b.w. p.o.*) during the same period before induction of ulcerative colitis.

2.12. Induction of ulcerative colitis in rats

All the animals were kept fasting overnight. Ulcerative colitis was induced by the infusion of acetic acid (3%, *v/v*, 5 mL/kg, *b.w.*) for 30s using a polyethylene tube, inserted through rectum into the colon up to a distance of 8 cm. 24 h later, animals were sacrificed; the colon was rapidly excised, macroscopically examined and homogenized in phosphate buffer saline for determining biochemical parameters. The remaining portion of colon was kept in 10% formalin for histopathological study. Blood was also collected in heparinized tubes. After centrifugation at 3000 g for 15 min, plasma was stored at −20 °C.

2.13. Assessments of colitis

For each animal, the distal portions of colon were removed and cut longitudinally, cleaned with physiological saline to remove fecal residues. Macroscopic inflammation scores are assigned based on the clinical features of the colon using an arbitrary scale ranging from 0-4 according to Millar et al.[19] as follows: 0 (no macroscopic changes), 1 (mucosal erythema only), 2 (mild mucosal oedema, slight bleeding or small erosions), 3 (moderate oedema, slight bleeding ulcers or erosions) and 4 (severe ulceration, edema and tissue necrosis).

2.14. Histopathological study

Immediately after sacrifice, tissue samples for the histopathological study were dehydrated in a 10% neutral buffered formalin solution, embedded in paraffin and used for histopathological examination. Sections of 5 µm thickness were cut, deparaffinized, hydrated and stained with hematoxylin and eosin. The colon sections were examined in blind fashion in all treatments.

2.15. Biochemical assays

2.15.1. Plasma scavenging activity

The free radical scavenging activity of plasma samples was measured using the DPPH radical method according to Brand-Williams et al.[20] Briefly, 100 µL of each plasma sample was added to 2 mL of 2,2-diphenyl-1-picrylhydrazyl (DPPH) in methanol solution (100 mM). After incubation at 37 °C for 30 min, 1 mL of chloroform was added and the solution was centrifuged at 3000 g for 10 min. The absorbance of clear supernatant was then determined at 517 nm using spectrophotometer. DPPH solution was used as control and the plasma scavenging activity (PSA), expressed in percentage, was calculated according to the following equation: PSA (%) =100 × (A_{517}(control) × A_{517}(sample)/A_{517}(control)

2.15.2. Lipid peroxidation measurement

Colon lipid peroxidation was determined by MDA measurement according to the double heating method.[21] Briefly, aliquots from colon tissue homogenates were mixed with BHT-TCA solution containing 1% BHT (w/v) dissolved in 20% TCA (w/v) and centrifuged at 1000 *g* for 5 min at 4°C. Supernatant was blended with 0.5 N HCl 120 mM TBA in 26 mM Tris and then heated at 80°C for 10 min. After cooling, absorbance of the resulting chromophore was determined at 532 nm using a UV-visible spectrophotometer (Beckman DU 640B). MDA levels were determined by using an extinction coefficient for MDA-TBA complex of 1.56 10^5 $M^{-1}cm^{-1}$.

2.15.3. Antioxidant enzymes activities assays

The activity of SOD was determined using modified epinephrine assays.[22] At alkaline pH, superoxide anion $O_2^{\bullet-}$ causes the autoxidation of epinephrine to adenochrome; while competing with this reaction, SOD decreased the adenochrome formation. One unit of SOD is

defined as the amount of the extract that inhibits the rate of adenochrome formation by 50%. Enzyme extract was added to 2 mL reaction mixture containing 10 µl of bovine catalase (0.4 U/µl), 20 µL of epinephrine (5 mg/mL) and 62.5 mM of sodium carbonate/bicarbonate buffer pH 10.2. Changes in absorbance were recorded at 480 nm.

CAT activity was assayed by measuring the initial rate of H_2O_2 disappearance at 240 nm.[23] The reaction mixture contained 33 mM H_2O_2 in 50 mM phosphate buffer pH 7.0 and CAT activity was calculated using the extinction coefficient of 40 $mM^{-1}cm^{-1}$ for H_2O_2.

The activity of GPx was quantified following the procedure of Flohé and Günzler.[24] Briefly, 1 mL of reaction mixture containing 0.2 mL of colonic supernatant, 0.2 mL of phosphate buffer 0.1 M pH 7.4, 0.2 mL of GSH (4 mM) and 0.4 mL of H_2O_2 (5 mM) was incubated at 37°C for 1 min and the reaction was stopped by the addition of 0.5 mL TCA (5%, w/v). After centrifugation at 1500 g for 5 min, aliquot (0.2 mL) from supernatant was combined with 0.5 mL of phosphate buffer 0.1 M pH 7.4 and 0.5 mL DTNB (10 mM) and absorbance was read at 412 nm. The activity of GPx was expressed as nmol of GSH consumed/min/mg protein.

2.15.4. Non-enzymatic antioxidants measurement

The total concentration of thiol groups (-SH) was performed according to Ellman's method.[25] Briefly, homogenates of colonic tissue were mixed with 800 µL of 0.25 M phosphate buffer (pH 8.2) and 100 µL of 20 mM EDTA, and the optical density was measured at 412 nm (A1). Then, 100 µL of 10 mM DTNB were added, incubated during 15 min and the absorbance of the sample was quantified at 412 nm (A2). The thiolgroups concentration was calculated from A1 to A0 subtraction using a molar extinction coefficient of 13.6×10^3 $M^{-1}\times cm^{-1}$. The results were expressed as µmo of thiol groups per mg of protein.

GSH was estimated in colonic tissue by the method of Sedlak and Lindsay.[26] Briefly, 500µL of tissue homogenate prepared in 20 mM EDTA, (pH 4.7) were mixed with 400µL of cold

distilled water and 100 µL of 50% TCA. The samples were shaken using vortex mixer and centrifuged at 1200×g during 15 min. Following centrifugation, 2 mL of supernatant were mixed with 400 µL of 400 mMTris–buffer (pH 8.9) and 10 µL of 10 mM DTNB. The absorbance was read at 412 nm against blank tube without homogenate.

2.15.5. H_2O_2 determination

The colon H_2O_2 level was performed according to Dingeon et al.[27] Briefly, in the presence of peroxidase, the hydrogen peroxide reacts with p-hydroxybenzoic acid and 4-aminoantipyrine leading to a quantitative formation of a quinoneimine which has a pink color detected at 505 nm.

2.15.6. Iron measurement

Colon non haem iron were measured colorimetrically using ferrozine as described by Leardi et al.[28] Briefly, the iron dissociated from transferrin-iron complex by a solution of guanidine acetate and reduced by ascorbic acid reacts with ferrozine to give a pink complex measured at 562 nm.

2.15.7. Calcium determination

Colonic calcium level was performed using colorimetric method according to Stern and Lewis.[29] Briefly, at alkaline medium, calcium reacts with cresolphtalein leading to a coloured complex measurable at 570 nm.

2.15.8. Protein determination

Protein concentration was determined according to Hartree[30] which is a slight modification of the Lowry method. Serum albumin was used as standard.

2.16. Statistical analysis

Data were analyzed by unpaired Student's t-test or one-way analysis of variance (ANOVA) and are expressed as means ± standard error of the mean (SEM). Data are representative of four independent experiments. All statistical tests were two-tailed, and a p value of 0.05 or less was considered significant.

3. Results

3.1. HPLC-PDA-ESI-MS/MS analysis

The combination of UV and MS spectral data allowed a tentative identification of 18 phenolic compounds distributed in three major groups; hydroxybenzoic acid derivatives, anthocyanins derivatives and flavonols derivatives (Fig. 1 and Table 1).

The only representative compound (peak 1; Tr = 6.71 min) in the first group was identified as hydroxybenzoic acid hexose based on its UV spectrum with a maximum at 254 nm. It exhibit a deprotonated $[M-H]^-$ ion at m/z 299 and a major fragment ion at m/z 137, corresponding to the loss of hexose moiety (162 amu).

The second group showed characteristic UV spectra of anthocyanins with maximum absorbance around 520 nm. Another feature for the identification of anthocyanins is based on the presence of doublet ions $[M-2H]^-$ and $[M-2H+H_2O]^-$ in negative mode.[31] In the present study, 14 anthocyanins were found in *M. communis* berries seeds. Peaks 2 and 3, eluted at 7.94 and 8.93 min, respectively, showed a pseudomolecular $[M-2H]^-$ ion at m/z 463 and the loss of hexose (162 amu) moiety resulted in a dominant fragment ion at m/z 301 indicative to delphinidin aglycon. Considering their elution order, peak 2 and 3 were tentatively identified as delphinidin-3-*O*-galactoside and delphinidin-3-*O*-glucoside.[31,32] By using the same procedure, peaks 5 (Tr = 10.33 min) was identified as delphinidin-3-*O*-rhamnoside. This compound exhibited a pseudomolecular ion $[M-2H]^-$ at m/z 447 and major fragment ion at m/z 301 [M-2H-146] corresponding to the loss of rhamnose residue (146 amu). By using the same procedure adopted for peaks 2 and 3, peaks 6 and 7 eluted at 12.27 and 15.95 min, respectively, were tentatively identified as delphinidin rutinoside and delphinidin-3-(6-coumaroyl)-glucoside. Both components showed a deprotonated molecular $[M-2H]^-$ ion at m/z 609 and a major fragment ion at m/z 301 indicative to delphinidin aglycon. The loss of

308 corresponding to the rahmnoside residue or coumaroylglucose allowed the identification of compound 6 as delphinidin rutinoside[33] and compound 7 as delphinidin-3-(6-coumaroyl)-glucoside.[34] Peak 8 eluted at 17.25 min exhibited a deprotonated molecular $[M-2H+H_2O]^-$ ion at m/z 493 and a major fragment ion at m/z 315 (petunidin aglycon) corresponding to the loss of hexose moiety (162 amu). This compound was tentatively identified as petunidin-3-*O*-glucoside.[31] Peak 9 (Tr = 20.38 min) showed a pseudomolecular ion $[M-2H+H_2O]^-$ at m/z 655 and the loss of 2 glucoside residues resulted in the formation of a major fragment ion at m/z 315 (petunidin aglycon). Consequently, peak 9 was identified as petunidin diglucoside.[31] Peak 10 eluted at 29.25 min had a petunidin aglycon (fragment m/z 315) and a pseudomolecular ion at $[M-2H]^-$ at m/z 563. The fragment loss was 248 which could be due to a malonyglucoside residue. This anthocyanin was identified as petunidin malonylglucoside. Peak 11 eluted at 30.66 min exhibited a pseudomolecular ion $[M-2H]^-$ at 621 and the loss of rhamnoside residue (308 amu) generates a major fragment ion at m/z 315 suggesting a petunidin aglycon. This component was identified as petunidin-3-*O*-rutinoside. Peak 13 (Tr = 30.78 min) had a peonidin aglycon (fragment m/z 299) and a pseudomolecular ion at $[M-2H+H_2O]^-$ at m/z 615. The fragment loss was 298 which could be due to a sambubioside residue. This component was tentatively identified as peonidin sambubioside.[35] Peaks 15 and 16 eluted at 39.94 and 41.07 min, respectively, showed a pseudomolecular ion $[M-2H+H_2O]^-$ at m/z 511 and a major fragment ion at m/z 311 (malvidin aglycon) corresponding to the loss of hexose residue (162 amu). Considering their elution order, these components were tentatively identified as malvidin-*O*-galactoside and malvidin-*O*-glucoside.[31] Peak 17 (Tr = 44.04 min) with a pseudomolecular ion $[M-2H+H_2O]^-$ at 641 give a major fragment ion at m/z 299 (peonidin aglycon) following the loss of 2 glucoside residues. This anthocyanin was identified as peonidin diglucoside. Peak 18 (Tr = 69.95 min) exhibited a pseudomolecular ion $[M-2H]^-$ at m/z 461 and a fragment ion at m/z 331 (petunidin aglycon). The fragment loss was

146 which could be due to a methyl pentose.[31]This component was identified as petunidin methyl pentose.

In addition to hydroxybenzoic acid derivative and anthocyanins derivatives, the *M. communis* berries seeds also revealed the presence of three flavonols derivatives. They were characterized based on their characteristic UV spectra (around 350 nm) and mass spectral data. The first flavonol was eluted at 9.04 min and exhibited a deprotonated $[M-H]^-$ ion at m/z 463 and the loss of a hexose moiety (162 amu) a major fragment ion at m/z 301 indicative to the quercetin aglycon. This component was identified as quercetin hexoside. Peak 12 eluted at 30.74 had a isorhamnetin aglycon (fragment m/z at 315) and a pseudomolecular ion $[M-H]^-$ at m/z 447. The loss of 132 amu corresponding to the pentose residue allowed identification of this flavonol as isorhamnetin pentoside. The latter compound (peak 14, Tr = 39.9 min) showed a pseudomolecular ion $[M-H]^-$ at m/z 461 and a fragment ion at m/z 315 (isorhamnetin aglycon) corresponding to the loss of rhamnose residue (146 amu). This flavonol was tentatively identified as isorhamnetin-*O*-rhamnoside.

3.2. Effect of MBSAE on luminol-amplified chemiluminescence in human neutrophils

MBSAE was tested on ROS production in response to fMLF and PMA. Results show that MBSAE significantly and dose-dependently inhibited luminol-amplified chemiluminescence in neutrophils stimulated with PMA (Fig. 2A) and fMLF (Fig. 2B).

3.3. Effect of MBSAE on fMLF and PMA-induced neutrophils superoxide anion production

As illustrated in figure 3, fMLF and PMA induced a potent experimental superoxide anion production by human neutrophils. However, MBSAE had no effect at these doses on cytochrome c reduction as well as NADPH oxidase activity.

3.4. Effect of MBSAE on H_2O_2 production in a cell free system

We next decided to evaluate the role of MBSAE on H_2O_2 production in a cell free system (Fig. 4). Results show that MBSAE significantly and dose-dependently inhibited luminol-amplified chemiluminescence stimulated with horseradish peroxydase (HRPO).

3.5. Effect of MBSAE on PMA-Induced MPO Release from Neutrophils

We also studied the effect of MBSAE on PMA-induced MPO expression by western blot analysis (Fig. 5). PMA *per se* clearly stimulated the expression of MPO. However MBSAE co-treatment significantly abolished PMA-induced MPO expression in a dose-dependent manner.

3.6. Qualitative and quantitative gross evaluation of colon lesions

The antiulcer activity of myrtle berries seeds aqueous extract on AA-induced ulcerative colitis is shown in Figure 6. Indeed, acetic acid administration produced extensive visible black hemorrhagic lesions, edema and necrosis of colon mucosa, accompanied by an increase of colonic affected area, score lesions and wet colon weight (Table 2). However, pre-treatment with MBSAE significantly and dose-dependently reduced these perturbations compared to colitis control group. The significant inhibition of ulcerative colitis in rats pretreated with the high dose of MBSAE was comparable to sulfasalazine, a standard drug used for curing colonic ulcer (Fig. 6 and Table 2).

3.7. Histopathological study

As shown in Figure 7, acetic acid-induced colitis was accompanied by a substantial structural changes such as congestion of vascular and epithelial cells of the mucosa and submucosa, alteration of surface coating, edema, necrosis and diffuse inflammatory cell infiltration in the mucosa and sub-mucosa. MBSAE pre-treatment greatly reduced the histopathological changes induced by acute acetic acid intoxication. Sulfasalazine, used as standard drug, also significantly protected against acetic acid-induced colon cells damage.

3.8. Effect of AA and MBSAE on colonic lipid peroxidation

Concerning the effect of AA and MBSAE on oxidative stress condition, we firstly studied the colonic lipid peroxidation. AA *per se* drastically increased the colonic MDA level when compared to control group. Pre-treatment with MBSAE significantly and dose-dependently decreased the colonic MDA level. Sulfasalazine, used as standard drug, also decreased the colonic lipid peroxidation compared to ulcerative group (Table 3).

3.9. Effect of AA and MBSAE on plasma scavenging activity

Acetic acid-induced ulcerative colitis significantly decreased the plasma scavenging activity as compared to control group (Fig. 8). However, pre-treatment with MBSAE significantly increased the PSA in a dose-dependent manner. Administration of sulfasalazine also increased the plasma scavenging activity when compared to colitis rats.

3.10. Effect of AA and MBSAE on antioxidant enzyme activities

The effect of acetic acid and MBSAE treatment on colon antioxidant enzymes activities was also investigated and the results are presented in figure 8. Acetic acid-induced ulcerative colitis was accompanied by a significant decrease in colon antioxidant enzyme activities such as SOD, CAT and GPx. This reduction was significantly and dose-dependently improved by MBSAE (25, 50 and 100 mg/kg, *p.o*) treatment as well as sulfasalazine at100 mg/kg, *p.o*. (Table 3).

3.11. Effect of MBSE on non-enzymatic antioxidants levels

We also showed that thiol groups and reduced glutathione levels were significantly reduced in the colons of acetic acid-treated rats. However MBSAE (25, 50 and 100 mg/kg, *b.w. p.o.*) or sulfasalazine (100 mg/kg, *b.w. p.o.*) pre-treatment significantly protected against this decrease when compared to colitis control group (Table 3).

3.12. Effect of MBSE on free iron, H_2O_2 and calcium levels

We further looked at the effect of AA and MBSAE on some intracellular mediators such as hydrogen peroxide, free iron and calcium. As expected, AA administration increased iron, H_2O_2 and calcium levels. MBSAE and sulfasalazine pre-treatment significantly protected against AA-induced intracellular mediators disturbances in a dose-dependent manner (Table 4).

4. Discussion

We firstly showed, in the present investigation, that the phytochemical study of MBSAE using HPLC-PDA-ESI-MS/MS analysis allowed us to identify 18 phenolic compounds, distributed in three major groups; hydroxybenzoic acid derivatives, anthocyanins derivatives and flavonols derivatives. These molecules especially anthocyanins have been previously identified in many plant extracts such as raspberry seeds,[37] blueberries,[38] elderberry[39] and Strawberry.[40] Therefore, owing mainly to their antioxidant and anti-inflammatory properties,36 anthocyanins are known to exhibit many beneficial health effects such as cardio-protective, neuro-protective, and anticancer activities.[39,41]

On the other hand, we tested the myrtle berry seeds aqueous extract on human neutrophils total ROS production, in response to chemotactic peptide (fMLF) and protein kinase C activator (PMA) stimulation, as well as on H_2O_2 accumulation in a cell free system. Importantly, we showed that MBSAE (0.62 ; 1.25 ; 2.5 and 5 ng/mL) treatment significantly inhibited luminol-amplified chemiluminescence in neutrophils and H_2O_2 production in a cell free system in a dose-dependent manner. At these concentrations, MBSAE did not affect neutrophils viability, determined through trypan blue exclusion test (data not shown). MBSAE had also no effect on cytochrome c reduction by human neutrophils stimulated with fMLF or PMA, suggesting that it does not affect NADPH oxidase activity or scavenge superoxide anions as previously described for pomegranate peel extract.[42] To test whether this inhibitory effect may reflect decreased neutrophil degranulation, or is related to decreased enzymatic activity, we performed a western blotting analysis of the supernatant from PMA-stimulated samples, using an antibody directed against human MPO. In this respect, we showed that MBSAE inhibited MPO activity in a concentration-dependent manner. These results suggest that MBSAE inhibits luminol-amplified chemiluminescence in neutrophils by

inhibiting neutrophil degranulation and H_2O_2 production. In fact the heme peroxidase enzyme myeloperoxidase (MPO) is released by activated neutrophils and monocytes, which used hydrogen peroxide (H_2O_2) to catalyze the production of the potent oxidants hypochlorous acid (HOCl), hypobromous acid (HOBr) and hypothiocyanous acid (HOSCN) from halide and pseudohalide (SCN^-) ions.[43] These oxidants have been implicated as key mediators of tissue damage in many human inflammatory diseases including inflammatory bowel disease.[43,44] In this respect, we further used, ulcerative colitis, as model of inflammatory bowel disease.

In vivo, we have shown that acute anal administration of acetic acid (3% v/v, 10 mL/kg, PC) clearly induced a severe ulcerations in the colonic mucosa. These morphological perturbations are accompanied by an increase in wet weight of the colon, which is considered as a reliable and sensitive indicator of the severity and extent of inflammatory response.[45]Acetic acid-induced colitis is a model characterized a severe activity of apoptosis, leading to epithelial cells noxious stimuli such as accumulation of phagocytic cells in the lamina propria, particularly neutrophils which release upon activation of a large amounts of reactive oxygen species that are cytotoxic to the mucosa cells.[46,47] In the histopathological study, we have shown that the acetic acid administration induces structural changes such as massive necrotic destruction of epithelium, submucosal edema, areas of haemorrhages and inflammatory cellular infiltration mainly neutrophils and macrophages. Indeed, the transepithelial resistance is reduced in the inflamed mucosa of ulcerative colitis.[48] However, the tissue destruction and necrosis are in part associated to the release of proteases, such as metalloproteinases and elastase which induced epithelial cells apoptosis leading to their basement membrane.[46,49] Our data also showed that sub-acute pre-treatment with MBSAE or sulfasalazine abolished all macroscopic and histological changes induced by acetic acid intoxication in a dose-related manner. This anti-inflammatory effect is in part attributed to the richness of MBSAE in anthocyanins. These later, are known to exert multiple protective effects against pleurisy in

rat model and capable of attenuating inflammation.[50] Anthocyanins treatment also downregulated expression of enzymes involved in inflammation in the lung,[50] endothelial cells[51] as well as Caco-2 intestinal cells.[52]

We also showed in the present study, that single anal administration of acetic acid induced an oxidative stress status in the colon mucosa assessed by an increase lipoperoxidation, decrease in plasma scavenging activity, depletion of antioxidant enzyme activities such as SOD, CAT and GPx, as well as deleterious effects on non-enzymatic antioxidants such as reduced glutathione and sulfhydryl groups. These data fully corroborated previous reports, indicating that acetic acid-induced colitis is accompanied by an oxidative balance perturbation.[53,54,55] Indeed colonic inflammation is characterized by infiltration of neutrophils that migrate to the colonic wall after aggression.[56] However the oxygen consumed by neutrophils is enzymatically converted to superoxide anion ($O_2^{\bullet-}$) by univalent transfer of two electrons from the cell NADPH of the hexose monophosphate pathway.[57] In addition, $O_2^{\bullet-}$ is the source of other ROS such as hydrogen peroxide (H_2O_2) and the hydroxyl radical $OH^{\bullet}$ known for its high toxicity.[58]

Biochemical lesions created by the ROS are multiple, inducing lipid peroxidation leading to membrane disruption, protein damages with fragmentation, aggregation, oxidation of sulfhydryl groups and a destruction of nucleic acids with DNA breakage and mutation.[44] More importantly, we demonstrated that MBSAE pre-treatment protects against acetic acid-induced oxidative stress in colon mucosa. In the present investigation we have shown that MBSAE is rich in phenolic compounds paticulary anthocyanins. However, these molecules are the primal source of the antioxidant ability of this plant by scavenging free radicals, such as hydroxyl radical ($OH^{\bullet}$), which is the major cause of lipid peroxidation.[59] Indeed, Cyanidin-*3*-glucoside and other anthocyanins from fruits and plant extracts were recently presented to have both *in vitro* and *in vivo* antioxidant properties.[60,61,62] To the best of our knowledge, our

report is the first where we identify the phenolic compounds myrtle berry seeds aqueous extract and study their action on ROS production by human neutrophils and MPO activity as well as their protective effet againt acetic acid-induced colon injuries.

MBSAE pre-treatment also abolished colon H_2O_2 and free iron accumulation induced by acetic acid administration. In fact, these two components catalyzed the highly toxic hydroxyl radical ($OH^{\bullet}$) production via the Fenton reaction, leading to membrane lipoperoxidation.[63,64] We also showed that a acetic acid intoxication induced an increase in colonic calcium level, while MBSAE protects against this homeostasis disturbance. Indeed, it well known that ROS can cause a rapid rise in calcium level in the cytoplasm of different cell types.[65,66,67] However, we can now suggest that MBSAE also acts in part through a complex mechanism in preventing the colic calcium accumulation. The same mechanism was previously described for other plant extracts rich in phenolic compounds such as *Vitis vinifera*,[68] *Artemisia campestris*[14] and *Matricaria recutita*.[64]

Conclusion

In conclusion, our data clearly demonstrate that Myrtle berry seeds aqueous extract exerts protective effects against acute acetic acid-induced injury in the rat colon owing in part to its antioxidant properties and its opposite effect on some intracellular mediators such as free iron, hydrogen peroxide and calcium.

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Declaration of interest

The authors alone are responsible for the content of this paper.

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Legend of figures

Figure 1: Representative LCMS-TIC of phenolic compounds in the aqueous extract of M. communis seeds (assignments of peaks are given in Table 1).

Figure 2: Effect of MBSAE on luminol-amplified chemiluminescence in human neutrophils. Human neutrophils (5×10^5) were incubated in the presence or absence of different MBSAE concentrations and stimulated with PMA(**A**) or fMLF (**B**). Luminol-amplified chemiluminescence was measured for 30 min (Data are presented as means ± S.E.M. of five independent experiments, *$p< 0.05$).

Figure 3 Effect of myrtle berries seed aqueous extract (MBSAE) on superoxide anion production by human neutrophils using cytochrome c reduction assay. Cells (1×10^6) were incubated in the presence or not of different MBSAE concentrations and stimulated with PMA (100 ng/mL) or fMLF (0.1 µM). Cytochrome c reduction was measured at 550 nm in a spectrophotometer for 10 min (Data are presented as means ± S.E.M. of five independent experiments, *$p< 0.05$).

Figure 4: Effect of MBSAE on luminol-amplified chemiluminescence in the presence or not of different concentrations myrtle berries seed aqueous extract (MBSAE) and stimulated with 2.5 U/mL horseradish peroxydase (HRPO). Luminol-amplified chemiluminescence was measured for 30 min (Data are presented as means ± SEM of five independent experiments, *$p< 0.05$).

Figure 5: Western blot analysis of PMA and different concentrations of myrtle berries seed aqueous extract (MBSAE) on MPO expression in human neutrophils. MPO protein was determined by immunoblotting with anti MPO antibody (Data are presented as means ± SEM of five independent experiments, *p< 0.05).

Figure 6: Effect of myrtle berries seed aqueous extract (MBSAE) and sulfasalazine on acetic acid (AA)-induced changes in colon morphology. Animals were treated with various doses of MBSAE (25, 50 and 100 mg/kg, *b.w., p.o.*), reference molecule (Sulfasalazine, 100 mg/kg, b.w., p.o) or bidistilled water, challenged with a single anal administration of AA (300 mg/kg, b.w., p.o.) (3%, v/v, 5 mL/kg, b.w.)or NaCl 9‰ for 24 hours. ***(A)*** *: H_2O + NaCl,* ***(B)*** *: AA + H_2O,* ***(C)*** *: AA +MBSAE (25 mg/kg, PC, p.o.),* ***(D)*** *: AA + MBSAE (50 mg/kg, PC, p.o.),* ***(E)*** *: AA +MBSAE (100 mg/kg, PC, p.o.),* ***(F)*** *: AA + Sulfasalazine (100 mg/kg, PC, p.o.).*

Figure 7: Effect of myrtle berries seed aqueous extract (MBSAE) and sulfasalazine on acetic acid (AA)-induced changes in colon histology. Animals were treated with various doses of MBSAE (25, 50 and 100 mg/kg, *b.w., p.o.*), reference molecule (Sulfasalazine, 100 mg/kg, b.w., p.o) or bidistilled water, challenged with a single anal administration of AA (300 mg/kg, b.w., p.o.) (3%, v/v, 5 mL/kg, b.w.)or NaCl 9‰ for 24 hours. ***(A)*** *: H_2O + NaCl,* ***(B)*** *: AA + H_2O,* ***(C)*** *: AA +MBSAE (25 mg/kg, PC, p.o.),* ***(D)*** *: AA + MBSAE (50 mg/kg, PC, p.o.),* ***(E)*** *: AA +MBSAE (100 mg/kg, PC, p.o.),* ***(F)*** *: AA + Sulfasalazine (100 mg/kg, PC, p.o.).*

Figure 8: Effect of myrtle berries seeds aqueous extract (MBSAE) and sulfasalazine on acetic acid (AA)-induced changes in plasma scavenging activity (PSA). Animals were treated with various doses of MBSAE (25, 50 and 100 mg/kg, *b.w., p.o.*), reference molecule (Sulfasalazine, 100 mg/kg, *b.w., p.o.*) or bidistilled water, challenged with a single anal administration of AA (300 mg/kg, b.w., p.o.) (3%, v/v, 5 mL/kg, b.w.) or NaCl 9‰ for 24 hours. The data are expressed as mean ± S.E.M. (n=10)

* :$p< 0.05$ compared to control group and #: $p < 0.05$ compared to colitis group.

Table 1: Characterisation of phenolic compounds of *Myrtus communis* berries seeds by HPLC-DAD-ESI-MS/MS

Peak no.	t_R (min)	λ max (nm)	$[M-H]^-$ or $[M-2H]^-$ (m/z)	Fragment ion (m/z)	Tentative Identification
1	6.71	254	299	137	Hydroxybenzoic acid hexose
2	7.94	276 ; 515	463	301	Delphinidin-3-*O*-galactoside
3	8.93	276 ; 520	463	301	Delphinidin-3-*O*-glucoside
4	9.04	354	463	301	Quercetin hexoside
5	10.33	276 ; 515	447	301	Delphinidin-3-*O*-rhamnoside
6	12.27	276 ; 515	609	301	Delphinidin rutinoside
7	15.95	276 ; 520	609	301	Delphinidin-3- (6 coumaroyl)-glucoside
8	17.25	278 ; 524	493	315	Petunidin-3-*O*- glucoside
9	20.38	278 ; 525	655	315	Petunidin diglucoside
10	29.25	278 ; 525	563	315	Petunidin malonylglucoside
11	30.66	525	621	315	Petunidin-3-*O*-rutinoside
12	30.74	278 ; 355	447	315	Isorhamnetin-*O*-pentoside
13	30.78	276 ; 516	615	299	Peonidin sambubioside
14	39.90	351	461	315	Isorhamnetin-*O*-rhamnoside
15	39.94	282 ; 527	511	331	Malvidin-*O*-galactoside
16	41.07	282 ; 527	511	331	Malvidin-*O*-glucoside
17	44.04	276 ; 516	641	299	Peonidin diglucoside
18	69.95	278 ; 525	461	331	Petunidin methyl pentose

Table 2: Effect of myrtle berries seedaqueous extract (MBSAE) and sulfasalazine on acetic acid (AA)-induced changes in cross lesion score and colon weight to length ratio. Animals were treated with various doses of MBSAE (25, 50 and 100 mg/kg, *p.o.*), reference molecule (Sulfasalazine 100 mg/kg, b.w, p.o) or bidistilled water, challenged with a single anal administration of AA (300 mg/kg, b.w., p.o.) (3%, v/v, 5 mL/kg, b.w.) or NaCl 9‰ for 24 hours. The data are expressed as mean ± S.E.M. (n=10)

* :$p< 0.05$ compared to control group and #: $p < 0.05$ compared to colitis group.

Treatment	Gross lesion score	Wet colon weight/length (mg/cm)	(% protection)
Control	00 ± 00	82.44 ± 4.49	--
Colitis	4.2 ± 0.20*	163.22 ±10.66*	--
Colitis + MBSAE 25	3.0 ± 0.31#	138.50 ± 3.43#	37.50
Colitis + MBSAE 50	2.4 ± 0.40#	116.31 ± 3.72#	50.00
Colitis + MBSAE 100	1.4 ± 0.50#	96.36 ± 5.57#	70.83
Colitis + Sulfasalazine	0.8 ± 0.37#	87.17 ± 2.84#	83.33

Table 3: Effect of myrtle berries seed aqueous extract (MBSAE) and sulfasalazine on acetic acid (AA)-induced colon oxidative stress. Animals were treated with various doses of MBSAE (25, 50 and 100 mg/kg, *b.w., p.o.*), reference molecule (Sulfasalazine, 100 mg/kg, b.w., p.o) or bidistilled water, challenged with a single anal administration of AA (300 mg/kg, b.w., p.o.) (3%, v/v, 5 mL/kg, b.w.)or NaCl 9‰ for 24 hours. MDA, nmol of MDA/mg protein; SOD, units/mg protein (one unit of the SOD activity is the amount of enzyme required to give 50% inhibition of epinephrine auto oxidation); CAT, µmol of H_2O_2 consumed/min/mg protein; GPx, nmol GSH oxidized/min/mg protein; -SH groups, µmol/mg protein; GSH, nmol of GSH/mg protein.

The data are expressed as mean ± S.E.M. (n=10)

* :$p< 0.05$ compared to control group and #: $p < 0.05$ compared to colitis group.

Treatment	MDA	SOD	CAT	GPx	-SH groups	GSH
Control	1.19 ± 0.17	14.26 ± 0.76	18.44 ± 0.84	24.81 ± 0.94	80.86 ± 3.61	22.30 ± 0.85
Colitis	4.24 ± 0.35*	7.63 ± 0.39*	8.05 ± 0.65*	12.52 ± 0.56*	47.91 ± 2.85*	11.64 ± 0.68
Colitis + MBSAE 25	2.80 ± 0.34#	9.72 ± 0.65#	11.22 ± 0.71#	16.92 ± 0.79#	64.14 ± 2.23#	16.98 ± 0.47#
Colitis + MBSAE 50	2.23 ± 0.31#	11.01 ± 0.80#	13.93 ± 0.84#	19.66 ± 0.64#	68.58 ± 3.79#	17.24 ± 0.98#
Colitis + MBSAE 100	1.71 ± 0.20#	12.19 ± 0.65#	15.84 ± 0.53#	21.10 ± 0.84#	72.59 ± 2.71#	19.26 ± 1.06#
Colitis + Sulfasalazine	1.46 ± 0.25#	13.18 ± 0.91#	16.14 ± 0.74#	22.37 ± 0.91#	77.69 ± 3.48#	20.58 ± 0.91#

Table 4: Effect of myrtle berries seed aqueous extract (MBSAE) and sulfasalazine on acetic acid (AA)-induced changes in colon free iron, H_2O_2 and calcium. Animals were pre-treated with various doses of MBSAE (250, 500 and 750 mg/kg, *p.o.*) and reference molecule (Sulfasalazine, 100 mg/kg, b.w, p.o) or bidistilled water, challenged with a single anal administration of AA (300 mg/kg, b.w., p.o.) (3%, v/v, 5 mL/kg, b.w.) or NaCl 9‰ for 24 hours. The data are expressed as mean ± S.E.M. (n=10)

* :p< 0.05 compared to control group and #: p < 0.05 compared to colitis group.

Treatment	Free iron (µmol/mg protein)	H_2O_2 (mmol/mg protein)	Calcium (mmol/mg protein)
Control	4.87 ± 0.34	0.79 ± 0.09	1.06 ± 0.07
Colitis	11.04 ± 0.31*	1.67 ± 0.18*	2.29 ± 0.07*
Colitis + MBSAE 25	7.71 ± 0.36$^{\#}$	1.25 ± 0.17$^{\#}$	1.87 ± 0.07$^{\#}$
Colitis + MBSAE 50	6.75 ± 0.29$^{\#}$	1.04 ± 0.11$^{\#}$	1.66 ± 0.09$^{\#}$
Colitis + MBSAE 100	5.55 ± 0.37$^{\#}$	0.94 ± 0.09$^{\#}$	1.46 ± 0.11$^{\#}$
Colitis + Sulfasalazine	5.00 ± 0.28$^{\#}$	0.87 ± 0.10$^{\#}$	1.11 ± 0.09$^{\#}$

Figure 1

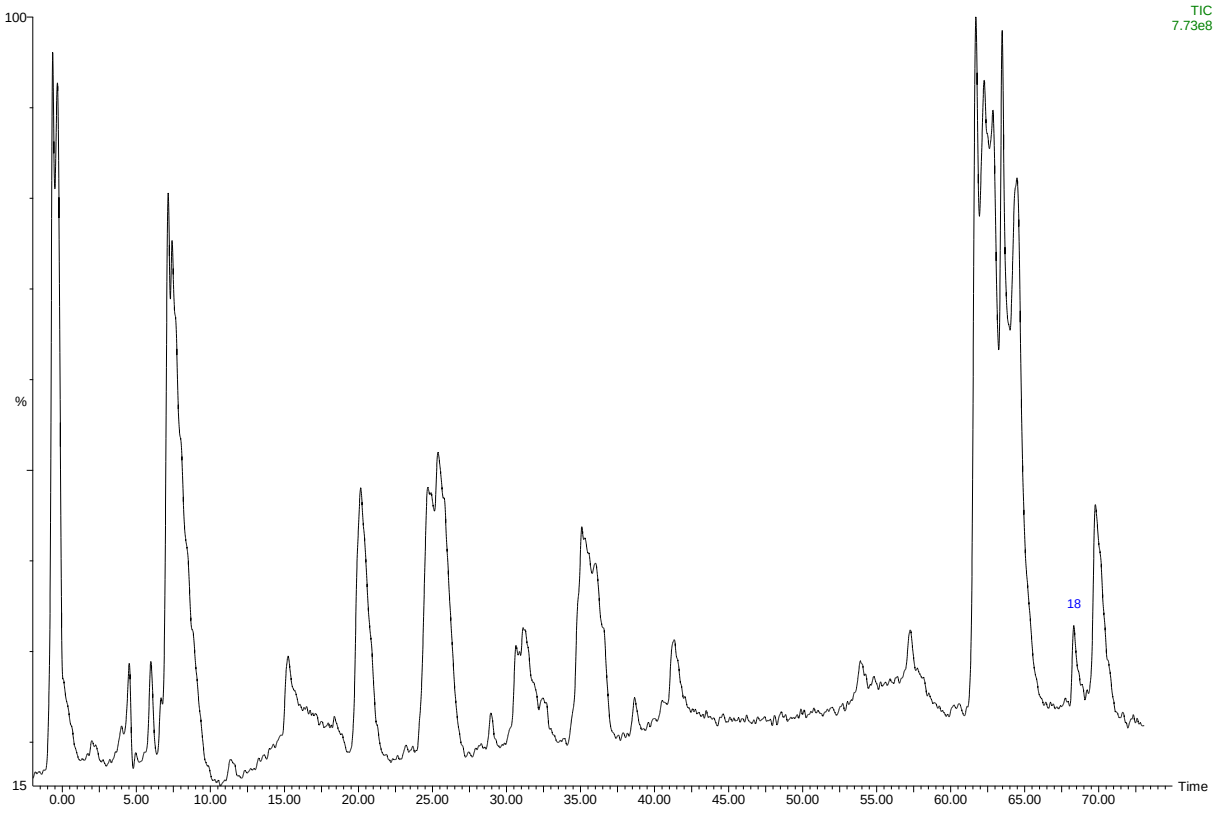

Figure 2

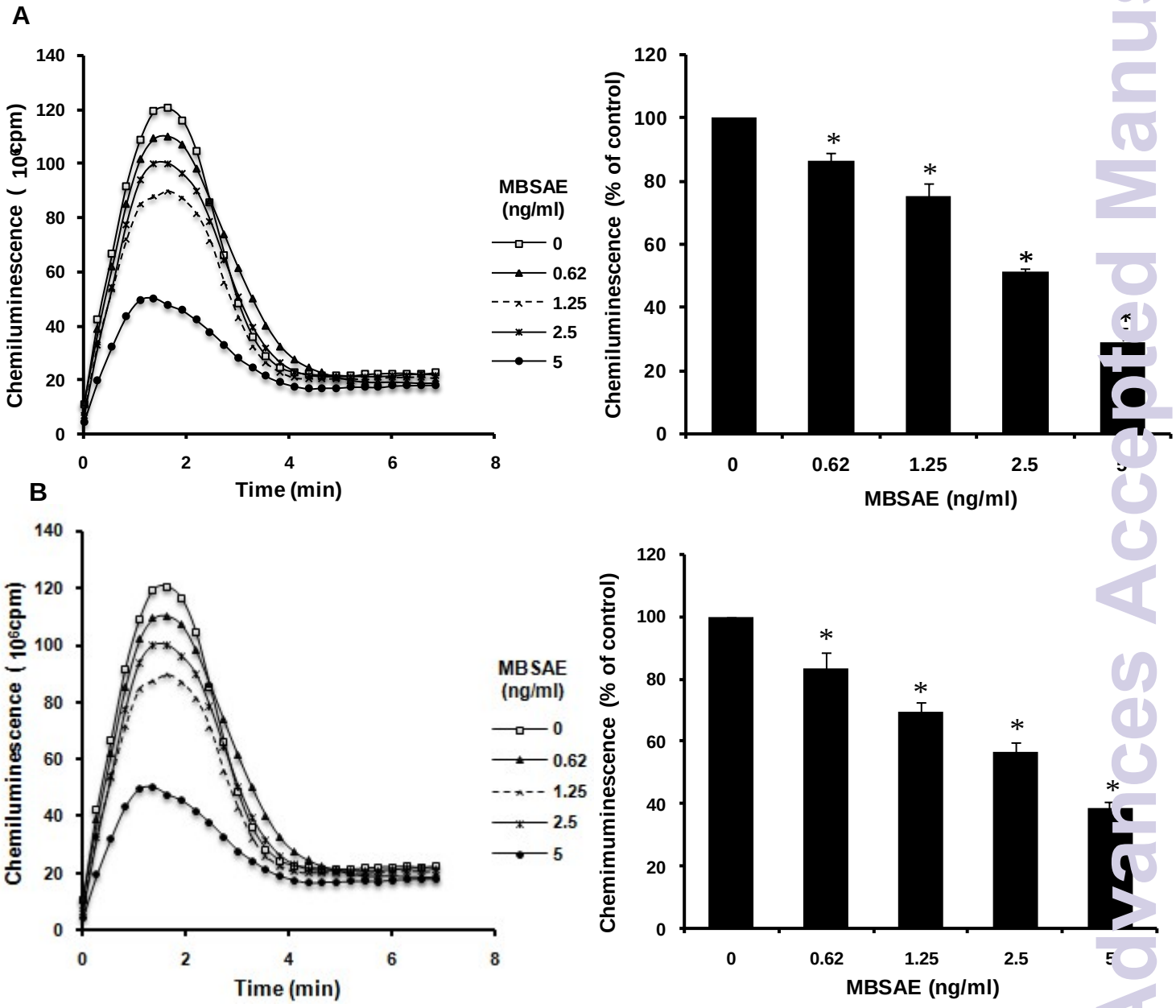

Figure 3

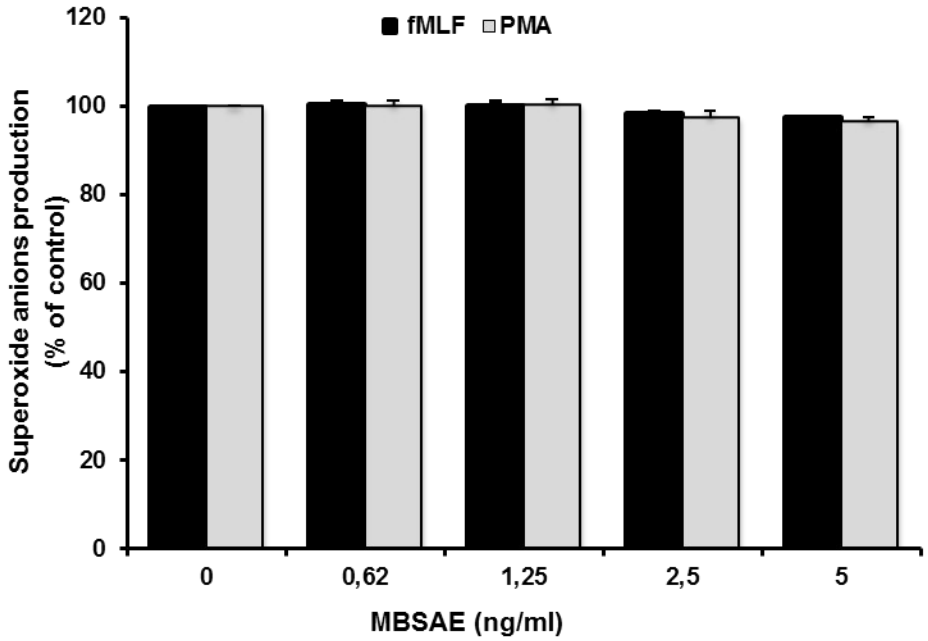

Figure 4

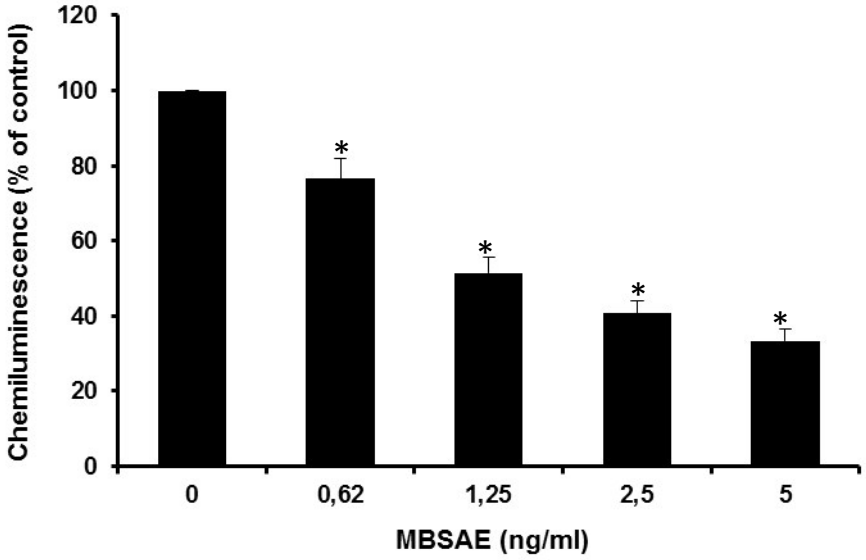

Figure 5

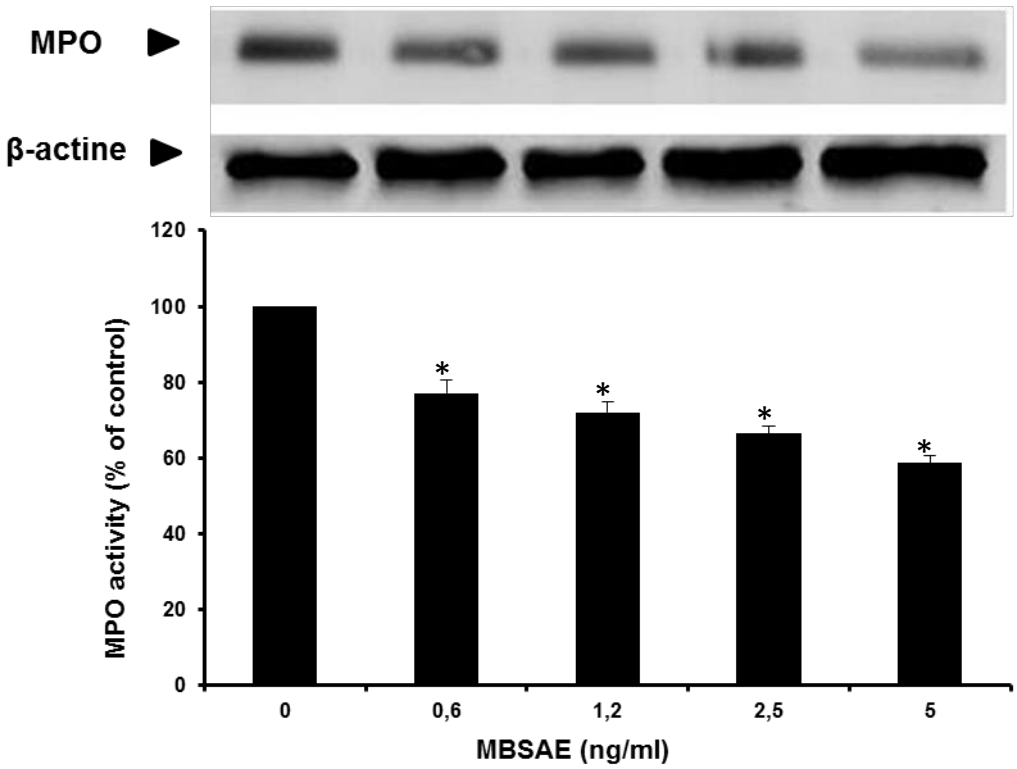

Figure 6

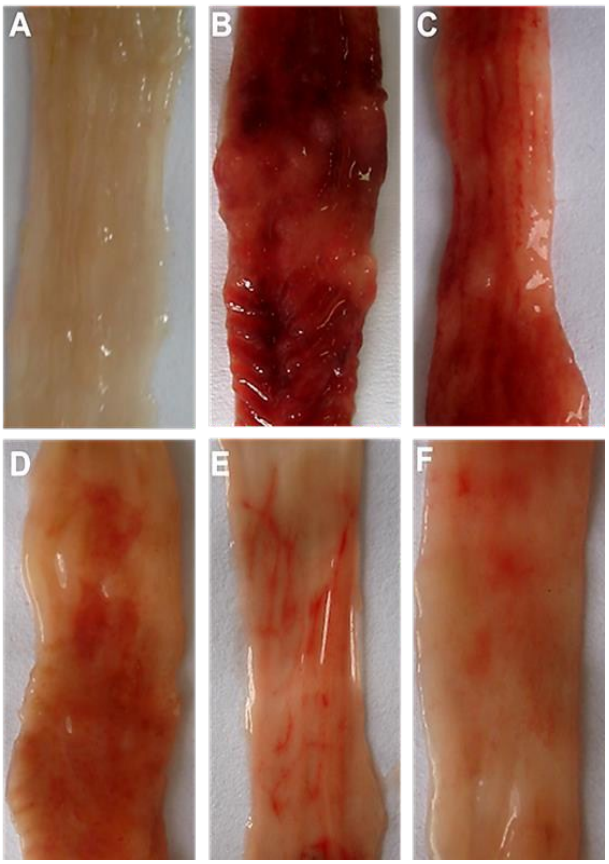

Figure 7

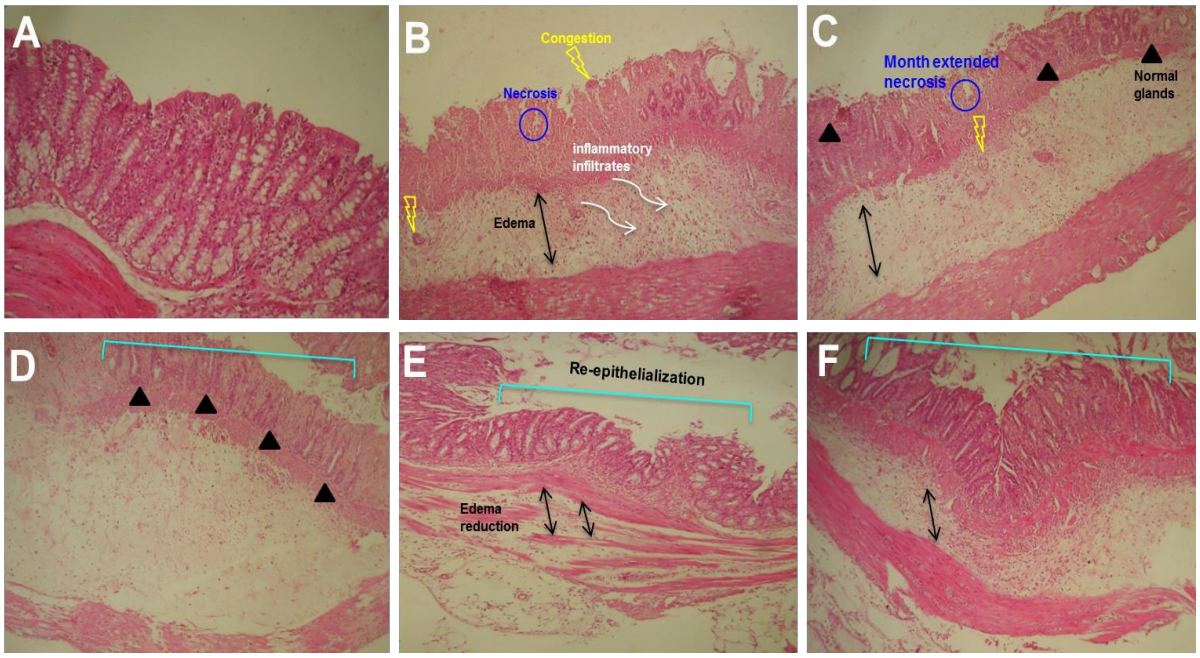

Figure 8