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Keyword: Myrtle berries, antioxidant, unsaturated fatty acids, free acidity, peptic ulcer
Fatty acids composition and mechanism of protective effects of myrtle berries seeds aqueous extract against alcohol-induced peptic ulcer in rat

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Running title: Antiulcer and antioxidant properties of MBSAE.
Abstract

This study aimed to investigate the anti-ulcer and antioxidant activities of the myrtle berries seeds aqueous extract (MBSAE) on peptic ulcer model induced by ethanol (EtOH) in male Wistar rats. MBSAE is rich in total polyphenols, total flavonoids and unsaturated fatty acids particularly linoleic (C18:2) and oleic acids (C18:1). MBSAE also exhibited an importance in vitro antioxidant activity using 2,2-diphenyl-1-picrylhydrazyl (DPPH) (IC50 = 172.1 µg/mL) and superoxide anion (IC50 = 200.24 µg/mL) scavenging activities. In vivo, MBSAE provide a dose-dependent protection against ethanol-induced gastric and duodenal macroscopic and histological alterations. Also, it inhibits the secretory profile disturbances and lipid peroxidation, preserve normal antioxidant enzyme activities and non-enzymatic antioxidant levels. More importantly, we showed that alcohol acute intoxication increased gastric and duodenal calcium, hydrogen peroxide (H₂O₂) and free iron levels while MBSAE treatment protected against intracellular mediators deregulation.

In conclusion, we suggest that MBSAE had a potent protective effects against alcohol-induced peptic ulcer in rat. This protection might be related in to part its antioxidant properties as well as its opposite effects on some studied intracellular mediators.

Key words: Myrtle berries, peptic ulcer, antioxidant, unsaturated fatty acids, free acidity.

Abbreviations: CAT, catalase ; DPPH, 2,2-diphenyl-1-picrylhydrazyl ; GA, gallic acid ; GPx, glutathione peroxidase ; H₂O₂, hydrogen peroxide ; FAM, Famotidine ; MBSAE, myrtle berries seeds aqueous extract ; MDA, malondialdehyde ; SOD, superoxide dismutase
Introduction

Peptic ulcer disease (PUD) is an illness that affects a considerable number of people worldwide. It develops when there is an imbalance between the aggressive and protective factors in the luminal surface of epithelial cells. The major causes of this disease are *Helicobacter pylori*, HCl, pepsins, non-steroidal anti-inflammatory drugs (NSAIDs), bile acids, ischemia, hypoxia, smoking and alcohol while the defensive factors include bicarbonate, mucus layer, mucosal blood flow, PGs as well as growth factors (Harold et al. 2006). However, in response to these numerous injurious luminal agents and irritants of both exogenous and endogenous origins, the stomach is a site of massive production and concentration of reactive oxygen species (ROS), far higher than other tissues or biological fluids (Graziani et al. 2005). The generation of ROS plays a major role in the development of multiple stomach pathologies, such as gastritis, peptic ulcerations or gastric adenocarcinoma (Oliveira et al. 2003). In this respect, gastric mucosal layers represent a dynamic barrier in counteracting the effects of noxious agents through a series of endogenous antioxidant defense systems. Indeed, increased oxidative stress is known to be associated to the aggressive factors-induced gastric mucosal damage (Kountouras et al. 2001; Guslandi 1987). The mechanisms underlying the ethanol-induced gastric and duodenal injuries have not yet been fully elucidated. Several naturally occurring antioxidant compounds were largely used to protect against gastric and duodenal diseases both in experimental and clinical situations. The plants and herbs are used to treat different gastrointestinal illnesses, including peptic ulcers without side effects in ayurvedic medicinal system (Jaime et al. 2006). Myrtle berries are among the fruits with high antioxidant power and rich in phenolic compounds such as anthocyanins and flavonoids and seem to have the highest antioxidant capacity among fruits and vegetables. Myrtle berries contain a wide array of phenolic compounds (Messaoud et al. 2012). This plant contains also fiber, sugars, tannins as well as many phenolic active
compounds (Hayder et al. 2004). Phytochemical analysis of the fruit seeds shows a wide variety of active compounds as well as fatty acid such as linoleic and oleic acids (Messaoud and Boussaid 2011).

In this context, we are interested to evaluate the protective effects of MBSAE against ethanol-induced ulceration in the gastro-duodenal mucosa as well as the mechanisms implicated in such protection.
Materials and Methods

Plant collection and extract preparation

Myrtle berries (Myrtus communis L.) were collected in October, 2014 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.

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 during 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.

Total phenolic content

Total phenolic content was determined by the colorimetric Folin-Ciucalteu method (Haseeb et al. 2006). Briefly, 500 µL of the extract was added to 10 ml of water and 0.5 ml of Folin-Ciucalteu reagent. After 5 min, 8 ml of 7.5% sodium carbonate solution was added. The reaction mixture was kept in the dark for 2 h and its optical density was measured at 765 nm using a UV-visible spectrophotometer (Beckman DU 640B). Gallic acid was applied as standard, and the results were expressed as mg of gallic acid equivalents per gram of dry weight (mg GAE/g DW).

Total flavonoids determination

Total flavonoid content was determined by the AlCl₃ colorimetric method (Djeridane et al. 2006). Briefly, 1 mL of the sample was mixed with 1 mL of 2% aluminium chloride solution.
After incubation for 15 min at room temperature, the optical density of the reaction mixture was evaluated at 430 nm. Quercetin was used as a citation standard and the total flavonoid content was expressed as mg of quercetin equivalent per gram of dry weight (mg QtE/g DW).

**Extraction and analysis of fatty acids**

Total lipids were extracted following the modified method of Bligh and Dyer (1959). Briefly, the air dried seeds fruits were kept in boiling water for 10 min to inactivate lipase, and then ground manually in the presence of chloroform/methanol (2:1, v/v). After washing with water and centrifugation at 3000 x g during 10 min, the organic layer containing lipids was recovered and dried under a nitrogen stream. Total fatty acids were methylated using sodium methoxide solution (Cecchi et al. 1985) and the resulting fatty acids methyl esters (FAMEs) were subsequently analyzed by gas chromatography (GC).

The GC analysis was conducted on a HP 6980 gas chromatograph (Agilent Technologies, Palo Alto, Ca, USA) equipped with a flame ionisation detector (FID). The FAMEs were separated on a RT-2560 capillary column (100 m x 0.25 mm i.d; 0.20 µm film thikeness). The oven temperature was kept at 170°C for 2 min, followed by a 3°C/min ramp to 240°C and finally held there for 15 min. Nitrogen was used as carrier gas et a flow rate of 1.2 mL/min. The injector and detector temperatures were maintained at 225°C. FAMEs were identified by comparison of their retention time with those of authentic standards.

**Free radical-scavenging activity on DPPH**

The antioxidant capacity of MBSAE was performed using 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging activity as previously described by Grzegorczyk et al. (2007). Briefly, various concentrations of MBSAE (20, 50, 100, 150, and 200 µg/mL) were added to 1 mL of 0.1 mM methanol solution of DPPH and incubated at 27°C during 30 min. The
optical density of the sample was quantified at 517 nm. DPPH radical-scavenging activity (RSA), expressed as a percentage, was estimated utilizing the following formula:

\[
\text{RSA (\%)} = \left( \frac{A_{\text{DPPH}} - A_{\text{control}}}{A_{\text{DPPH}}} \right) \times 100
\]

Quercetin was used as a reference molecule in the same concentration as the test extract.

All the analyses were executed in triplicate. The efficacy concentration 50 (EC\textsubscript{50}) value was determined as the concentration (in µg/mL) of the compound required to scavenge 50% of the DPPH radical.

**Superoxide anion scavenging activity**

The superoxide anion (O\textsubscript{2}•\textsuperscript{-}) scavenging activity was performed according to Marklund and Marklund (1974). Briefly, 0.2 ml various concentrations of MBSAE were added to 5.7 mL of 50 mM Tris-HCl buffer (pH 8.2). The mixture was incubated at 25°C during 10 min and then added to 0.1 ml of 6 Mm pyrogallol (dissolved in 10 mmol/L HCl). The absorbance of the reaction mixture was determined at 320 nm and the superoxide anion scavenging activity (SASA) was calculated using the following formula:

\[
\text{SASA (\%)} = \left[ 1 - \frac{A_1 - A_2}{A_0} \right] \times 100
\]

A\textsubscript{0} is the autoxidation rate of pyrogallol for control (the change of the absorbance), A\textsubscript{1} is the oxidation rate of pyrogallol for samples, and A\textsubscript{2} is the absorbance of the sample blank.

**Antiulcerogenic activity of MBSAE**

**Animals and treatment**

Adult male Wistar rats (weighing 220-240 g; housed five per cage) were purchased from Pasteur Institute of Tunis and used in accordance with the local ethic committee of Tunis University for use and care of animals in accordance with the NIH recommendations. They were provided with food (standard pellet diet- Badr Utique-TN) and water \textit{ad libitum} and
maintained in animal house at controlled temperature (22 ± 2°C) with a 12 hours light-dark cycle. The rats were divided into seven groups of 10 animals each. Groups 1 and 2 were served as controls and had bidistilled water (5 mL/kg, b.w., p.o.). 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 and group 7 were pre-treated respectively with famotidine (10 mg/kg, b.w., p.o.) and gallic acid (50 mg/kg, b.w., p.o.). The period of pretreatment was 15 days.

Animals were fasted for 24 h before the last administration of MBSAE or reference molecules. After 2 hours, each animal, except those of groups 1 and 2, was intoxicated by acute administration of EtOH (4 g/kg, b.w., p.o.). Sixty min later, animals were sacrificed. Blood was collected in heparinized tubes. After centrifugation at 3 000 g during 15 min, plasma was treated for PSA determination.

**Evaluation of gastric and duodenum mucosal damage.**

The stomach and duodenum of each animal were removed and opened along its greater curvature. The tissues were gently rinsed in NaCl 0.9 %. The lesions in the gastric and duodenum mucosa were macroscopically examined and the photographs of hemorrhagic erosions were acquired with a Photometric Quantic digital camera. Ulcer indexes were determined as the sum of the lengths of the whole gastric and duodenum lesions (in mm²). Two independent, blinded observers performed the measurements of lesion lengths.

**Histopathological analysis**

Immediately after sacrifice, small pieces of stomach and duodenum were harvested and washed with ice cold saline. Tissue fragments were then fixed in a 10% neutral buffered formalin solution, embedded in paraffin and used for histopathological examination. 5 µm
thick sections were cut, deparaffinized, hydrated and stained with hematoxylin and eosin (HE). The sections were examined in blind fashion for all treatments.

**Determination of pH, titrable acidity and volume of gastric juice**

After stomachs opening, the gastric contents were collected and centrifuged for 10 min at 2000 g. The total volume of the gastric content was expressed as ml/100 g body weight (Shay et al. 1974). One ml of the supernatant was diluted to 10 ml with distilled water and the total acid content was determined by titrating with sodium hydroxide (0.01 N) using phenolphthalein as indicator and expressed as mEq/l/100 g (Li et al. 2002). The pH values were measured using pH meter.

**Stomach and duodenum mucosa preparation**

After the macroscopic analyses, the stomach and duodenum mucosa were rapidly excised and homogenized in phosphate buffer saline (KH$_2$PO$_4$/K$_2$HPO$_4$, 50 mM, pH 7.4) with Potter–Elvehjem homogenizer. After centrifugation at 10000 g for 10 min at 4°C, supernatants were used for biochemical determination of protein, free iron, H$_2$O$_2$, calcium, SH- groups, MDA and antioxidant enzyme activities.

**Lipid peroxidation measurement**

Lipid peroxidation was determined by MDA measurement according to the double heating method (Draper and Hadley 1990). Briefly, aliquots from gastric and duodenal mucosa 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 and 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 using an extinction coefficient for MDA-TBA complex of $1.56 \times 10^5$ M$^{-1}$ cm$^{-1}$.

**Plasma scavenging activities**

The free radical scavenging activities of plasma was measured using the DPPH radical method according to Brand-Williams et al. (1995). Briefly, 100 µL of 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 (Beckman DU 640B). DPPH solution was used as a control and the plasma scavenging activities (PSA), expressed in percentage, was calculated according to the following equation:

$$\text{PSA} \ (%) = 100 \times \frac{A_{517}(\text{control}) \times A_{517}(\text{sample})}{A_{517}(\text{control})}$$

**Antioxidant enzyme activities**

The activity of SOD was determined using modified epinephrine assays (Misra and Fridovich 1972). At alkaline pH, superoxide anion $O_2^{•-}$ 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. Absorbance were recorded at 480 nm. CAT activity was assayed by measuring the initial rate of $H_2O_2$ disappearance at 240 nm (Aibi 1972). 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 $H_2O_2$. 

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The activity of GPx was quantified following the procedure of Flohé and Günzler (1984). Briefly, 1 mL of reaction mixture containing 0.2 mL of gastric or duodenum 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 \( \text{H}_2\text{O}_2 \) (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.

**Non-enzymatic antioxidants measurement**

The total concentration of thiol groups (-SH) was performed according to Ellman's method (Ellman 1959). 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 thiol groups concentration was calculated from A1 to A0 subtraction using a molar extinction coefficient of \( 13.6 \times 10^3 \text{ M}^{-1} \times \text{cm}^{-1} \). The results were expressed as µmol of thiol groups per mg of protein.

GSH was estimated in colonic tissue by the method of Sedlak and Lindsay (1968). 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 mM Tris–buffer (pH 8.9) and 10 µL of 10 mM DTNB. The absorbance was read at 412 nm against blank tube without homogenate.

**H\textsubscript{2}O\textsubscript{2} determination**


The gastric and duodenal \( \text{H}_2\text{O}_2 \) levels were performed according to Dingeon et al (1975). Briefly, the hydrogen peroxide reacts with \( \text{p-hydroxybenzoic acid} \) and \( \text{4-aminoantipyrine} \) in the presence of peroxidase leading to the formation of quinoneimine that has a pink color detected at 505 nm.

**Iron measurement**

The gastric and duodenal non haem iron were measured colorimetrically using ferrozine as described by Leardi et al (1998). Briefly, the iron dissociated from transferrin-iron complex by a solution of guanidine acetate was reduced by ascorbic acid and reacted with ferrozine leading to the formation of pink complex measured at 562 nm.

**Calcium determination**

The gastric and duodenal calcium levels were performed using a colorimetric method according to Stern and Lewis (1957). However at alkaline medium, calcium reacted with cresolphthalein leading to a colored complex measurable at 570 nm.

**Protein determination**

Protein concentration was determined according to Hartree (1972), as is a slight change of the Lowry method (Lowry et al. 1951). Serum albumin was used as standard.

**Statistical analysis**

The data were analyzed by unpaired Student's \( t \)-test and were expressed as means ± standard error of the mean (S.E.M.). The data are representative of 10 independent experiments. All statistical tests were two-tailed, and a \( p \) value of 0.05 or less was considered significant.
Results

Total polyphenols and flavonoids contents
The aqueous extract of myrtle berry seeds aqueous extract was firstly investigated for their phenolic compounds contents. As shown in table 1, the MBSAE exhibited high levels of total phenolics (147.56 ± 1.97 mg GAE/g DW) as well as total flavonoids (85.06 ± 2.31 mg QtE/g DW).

Fatty-Acid Composition
The proportional composition of the analyzed fatty acids revealed that the berry seeds of Myrtus communis was mainly constituted by the unsaturated fatty acids (88.51%) with linoleic acid (C18:2) and oleic (C18:1) being the main ones (Table 2). Palmitic acid (C16:0) was found as the most abundant fatty acid in the saturated fraction. When compared with earlier compositional studies, the same profile (C18:2 > C18:1) has been described by Aidi Wannes et al (2010), but differed from those reported by Cakir et al (2004), where oleic acid was found as the main fatty acid in Turkish specimen. In both studies, palmitic acid was found as the main saturated fatty of M. communis seeds, which is consistent with the our results. However, linolelaidic acid (tans, trans-C18:2) was reported herein for the first time. Its presence may be due to thermal isomerisation of linoleic acid.

In vitro antioxidant capacity
Concerning the antioxidant capacity, we have found that the radical-scavenging activity of MBSAE against superoxide anion and DPPH radicals increased significantly in a dose-dependent manner (data not shown). The IC50 values corresponding to the amount of the
fraction required to scavenge 50% of DPPH and O$_2^-$ radicals are respectively 172.1 and 200.24 µg/mL. However, regarding quercetin, used as reference molecule, the IC$_{50}$ values are 60.84 and 124.9 µg/mL, respectively for DPPH and O$_2^-$ radicals.

**Qualitative and quantitative macroscopic evaluation of MBSAE anti-ulcer activities**

The macroscopic examination of the glandular part of the stomach and duodenum was carried out on the opening of the gastro-duodenal segments. Animals intoxicated with ethanol showed an extensive elongated thick, dark red and black bands of hemorrhagic lesions on the glandular part of the stomach as shown in figure 1. In addition, the EtOH administration induced the formation of macroscopically evident lesions along the entire duodenum (Figure 1). However, MBSAE, famotidine and GA pre-treatment significantly protected gastric and duodenal mucosa against alcohol induced injury (Table 3).

**Histopathological evaluation of gastric and duodenal lesions**

Histological observation of ethanol-induced gastric and duodenal lesions in EtOH group showed a comparative extensive congestion, surface coating alteration, necrotic lesions, edema, epithelial and vascular cells alteration, haemorrhage and hyperaemia as well as inflammatory cell infiltration in the stomach and duodenum (Figure 2) mucosa and submucosa. Pretreatment with MBSAE presents a clear dose-dependent protection of the gastric and duodenal mucosa as seen by reduction of lesions, mucosal and submucosal edema as well as leucocytes infiltration. A similar protective effect had also observed in gallic acid and famotidine pretreated rats.
Evaluation of pH, titrable acidity and gastric juice volume

As shown in table 4, the stomach and duodenal ulceration were accompanied by a significant decrease of pH values as well as an increase of volume and titrable acidity of the gastric juice. MBSAE administration significantly restored, all these parameters in a dose-dependent manner. In addition, famotidine and gallic acid also protected against the secretory profile disturbance.

Effects on lipid peroxidation

To investigate the implication of oxidative stress in the antiulcerogenic effect of MBSAE, stomach and duodenal mucosa were firstly assessed for MDA determination. As expected, EtOH administration significantly increased stomach and duodenal MDA levels. Alcohol-induced lipoperoxidation was significantly reversed by MBSAE, famotidine or gallic acid pre-treatment in a dose-dependent manner (Figure 3).

Effects on plasma scavenging activity

EtOH administration significantly decreased the plasma scavenging activity as compared to control group (Figure 4). By contrast, PSA percentage was significantly and dose-dependently increased after MBSAE pre-treatment. A similar effects were also observed for famotidine and gallic acid, used as reference molecules.

Effects on non-enzymatic antioxidants

We also studied the gastric and duodenal non-enzymatic antioxidants levels (Figure 5). As expected, we showed that alcohol intoxication significantly decreased stomach and duodenal thiol groups (A) and reduced glutathione (B) contents. MBSAE per se exhibited significant
and dose-dependent restoration of all these parameters. The high dose of our extract (100 mg/kg) exerts a similar effects of gallic acid and more important of those of famotidine.

Effects on antioxidant enzyme

On other hand we examined the effect of MBSAE and EtOH treatment on antioxidant enzyme activities (Figure 6). As expected, gastric and duodenal injuries were accompanied by a significant decrease of superoxide dismutase (A), catalase (B) and glutathione peroxidase (C) activities. Myrtle extract treatment significantly and dose-dependently corrected the enzyme activities decrease induced by alcohol administration. This effect was also significantly corrected by subacute famotidine and gallic acid pre-treatment.

Effects on free iron, H$_2$O$_2$ and calcium

We further looked at the EtOH and MBSAE on intracellular mediators such as hydrogen peroxide, free iron and calcium levels in gastric mucosa (Table 5). Alcohol group showed a significant decrease in free iron, H$_2$O$_2$ and ionizable calcium levels in gastric and duodenal tissues when compared to negative control group. However, the MBSAE, gallic acid and famotidine treatment significantly and dose-dependently reduced the EtOH-induced intracellular mediators deregulation.
Discussion

In the present work, we investigated the protective effects of myrtle berries seeds aqueous extract on EtOH-induced peptic ulcer in rats as well as characterized disruptions in intracellular mediators that may subserve such protection.

Our phytochemical study firstly revealed that MBSAE presents a powerful scavenging action against DPPH radical and superoxide anion with lower IC$_{50}$ values (172.1 and 200.24 µg/mL, respectively). However, similar free radical-scavenging activities were previously observed for other plant extracts but lesser than MBSAE (Kelebek et al. 2009; Zhao et al. 2011). The antioxidant activity of MBSAE could be, in part, attributed to its high phenolic compounds levels. In this context, our data also suggest that MBSAE presents a high concentration of total polyphenols (147.56 ± 1.97 mg GAE/g), flavonoids (85.06 ± 2.31 mg QE/g) as well as polyunsaturated fatty acids (PUFAs). In fact, six fatty acids were identified in myrtle berries seeds, which linoleic acid (80.78%) and oleic acid (6.34%) are the two major compounds. Indeed, PUFAs from the n-6 and n-3 families are components of cell membrane phospholipids. However, these molecules, owing to their anti-inflammatory (Knoch et al. 2009) and antioxidant properties (Messaoud and Boussaid 2011), are implicated in the regulation of balance redox, cardiovascular activity, blood pressure, hormonal activity, kidney function (Williams 2000) and platelet aggregation (Haug et al. 2007).

*In vivo*, we firstly showed that alcohol administration clearly altered the gastric and duodenal mucosa and submucosa. These lesions are accompanied by edema, epithelial and vascular cells alteration, necrosis and leucocytes infiltration of the submucosal layer. Our data are in line with previous report using EtOH as ulceration inducer (Awaad Amani et al. 2013; Bode and Bode 1997; Meze 1985). Several mechanisms are implicated in the development of alcohol-induced lesions in the mucous membranes (Awaad Amani et al. 2013). However,
alcohol consumption can directly disrupt the integrity of the epithelial lining. In addition, EtOH-induced release of harmful signaling molecules, such as cytokines, histamine and leukotrienes. However, these molecules are capable of inducing damage in the small blood vessels and/or capillaries in the gastric and intestinal mucosa (Bode and Bode 1997). Subacute treatment with MBSAE protects against gastric and duodenal lesions induced by EtOH administration and allowed to the reduction of morphological and histopathological observed signs. This therapeutic effect, using MBSAE high dosage, is more effective than reference molecules such as famotidine (78.16 and 85.46%) and gallic acid (68.77 and 83.72%), respectively for the stomach and duodenum mucosa. Other bioactive molecules have been also studied for their anti-ulcer effects, such as L-citrulline (Liu et al. 2012) and ascorbic acid (Rezvanjoo et al. 20130). The ulcerogenic activity of ethanol is also linked to its ability to dissolve the gastric mucus, reducing the transmucosal potential, simultaneously increasing the flow of Na\(^+\) and H\(^+\) ions as well as the stimulation of histamine release, the H\(^+\) ions and pepsin (Szabo et al. 1987). In this context, we have shown, in the present study that EtOH administration leads to a significant decrease in pH values and increase in free acidity and volume of gastric juice. This study shows that pretreatment with famotidine, gallic acid and MBSAE (25, 50 and 100 mg/kg) significantly corrected these disorders in the secretory profile in a dose dependent manner. These results are consistent with some previous reports using other medicinal plants such as Citrus aurantium and Pithecellobium dulce (Moraes et al. 2009; Megala et al. 2012).

The role of neutrophils in the ulcerogenic effects of alcohol has been demonstrated in animal models. The mechanisms involved are the adhesion of neutrophils to the endothelium and their activation (Thiéfin et al. 2004). In this respect, myrtle berries seeds extract has been shown to attenuate the ROS production and myeloperoxidase expression in human neutrophils (Jabri et al. 2015).
The alcohol poisoning is also characterized by a clear redox balance deregulation of the gastric and duodenal mucosa, proved by inducing lipid peroxidation as evidenced by an increased rate of MDA, falling of plasma scavenging activity (PSA) and a decrease of the antioxidant enzyme activities such as superoxide dismutase, catalase and glutathione peroxidase as well as non-enzymatic antioxidants levels such as thiol groups and reduced glutathione. These data fully corroborated many previous reported, *in vivo*, with rat or mouse models (Alimi et al. 2010; Jelovac et al. 1999) and *in vitro* using macrophages in culture (Tanaka et al. 2009). Indeed, the pathogenesis of gastric and duodenal lesions leads to the generation of reactive oxygen species (ROS) that appear to play a key role in lipid peroxidation of the gastric and duodenal mucosa cells, and accompanied by degradation of the antioxidants enzyme activity (Konturek et al. 2005). Importantly, we demonstrated that the MBSAE protects against EtOH-induced oxidative stress in the gastric and duodenal mucosa. EtOH-induced gastric and duodenal oxidative stress have been shown to be attenuated by many medicinal plant extracts such as *Opuntia ficus* (Alimi et al. 2010), *Piper aduncum* (Arroyo et al. 2013), *Amukkara choornam* (Patra et al. 2014) and *Artemisia campestris* (Sebai et al. 2014). According to the study done in our laboratory, phytochemical analyzes of MBSAE by HPLC-PDA-MS allowed to a tentative identification of 18 phenolic compounds distributed in three major groups; hydroxybenzoic acid derivatives, anthocyanins derivatives and flavonols derivatives (Jabri et al. 2015). These molecules have been clearly shown for their antioxidant activities (Kavitha et al. 2005; Abdulazeez et al. 2016).

The richness of MBSAE in polyunsaturated fatty acids, gives it a powerful antioxidant and anti-inflammatory properties. Indeed, PUFAs may act as an antioxidant by reducing lipid peroxides and play a role in regulating oxidative stress through the enhancing of oxidative burden induced by lipid peroxidation and activating the antioxidant enzymes (Rebiger et al. 2016; Nguemeni et al. 2010). The anti-inflammatory effects of PUFAs results in activating the
AMP-activated protein kinase, inducing the synthesis of anti-inflammatory lipid mediators like resolvins and protectins and inhibiting the conversion of arachidonate acids to the proinflammatory lipid intermediates, interrupting the NF-κB signaling pathway (Chang et al. 2013).

More importantly, our results also suggest that pretreatment with MBSAE protects against overloading of cells of the gastric and duodenal mucosa by free iron and H₂O₂ induced by acute administration of EtOH. However, free iron and hydrogen peroxide are the two components of the Fenton's reaction, which is involved in the generation of hydroxyl radical (OH•) (Li et al. 2008). However, this later is the most powerful oxidant that can attack the molecular structures and thus play a major role in oxidative damage (Nobushi et al. 2010). Living organisms so, develop a complex endogenous and exogenous antioxidant defense system to block the production of this harmful radical (Jomova and Valko 2011). This study also showed an increase in gastric and duodenal ionizable calcium in response to oxidative stress induced by ethanol administration. This result corroborated several previous studies (Davidson and Duchen 2006; Ermak and Davies 2002). However, we can now speculate that MBSAE, exerts its beneficial effect by chelating free iron and scavenging H₂O₂ leading to calcium homeostasis. The same mechanism has been previously described in aspirine-induced gastric ulcer (Sebai et al. 2014), castor oil-induced Diarrhea in rat (Jabri et al. 2016a) as well as intestinal ischemia/reperfusion injuries (Jabri et al. 2016b).
**Conclusion**

In conclusion, our data clearly demonstrate that MBSAE exerts protective effects against alcohol-induced gastric and duodenal ulceration owing in part to its antioxidant properties primarily related to the presence of polyunsaturated fatty acids and high amount of phenolic compounds.

**Acknowledgements**

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**Declaration of interest**

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


Table 1: Total polyphenols and flavonoids contents and IC50 values of the DPPH and superoxide anion free radicals scavenging assay of the MBSAE and quercetin.

<table>
<thead>
<tr>
<th></th>
<th>Total phenolic content (mg GAE/g)</th>
<th>Total flavonoids content (mg QE/g)</th>
<th>DPPH IC50 (µg/ml)</th>
<th>Superoxide anion IC50 (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MBSE</td>
<td>147.56 ± 1.97</td>
<td>85.06 ± 2.31</td>
<td>172.1</td>
<td>200.24</td>
</tr>
<tr>
<td>Quercetin</td>
<td>nt</td>
<td>nt</td>
<td>60.84</td>
<td>124.9</td>
</tr>
</tbody>
</table>
**Table 2:** Fatty acid profile (% total fatty acids) of the total lipids of *M. communis* berries seeds

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Palmitic (C16:0)</td>
<td>6,03</td>
</tr>
<tr>
<td>Stearic (C18:0)</td>
<td>3,28</td>
</tr>
<tr>
<td>Oleic C18:1</td>
<td>6,34</td>
</tr>
<tr>
<td>Linoleic (<em>cis, cis</em>-C18:2)</td>
<td>80,78</td>
</tr>
<tr>
<td>Linoleaidic (<em>trans, trans</em>-C18:2)</td>
<td>1,39</td>
</tr>
<tr>
<td>Arachidic (C20:0)</td>
<td>0,59</td>
</tr>
</tbody>
</table>
Table 3: Effect of myrtle berries seeds aqueous extract (MBSAE), famotidine (FAM) and gallic acid (GA) on gastric and duodenal macroscopic alterations induced by EtOH: ulcer index and Percentage of protection (%). Animals were pretreated with various doses of MBSAE (25, 50 and 100 mg/kg, p.o.), FAM (20 mg/kg, b.w., p.o.), GA (50 mg/kg, p.o) or vehicle (H₂O). The data are expressed as mean ± S.E.M. (n=10) * : p < 0.05 compared to control group and #: p < 0.05 compared to EtOH group.

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Stomach</th>
<th>Duodenum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ulcer area (mm²)</td>
<td>Percentage of protection (%)</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>--</td>
</tr>
<tr>
<td>EtOH</td>
<td>98.00 ± 5.21*</td>
<td>--</td>
</tr>
<tr>
<td>EtOH + MBSAE-25</td>
<td>70.40 ± 2.01#</td>
<td>28.16%</td>
</tr>
<tr>
<td>EtOH + MBSAE-50</td>
<td>44.40 ± 1.88#</td>
<td>54.69%</td>
</tr>
<tr>
<td>EtOH + MBSAE-100</td>
<td>19.20 ± 0.86#</td>
<td>80.40%</td>
</tr>
<tr>
<td>EtOH + Famotidine</td>
<td>21.40 ± 1.72#</td>
<td>78.16%</td>
</tr>
<tr>
<td>EtOH + Gallic acid</td>
<td>30.60 ± 1.74#</td>
<td>68.77%</td>
</tr>
</tbody>
</table>
Table 4: Effect of myrtle berries seeds aqueous extract (MBSAE), famotidine (FAM) and gallic acid (GA) on EtOH-induced disturbance on pH, titrable acidity and volume of gastric juice. Animals were pretreated with various doses of MBSAE (25, 50 and 100 mg/kg, p.o.), FAM (20 mg/kg, b.w., p.o.), GA (50 mg/kg, p.o) or vehicle (H₂O). The data are expressed as mean ± S.E.M. (n=10) * : p < 0.05 compared to control group and #: p < 0.05 compared to EtOH group.

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>pH of gastric juice</th>
<th>Gastric volume (ml/100g)</th>
<th>Titrable acidity (mEq/L/100g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4.58 ± 0.22</td>
<td>2.67 ± 0.2</td>
<td>97.67 ± 1.28</td>
</tr>
<tr>
<td>EtOH</td>
<td>2.40 ± 0.24*</td>
<td>3.91 ± 0.17*</td>
<td>122.07 ± 2.16*</td>
</tr>
<tr>
<td>EtOH + MBSAE-25</td>
<td>3.84 ± 0.23#</td>
<td>3.17 ± 0.08#</td>
<td>112.37 ± 3.59#</td>
</tr>
<tr>
<td>EtOH + MBSAE-50</td>
<td>3.94 ± 0.16#</td>
<td>2.99 ± 0.08#</td>
<td>109.46 ± 2.86#</td>
</tr>
<tr>
<td>EtOH + MBSAE-100</td>
<td>4.12 ± 0.16#</td>
<td>2.65 ± 0.11#</td>
<td>100.07 ± 4.61#</td>
</tr>
<tr>
<td>EtOH + Famotidine</td>
<td>4.26 ± 0.29#</td>
<td>2.49 ± 0.13#</td>
<td>92.30 ± 3.38#</td>
</tr>
<tr>
<td>EtOH + Gallic acid</td>
<td>4.028 ± 0.37#</td>
<td>2.738 ± 0.12#</td>
<td>105.51 ± 3.68#</td>
</tr>
</tbody>
</table>
Table 5: Effect of myrtle berries seeds aqueous extract (MBSAE), famotidine (FAM) and gallic acid (GA) on EtOH-induced disturbance in gastric and duodenal H$_2$O$_2$, free iron and calcium levels. Animals were pretreated with various doses of MBSAE (25, 50 and 100 mg/kg, p.o.), FAM (20 mg/kg, b.w., p.o.), GA (50 mg/kg, p.o) or vehicle (H$_2$O). The data are expressed as mean ± S.E.M. (n=10) *: p < 0.05 compared to control group and #: p < 0.05 compared to EtOH group

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>H$_2$O$_2$ (mmol/mg protein)</th>
<th>Free iron (µmol/mg protein)</th>
<th>Calcium (mmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Stomach</td>
<td>Duodenum</td>
<td>Stomach</td>
</tr>
<tr>
<td>Control</td>
<td>0.24 ± 0.011</td>
<td>0.17 ± 0.011</td>
<td>17.80 ± 1.41</td>
</tr>
<tr>
<td>EtOH</td>
<td>0.54 ± 0.014*</td>
<td>0.36 ± 0.028*</td>
<td>40.24 ± 1.83*</td>
</tr>
<tr>
<td>EtOH + MBSAE-25</td>
<td>0.39 ± 0.012#</td>
<td>0.28 ± 0.021#</td>
<td>31.60 ± 1.60#</td>
</tr>
<tr>
<td>EtOH + MBSAE-50</td>
<td>0.33 ± 0.012#</td>
<td>0.23 ± 0.023#</td>
<td>25.21 ± 1.89#</td>
</tr>
<tr>
<td>EtOH + MBSAE-100</td>
<td>0.28 ± 0.009#</td>
<td>0.19 ± 0.009#</td>
<td>21.86 ± 1.30#</td>
</tr>
<tr>
<td>EtOH + Famotidine</td>
<td>0.32 ± 0.011#</td>
<td>0.22 ± 0.019#</td>
<td>23.43 ± 1.76#</td>
</tr>
<tr>
<td>EtOH + Gallic acid</td>
<td>0.27 ± 0.011#</td>
<td>0.19 ± 0.015#</td>
<td>19.76 ± 1.22#</td>
</tr>
</tbody>
</table>
Legends of figures

Figure 1: Gastric (a) and duodenal (b) morphology showing the protective effects of myrtle berries seeds aqueous extract (MBSAE), famotidine (FAM) and gallic acid (GA) on EtOH-induced ulcer. Animals were treated with various doses of MBSAE (25, 50 and 100 mg/kg, p.o.), FAM (20 mg/kg, b.w., p.o.), GA (50 mg/kg, p.o) or vehicle (H₂O). (A) H₂O + NaCl; (B) H₂O + EtOH; (C, D and E) MBSAE (25, 50 and 100 mg/kg, p.o., respectively) + EtOH; (F) Famotidine (20 mg/kg, PC, p.o.) + EtOH; (G) Gallic acid (50 mg/kg, PC, p.o.) + EtOH.

Figure 2: Gastric (a) and duodenal (b) histology showing the protective effects of myrtle berries seeds aqueous extract (MBSAE), famotidine (FAM) and gallic acid (GA) on EtOH-induced histological alteration in stomach and duodenum. Animals were treated with various doses of MBSAE (25, 50 and 100 mg/kg, p.o.), FAM (20 mg/kg, b.w., p.o.), GA (50 mg/kg, p.o) or vehicle (H₂O). (A) H₂O + NaCl; (B) H₂O + EtOH; (C, D and E) MBSAE (25, 50 and 100 mg/kg, p.o., respectively) + EtOH; (F) Famotidine (20 mg/kg, PC, p.o.) + EtOH; (G) Gallic acid (50 mg/kg, PC, p.o.) + EtOH.

Figure 3: Effect of MBSAE, famotidine (FAM) and gallic acid (GA) on EtOH-induced disturbances in gastric and duodenal MDA level. Animals were pretreated with various doses of MBSAE (25, 50 and 100 mg/kg, p.o.), FAM (20 mg/kg, b.w., p.o.), GA (50 mg/kg, p.o) or vehicle (H₂O). The data are expressed as mean ± S.E.M. (n=10) *: p < 0.05 compared to the control group and #: p < 0.05 compared to the EtOH group.

Figure 4: Effect of MBSAE, famotidine (FAM) and gallic acid (GA) on EtOH-induced disturbances in plasma scavenging activity (PSA). Animals were pretreated with various doses of MBSAE (25, 50 and 100 mg/kg, p.o.), FAM (20 mg/kg, b.w., p.o.), GA (50 mg/kg, p.o) or vehicle (H₂O). The data are expressed as mean ± S.E.M. (n=10) *: p < 0.05 compared to the control group and #: p < 0.05 compared to the EtOH group.
**Figure 5**: Effect of MBSAE, famotidine (FAM) and gallic acid (GA) on EtOH-induced disturbances in gastric and duodenal non-enzymatic antioxidants levels: Thiol groups (A) and reduced glutathione (B). Animals were pretreated with various doses of MBSAE (25, 50 and 100 mg/kg, *p.o.*), FAM (20 mg/kg, b.w., *p.o.*), GA (50 mg/kg, p.o) or vehicle (H₂O). The data are expressed as mean ± S.E.M. (*n=10*) *: p < 0.05 compared to the control group and #: p < 0.05 compared to the EtOH group.

**Figure 6**: Effect of MBSAE, famotidine (FAM) and gallic acid (GA) on EtOH-induced disturbances in gastric and duodenal antioxidant enzyme activities: SOD (A), CAT (B) and GPx (C). Animals were pretreated with various doses of MBSAE (25, 50 and 100 mg/kg, *p.o.*), FAM (20 mg/kg, b.w., *p.o.*), GA (50 mg/kg, p.o) or vehicle (H₂O). The data are expressed as mean ± S.E.M. (*n=10*) *: p < 0.05 compared to the control group and #: p < 0.05 compared to the EtOH group.
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167x149mm (300 x 300 DPI)
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Figure 4: Effect of MBSAE, famotidine (FAM) and gallic acid (GA) on EtOH-induced disturbances in plasma scavenging activity (PSA). Animals were pretreated with various doses of MBSAE (25, 50 and 100 mg/kg, p.o.), FAM (20 mg/kg, b.w., p.o.), GA (50 mg/kg, p.o) or vehicle (H2O). The data are expressed as mean ± S.E.M. (n=10) * : p < 0.05 compared to the control group and #: p < 0.05 compared to the EtOH group.
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109x202mm (300 x 300 DPI)