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**Pharmacological effects of *Myrtus communis* L. on the  
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## ملخص

الريحان (*M. communis* L.) ينتمي إلى عائلة Myrtaceae وهو شجيرة واسعة الانتشار في منطقة البحر الأبيض المتوسط. يهتم هذا العمل أساسا بدراسة تأثيرات هذا النبات و استخدامه الشائع في الطب التقليدي لبعض الحالات الفيزيولوجية المرضية للجهاز الهضمي. أظهرت نتائج التحليل الكيميائي للزيوت الأساسية لأوراق نبات الريحان أن هذه الأخيرة غنية جدا ب  $\alpha$ -pinene (54.1%) و 1.8-cineole (26.5%) وكانت المستخلصات المثنولية المختلفة لكل من أوراق و ثمار النبتة غنية بمركبات عديدة الفينول التي تم اختبارها. أظهر تحليل HPLC الأولي لكل من المستخلصات المثنولية لأوراق و ثمار النبتة وجود حمض الغاليك، حمض الإيلاجيك، حمض الجانتيسيك، حمض هيدروكسي البنزويك والكيرسيتين كمكونات أساسية في الأوراق وحمض الغاليك وحمض الجانتيسيك وحمض الفانيليك في الثمار. تم تقييم التأثيرات المضادة للإسهال لكل من الزيوت الأساسية ومختلف مستخلصات الأوراق لدى الفئران باستخدام جرعات وآليات مختلفة. قد تبين أن لهذه العناصر تأثيرات فعالة مضادة للإسهال . نفس هذه العناصر كانت لها تأثيرات قوية مضادة للتقرح في معدة الجرذان . قد ينجم هذا التأثير المضاد للتقرح ولو جزئيا عن النشاط المضاد للأكسدة. في الواقع، أظهرت كل من الزيوت الأساسية و المستخلصات تأثيرات مضادة للأكسدة في الجسم و في المختبر مع معظم التجارب المجراة، كما قد تعود آليات التأثيرات كذلك إلى تحفيز إفراز المخاط. أظهرت النتائج التجريبية لالتهاب القولون والتقرح أن للمستخلص تأثيرات مضادة للالتهابات و للتقرح عن طريق التصدي لحالة الأكسدة المرتبطة بالالتهاب، و بتنشيط مؤشرات الالتهاب ( $\alpha$  TNF، IL-1 $\beta$ ، iNOS، ICAM-1 و CIN-1). يوفر هذا العمل لأول مرة الأسس العلمية للاستخدام التقليدي الواسع لهذا النبات فيما يخص التعامل مع أمراض الجهاز الهضمي.

**الكلمات الدالة:** الريحان، الزيوت الأساسية، إفراغ المعدة، العبور المعوي، الإسهال، القرحة، التهاب القولون.

## Abstract

Myrtle or *Myrtus communis* L. (*M. communis* L.) belongs to the Myrtaceae family. It is a widespread shrub in the Mediterranean area. This work was mainly concerned with the effects of this widely used plant in traditional medicine on some physiopathologic states of the gastrointestinal tract. The phytochemical analysis of the essential oil from the leaves revealed that the latter is very rich in  $\alpha$ -pinene (54.1%) and 1,8-cineole (26.5%). The different methanolic fractions of both leaves and berries were also rich in the tested polyphenolic compounds. The preliminary HPLC analysis of both the methanolic extracts from the leaves and berries showed the presence of gallic acid, ellagic acid, gentisic acid, hydroxy benzoic acid and quercetin as the main constituents in the leaves and gallic acid, gentisic acid and vanillic acid in berries. The antidiarrhoeal effects of both essential oil and different extracts from the leaves were assessed in mice using different doses and different mechanisms. It was found that these elements had effective antidiarrhoeal actions. The same agents also had potent antiulcerogenic effects in the rat stomach. This antiulcerogenic effect may result at least in part from the antioxidant activity. Indeed, both essential oil and extracts showed antioxidant effects *in vivo* and *in vitro* with most tests. The mechanisms of action may also be due to the stimulation of mucus secretion. The results from the experimental colonic inflammation and ulceration showed that the extract has anti-inflammatory and antiulcerogenic effects by counteracting the oxidative status associated with inflammation, and by inhibiting the pro-inflammatory markers (IL-1 $\beta$ , TNF  $\alpha$ , iNOS, ICAM-1, and CIN-1). This work provides for the first time the scientific bases for the worldwide traditional use of this plant in the management of the digestive system ailments.

**Keywords:** *Myrtus communis* L., essential oil, gastric emptying, intestinal transit, diarrhoea, ulcer, colitis.

## List of abbreviations

<b>5-ASA:</b>	5-aminosalicylic acid
<b>ABTS:</b>	2,2'-Azino-bis (3-ethylbenzenothiazoline -6- sulfonic acid)
<b>APC:</b>	Antigen presenting cell
<b>AqE:</b>	Aqueous extract
<b>BHT:</b>	Butylated hydroxytoluene
<b>BMDM</b>	Bone marrow derived macrophage
<b>CAT:</b>	Catalase
<b>CD:</b>	Crohn disease
<b>CHE:</b>	Chloroform extract
<b>CINC-1:</b>	Cytokine-induced neutrophil chemoattractant-1
<b>CMC:</b>	Carboxymethyl cellulose
<b>CNS:</b>	Central nervous system
<b>Cox-2:</b>	Cyclooxygenase
<b>DAI:</b>	Disease activity index
<b>DC:</b>	Dendritic cell
<b>DMEM:</b>	Dulbecc's Modified Eagle Medium
<b>DMSO:</b>	Dimethyl sulfoxide
<b>DNBS:</b>	Dinitrobenzene sulfonic acid
<b>DPPH:</b>	2, 2-diphenyl-1-picryl- hydrazyl
<b>DSS:</b>	Dextran sulfate sodium
<b>DTNB:</b>	5, 5' -dithio-bis (2-nitrobenzoic acid)
<b>EAE:</b>	Ethyl acetate extract

<b>ENS:</b>	Enteric nervous system
<b>FCS:</b>	Foetal calf serum
<b>FRAP:</b>	Ferric reducing antioxidant power assay
<b>FTC:</b>	Ferric thiocyanate
<b>GADHP:</b>	Glyceraldehyde-3-phosphate dehydrogenase
<b>GC-MS:</b>	Gas Chromatography coupled to Mass Spectrometer
<b>GI:</b>	Gastrointestinal tract
<b>GPX:</b>	Glutathione peroxidase
<b>GSH:</b>	Glutathione
<b>GSSG:</b>	Glutathione disulfide oxidized
<b>HE:</b>	Hematoxylin
<b>HPLC:</b>	High-performed liquid chromatography
<b>HTAB:</b>	Hexadecyl trimethyl ammonium bromide
<b>HZ:</b>	Hertz
<b>IBD:</b>	Inflammatory bowel disease
<b>IC<sub>50</sub>%:</b>	Inhibitory concentration for 50% of activity
<b>ICAM-1:</b>	Inter-Cellular Adhesion Molecule
<b>IFN<math>\gamma</math>:</b>	Interferon-Gamma
<b>IL:</b>	Interleukin
<b>iNOS:</b>	inducible NOS
<b>JAK:</b>	Janus kinase
<b>LDL:</b>	Low density lipoprotein
<b>LPO:</b>	Lipid peroxidation

<b>LPS:</b>	Lipopolysaccharide
<b>MAPKs:</b>	Mitogen-activated protein kinase
<b>MBF:</b>	Mucosal blood flow
<b>MCP:</b>	Monocyte chemoattractant protein
<b>ME:</b>	Methanolic extract
<b>MEO:</b>	Myrtle essential oil
<b>MIP:</b>	Macrophage inflammatory protein
<b>MΦ:</b>	Macrophage
<b>MPO:</b>	Myeloperoxidase
<b>MMP:</b>	Matrix metalloproteinase
<b>MUC:</b>	Mucin
<b>NADPH:</b>	Nicotinamide adenine dinucleotide phosphate
<b>NF-κB:</b>	Nuclear factor-Kappa B
<b>NGF:</b>	Nerve growth factor
<b>NK:</b>	Natural killer cell
<b>NO:</b>	Nitric oxide
<b>NOS:</b>	Nitric oxide synthase
<b>NSAIDs:</b>	Non-steroidal anti-inflammatory drugs
<b>OECD:</b>	Organisation for Economic Co-operation and Development
<b>PAP:</b>	Platelet activating factor
<b>PBS:</b>	Phosphate buffered saline
<b>PCR:</b>	Polymerase chain reaction
<b>PG:</b>	Prostaglandin

<b>PK:</b>	Protein kinase
<b>qPCR:</b>	Real time quantitative PCR
<b>RNS:</b>	Reactive nitrogen species
<b>ROS:</b>	Reactive oxygen species
<b>RT-PCR:</b>	Reverse transcriptase-PCR
<b>SOD:</b>	Superoxyde dismutase
<b>STAT:</b>	Signal transducer and activator of transcription
<b>TBA:</b>	Thiobarbutiric acid
<b>TBARs:</b>	TBA-reactive species
<b>TCA:</b>	<b>Trichloro-acetic acid</b>
<b>TGF-<math>\beta</math>:</b>	Transforming growth factor
<b>TH:</b>	T helper cell
<b>TLR:</b>	Toll-like receptor
<b>TNBS:</b>	Trinitrobenzene sulfonic acid
<b>TNF:</b>	Tumor necrosis factor
<b>Treg:</b>	Regulatory T cell
<b>UC:</b>	Ulcerative colitis
<b>WHO:</b>	World Health Organization



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# **Introduction and aims**

The human digestive system is a very important and complex system. It consists of a hollow tube and some associated organs that assist in its functional duties. The main role of the digestive system is to digest the ingested food and beverages, to absorb their important constituents and to get rid of unexplored and some waste contents of the body. It usually resists the different contaminants and noxious agents through a sophisticated system of defense. The ability of the gastrointestinal tract to withstand the injurious factors relies on several important protective factors.

However, when these are exceeded by the noxious agents, real disturbances of the normal physiology of the tract occur and may lead to life threatening diseases, such as ulcers, diarrhoea and cancers.....

Ulcers occur along the gastrointestinal tract and can be classified into the upper gastrointestinal ulcers, including duodenal ulcers, gastric ulcers and oesophageal ulcers. The lower gastrointestinal ulcers include ulcerative colitis and crohns disease. The gastrointestinal ulcers can be defined as lesions that occur in the mucosal part of the tract under sevral factors, such as stress, acid, bacterial, genetic and immunological factors depending on the affected part of the tract.

Diarrhoea may be defined as a discharge of semisolid or watery faecal matter three or more times per day. The above mentioned ailments of digestive tract are among the most spread diseases of the system and represent a leading cause of morbidity and mortality especially in the developing countries where they are responsible for the death of millions of people each year.

The herbal medecin has a long therapeutical history for the management of human and animal health and is still being widely used by a large population of the world. Indeed, some of these plants are of real medicinal values being the source of many modern drugs.

The World Health Organization (WHO) acknowledged the use of herbal medicine and has considered it as an important part of the world health care system. *Myrtus communis* L. (*M. communis* L.) is an evergreen endemic shrub of the Mediterranean area and it is widely used by the local population of both industrial and medicinal traitement. In Algeria, it is also used for the relief and treatment of several disorders such as diabetis, respiratory diseases and the gastro intestinal disorders. However, the scientific bases of this traditional use of this plant in diarrhoeal and ulcerative diseases are lacking.

Thus, the aims of this study were:

- Determination of polyphenols and phytoanalysis of the plant extracts.
- Extraction and analysis of the plant essential oil.
- Evaluation of the acute oral toxicity of the plant extracts and essential oil.
- To study the effect of *M.communis* L. extracts and essential oil on:
  - \*Gastrointestinal motility and diarrhoea
  - \*Ethanol-induced gastric ulceration in rats
- Evaluation of the *in vitro* antioxidant activity of plant extracts and essential oil.
- Evaluation of the *in vivo* antioxidant activity of plant extracts and essential oil.
- To perform *in vivo* studies on the trinitrobenzene sulphonic acid (TNBS) inducing colitis in rats.

# Literature review

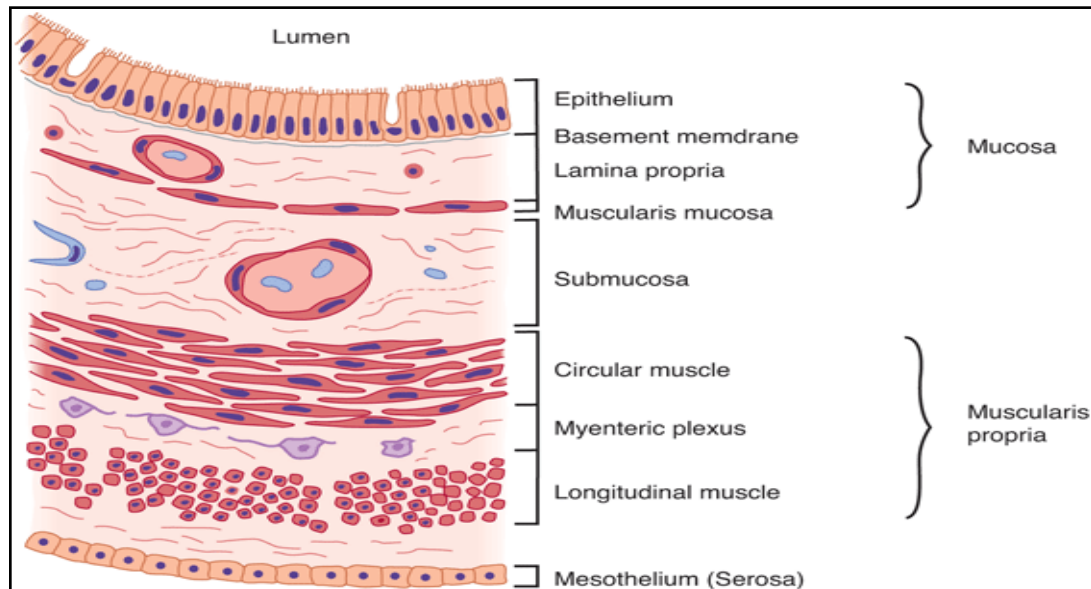
## 1 Structure of the normal gastrointestinal tract

The principal function of the gastrointestinal (GI) tract is to ensure the proper digestion and absorption of nutrients and the expulsion of undigested residue and unwanted waste. This complex process requires the coordinated propulsion of endoluminal content along the length of the GI tract, which in turn relies on the activity of specialized cells, including smooth muscle cells, and a hierarchy of intrinsic and extrinsic neurons to regulate the motor programs (Knowles *et al.*, 2013). The gastrointestinal tract also serves as a barrier from the directly contiguous external and internal environment. The GI tract is also one of the largest endocrine organs and plays a critical role in the regulation of satiety and weight control (Urbanska *et al.*, 2016).

### 1.1 Organization and cellular arrangements in the gastrointestinal tract

The digestive system consists of the alimentary tract or canal and the associated organs such as, teeth, salivary glands, the liver and the pancreas. The alimentary tract is a hollow tube that extends from the mouth to the anus. It is divided into the mouth, pharynx, esophagus, stomach, duodenum, jejunum, ileum, colon, rectum, and the anus. The duodenum, jejunum and ileum form the small intestine, whereas the colon is sometimes called the large intestine. In some parts of the tube, a large number of glands in its wall empty their secretions in the lumen. Moreover, the associated glandular glands, such as the salivary glands, the pancreas and the liver also empty their secretions in the lumen. The structure of the tract varies from one region to another, but there are similarities in the four main layers that constitute its wall. These are from the inside to the outside, the mucosa, the submucosa, the muscularis externa, and the serosa that covers the wall of the tract except the esophagus and the distal rectum (Figure 1). The mucosa is composed of the epithelium, the lamina propria, and the muscularis mucosae. The epithelium is a single layer of specialized cells that line the lumen; the most abundant are cells called absorptive enterocytes, enteroendocrine cells and mucin-secreting cells. Beside this, in the gastric epithelium specialized cells produce protons, pepsins and bicarbonate. The nature of the epithelium changes from one part to another in the tract. Beneath the epithelium, the lamina propria is a connective tissue rich in glands and contains nerve fibers, capillaries, and lymph vessels and nodules. The muscularis mucosa is a thin layer of smooth muscle. The submucosa is a connective tissue that contains larger nerve trunks, blood vessels, and lymph vessels, together with the first plexus of the enteric nervous system (ENS); the submucosal plexus. The muscularis externa consists of two muscular

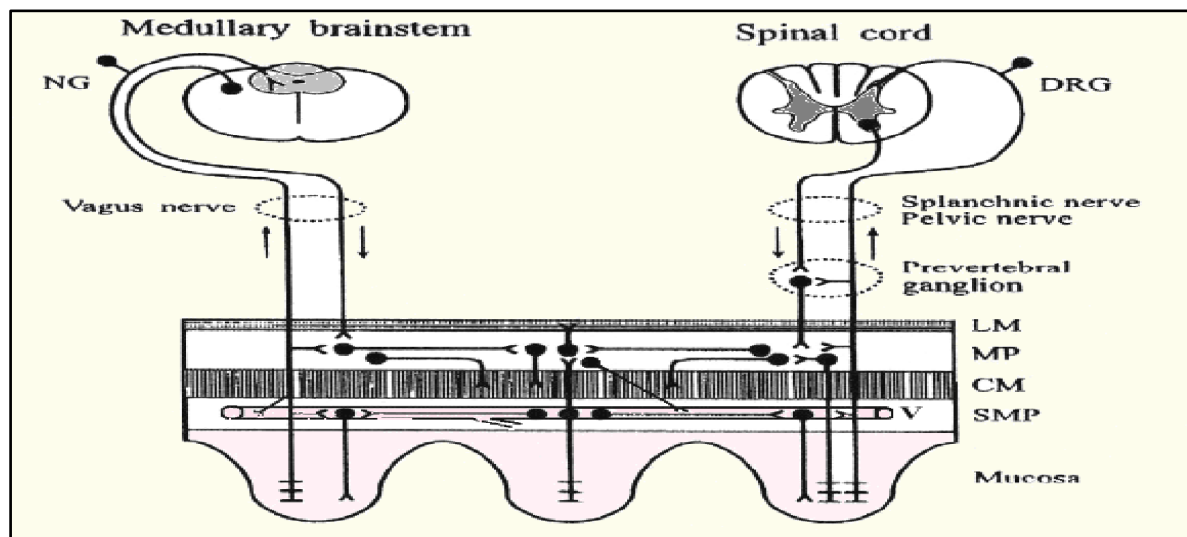
layers; the inner circular layer and the outer longitudinal layer. Between the two layers lies the second plexus of the ENS; the myenteric plexus. The last or the outermost layer is the serosa or adventitia. It consists of a layer of squamous mesothelial cells and it is a part of the mesentery (Rhoades and Pflanzer, 1996).



**Figure 1:** Organization of the wall of the intestine into functional layers (Ganong, 2010)

## 1.2 Neural control of the gastrointestinal tract

The enteric nervous system is a morphologically and functionally complex system that controls, largely independent of central and peripheral nervous system input, virtually all gut functions. It is comprised of a very large number of neuronal and glial cells (about the same number as are in the mammalian spinal cord). It is organized into two major plexuses: the myenteric (Auerbach's plexus), which extends along the entire length of the GI tract, and the submucosal (Meissner's plexus), which is present from the stomach to the rectum (Figure 2). Enteric neurons can be classified into functionally distinct sub populations (e.g. intrinsic primary afferent neurons, motor neurons, and interneurons) synaptically linked in reflex circuitries. The central nervous system (CNS) can modulate some intrinsic reflexes, particularly in the esophagus, stomach and rectum, via sympathetic and parasympathetic pathways. However, the ENS has the ability to control most gut functions independent of CNS input, including secretion, absorption, vascular tone, and motility (Furness, 2012).



**Figure 2:** Schematic diagram showing the multiple innervation of the gastrointestinal tract (Adapted from Holzer *et al.*, 2001). LM: longitudinal muscle; CM: circular muscle; MP: myenteric plexus; SMP: submucosal plexus; DRG: dorsal root ganglion; NG: nodose ganglion; V: vessel

### 1.3 Gastrointestinal circulation

The circulation of the stomach, small and large intestines, pancreas and liver is called the splanchnic circulation. The heart pumps blood to these organs through arteries that branch from the abdominal aorta. The blood that leaves the stomach, intestines, and pancreas goes next to the liver by way of the portal vein. The significance of this vascular organization is that many absorbed digestion products are subject to metabolic processing by the liver before being distributed to the cells of the body. The splanchnic circulation receives almost one fourth of the cardiac output. Its resting flow rate is about 1400 ml per minute, which is greater than that of any of the other major peripheral circulations. Flow of blood through the splanchnic circulation increases when a meal is eaten, this increase facilitates the removal of the digestion products from the digestive tract. In addition, extra oxygen and other nutrients are supplied to meet the enhanced demands for energy imposed by the increased muscular, secretory and absorptive activities that occur after a meal (Rhoades and Pflanzer, 1996).

### 1.4 The stomach

The stomach can be divided functionally into 3 parts, proximal stomach (cardia, fundus and proximal corpus), distal stomach (distal corpus and antrum) and pylorus. The gastric mucosa contains many deep glands. In the *pyloric* and cardiac regions, the glands secrete mucus. In the body of the stomach, including the fundus, the glands contain parietal



cells, which secrete hydrochloric acid and intrinsic factor, and chief (zymogen, peptic) cells, which secrete pepsinogens. These secretions mix with mucus secreted by the cells in the necks of the glands. Mucus is also secreted along with  $\text{HCO}_3^-$  by mucus cells on the surface of the epithelium between glands. The stomach has a very rich blood and lymphatic supply. Its parasympathetic nerve supply comes from the vagi and its sympathetic supply from the celiac plexus. Stomach contains three muscle layers: outer longitudinal muscle, intermediate oblique muscle layer, while the myenteric plexus is located in between the circular and longitudinal muscle layer (Ganong, 2010).

Central and peripheral stimuli of gastric acid secretion are mediated through activation of histaminergic, gastrinergic, and cholinergic pathways coupled to intracellular second messenger systems that determine the trafficking and activity of  $\text{H}^+\text{K}^+$ -ATPase, the proton pump of the parietal cell. The major stimulants of acid secretion are histamine, gastrin, and acetylcholine, whereas, the main inhibitor is somatostatin (Ganong, 2010)

### 1.5 The intestines

The small **intestine** is the part of the gastrointestinal tract between the stomach and the large intestine, and is where most of the end absorption of food takes place. The small intestine has three distinct regions: the duodenum, jejunum, and ileum. The internal walls of the small intestine are covered in finger-like tissue called villi. Each of these villi is covered in even smaller finger-like structures called microvilli. These villi and microvilli increase the surface area available for the absorption of nutrients. The primary function of the small intestine is the absorption of nutrients and minerals from food. The small intestine also supports the body's immune system.

In the **colon**, the surface of the mucosa is flat and its architecture is characteristic with crypts as straight tubes, in parallel alignment. The crypt base rests upon a layer of smooth muscle cells, the muscularis mucosae, which separates the mucosa from the submucosal connective tissue. The distance between the crypts and the internal diameter of the crypts is constant. The crypt architecture is maintained throughout the colon, except in the presence of lymphoid collections, in zones of transition to small intestinal mucosa (ileocecal valve) or to squamous epithelium (the anorectum) and in normally occurring grooves in the surface. Many mucous glands secrete mucus into the hollow lumen of the large intestine to lubricate its surface and protect it from rough food particles (McGuiness, 2010; Ganong, 2010).

The main function of the colon is absorption of water,  $\text{Na}^+$  and other minerals. By removal of about 90% of the fluid, it converts the 1000-2000 mL of isotonic chyme that enters it each day from the ileum to about 200-250 mL of semisolid feces. There are no villi on the mucosa. The colonic glands are short inward projections of the mucosa that secrete mucus (Ganong, 2010; McGuiness, 2010).

## **2 Peptic ulcer**

Peptic ulcer forms in the stomach or upper part of the small intestine and is the most frequent upper gastrointestinal acid-related disease of the digestive system, significantly affecting millions of people worldwide. Peptic ulcers frequently occur along the lesser curvature of the antral end of the stomach or, more rarely, in the lower end of the esophagus where stomach juices frequently reflux (Kansara and Sakhreliya, 2013). Gastric ulcer is predominantly characterized by damage to the gastric mucosa in the stomach lining, resulting in abdominal pain, possible bleeding, chest pain, fatigue, vomiting and weight loss and other gastrointestinal symptoms (Awaad *et al.*, 2013; Son *et al.*, 2015).

### **2.1 Epidemiology**

The annual incidence rates of peptic ulcer diseases for the last decades were 0.1-0.19% for physician diagnosis (Sung *et al.*, 2009). Based on 31 published papers, in the last 3 decades that had reported incidence rate estimates for peptic ulcer in the general population, it was found that the incidence rate was 1 case per 1000 person-years (Lin *et al.*, 2011). In a study from Sweden in which the symptomatic and asymptomatic peptic ulcer disease was considered the prevalence of peptic ulcer was 4.1%, where 19.5% of peptic ulcer disease were asymptomatic (Aro *et al.*, 2006).

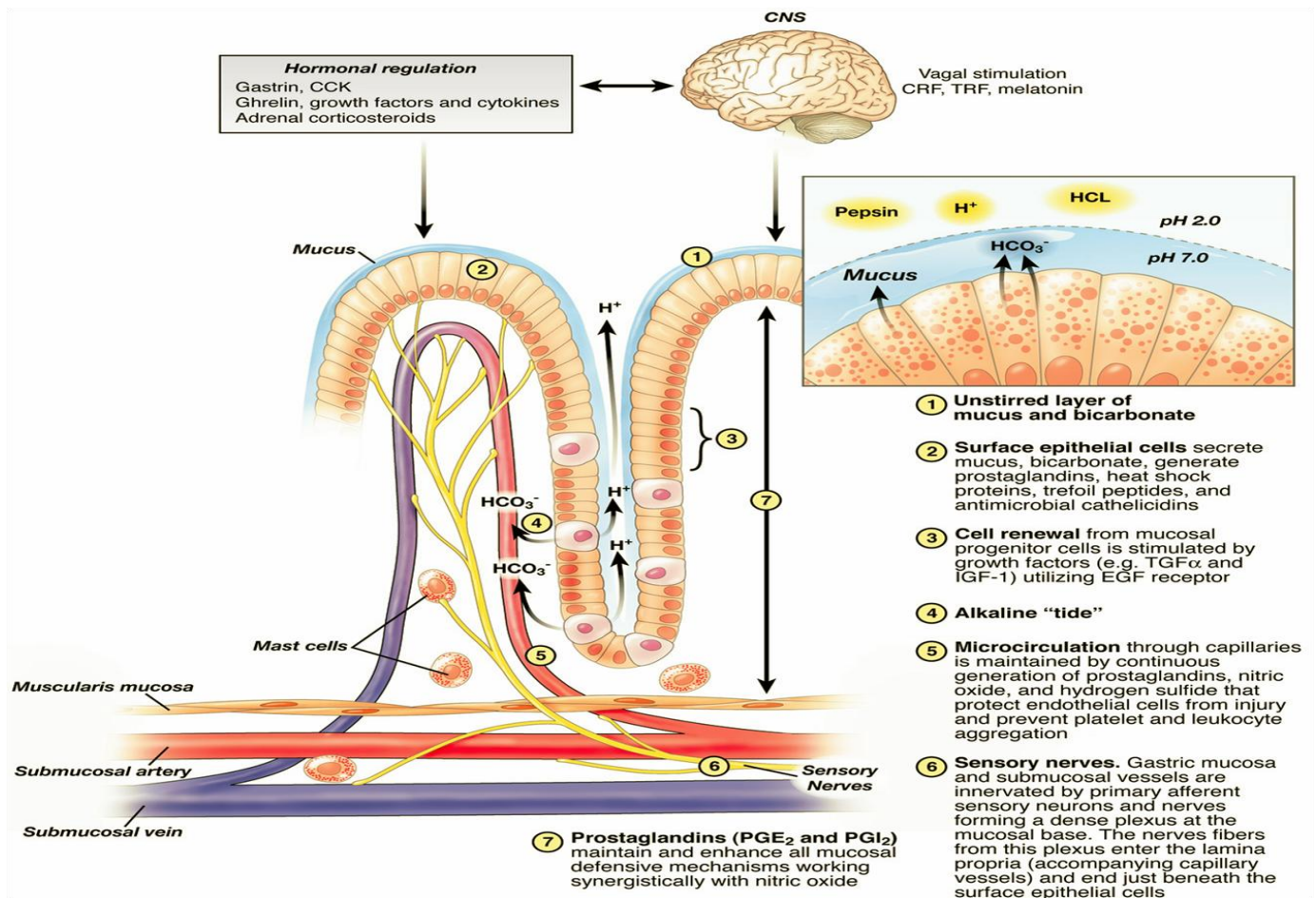
Every year, 4 million people are diagnosed with gastric ulcer disease around the world (Zelickson *et al.*, 2011). Complications are encountered in 10%-20% of these patients and 2%-14% of the ulcers will perforate (Lau *et al.*, 2010). An estimated 6000 people die every year because of the complications associated with stomach ulcer. 40,000 people undergo surgery in order to get relief from the persistent symptoms of ulcer annually. An estimated 15,000 deaths occur as consequence of peptic ulcer disease (Sandhya *et al.*, 2013).

## 2.2 Aetiology

The multifactorial etiology of gastric ulcer includes bacterial infection, excessive alcohol intake, emotional stress, free radicals, the use of steroidal and non-steroidal anti-inflammatory drugs (NSAIDs), and nutritional deficiencies that disrupt the gastric mucosal barrier and make it vulnerable to normal gastric secretions. Ulcerogenic risk factors, such as excessive alcohol consumption and use of NSAID drugs, cause dispersal of the protective mucus gel and the phospholipid bilayer, resulting in acid back diffusion and mucosal injury secretions (Son *et al.*, 2015).

## 2.3 Pathophysiology of ulcers

The pathophysiology of ulcer is due mainly to an imbalance between aggressive factors (acid, pepsin, *H. pylori*, NSAIDs and local mucosal defensive factors (mucus, blood flow, endogenous prostaglandins (PGs) secretion, nitric oxide (NO), antioxidant and bicarbonate etc). The integrity of gastro-duodenal mucosa is maintained through a homeostatic balance between these aggressive and defensive factors (Sumbul *et al.*, 2010; Kansara and Sakhreliya, 2013). The gastric and duodenal mucosa is covered by mucus and bicarbonate in order to protect against gastric acid. Mucus and bicarbonate are secreted by gastric epithelium and by Brunner's glands in the duodenum. The epithelium also has a role in acid protection. The apical cell membranes and the tight junctional complexes between the surface cells limit the penetration of hydrogen ion into the mucosa. In addition, mucosal blood flow transports nutrients and oxygen and bicarbonate to the surface to neutralise acid (Figure 3) (Allen and Flemstrom, 2005).



**Figure 3:** Gastroduodenal mucosal defense mechanisms (Laine *et al.*, 2008).

## 2.4 Models of gastric ulcer

There are several models that are used to evaluate antiulcer drugs. However, the choice of a suitable model has proven to be difficult as each model has significant advantages as well as disadvantages. The choice of a particular model is sometimes influenced by local resources, the objectives of the study, the hypothesis being tested, or research questions being answered by the researcher. The choice of model may also depend on the relevance to the type of peptic ulcer disease under investigation (Adinortey *et al.*, 2013; Thabrew and Arawwawala, 2016).

Peptic ulcers can be induced by physiological, pharmacological or surgical manipulations in several animal species. However, most experiments in peptic ulcer studies are carried out in rodents. Several models are used experimentally for testing or evaluating anti-peptic ulcer activity of drugs/agents and the main of these are:

### 1. Ethanol-induced gastric ulcers

2. NSAIDs- (indomethacin, aspirin, and ibuprofen) induced gastric ulcers.
3. Water-immersion stress or cold-water-restraint or cold-restraint stress.
4. Acetic acid-induced gastric ulcers.
5. Histamine-induced gastric ulcers.
6. Reserpine-induced gastric ulcers.
7. Serotonin-induced gastric ulcers.
8. Pylorus-ligated-induced peptic ulcers (Adinortey *et al.*, 2013; Thabrew and Arawwawala, 2016).

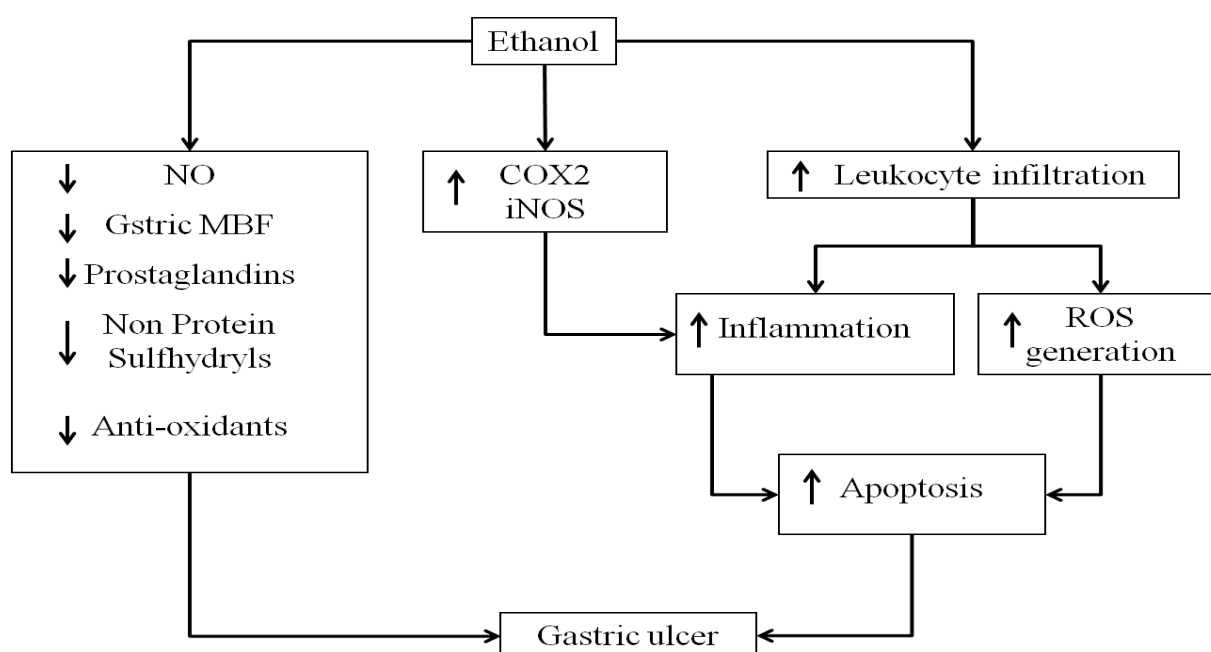
### **Ethanol-induced gastric ulcer model**

Alcohol consumption may results in several diseases including gastric ulcer. Because of its damaging effect, ethanol has been explored as a model in gastric ulcer. It is one of the must widely used models in testing gastroprotective agents. The mechanisms of ethanol-inducing ulcer are diverse. It may directly affect the gastric mucosa by reducing bicarbonate and mucus secretions and exposing it to the damaging effect of acid and pepsin or by acting on neurohormonal factors that stimulate acid secretion (Bode and Bode, 1997). Ethanol may also decrease blood flow (Adinortey *et al.*, 2013).

One crucial mechanism through which ethanol provokes gastric ulceration is via increased oxidative stress and reducing the antioxidant system (Hirokawa *et al.*, 1998; Samonina *et al.*, 2004). ROS are responsible for oxidation of tissues leading to lipid peroxidation and tissue damage. They are also responsible for oxidation of bases in cellular DNA making them mutagenic, cytotoxic and cross linking agents, which in turn causes uncontrolled expression of certain genes causing increased multiplication of cells leading to cancer. Antioxidants seemed to have protective role in gastric ulcers. Stress causes both sympathetic (causes direct arteriolar vasoconstriction) and parasympathetic (induces an increased motility and muscular contraction) stimulation of stomach leading to local hypoxia and near or actual “ischemia”. The ischemic condition caused an increase in the level of  $H_2O_2$  (by the action of SOD), which, in conjugation with  $O_2$  generates OH via the methyl catalyzed Haber-Weiss reaction. Hydroxyl radicals thus generated oxidizes important cellular constituents such as structural and functional proteins membrane lipids and depletes

glutathione. Lipid peroxidation causes loss of membrane fluidity, impaired ion transport and membrane integrity and finally loss of cellular functions (Suzuki *et al.*, 2012).

In addition, ethanol causes a massive intracellular accumulation of calcium leading to cell death (Wong *et al.*, 1991). Moreover, ethanol relaxed the pyloric sphincter and increased gastric emptying which may result gastric damage (Dinoso *et al.*, 1972). The ethanol-induced ulcer model looks much more appropriate for evaluating cytoprotection and/or antioxydant agents and the general pathways of ethanol-inducing gastric ulcer could be summarized in Figure 4.



**Figure 4:** Main pathways of ethanol-inducing gastric ulcer

## 2.5 Treatment

Peptic ulcer disease is a serious gastrointestinal disorder that requires a well-targeted therapeutic strategy. For over a century, peptic ulcer disease has been one of the leading causes of gastrointestinal surgery, with high morbidity and mortality rates. Several classes of pharmacological agents have proved to be effective in the management of the acid peptic disorders. These groups include: antacids (aluminum hydroxide, magnesium trisilicate), acid suppressive agents (Antisecretory drugs) which include proton pump H<sup>+</sup>/K<sup>+</sup> ATPase inhibitors (omeprazole, lansoprazole), histamine H<sub>2</sub> receptor antagonist (cimetidine, ranitidine) and anticholinergic (pirenzepine), cytoprotective agents (sucralfate and

prostaglandin analogs (misoprostol), antimicrobials for eradication of *H. pylori* (amoxicillin, clarithromycin) and Triple therapy (one week triple therapy consisting of a proton pump inhibitor such as Omeprazole and the antibiotics Clarithromycin and Amoxicillin) (Waller *et al.*, 2005). Although these drugs are effective, they produce many adverse effects (headache, diarrhoea, abdominal pain and nausea), thus limiting their use. In recent years, there has been a growing interest in alternative therapies, especially those from plants due to their perceived relative lower side effects (Adinortey *et al.*, 2013; Sriniva *et al.*, 2013; Al-Snafi, 2016). Indeed, a large number of medicinal plants and their secondary metabolites possess beneficial effects in gastric diseases especially peptic ulcer diseases and in intestinal disease especially diarrhoea and ulcerative colitis, these plants include:

***Boswellia* (*Boswellia serrata*)** which is reported to be effective in IBD. *Boswellia serrata* represses the formation of leukotriene via inhibition of 5-lipoxygenase with the action of two *boswellia* acids, namely 11-keto- $\beta$ -boswellic acid and acetyl-11-keto- $\beta$ -boswellic acid (Gupta *et al.*, 2001). ***Triticum aestivum*:** Treatment of patients with wheat grass juice (*Triticum aestivum*) (as food, or as a drug), was associated with significant reduction in the overall disease activity index and in the severity of rectal bleeding. Apart from nausea, no other serious side effects were noticed. *Triticum aestivum* L. (Poaceae) juice appeared effective and safe as a single or adjuvant treatment of active distal UC (Ben-Arye *et al.*, 2002). ***Aloe vera* (Xanthorrhoeaceae):** is an herbal preparation with significant anti-inflammatory effects. Oral administration of *aloe vera* produced a clinical response more often than placebo; it also reduced the histological disease activity and appeared to be safe (Langmead *et al.*, 2004). **Green tea (*Camellia sinensis* L.)** was found to be effective in the treatment of ulcerative colitis. Both diarrhoea and loss of body weight can be significantly attenuated by the treatment with green tea extract. The mechanism of action was associated to remarkable amelioration of the disruption of the colonic architecture, significant reduction of colonic myeloperoxidase (MPO) and tumor necrosis factor-alpha (TNF- $\alpha$ ) production (Mazzon *et al.*, 2005). *Zingiber Officinale* Roscoe (Zingiberaceae) extract showed a valuable effect against acetic acid-induced ulcerative colitis possibly by its antioxidant and anti-inflammatory properties (El-Abhar *et al.*, 2008). ***Ginkgo biloba* L.** (Ginkgoaceae) was found to suppress the activation of macrophages and can be used to both prevent and treat mouse colitis (Kotakadi *et al.*, 2008). Administration of ***Garcinia cambogia* Desr.** (Clusiaceae) extract to colitic rats significantly improved the macroscopic damage and caused substantial

reductions in increases in MPO activity, COX-2 and iNOS expression. In addition, garcinia extract treatment was able to reduce PGE<sub>2</sub> and IL-1beta colonic levels (dos Reis *et al.*, 2009).

### **3 Inflammatory bowel disease (IBD)**

Immune responses in the digestive tract are tightly regulated to ensure host protective immunity and prevent the development of immune-mediated pathologies. The host immune system must efficiently respond to invading pathogenic microorganisms, while simultaneously and specifically blocking these mechanisms in response to antigens derived from the abundant commensal gut flora and the alimentation. The integrity of the intestinal mucosa is crucial and acts as a physical barrier, but additional tolerance mechanisms are required to prevent the excessive inflammation that can lead to the rupture of intestinal homeostasis observed in IBDs (Pedros *et al.*, 2016).

Inflammatory bowel disease is a spontaneous chronic, relapsing, and remitting disorder of the gastrointestinal tract (GIT) that comprises two major subtypes: ulcerative colitis (UC) and Crohn's disease (CD). UC was first described in the mid-1800s, whereas CD was first reported later, in 1932, as "regional ileitis" (Danese and Fiocchi, 2011). UC and CD are characterized by chronic intestinal inflammation resulting in recurrent episodes of disease exacerbations with associated abdominal pain, diarrhoea, weight loss and rectal bleeding (Allegretti *et al.*, 2014). IBD considerably reduces patients' health-related quality of life and influences their professional, social and personal lifestyle. Due to the early onset and chronic character of the disease, patients have to deal with their disorder throughout their lifetime (Rencz *et al.*, 2015).

The inflammation in UC is confined to the colonic mucosa, beginning in the rectum and extending proximally in a continuous fashion. The inflammation in CD is transmural and can involve any part of the gut. Both UC and CD develop during childhood or adolescence in up to 25% of patients, with UC often having an earlier age of onset than CD (Griffiths *et al.*, 2004).

#### **3.1 Ulcerative colitis**

Ulcerative colitis (UC) is an inflammatory bowel disease that primarily affects the colonic mucosa. In its most limited form it may be restricted to the distal rectum, while in its most extended form, the entire colon is involved (Friedman and Blumberg, 2005).



UC can occur in both sexes and in any age group but most often begins in people between 15 and 30 years of age. The exact causes of UC are still not clear but different factors have been postulated as possible etiologic agents. They are genetic factors, infective agents, immunological basis, medications and pathological factors (Berardi, 2000).

In patients with UC, ulcers and inflammation of the inner lining of the colon lead to symptoms of poor appetite, weight loss, bloody diarrhoea, passage of pus and mucus, and abdominal cramping during bowel movements (Baumgart and Sandborn, 2007; Awaad *et al.*, 2013). Clinical signs of the disease may be mild, moderate or severe:

- Mild: Less than four stools per day, with or without blood, with no systemic disturbance and a normal erythrocyte sedimentation rate (ESR).
- Moderate: More than four stools per day with minimal systemic disturbance.
- Severe: More than six bloody stools per day, with the evidence of systemic disturbance as fever, tachycardia, anemia, or ESR of more than 30 (Awaad *et al.*, 2013).

### **3.1.1 Epidemiology**

Approximately 10 individuals per 100 000 per year are diagnosed with UC. The incidence of UC varies depending on geography, and is most common in Western countries, including the US. The highest incidence and prevalence of IBD are seen in the populations of Northern Europe and North America (USA: 1.4 millions) and the lowest in continental Asia (Cui *et al.*, 2014), where ulcerative colitis is by far the most common form of inflammatory bowel disease (Danese and Fiocchi, 2011). The prevalence of IBD is of 150-250 per 100000 populations (Halpin and Ford, 2012) and about 0.4% in the population in North America and Europe (Loftus, 2004).

### **3.1.2 Aetiology and risk factors**

A westernized environment and lifestyle is linked to the appearance of inflammatory bowel disease, which is associated with diets high in fat and sugar, medication use, stress, and high socioeconomic status (Danese and Fiocchi, 2011). Although the precise etiology of IBD still remains obscure, the accepted hypothesis is that in genetically predisposed individuals the commensal luminal flora trigger an inappropriate, overactive mucosal immune response causing intestinal tissue damage that is further modified by specific environmental factors

(e.g., smoking)(Magyari *et al.*, 2014). Evidence from both UC patients and animal models of colitis suggest that UC is influenced by a complex interaction of genetic, environmental, and immunologic factors:

#### 3.1.2.1 Genetic susceptibility

Although the exact pathogenesis of IBD remains unknown, part of the underlying mechanism is a deregulated host immune response to intestinal flora, in genetically susceptible individuals. The greatest risk for developing IBD is having a family history of the disease. The greatest risk is seen in monozygotic twins and if both parents suffer from IBD. The estimated relative risk to a sibling of a patient with IBD is 13-36 and 7-17 for CD and UC respectively (Abegunde *et al.*, 2016).

#### 3.1.2.2 Environmental influences

Environmental factors such as smoking, infection, drugs, stress, air pollution, water pollution, diet, and food additives have been investigated in IBD and other autoimmune diseases. These factors have been collectively referred to as exposomes. The term exposome refers to all possible environmental exposures on a human being from conception to death (Abegunde *et al.*, 2016). The clarification of the pathophysiological mechanisms in relation with the environmental effect on the incidence of IBD can lead to more effective prevention and/or treatment of disease. Environmental factors are suggested to affect not only the occurrence but also the phenotype of the diseases. Crohn's disease and ulcerative colitis are multifactorial diseases that are manifested after disruption of a genetic predisposed individual and its intestinal microflora through an environmental stimulus. Urbanization and industrialization are associated with IBD. Epidemiological data, clinical observations and family/immigrants studies indicate the significance of environmental influence in the development of IBD. Some environmental factors have a different effect on the subtypes of IBD. Smoking and appendectomy is negatively associated with UC, but they are aggravating factors for CD (Legaki and Gazouli, 2016; Abegunde *et al.*, 2016). Nonsteroidal anti-inflammatory drugs trigger exacerbation of UC or can induce the disease because of the decreased production of mucosal prostanoids and increased adherence and migration of the leukocytes. Oral contraceptive pills as well as the inadequacy of vitamin D leads to an increased risk for IBD and a more malignant course of disease. Moreover, other factors such as air pollution, psychological factors, sleep disturbances and exercise influence the development and the course of IBD. A westernized high fat diet, full of refined carbohydrates

is strongly associated with the development of IBD, contrary to a high in fruit, vegetables and polyunsaturated fatty acid-3 diet that is protective against these diseases (Legaki and Gazouli, 2016).

#### 3.1.2.3 **Microbiota**

The intestinal microbial community plays an important role for the host, as it carries out many useful functions including the digestion of substrates that host enzymes are unable to digest; the production of vitamins and short chain fatty acid; the formation of enteric immune system; and the protection of enteric homeostasis repressing the growth of harmful microorganisms (Legaki and Gazouli, 2016). Changes in the gut microbiota have been repeatedly reported in patients with IBD, with certain changes clearly linked to either CD or UC (Marchesi *et al*, 2016). Patients with IBD present a different composition in their intestine characterized by a reduction in their microbial diversity, specifically reduction of the dominant members of the gut microbiota. This altered balance in the gut microbiota constituents, called dysbiosis, causes functional changes that seem to be involved in the pathophysiology of many diseases, including IBD (Legaki and Gazouli, 2016).

#### 3.1.2.4 **Immunological defects**

In IBD, the immune defense against intestinal microbes fails at two levels: (1) the epithelial mucosal barrier is impaired and (2) the innate and acquired host immune responses are altered. The immunopathogenesis of IBD occurs in three temporally distinct stages: (1) penetration of luminal contents into underlying tissues which may be facilitated by environmental factors such as infection or inherent defects in mucosal barrier, (2) impaired clearance of foreign material from the bowel wall which may be due to defective secretion of pro-inflammatory cytokines by macrophages and (3) a compensatory acquired immune response which leads to a chronic inflammatory response and gives rise to characteristic IBD lesions (Matricon *et al.*, 2010).

An inappropriate immune response to normal luminal components is proposed to increase the risk of UC. In the healthy gut, commensal bacteria live symbiotically with the host. It is hypothesized that exposure to commensal bacteria down-regulates the inflammatory genes and blocks activation of the Nuclear Factor Kappa-B (NF- $\kappa$ B) pathway, thus inhibiting the inflammatory immune response of the gut to the microbes. In UC, this tolerance is lost.

Exposure to luminal microflora triggers an inflammatory response by the cells lining

the mucosa, leading to chronic, destructive immune responses. Uncontrolled immune responses can impair gut barrier function (Zhang *et al.*, 2014).

In the healthy gut, the presence of tight junctions and a colonic mucus layer provides an effective barrier against luminal microbes and antigens. The intestinal epithelial cells also have developed control mechanisms that limit inappropriate activation of immune responses. However, when microbes cross the mucosal barrier, they may come into direct contact with immune cells, thereby intensifying the permeability defect, and establishing a self-sustaining cycle of mucosal inflammation. Consequently, immunoregulatory defects and microbial exposure increase the risk of colitis development (Zhang *et al.*, 2014).

### 3.1.3 Pathophysiology of ulcerative colitis

The innate immune system comprises the epithelial cell barrier, macrophages, monocytes, neutrophils, dendritic cells (DCs), natural killer (NK) cells, eosinophils and basophils. When innate immunity fails to counteract the pathogen aggression, the adaptive immune response is triggered. In this process, DCs get into the mesenteric lymph nodes, and then they present the antigen to naive T cells and, depending on the factors released by DCs, induce the T cell differentiation (Figure 5) (Cerovic *et al.*, 2013). Depending on the effector cytokines produced by APCs, naive T cells have the potential to differentiate into different T helper (Th) subtypes. IL-12 generates Th1 cells, IL-4 promotes Th2 differentiation, IL-10 and TGF- $\beta$  induce regulatory T cells (Treg), whereas IL-6, IL-1 $\beta$  and TGF- $\beta$  promote Th17 cells (Vezza *et al.*, 2016). In addition to Th cells, neutrophils are also able to infiltrate the inflamed mucosa and generate several pro-inflammatory cytokines, and induce oxidative reactions, thus modifying the redox balance in the gut mucosa, which can collaborate to maintain the inflammatory status through the upregulation of redox-sensitive signaling pathways and transcription factors (Piechota-Polanczyk and Fichna, 2014).

The pathogenesis of ulcerative colitis is believed to be related to a dysregulated proinflammatory response to commensal gut bacteria. Due to mutations, some mucosal defense mechanisms are disrupted. MUC<sub>2</sub> is the most abundant protective mucin that coats the epithelium and prevents entry of microbes. When the disease is active, the lamina propria of the mucosa becomes heavily infiltrated with a mixture of acute and chronic inflammatory cells. There is a predominant increase in mucosal IgG production, evidence of complement activation, and activation of macrophages and T cells. This immunological activity is associated with the release of a vast array of cytokines, kinins, leukotriens, platelet activating

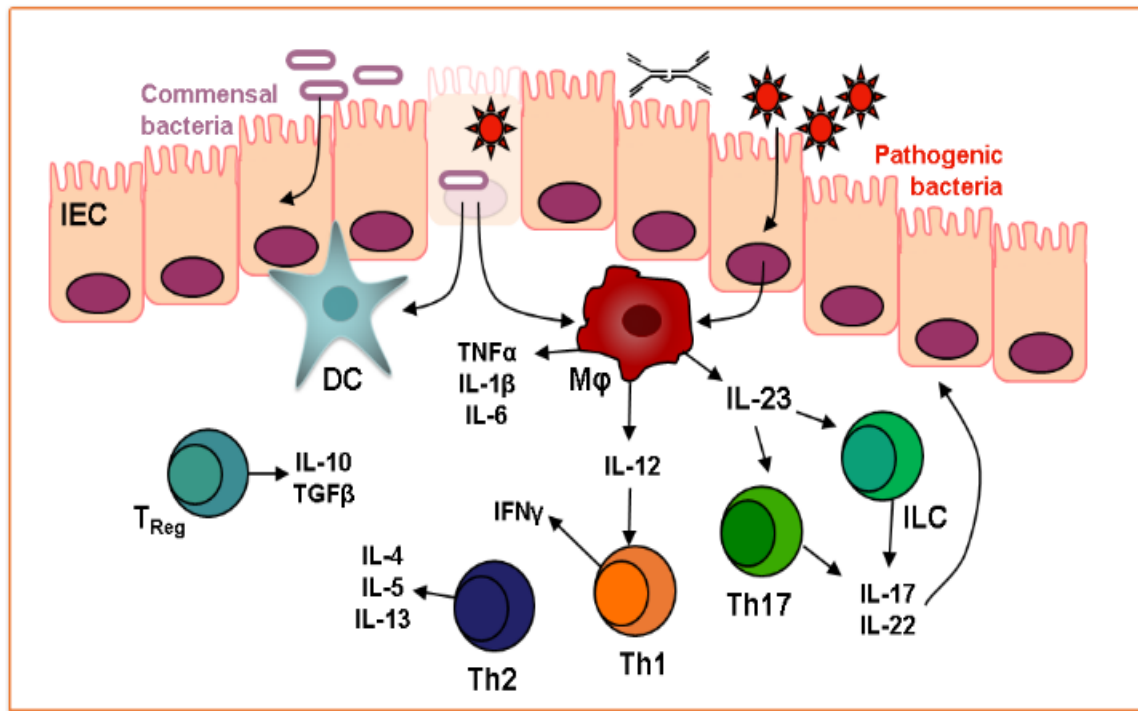
factor (PAF) and reactive oxygen metabolites (Ardizzone, 2003). When defense mechanisms are depressed, uncontrolled microbial proliferation can occur, and NF- $\kappa$ B-dependent genes are stimulated to produce pro-inflammatory cytokines like tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin-1(IL-1), IL-6, IL-17, IL-13 and the chemokine IL-8 and MCP-1. These pro-inflammatory mediators are highly expressed in the intestinal mucosa in areas of active CD and UC and may be important mediators of inflammation in IBD. TNF- $\alpha$  promotes inflammation by the production of IL-1 $\beta$  and IL-6, the expression of adhesion molecules, the proliferation of fibroblasts, the activation of procoagulant factors, the cytotoxicity of the acute phase response, and inhibition of apoptosis (Begue *et al.*, 2006). IL-6 induces NF- $\kappa$ B activation and then enhanced expression of the intercellular adhesion molecule, which is important in IBD pathogenesis and most likely in extraintestinal manifestations of the disease (Múzes *et al.*, 2012). IL-17, which is mainly produced by Th17 cells, is acting as a key mediator in delayed-type immune reactions by increasing chemokine production and recruiting monocytes and neutrophils to the inflammatory site (Yao *et al.*, 1995). IL-17 levels were increased in UC compared to controls (Strober and Fuss, 2011). IL-13 is a typical Th2 cytokine which is responsible for epithelial barrier damage and alteration of the tight junctions, with a consequent impairment of the mucosal permeability (Roda *et al.*, 2010). These mediators not only serve to amplify the immune and inflammatory response, but they also have direct effects on epithelial function, on endothelial function (which may increase permeability and lead to ischaemia), and on repair mechanisms, thus increasing collagen synthesis (Ardizzone, 2003; Roda *et al.*, 2010). In addition, many of the cytokines (interleukins 1 and 6, tumor necrosis factor) will activate an acute phase response, resulting in fever and a rise in serum acute phase proteins (Ardizzone, 2003). Moreover, increased expression of chemokines in the active phases of IBD was observed, such as, IL-8, MCP-1 and MCP-3, and MIP, which are responsible for the recruitment of different leukocyte effector populations, by controlling their adhesion and migration across the endothelium in sites of inflammation (Vezza *et al.*, 2016).

Another potentially significant component in the tissue injury that occurs in IBD is the activation of matrix metalloproteinases (MMP). MMP are a group of tissue proteinases that exhibit preferred cleavage specificity for the N-terminal side of hydrophobic residues (MacDermott, 1999). Studies have revealed high levels of extracellular MMP in areas of ulceration in the intestines of humans with IBD (MacDonald *et al.*, 2000). In addition, a number of different growth factors may play an important role in IBD. For example, keratinocyte growth factor is produced by gut stromal cells and is activated by

proinflammatory cytokines. Keratinocyte growth factor has been implicated in the crypt hyperplasia noted in areas of inflammation in IBD (Hendrickson *et al.*, 2002).

On the other hand, some cytokines have anti-inflammatory effects, such as IL-10, IL-4, TGF- $\beta$  and IL-11. IL-10 is produced by T cells, B cells, and monocytes; in presence of an antigenic stimulus, IL-10 inhibits the production of IL-1 $\beta$ , IL-6, and TNF- $\alpha$  (Roda *et al.*, 2010; Bouguen *et al.*, 2011). IL-4 is a stimulatory molecule for both B and T cells, and it is produced mainly by activated lymphocytes. Its main role stands in the inhibition of the formation of colonies of macrophages, of the production of H<sub>2</sub>O<sub>2</sub> by monocytes, and of the release of mediators such as IL-1 $\beta$  and TNF- $\alpha$ . The levels of IL-4 and IL-4 mRNA were reduced in IBD, demonstrating the loss of balance between anti and pro-inflammatory cytokines in favor of the pro-inflammatory ones (Andoh *et al.*, 2008). TGF- $\beta$  is an inhibitory cytokine, a key regulator in the maintenance of immune and inflammatory responses (Lawrance *et al.*, 2001). IL-11 is a cytokine originated from mesenchymal cell. It exhibits potent anti-inflammatory activity on macrophages and T cells by inhibiting the secretion of pro-inflammatory cytokines and has shown beneficial effects on intestinal mucosa in several animal IBD models (Bouguen *et al.*, 2011).

Overproduction of nitric oxide (NO) is implicated in the pathophysiology of UC. As much as 100-fold increases in luminal NO have been detected in patients with active UC. Excess NO can cause reduced colonic tone, intestinal hyperaemia and tissue damage, due to formation of nitric oxide reactive products such as peroxynitrite (Southey *et al.*, 1997).



**Figure 5:** Main cellular populations and mediators in intestinal hemostasis and pathogenesis of inflammatory bowel disease. [bitesizedimmunology.org/immune.dysfunction/inflammatory-bowel-disease](https://www.bitesizedimmunology.org/immune.dysfunction/inflammatory-bowel-disease). Treg: regulatory T cells; DC: Dendritic cell; Mφ: Macrophage; Th: T helper cell

### 3.1.4 Animal models of intestinal inflammation

A variety of mammalian models have been used to study acute and chronic intestinal inflammation. Mice are considered a good animal model as their intestinal development is relatively similar to the human intestine and they have many of the same immune responses and genes. Rat models have the advantage of being larger than mice thus allowing the acquisition of larger samples for analysis. Pigs are commonly used as an alternative monogastric mammalian model, as their intestinal function and morphology is similar to human beings. Non-human primates provide the best and most comparable data to people due to their high degree of genetic and physiological similarity to the human intestine (Jiminez *et al.*, 2015).

Different models of experimental IBD have been developed to investigate the etiology of the disease, and examine the efficacy of new therapeutic options on disease treatment. These experimental models can be classified into four major categories: genetic, immunological, bacterial and chemical (Elson *et al.*, 1995; Yan *et al.*, 2009).

**Genetic models:** Gene knockout and transgenic models are two main genetic models, which are increasingly being used to investigate the effect of host-related factors on disease development (e.g specific components of innate and adaptive immune system, for example IL-10 deficiency) (Elson *et al.*, 1995; Hoffmann *et al.*, 2009).

**Immunological models:** These models are used to investigate the role of specific immune cell populations in IBD pathology. In immunological models, a specific subtype of immune cells is introduced to immunodeficient mice, to investigate their roles and effects in disease progression or treatment (Elson *et al.*, 1995; Ding *et al.*, 2005)

**Bacterial models:** In this model, germ free, specific pathogen free or immunodeficient mice are exposed to a specific bacteria (pathogen or commensal), to investigate the potential beneficial or adverse effects of different bacterial strains in IBD development or treatment (Nell *et al.*, 2010).

**Chemical models:** Gastrointestinal inflammation can be induced by administering specific chemicals to animals. Presently, using trinitrobenzene sulfonic acid (TNBS) and DSS are the most common chemicals used to induce colitis in mice and rats. Both act via acute destruction of the intestinal barrier. Acetic acid, oxazolone, carragenin, sodium hydroxide and azoxymethane have also been used, but to a lesser extent than DSS and TNBS (MacPherson and Pfeiffer, 1978; Moyana and Lalonde, 1990). The effectiveness of the chemical agent in inducing injury varies according to the concentration, the way of administration and the presence of microorganisms in the lumen (Jiminez *et al.*, 2015).

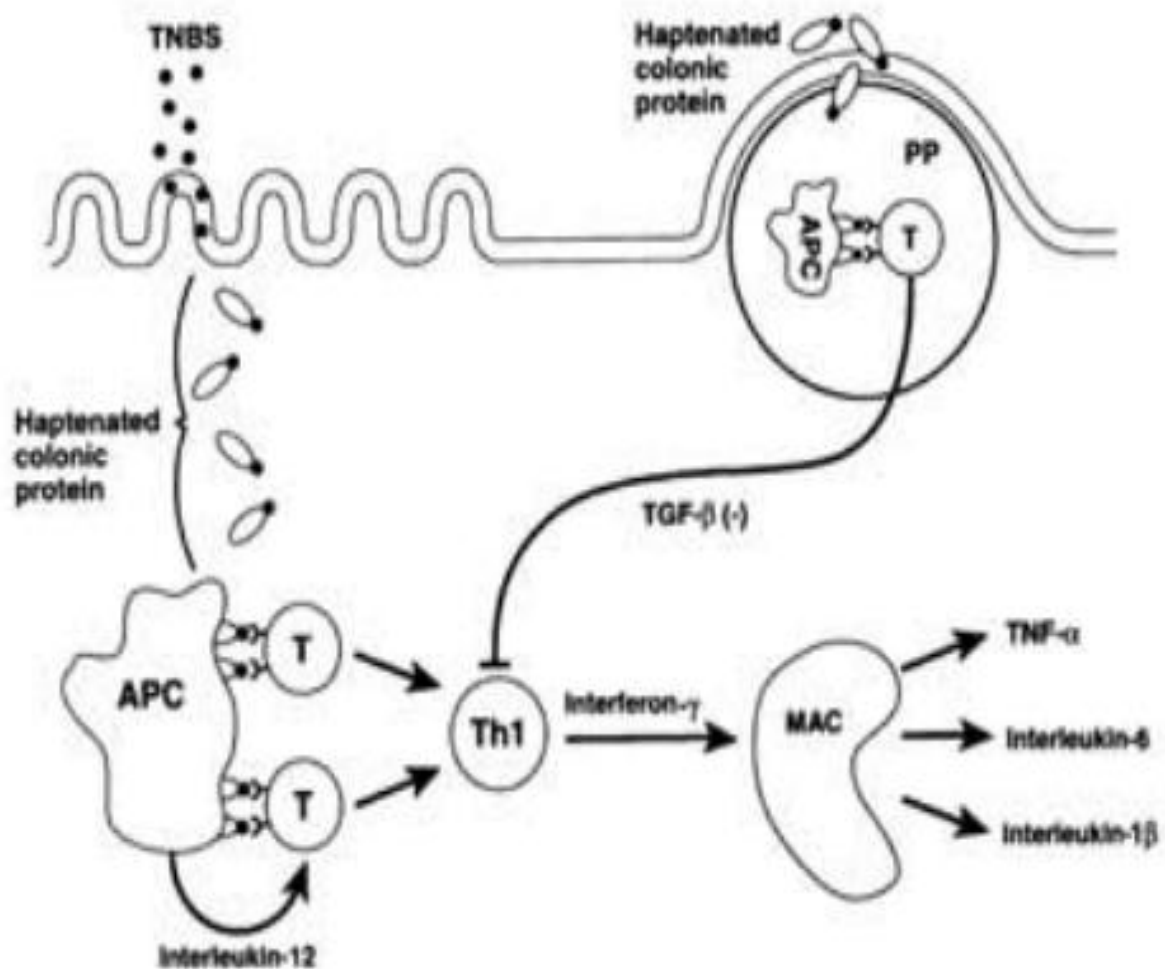
- **Trinitrobenzene sulfonic acid model**

The 2,4,6-trinitrobenzenesulfonic acid (TNBS) model was originally described by Morris *et al.* (1989). TNBS is a nitroaryl oxidizing acid with extreme oxidising properties. TNBS (0.5 to 4.0 mg for mice and 10 to 30 mg for rats) is dissolved in 45% or 50% ethanol. This TNBS-ethanol mixture induces intestinal inflammation by altering host proteins through the formation of covalent bonds with trinitrophenyl haptens of TNBS. It has been shown that rectal administration of TNBS in 40–50 % ethanol leads to severe colonic damage characterized by colon shortening, intestinal haemorrhage, epithelial necrosis causing crypt architecture destruction, and transmural inflammation accompanied by an elevated Th1 immune response within the colon (Jiminez *et al.*, 2015). Moreover, ethanol also acts as an irritant that contributes to the damage of the epithelial barrier (Jiminez *et al.*, 2015). The



damage is associated with high myeloperoxidase activity, mainly as a reflection of neutrophilic infiltration into the damaged tissue (Veljaca *et al.*, 1995).

As a hapten, TNBS can bind to the endogenous proteins, giving rise to hapten protein formation, which induces the interleukin-12 (IL-12) and T helper 1-mediated local immunological response. The activated macrophages produce inflammatory mediators, such as tumor necrosis factor- $\alpha$ , IL-6 and IL-1 $\beta$  (Figure 6)(Ishiguro *et al.* 2010; Strober and Fuss, 2011), which in turn result in transmural inflammation with weight loss and diarrhoea. TNBS may reduce mucosal hydrophobicity by reacting with the surface-active phospholipids of the colonic mucosa. Reduced hydrophobic integrity of the colonic mucosa may contribute to TNBS-induced colonic inflammation (Tatsumi and Lichtenberger, 1996). The TNBS model has many advantages, such as the simple process and the short duration of the experiment. Moreover, it is widely applicable to vertebrates, including mice, rats, guinea pigs and rabbits, and can be used either acutely with a single TNBS treatment or chronically with the repeated administration of TNBS (Terai *et al.* 2014). The colonic content of malondialdehyde (a major product of lipid peroxidation) is increased in both IBD patients and experimental animal models (Girgin *et al.*, 2000). Increases in myeloperoxidase activity, and the subsequent generation of nitric oxide, have also been demonstrated in biopsies of inflamed colon and both are related to the progression of the disease (Cooke and Davidge, 2002). Excessive NO production can accelerate the progression of UC. The increase in highly toxic ROS may exceed the limited antioxidant defense system of the intestines, thereby contributing to intestinal injury in UC patients (Babbs, 1992). Glutathione (GSH) is the most important intracellular defense against oxidative stress and is essential for both the functional and structural integrity of the gut. GSH is often depleted during inflammation, and GSH-deficient mice show severe degradation of the colonic mucosa, develop diarrhoea, and lose body weight (Joo *et al.*, 2015)



**Figure 6:** Mechanisms of colitis induction and tolerance in trinitrobenzene sulfonic acid (TNBS) colitis (Strober *et al.*, 1998). APC: Antigen presenting cell; MAC: Macrophage; TGF: Transforming growth factor.

### 3.1.5 Treatment

Currently, there is no effective therapy to cure the disease but the main stream treatment depends on reduction of the abnormal inflammation in the colon lining and thereby relieves the symptoms of diarrhoea, rectal bleeding, and abdominal pain. The treatment depends on the severity of the disease; therefore treatment is adjusted for each individual (Botoman *et al.*, 1998).

#### 3.1.5.1 Conventional Treatments

The four major types of conventional treatments include anti-inflammatory, immunomodulatory, antibiotic, and biologic agents.

Corticosteroids are anti-inflammatory agents used in IBD treatment. Corticosteroids, such as prednisone and 5-aminosalicylic acid (5-ASA), are the most common conventional medicines in UC treatment. Additionally, long-term consumption of corticosteroids can affect wound healing and increase the risk of osteoporosis and hyperglycemia. Most people with mild or moderate ulcerative colitis are treated with corticosteroids (dexamethasone) to reduce inflammation and relieve symptoms (Hanauer *et al.*, 2004). Nearly 25% of patients with UC requiring steroids therapy become steroid-dependent after one year, and virtually all develop steroid-related adverse events (Faubion *et al.*, 2001). These Immunomodulators have clinical limitations for use, and can also induce serious side effects, especially by suppressing bone marrow, causing pancreatitis, and inducing allergy symptoms, abdominal pain, rash, and fever (Awaad *et al.*, 2013; Jiminez *et al.*, 2015).

Antibiotics, such as Ciprofloxacin and Rifaximin, are also considered as potential options in UC treatment. Some antibiotics showed the ability to attenuate the immune response by suppressing the luminal and pathogen bacteria; however the findings about their efficacy are controversial (Venturi *et al.*, 1999).

Some examples of biologic agents with anti-IBD potentials are the monoclonal antibodies that are targeted against TNF- $\alpha$  (infliximab), leukocyte adhesion molecules (MLN02 and alicaforsen), Th1 polarisation, T-cell activation or NF- $\kappa$ B signaling (Ardizzone and Bianchi, 2005).

It is generally accepted that patients with extensive colitis require surveillance colonoscopy to detect dysplasia approximately 8 years after diagnosis. After initiation of surveillance colonoscopy, repeat examinations should follow every 1 to 2 years. The risk of developing colon cancer in Swedish patients diagnosed before 15 years of age was 1% after 15 years, 6.5% after 20 years, and 15% after 25 years (Hendrickson *et al.*, 2002).

#### **3.1.5.2 Herbal therapy**

Herbal therapy has become increasingly popular in persons with digestive disorders, especially when conventional therapies fail to improve their symptoms. Some of the most studied natural components with potential benefits in UC treatments are prebiotics and phenolic compounds. Phenolic compounds are group of natural components with anti-oxidative and anti-inflammatory actions (Thavorn *et al.*, 2014; Farzaei *et al.*, 2015).

A survey of 539 patients attending an outpatient clinic in Spain showed that nearly two-thirds (61.6%) of patients with digestive disorders had used herbal therapies in the past year, and approximately 80% of these users were satisfied with the results these therapies yielded (Thavorn *et al.*, 2014). Plant extracts used for the treatment of ulcerative colitis include: Aloe vera; *Boswellia serrata*; Tormentil (Rosaceae); Wheat grass (*Triticum aestivum*); Germinated barley (*Hordeum vulgare*); *Zingiber Officinale* Roscoe (Zingiberaceae); *Rheum tanguticum*; Green tea (*Camellia sinensis*) (Thavorn *et al.*, 2014; Triantafyllidi *et al.*, 2015)

Polyphenols, the most abundant phytochemicals, have been associated with anti-inflammatory, antioxidant, immunomodulatory, and apoptotic properties. Locally reducing oxidative stress, they can further act on cellular targets, altering gene expression related to inflammation, including NF- $\kappa$ B, Jak/STAT, and MAPKs, suppressing downstream cytokine formation (e.g., IL-8, IL-1 $\beta$ , and TNF- $\alpha$ ), and boosting the bodies' own antioxidant status (SOD, and GPx). However, potential adverse effects such as acting as prooxidants, or perturbations of efflux transporters should also be considered (Kaulmann and Bohn, 2016).

Altered immune response is associated with an increased release of pro-inflammatory cytokines, including IFN, TNF $\alpha$ , IL-6, IL-1 $\beta$ , GM-CSF and IL-17A, chemokines, such as IL-8, MIP-2 and MCP-1, and adhesion molecules, such as ICAM-1. Different studies are performed to show the ability of flavonoids to regulate the altered immune response that occurs in intestinal inflammation. For instance, the administration of flavonoids, such as cardamonin, chrysin, glabridin, quercitrin, naringenin or rutin, in the DSS model remarkably decreased the increased levels of the different cytokines evaluated in the inflamed colon (Vezza *et al.*, 2016). These immunomodulatory properties exerted by the flavonoids have also been confirmed when *in vitro* experiments were performed in different cell types involved in the immune response: epithelial cells, monocytes/macrophages, T cells, and dendritic cells. For instance, the incubation of LPS-activated macrophages, RAW 264.7 and BMDM cells with quercetin or baicalin resulted in reduced levels of IL-1 $\beta$  and TNF $\alpha$  when compared with stimulated cells without flavonoid treatment (Comalada *et al.*, 2005). Similarly, rutin was able to significantly reduce increased IL-1 $\alpha$  levels produced by DSS-stimulated pM' cells, obtained from mouse peritoneal exudate. In addition, quercetin exerts anti-proliferative effects by reducing IFN and TNF $\alpha$  production in concavalin A-stimulated purified T lymphocytes isolated from rat splenocytes (Vezza *et al.*, 2016).

## **4 Gastrointestinal motility**

### **4.1 Gastric motility**

When food enters the stomach, the organ relaxes to accommodate this increase in volume. This receptive relaxation allows storage of the meal without a concomitant increase in intragastric pressure. As a result, the human stomach increases in size from its empty volume of about 50 ml to a filled volume of 1500 ml or more. This relaxation of the gastric musculature is triggered by the movement of the pharynx and esophagus and is mediated via a vagal reflex and the release of NO. It is followed by peristaltic contractions that mix food and squirt it into the duodenum at a controlled rate. Ingested food may remain unmixed for up to 1 h after eating. Fats tend to form an oily layer on the top of the other gastric contents and their emptying is slowest. Liquids are emptied rapidly, whereas solid food and large or indigestible particles remain in the stomach for even longer time (Ganong, 2010).

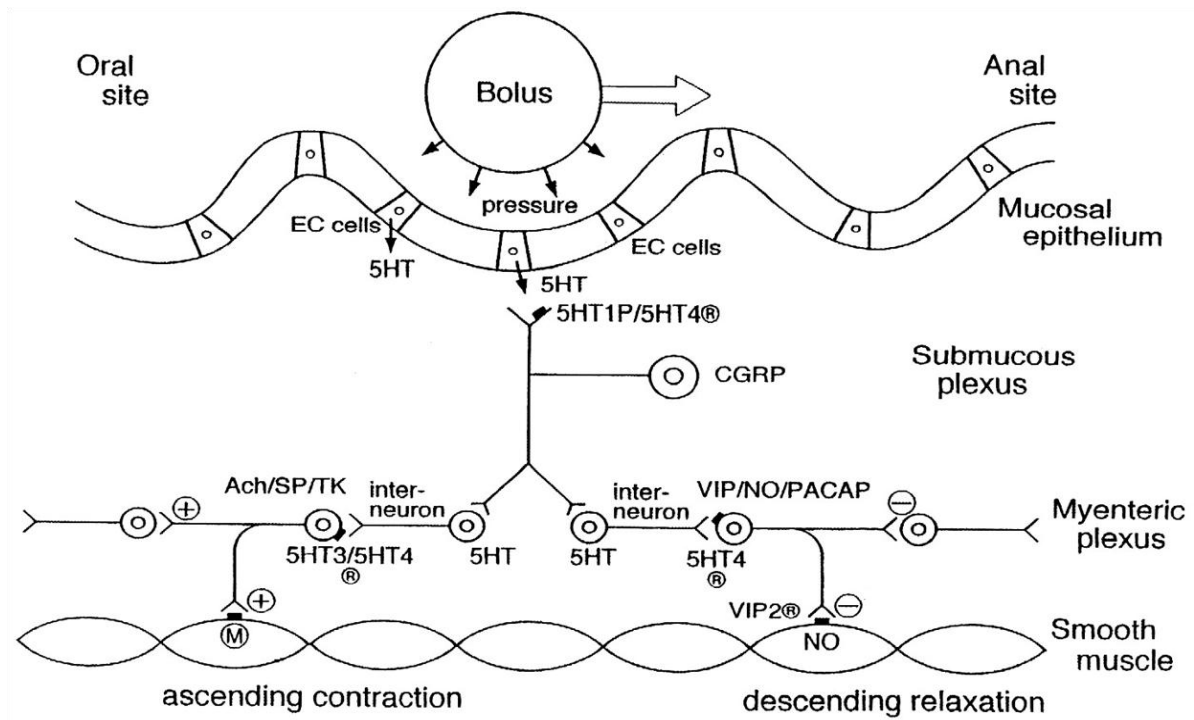
### **4.2 Intestinal motility**

There are two main types of movements, segmentation contractions and peristaltic waves. Both can take place in the absence of extrinsic innervation, but require an intact myenteric plexus. Segmentation contractions are ringlike contractions that appear at fairly regular intervals along the gut, then disappear and are replaced by another set of ring contractions in the segment between the previous contractions. These movements permit the movement of the chyme to and fro and increase its exposure to the mucosal surface (Ganong, 2010). A model of the peristaltic reflex mechanism in the rat intestine was proposed (Figure 07). Peptides involved in the endocrine and enteric nervous system as well as in the central nervous system exert concerted action on gastrointestinal motility. Mechanical and chemical stimuli which induce peptide release from the epithelial endocrine cells are the earliest step in the initiation of peristaltic activities. Gut peptides exert hormonal effects, but peptide-containing stimulatory (ACh, substance P, tachykinin) and inhibitory (VIP, PACAP, ATP, NO) neurons are also involved in the induction of ascending contraction and descending relaxation, respectively (Fujimori and Inui, 2000).

Activation of inhibitory motor neurons to circular muscle elicits a hyperpolarization and relaxation of smooth muscle. The inhibitory neurotransmitters participate in peristalsis by promoting the inhibitory descending reflex, which is aimed to facilitate propulsion of the chyme towards the anus. There is a unique relationship between the inhibitory transmitters in

that NO regulates the release of VIP and PACAP from the myenteric neurons and VIP and PACAP in turn enhance the production of NO in smooth muscle (Bornstein *et al.*, 2004). The increased intraluminal pressure caused by the bolus stimulates 5-hydroxytryptamine (5-HT) release from the epithelial EC cells. 5-HT acts on the 5-HT<sub>1P</sub> and/or 5-HT<sub>4</sub> receptors on the CGRP containing primary sensory neurons located in the submucosa plexus. This sensory neuron is coupled to 5-HT containing interneurons in the myenteric plexus and then the neurons are coupled to ascending excitatory motor neurons and descending inhibitory motor neurons (Fujimiya and Inui, 2000).

Colonic motility displays a circadian trend, and it is mostly present during daylight hours, with significant peaks after morning awakening, sudden awakening and after meals. In healthy individuals, the colon receives semi-solid contents from the terminal ileum and, by means of both segmentation (also helped by the peculiar anatomic aspect represented by the haustrae) and propulsion; it compacts and propels these contents aborally. Colonic propulsion is mainly represented by two kind of waves: the so-called high-amplitude propagated contractions that mainly move large quantities of solid contents aborally and start the defecatory reflexes, and the low-amplitude propagated contractions, whose function is principally devoted to the transport of liquid contents and gas, and which is associated with the emission of flatus and is often elicited by colonic distension. Once the colonic content reaches the rectum, the distension of the rectal ampulla and the sampling reflex of the anal sphincter send the relevant stimuli to the subject's brain and, if socially appropriate, he/she will decide whether to evacuate (Bassotti *et al.*, 2014).



**Figure 7:** Mechanism of peristaltic reflex of the rat intestine (Fujimiya and Inui, 2000). VIP: vasopressin intestinal peptide; NO: nitric oxide; PACAP: pituitary adenyl cyclase activating peptide; CGRP: calcitonin gene related peptide; TK: tachyinin; SP: substance P; 5-HT: 5-hydroxytryptan; EC: enterochromaffin cells; Ach: acetylcholine.

## 5 Diarrhoea:

Diarrhoea is a worldwide health problem. It is one of the leading causes of morbidity and mortality especially in children. In developing countries, diarrhoeal disease accounts for an estimated 17.5-21 % of all deaths in children under the age 5 years, equivalent to 1.5 million deaths per year (Boschi-Pinto *et al.*, 2008).

Diarrhoea can be defined as a gastrointestinal infection with the passage of loose or watery stools, three or more times in 24 hours. It occurs in all races, sexes, ages and geographic areas particularly in sites with poor sanitation and hygiene, including the lack of safe drinking water. It is mainly caused by many bacterial, viral and parasitic organisms (WHO, 2014).

## 5.1 Classification of diarrhoea

Several classifications have been attributed to diarrhoea based on duration, aetiological factors, pathophysiological mechanisms and systemic diseases contributing to diarrhoea. In 2014, the WHO classified diarrhoea in children to:

- Acute diarrhoea which is defined as an episode of diarrhoea that lasts less than 14 days. This type of diarrhoea induces dehydration and contributes to malnutrition; and the death of the affected children is usually due to dehydration.
- Persistent diarrhoea lasts 14 days or more. Up to 20% of episodes become persistent and it often provokes nutritional problems and contributes to death in children.
- Dysentery is diarrhoea with blood in the stool, with or without mucus. The main cause of dysentery is *Shigella* bacteria and a child may have both watery diarrhoea and dysentery.

Another classification based on duration was also proposed, according to which diarrhoea is classified into:

- Acute watery diarrhoea: It starts acutely and lasts for less than 14 days. The main causes of this type are rotavirus, *Escherichia coli*, *Shigella*, *Campylobacter jejuni* and *Cryptosporidium*.
- Persistent diarrhoea: Begins acutely but usually has longer duration (at least 14 days). Noticeable weight loss is frequent. *E. coli*, *Shigella* and *cryptosporidium* are the main infectious agents.
- Chronic diarrhoea: It is a common condition that lasts more than 4 weeks. It is due to non-infectious agents but may be caused by inherited metabolic disorders, such as sensitivity to gluten. It occurs in males more than females with a percentage of 3 to 5 of the general population worldwide (Frank-Briggs *et al.*, 2012).

## 5.2 Pathophysiology of diarrhoea

### 5.2.1 Normal physiology

Under normal physiological conditions, approximately 8 L of fluids reach the upper small bowel. This includes 2 L of ingested fluids and the remaining 6 L from salivary, gastric, biliary, and pancreatic secretions. Most of this fluid is reabsorbed before reaching the distal small bowel so that only about 1 L of fluid enters the colon. The colon reabsorbs almost all of



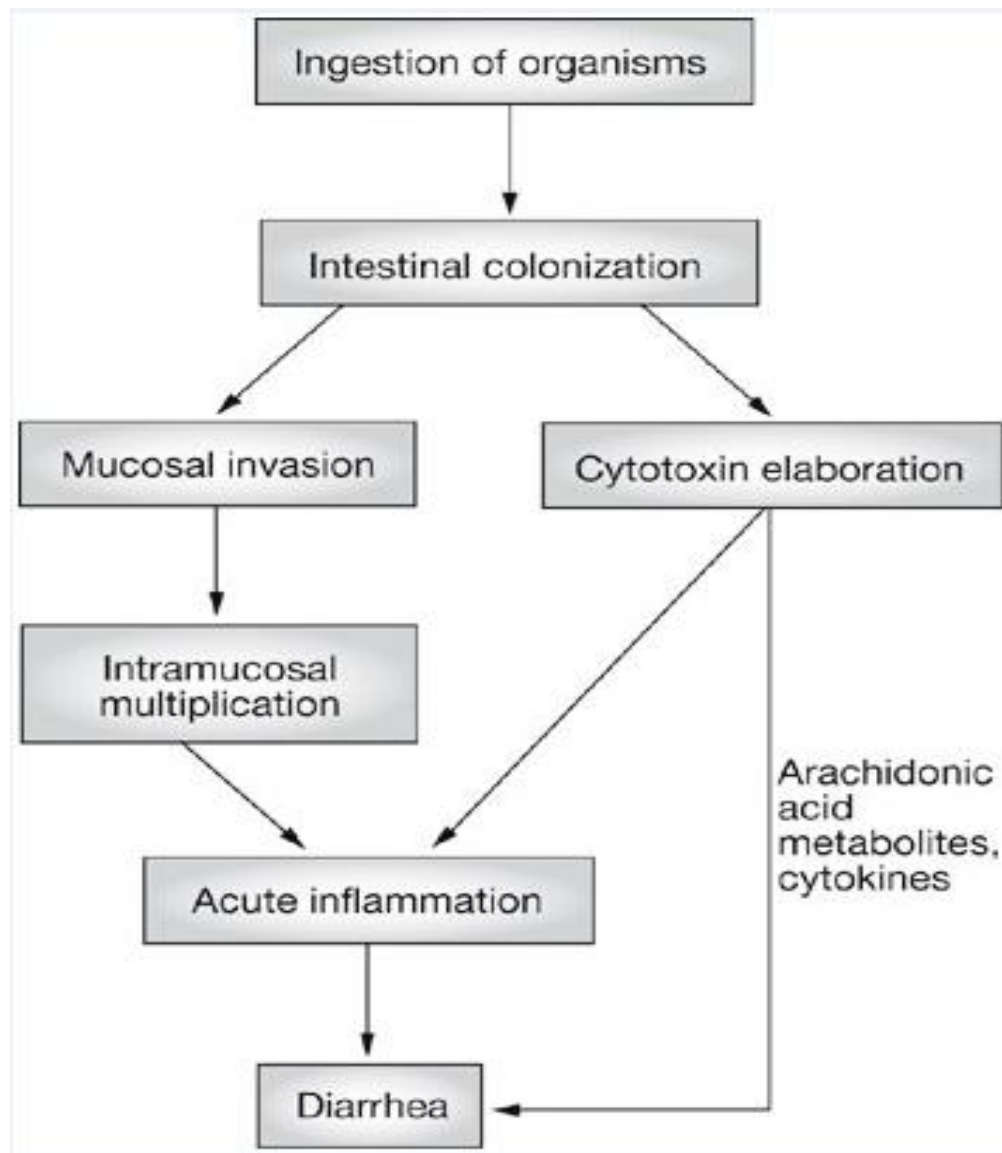
this fluid and the remaining; usually less than 200 ml is excreted in the stool. The colon has the capacity to reabsorb up to a maximum of 3-4 liters of fluid and thus to salvage much of the fluid that might be lost in small intestinal malabsorptive conditions (Navaneethan and Giannella, 2008). In the small intestine, water and electrolytes are simultaneously absorbed by the villi and secreted by the crypts of the bowel epithelium. This causes a two-directional flow of water and electrolytes between the intestinal lumen and the blood. Since fluid absorption is normally greater than fluid secretion, the net result is fluid absorption (WHO, 1992).

Sodium and water absorption by enterocytes is mediated by an active, adenosine triphosphate (ATP)-dependent active sodium (Na) pump (Na, K-ATPase) located on the basolateral membranes of intestinal crypt and villus cells (Binder, 2005). In the intestine, solute movement creates the osmotic force for fluid movement. Na absorption drives fluid reabsorption, while active Cl secretion contributes to water secretion in secretory diarrhoea. Small intestinal Na absorption is mediated primarily by two mechanisms: a glucose- or amino acid-stimulated cotransport in which Na accompanies the other solute and a coupled Na-Cl mechanism. The latter is a combination of Na-H exchange and Cl-HCO<sub>3</sub> exchange (Navaneethan and Giannella, 2008). Usually, more than 90% of the fluid entering the small intestine is absorbed, so that about 1 litre reaches the large intestine. There, further absorption occurs, only 100 to 200 millilitres of water being excreted each day in formed stools. Any change in the two-directional flow of water and electrolytes in the small intestine (*i.e.* increased secretion, decreased absorption, or both) results in either reduced net absorption or actual net secretion and causes an increased volume of fluid to enter the large intestine. When this volume exceeds the limited absorptive capacity of the large intestine, diarrhoea occurs (WHO, 1992). Any disturbance in the coordinated flux of water and ions and motility can result in the clinical syndrome of diarrhoea (Navaneethan and Giannella, 2008).

### **5.2.2 Physiological disturbances in diarrhoea**

The pathophysiology of diarrhoea can be classified based on the mechanism into secretory, osmotic, inflammatory, iatrogenic/drug related, and functional/motility-related diarrhoea. Diarrhoeal syndromes result from disturbances in any of the basic pathophysiological processes including osmosis, active secretion, exudation or inflammation, and altered motility (Field, 2003). Osmotic forces contribute to diarrhoea when poorly absorbable solutes remaining in the gastrointestinal lumen retain water and electrolytes resulting in reduced water reabsorption. The secretory diarrhoea is associated with an

activation of Cl<sup>-</sup> channels, causing Cl<sup>-</sup> efflux from the cell, resulting in massive secretion of water into the intestinal lumen and profuse watery diarrhoea (Jebakumar *et al.*, 2011). Active secretion can play a vital role in the pathophysiology of diarrhoea as evidenced in cholera or in celiac disease. Other secretory stimuli include other bacterial enterotoxins, hormones from endocrine neoplasms, dihydroxy bile acids, hydroxylated fatty acids, and inflammatory mediators (Navaneethan and Giannella, 2008). Exudation can contribute to diarrhoea when the intestinal epithelium's barrier function is compromised by loss of epithelial cells or disruption of tight junctions as occurs in invasive diarrhoea due to *Shigella*/*Salmonella* (Navaneethan and Giannella, 2008) and inflammatory disease process as in ulcerative colitis or Crohn's disease (Binder, 2009). Motility disturbances can result in diarrhoea as occurring in thyrotoxicosis and opiate withdrawal. Also slowing of the motor function of the small intestine can result in bacterial overgrowth and hence diarrhoea (Figure 8)(Camilleri, 2004).



**Figure 8:** Schema illustrating mechanisms of pathogenesis for invasive and cytotoxin-mediated bacterial inflammatory diarrhoea (Navaneethan and Giannella, 2008).

### 5.3 Treatment of diarrhoea

The treatment of patients with diarrhoea is based on the major features of the disease and an understanding of the underlying pathogenetic mechanisms. The main principles of treatment are as follows:

\* Watery diarrhoea requires replacement of fluids and electrolytes- irrespective of its etiology. Feeding should be continued during all types of diarrhoea to the greatest extent possible, and should be increased during convalescence so as to avoid any adverse effect on nutritional status.

\* Antimicrobials and antiparasitic agents should not be used routinely; most episodes, including severe diarrhoea and diarrhoea with fever, do not benefit from such treatment. The exceptions are:

- Dysentery, which should be treated with an antimicrobial effective for *Shigella*.
- Suspected cholera with severe dehydration;
- Persistent diarrhoea, when trophozoites or cysts of *Giardia* or trophozoites of *E. histolytica* are seen in faeces or intestinal fluid, or when pathogenic enteric bacteria are identified by stool culture (WHO, 1992).

Polyphenols may be used in controlling non-inflammatory diarrhoeal states. In the large intestine, colonic bacteria are known to act enzymatically on the polyphenolic backbone of the remaining unabsorbed polyphenols (90–95% of the total polyphenol intake), sequentially producing metabolites with different physiological significance. In addition, polyphenols may be converted by the colonic microbiota to bioactive compounds that can affect the intestinal ecology and influence host health (Cardona *et al.*, 2013).

A polyphenol extract from apples was found to have an inhibitory effect on cholera toxin induced diarrhoea, in a dose-dependent fashion. This effect could be seen as long as 10 min after toxin injection. The fraction containing highly polymerized catechins most effectively inhibited the toxin-mediated fluid secretion (Loeb *et al.*, 1989). A clinical trial in a pediatric population with acute diarrhoea evaluated the effects of a tannin-rich carob pod powder, which contained 21.2% polyphenols (Loeb *et al.*, 1989). The carob pod powder recipients experienced cessation of diarrhoeal symptoms approximately 1.5 days earlier than controls. A variety of other compounds used as antidiarrheals in traditional medicine have been screened for their active ingredient, and polyphenol components have been identified as the bioactive molecules (Dryden *et al.*, 2006).

## **6 The plant in study: *Myrtus communis* L.**

*Myrtus communis* L. is one of the important aromatic and medicinal species belonging to the Myrtaceae family which includes approximately 100 genera and 3000 species growing in temperate, tropical and subtropical regions. It is native to Southern Europe, North Africa and Western Asia and also distributed in South America, North western Himalaya and Australia (Males *et al.*, 2006; Nassar *et al.*, 2010; Sumbul *et al.*, 2011). In

Algeria, *M. communis* L. is widespread especially in the Tell Atlas and in the coastal regions (Quezel and Santa, 1963). It is commonly known under the name of El-Reihan or Hlamouche (Baba Aïssa, 2011).

### **6.1 Botanical description**

An evergreen shrub, generally 3–7-m high. It has stiff branches and reddish twigs, with dark glossy green leaves. The attractive flowers are white or pinkish and very fragrant. Fruits are round, reddish-blue to violet berries. The whole plant is very aromatic (Charles, 2013). The stem of the plant is branched and dark green leaves are glossy, glabrous, coriaceous, opposite, paired or whorled, ovate to lanceolate with stiff structure, aromatic entire margined, acuminate and 2.5-3.8 cm long, glands absent in the lamina. It has axillary white or pinkish and very fragrant flowers on slender peduncles; medium sized about 2 cm in diam., stiff having yellow anthers. The petals are pure white with glands and somewhat tomentose margin covered with fine hairs. They give off a sweet fragrant smell. Flowers are star-like, white or pinkish and very fragrant (Sumbul *et al.*, 2011; Charles, 2013). The round blue-black berry fruit contains several seeds. The pollination is done by insects, and the seeds are dispersed by birds that eat the berries (Satyavati *et al.*, 1976). Myrtle fruit is a multiseeded berry, spherical pea-sized, orbicular or ovoid-ellipsoid, blue-black or white seeds. They are of varying sizes (0.7-1.2 cm) and shapes. The glabrous berry has rounded (vaselike) shape with a swollen central part and remnants of persistent 4-5 partite calyx at the outer part. The developed fruits (berries) are initially pale green, then turn deep red and finally become dark indigo when fully mature (Figure 9). They are bitter when unripe, sweet when ripe. It can grow in damp places, shades as well as full sun up to 800 m altitudes. Its blooming time is summer (Sumbul *et al.*, 2011).

Systematic of myrtle is as the follows (Quezel and Santa, 1962).

Kingdom: Plantae

Phyllum: Trachephyta

Class: Magnoliopsida

Order: Myrtaceae

Genus: Myrtus Species: *Myrtus communis* L.



**Figure 9:** *Myrtus communis* L. plant (Sumbul *et al.*, 2011)

## 6.2 Traditional uses

Different parts of this herb such as its berries, branches, leaves and fruits have been used extensively as a folk. Generally speaking, this herb has been used traditionally for the treatment of diarrhoea, peptic ulcers, hemorrhoids, inflammation, bleeding, headache, palpitation, leucorrhoea, urethritis, epistaxis, conjunctivitis, and excessive perspiration, pulmonary and skin diseases (Evans, 2002). Myrtle edible products are effective in treating urinary tract infections, digestive problems, bronchitis, sinusitis and dry cough. The essential oil in topical products is used for rheumatoid pain. Fruit has carminative properties and effective in the treatment of diarrhoea, dysentery, hemorrhoid, internal wounds and rheumatism (Baharvand-Ahmadi *et al.*, 2015). Myrtle extensive use in traditional medicine and also the entry of its products (such as topical ointments and drops) to the pharmaceutical industry provoke the need for further knowledge of the herb's different aspects such as phytochemical, pharmacological and toxicological properties (Alipour *et al.*, 2014).

### 6.3 Chemical composition

The plant contains many biologically active compounds. Myrtle essential oil, extracted from its leaves, branches, fruits and flowers through steam distillation, is yellow or greenish yellow with a refreshing odour (Sumbul *et al.*, 2011). The oil has been widely investigated; its composition is quite variable depending on the geographic region of production, the season of harvest and the length of distillation (Tuberoso *et al.*, 2006; Sumbul *et al.*, 2011).

The major constituents of the leaf essential oil composition are  $\alpha$ -pinene and 1,8-cineole. The main essential oil components in the flower are  $\alpha$ -pinene and 1,8-cineole, as well as other compounds including limonene, eugenol,  $\alpha$ -terpineol, linalool and methyleugenol. 1,8-Cineole is the dominant component in stem essential oil and it is followed by  $\alpha$ -pinene, E-  $\beta$ -ocimene and linalool (Aidi Wannes *et al.*, 2010). However, in most regions, terpenoid compounds (1,8-cineole,  $\alpha$ -pinene, myrtenyl acetate, limonene, linalool,  $\alpha$ -terpinolene) are the major constituents found in the essential oil obtained from the leaves (Bradesi *et al.*, 1997; Aidi Wannes *et al.*, 2010; Berka-Zougali *et al.*, 2010; Hassiotis and Lazari 2010; Ghasemi *et al.*, 2011). Eucalyptol is reported as the major constituent of myrtle leaf essential oil grown in northern Cyprus as well (Akin *et al.*, 2012; Aleksic and Knezevic, 2013). Myrtle berries volatile oil also contains large amounts of monoterpene hydrocarbons and oxygenated monoterpenes with  $\alpha$ -pinene, 1,8-cineole, geranyl acetate and linalool as the main components (Aidi Wannes *et al.*, 2010; Barboni *et al.*, 2010).

*M. communis* L. extracts profile constitutes polyphenolic compounds, which are grouped in three major chemical classes: phenolic acids, tannins and flavonoids such as quercetin, catechin and myricetin derivatives, coumarins, myrtucommulone (MC) A and B, semimytucommulone (S-MC) (which are unique oligomeric, nonprenylated acylphloroglucinol compounds), galloyl-glucosides, ellagitannins, galloyl-quinic acids, caffeic, gallic and ellagic acids (Yoshimura *et al.*, 2008; Asif *et al.*, 2011; Sumbul *et al.*, 2011; Akin *et al.*, 2012). Polyphenolic composition of the berries was characterized by high concentrations of flavonol glycosides, flavonols and flavanols. The major fatty acids of berries were reported as linoleic, palmitic, oleic and stearic acids (Aidi Wannes *et al.*, 2010; Barboni *et al.*, 2010). Berries are also reported to contain tannin, resins, citric, malic and caffeic acids, sugar, anthocyanin arabinosides, anthocyanin glucosides, kaempferol, quercetin, myricetin 3-o-glucoside, myricetin 3, 3-di-ogalactoside amongst others (Sumbul *et al.*, 2011; Akin *et al.*, 2012; Alipour *et al.*, 2014).

## 6.4 Pharmacological properties

### 6.4.1 Antioxidant

Several reports describe the antioxidant activities of different extracts and compounds obtained from myrtle (Romani *et al.*, 2004; Gardeli *et al.*, 2008; Hayder *et al.*, 2008; Rosa *et al.*, 2008; Tuberoso *et al.*, 2010). The total phenolic content and flavonoids from methanolic, ethanolic and aqueous extracts of Myrtle leaves and berries were evaluated for their antioxidant activities (Amensour *et al.*, 2009; Amira *et al.*, 2012). In general, the leaf extracts had higher antioxidant activity than the berries and a positive correlation was observed between the phenolic content and the antioxidant capacity (Amensour *et al.*, 2009). The methanolic extract of the different parts of the plant were also found to have higher antioxidant activity than the leaf and flower essential oil (Aidi-Wannes *et al.*, 2010). The antioxidant properties and composition of the ethanol extracts obtained from myrtle berries have been studied as well and mainly focused on their main compounds, anthocyanins and flavonols, which are generally considered to be responsible for antioxidant activity (Alamanni and Cossu, 2004; Romani *et al.*, 2004; Montoro *et al.*, 2006; Tuberoso *et al.*, 2010). Various known antioxidants like flavonoids, tannins (Romani *et al.* 2004) and  $\alpha$ -tocopherol have been isolated from myrtle extracts. *M. communis* L. also exhibit the biological activities of tannins including anticancer and antioxidant activities (Romani *et al.*, 2004).

Flavonoids and anthocyanins in berries extract were checked for antioxidant activity by TEAC assay and the free radical activity. The myrtle extract showed interesting free radical scavenging activity (Montoro *et al.*, 2006). Radical scavenging activity of essential oil was studied by collecting the plant samples from two distant localities. Both oils exhibited moderate DPPH (2, 2-diphenyl-1-picrylhydrazyl) scavenging activity (Mimica-Dukic *et al.*, 2010).

### 6.4.2 Anti-inflammatory

Several studies have indicated the anti-inflammatory properties of the essential oil of *M. communis* in animal models (Rossi *et al.*, 2009; Hosseinzadeh *et al.*, 2011; Maxia *et al.*, 2011; Amira *et al.*, 2012). Rossi *et al.* (2009) showed that the injection of myrtucommulone from *Myrtus communis* had potent anti-inflammatory activity *in vivo* by reducing paw oedema and pleurisy induced by carrageenan. Similarly, Amira *et al.* (2012) noticed an inhibition in carragenan induced paw oedema in mice following the gavage of *Myrtus communis* aqueous



berries extract. Hosseinzadeh *et al.* (2011) evaluated the anti-inflammatory effect of aqueous and ethanolic extracts of the aerial parts of *M. communis* L. using xylene-induced ear oedema and cotton pellet tests. Both extracts had anti-inflammatory effects in acute and chronic inflammation, as well as, antinociceptive action in mice. In addition, Feisst *et al.*, (2005) found that Myrtucommulone, semimyrtucommulone and nonprenylated acylphloroglucinols present in the leaves of *M. communis* L., potently suppress the biosynthesis of eicosanoids by direct inhibition of cyclooxygenase-1 and 5-lipoxygenase *in vitro* and *in vivo*. Furthermore, topical use of the essential oil of *M. communis* is reported to cause a significant decrease in the ear oedema and cotton pellet-induced granuloma (Maxia *et al.*, 2011).

Other pharmacological studies on *Myrtus communis* have been focussing on antidiabetic activity. Elfellah *et al.* (1984) found that the ethanol-water extract of *M. communis* had anti-hyperglycaemic effect in mice. Similarly, a hypoglycaemic activity in alloxan-diabetic rabbits (Sepici-Dincel *et al.*, 2004) and rats (Malekpour *et al.*, 2012) was also observed.

Mansouri *et al.* (2001) evaluated the antibacterial activity of methanol crude extract of *M. communis* against 10 laboratory strains of microorganisms, including 6 Gram positive (*Staphylococcus aureus*, *Micrococcus luteus*, *Streptococcus pneumoniae*, *S. pyogenes*, *S. agalactiae* and *Listeria monocytogenes*) and 4 Gram negative bacteria (*Escherichia coli*, *Proteus vulgaris*, *Pseudomonas aeruginosa* and *Campylobacter jejuni*). The crude extract inhibited the growth of all tested bacteria except *C. jejuni*. When tested against 150 strains isolated from burns (predominantly *P. aeruginosa* and *S. aureus*), aqueous leaves extracts of *M. communis* gave an excellent effect on bacterial growth and their effects were located within the limits of antibiotic effects (Al-Saimary *et al.* 2002)

To our knoweldge, one study was devoted to the anti-ulcerogenic effects of *M. communis* berries in rats (Sumbul *et al.*, 2010). Results from this study showed a reduction in ulcer index in both methanolic and aqueous extracts, as well as, a decrease in gastric juice volume; total acidity and an increase in gastric wall mucus content. Myrtle oral paste had a therapeutic effect on reccurent aphthous stomatitis, an ulcerative disorder of the oral cavity (Babae *et al.*, 2010).

# **Materials and methods**

## **1 Materials**

### **1.1 Chemicals**

All chemicals were of analytical grade and purchased from sigma (St Louis, MO, USA) or Fluka Chemical Co. (Buchs, Switzerland).

### **1.2 Plant material**

The fresh leaves and berries of *M. communis* L. were collected from Jijel (North-East of Algeria) in November, 2014. The taxonomic identity of the plant was done by Professor Gonzalez-Tejero and Casares-Porcel Department of Botany, University of Granada, Spain and a voucher number ML 11/14 was deposited at the Laboratory of Phytotherapy Applied to Chronic Diseases, University Setif 1. The collected plant was dried under shade.

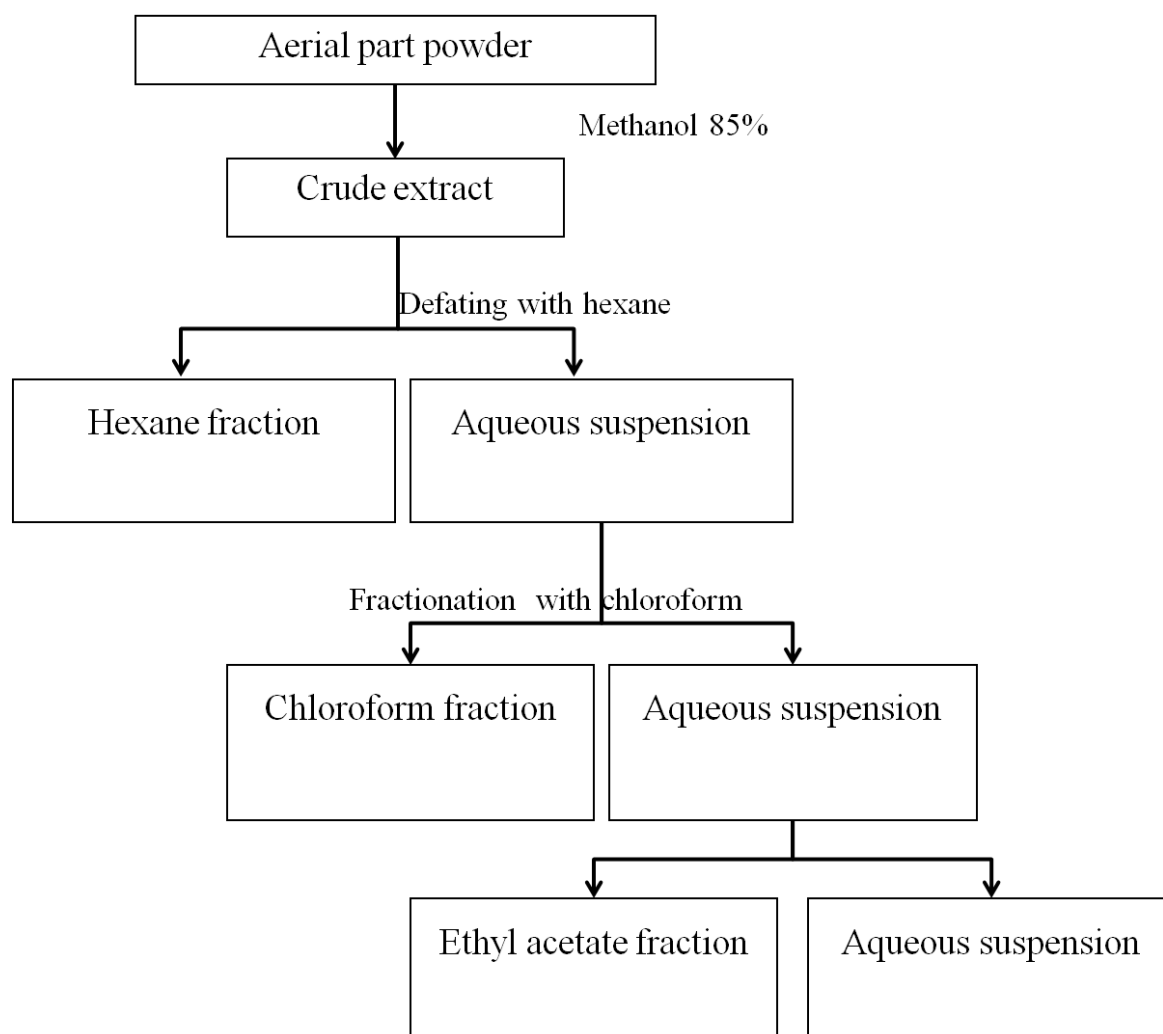
### **1.3 Animals**

Male Swiss white mice (Pasteur Institute, Algiers, Algeria), weighing between 25 and 30 g, were used in this study. They were initially housed in groups in cages and had free access to water and food ad libitum for a week. In all studies, the animals (with exception for the antidiarrhoeric test) were fasted for 18-20 h with free access to water until 60 min before the start of the experiment. During the fasting period, the animals were placed individually in cages with wide-mesh wire bottoms to prevent coprophagy.

## **2 Methods**

### **2.1 Extraction and fractionation**

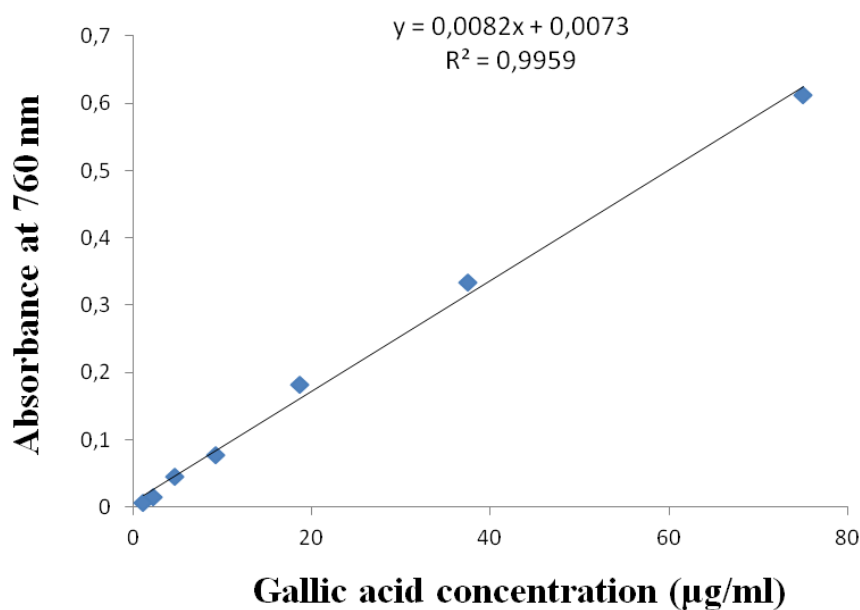
The extraction procedure was conducted as described by Markham (1982) with slight modification. The dried powder of *M. communis* L. leaves and berries was extracted with methanol (85%) at room temperature for 3 days. The resulting suspension was then filtered and concentrated by evaporation at 50 °C and fractionated by successive washing with different solvents of increasing polarity (hexane, chloroform and ethyl acetate). Each fraction was evaporated to dryness to obtain the following fractions: methanol extract (ME), hexane extract (HE), chloroform extract (CHE), ethyl acetate extract (EAE) and the remaining aqueous extract (AqE). The extracts were stored at 4 °C until use (Figure 10).



**Figure 10:** Fractionation steps of the crude extract of *Myrtus communis* L. with slight modifications (Markham, 1982).

## 2.2 Determination of total phenolic content

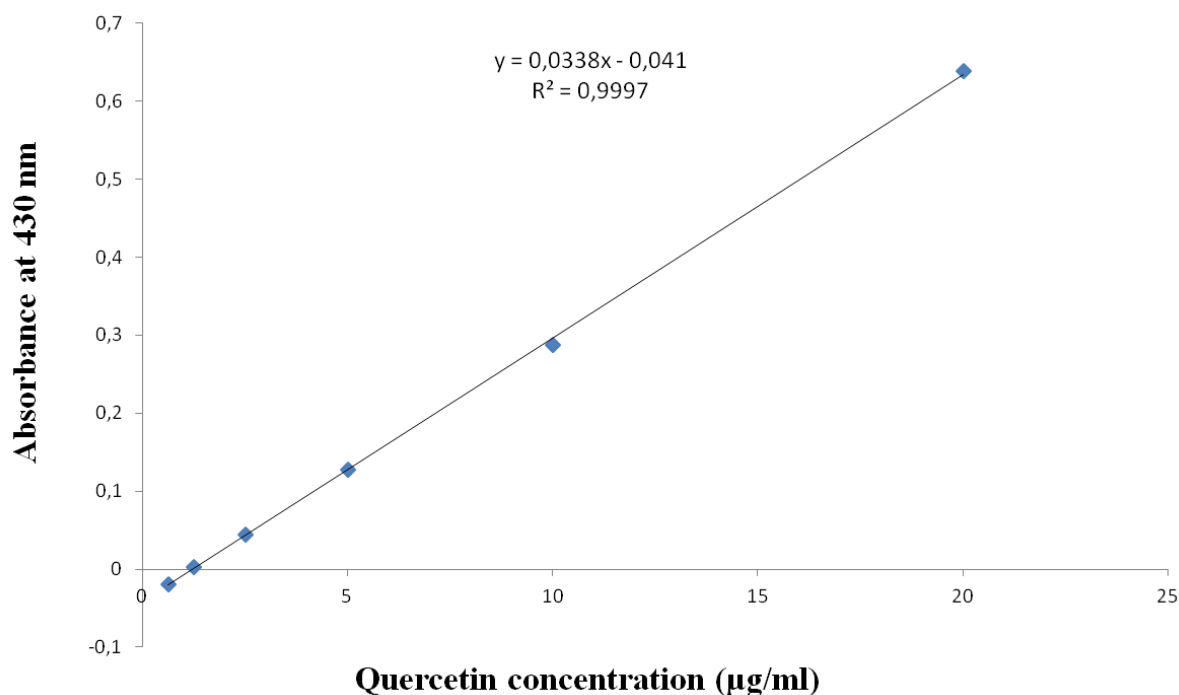
Total phenolic content was assessed by Folin Ciocalteu reagent as described by Li *et al* (2007). A volume of 100  $\mu$ l of each extract was mixed with 500  $\mu$ l of Folin Ciocalteu reagent (diluted 10 times). After 4 min, 400  $\mu$ l of 7.5% of  $\text{Na}_2\text{CO}_3$  solution was added. The final mixture was shaken and incubated in dark at room temperature for 1 hour and the absorbance of the reaction mixture was measured at 760 nm. The amount of total polyphenols in different extracts was determined from a standard curve of gallic acid (Figure 11). The results were expressed as mg of gallic acid equivalent (GAE) per gram of dried plant extract.



**Figure 11:** Standard curve of gallic acid for the determination of total polyphenols in *M.communis* L. extracts. Each value represents mean  $\pm$  SD (n=3).

### 2.3 Determination of total flavonoid content

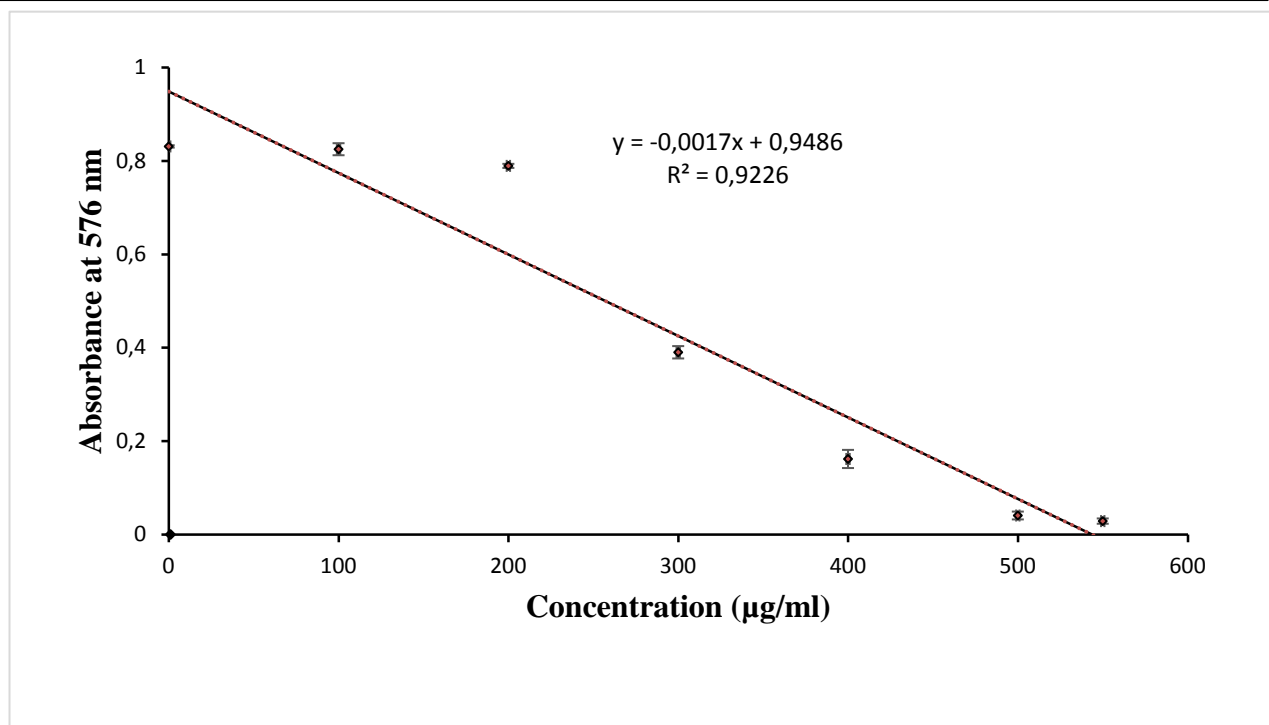
Total flavonoid content was determined using aluminum chloride assay (Bahorun *et al.*, 1996). Briefly, 1 ml of each tested extract or standard (quercetin) were mixed with 1 ml of  $\text{AlCl}_3$  (2%). After 10 min of incubation, the absorbance against a prepared blank was measured at 430 nm. The results were expressed as quercetin equivalent per gram of dry plant extract weight (mg QE/g DW) using a calibration curve of quercetin (Figure 12).



**Figure 12:** Standard curve of quercetin for the determination of flavonoids in *M. communis* L. extracts. Each value represents mean  $\pm$  SD (n=3).

## 2.4 Determination of total tannins content

This was achieved by testing the capacity of the different extracts to precipitate haemoglobin from fresh bovine blood according to the method described by Bate-Smith (1973). Briefly, a volume of each plant extract was mixed with an equal volume of hemolysed bovine blood (absorbance = 1.6). After 20 minutes of incubation at room temperature, the mixture was centrifuged at 4000 rpm, and the absorbance of the supernatant was measured at 576 nm and the results were expressed as mg equivalent tannic acid per gram of extract dry weight (mg TAE/g DW) using a calibration curve of tannic acid (Figure 13).



**Figure 13:** Standard curve of tannic acid for the determination of tannins in *M. communis* L. extracts. Each value represents mean  $\pm$  SD (n=3).

## 2.5 HPLC-MS analysis

Agilent Technology of 1260 Infinity HPLC System was coupled with 6210 Time of Flight (TOF) LC/MS detector and ZORBAX SB-C18 (4.6 x100mm, 3.5µm) column. Mobile phases A and B were ultra-pure water with 0.1% formic acid and acetonitrile (not very clear), respectively. Flow rate was 0.6 mL min<sup>-1</sup> and column temperature was 35°C. Injection volume was 10 µL. The solvent program was as follow: 0. min 10% B; 0-1.min 10% B; 1-20.min 50% B; 20-23.min 80% B; 23-25.min 10% B; 25-30. min 10% B. Ionization mode of HPLC-TOF/MS instrument was negative and operated with a nitrogen gas temperature of 325 °C, nitrogen gas flow of 10.0 L min<sup>-1</sup>, nebulizer of 40 psi, capillary voltage of 4000 V and finally, fragmentor voltage of 175 V. For sample analysis, dried crude extracts from *M. communis* leaves and berries (200 ppm) were dissolved in methanol at room temperature. Samples were filtered passing through a PTFE (0.45 µm) filter by an injector to remove particulates (Abay *et al.*, 2015).

## 2.6 Extraction and GC-MS analysis of essential oil

The volatile components of *M. communis* L. leaves were determined and identified using GC/MS analyses. The analyses were carried out with a Varian CP-3800 gas-chromatograph equipped with a DB-5 capillary column (30 m 0.25 mm; coating thickness 0.25 mm) and a Varian Saturn 2000 ion trap mass detector. The analytical conditions were as

follows: injector and transfer line temperatures 220 and 240°C, respectively; oven temperature programmed from 60 to 240°C at 3°C/min; carrier gas helium at 1 ml/min; injection of 0.2 ml (10% hexane solution); split ratio 1:30. The identification of the constituents was based on the comparison of the retention times with those of authentic samples, comparing their linear retention indices relative to a series of n-hydrocarbons, and on computer matching against commercial (NIST 98 and ADAMS) and homemade library mass spectra built up from pure substances and constituents of known oils and MS literature data (Adams, 1995; Masada, 1976; Swigar and Silverstein, 1981).

## **2.7 Acute oral toxicity**

Acute oral toxicity of *M. communis* L. extracts and essential oil was performed using few animals according to the limit test recommendation of the Organization of Economic Cooperation and Development (OECD), guideline 423 (2001). The oil was administered to the first animal at a single oral dose (2000 mg/kg), whereas the extracts were administered at 2 single oral doses (2000 and 5000 mg/kg). The animals were not fed for three hours following administration. Gross behavioral and toxic effects (restlessness, agitation, dullness, writhing etc.) were observed at short intervals for 24 h. As these animals did not die, two more animals were treated in the same way. After 14 days mice were sacrificed and all the organs were removed for gross pathological examination.

## **2.8 Ethanol-induced gastric ulceration in rats**

The animals were divided into 14 groups, consisting of six rats each. Each rat in each group was subsequently separately placed in a cage. Group 1 rats were treated with CMC (1.5%) as negative control. Group 2 received 5 mg/kg ranitidine (positive control). Groups 3, 4 and 5 were treated with ME at doses 50; 250 and 500 mg/kg. Groups 6, 7 and 8 received 50, 250 and 500 mg/kg of CHE extract. Groups 9, 10 and 11 received 50, 250 and 500 mg/kg of EAE extract. Groups 12, 13 and 14 were treated with EO at doses 25, 250 and 500 mg/kg. 60 minutes after the oral respective treatments (5 ml/kg), ulceration was induced by intragastric instillation of 70% ethanol (0.5 ml/200 g). Thirty minutes later, the animals were sacrificed with cervical dislocation and each stomach was incised along the greater curvature, photographed and macroscopically examined for linear haemorrhagic lesions in the glandular region. The length (mm) of each lesion was determined and each length was summed per stomach. The sum of length (mm) of all lesions for each stomach was used as the ulcer index



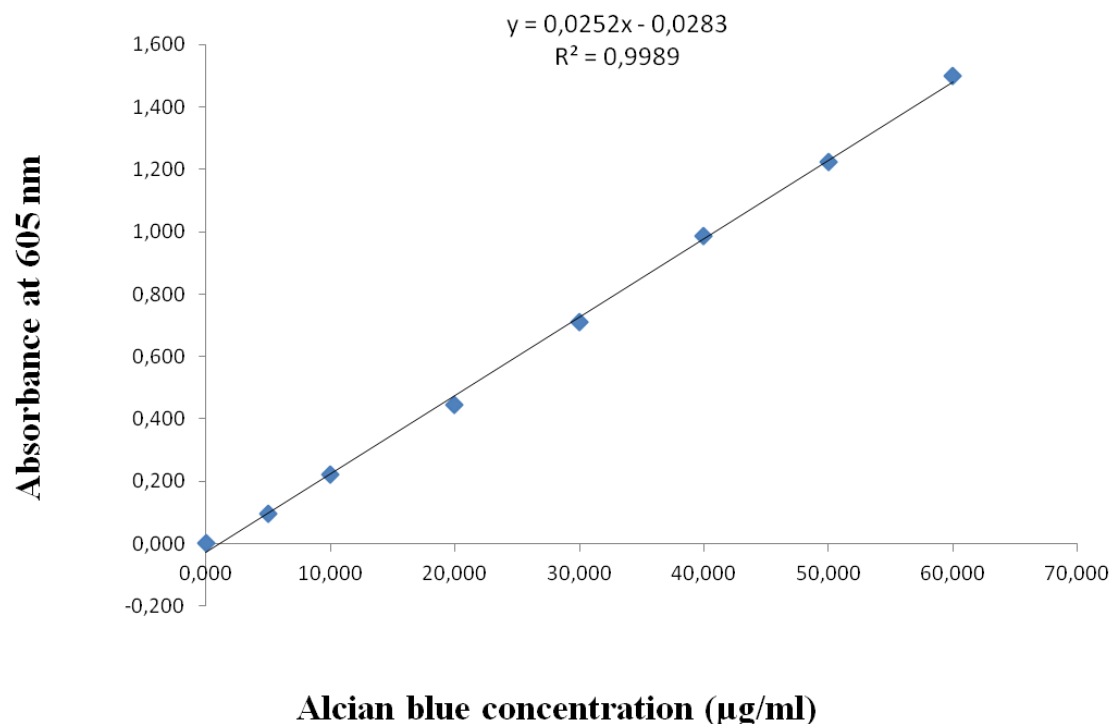
(UI). The percentage inhibition was calculated by the following formula: %inhibition =  $\frac{\text{UI control} - \text{UI treated}}{\text{UI control}} \times 100$ .

## **2.9 Histopathological examinations**

For microscopic analysis, a portion of stomach from each experimental group was fixed in 10% formalin and dehydrated with mixtures of increasing grades of ethanol, clarified in xylene using a tissue processor (MTP-SLEE, Mainz, Germany) and embedded in paraffin. After processing, sections of 5  $\mu\text{m}$  were obtained with a standard microtome and were stained with hematoxylin and eosin (HE). The tissue sections were examined by a pathologist without knowledge of the experimental groups for presence of any negative features, such as edema, erosion, ulceration and necrosis. The slides were later photographed.

## **2.10 Determination of mucus in gastric layer**

Adherent mucus measurement was carried out in rats according to the alcian blue dye method described by Corne *et al.* (1974), this cationic dye binds to glycoproteins and soluble mucopolysaccharides into insoluble complexes without penetrating mucosal cells. The animals grouped into 14 groups as described in the previous experimental design. Each glandular portion of the stomach was immediately immersed in 10 ml of the 0.1% alcian blue solution (0.16 M sucrose/0.05 M sodium acetate, pH 5.8). At the end of the 2 h-period, the unbound dye was removed by two successive washings, first for 15 min. and then for 45 min with 10 ml of 0.25 M sucrose. The mucus-bound dye was then eluted by immersion of the tissue in 0.5 M magnesium chloride and shaken for 2 h. Four milliliters of the blue extract was then briefly shaken with an equal volume of diethyl ether in order to dissolve possible particles, which could interfere with the spectrophotometric determination. The absorbance of the aqueous phase was then read at 605 nm (Shimadzu UV/Vis-1601 Spectrophotometer, Japan). The amount of alcian blue extracted per gram of wet glandular tissue was then calculated by linear regression with a calibration curve obtained from standard serial dilutions of different concentrations of the dye (Figure 14), and results were expressed as  $\mu\text{g}$  of alcian blue/g of tissue.



**Figure 14:** Standard curve of alcian blue for the determination of mucus in rat gastric tissue. Each value represents mean $\pm$ SD (n=3).

## 2.11 Evaluation of *in vivo* antioxidant activity

### 2.11.1 Preparation of homogenate

After the sacrifice, the stomach from each animal was dissected out, opened along the great curvature, washed with ice-cold saline, blotted with filter paper and the glandular portion was cut, weighed and homogenized in 50 mM Tris HCl buffer (pH 7.4) using dounce homogenizer in ice-cold condition to obtain 10 % (w/v) homogenate. Then the homogenate was centrifuged at 4000 g at 4 °C for 15 min and the supernatant was collected and stored at -20 °C for the following biochemical parameters: estimation of lipid peroxidation (MDA), reduced glutathione (GSH), catalase (CAT), Superoxide dismutase (SOD) and total proteins.

### 2.11.2 Estimation of gastric total proteins content

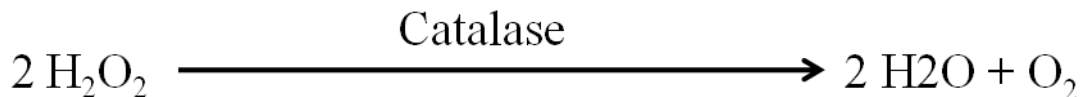
Gastric total proteins were determined by the the method of Gornall *et al.* (1949) using the Biuret kit total protein reagent (potassium iodide, potassium sodium tartarate, copper sulphate and sodium hydroxide). Proteins give a blue-violet color with copper sulphate in alkaline medium. In brief, 1 ml of Biuret reagent was mixed with 25  $\mu$ l of the tissue homogenate or standard (bovine serum albumen), then the mixture was incubated at room temperature for 10 min. The absorbance was then read at 540 nm. Total protein amount was calculated according the following formula:

$$\text{Total protein (mg/ml)} = (\text{Abs of sample} / \text{Abs of standard}) \times n$$

Where n is standard concentration.

### 2.11.3 Estimation of catalase (CAT) activity

Catalase activity was measured by the method of Clairborne (1985) with slight modification. The principle of this assay is based on the hydrogen peroxide breakdown in the presence of catalase according to the following reaction:

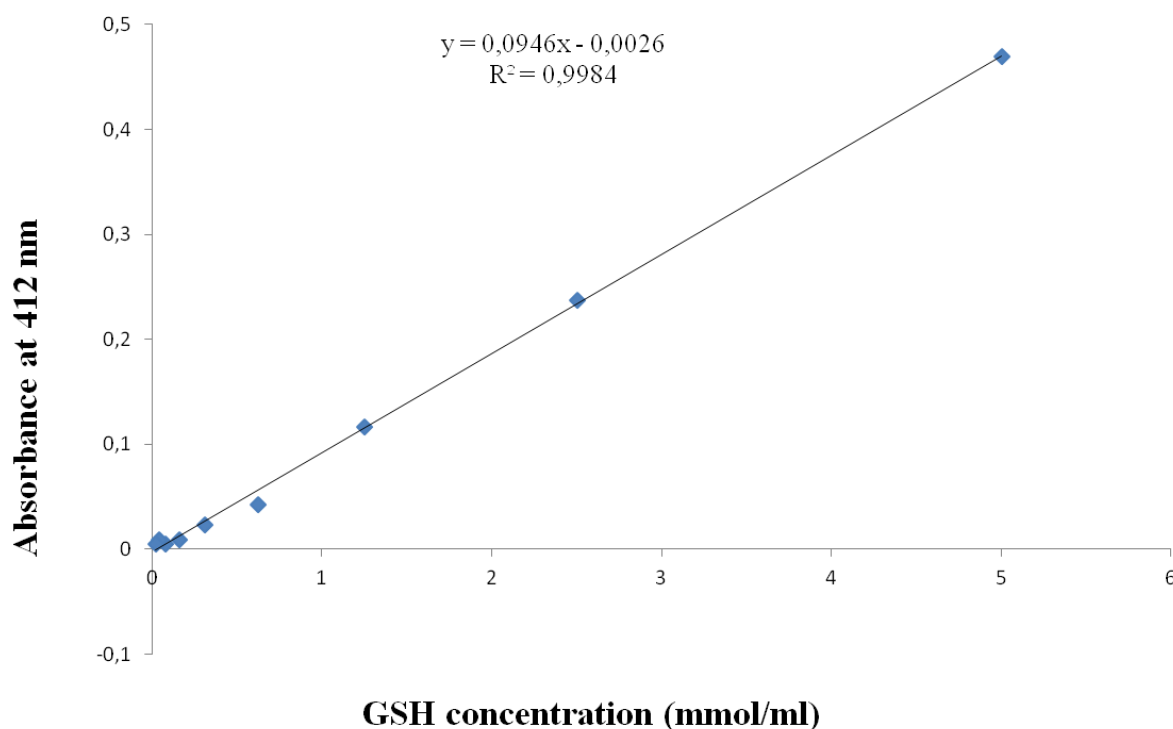


A solution of 19 mM  $\text{H}_2\text{O}_2$  (2.9 mL) in 50 mM phosphate buffer pH 7.4 was put into a quartz cuvette, 50  $\mu$ L of tissue homogenate was added. The rate of decomposition of  $\text{H}_2\text{O}_2$  in presence of CAT was monitored spectrophotometrically at 240 nm immediately and at every 15 seconds for 1 min; the enzymatic activity was expressed as  $\mu\text{M H}_2\text{O}_2/\text{min/mg protein}$ .

### 2.11.4 Assessment of reduced glutathione (GSH)

Reduced glutathione (GSH) was measured by the method of Ellman (1959). The assay is based on the oxidation of GSH by 5, 5' -dithio-bis (2-nitrobenzoic acid) (DTNB)(Ellman's reagent). DTNB and GSH react to generate 2-nitro-5-thiobenzoic acid (TNB) which has an intense yellow color and maximum absorbance at 412 nm (Wendel and Cikryt, 1980). For this assay, 50  $\mu$ l of the tissue homogenate was diluted in 10 ml of phosphate buffer (0.1 M, pH 8). To 3 ml of the mixture of dilution, 20  $\mu$ l of DTNB (0.01 M) were added and after 5 min of incubation, the yellow color developed was read at 412 nm. The concentration of GSH

expressed in mmol/g of tissue was extrapolated from a curve of standard concentrations of GSH (Figure 15) realized in the same conditions.

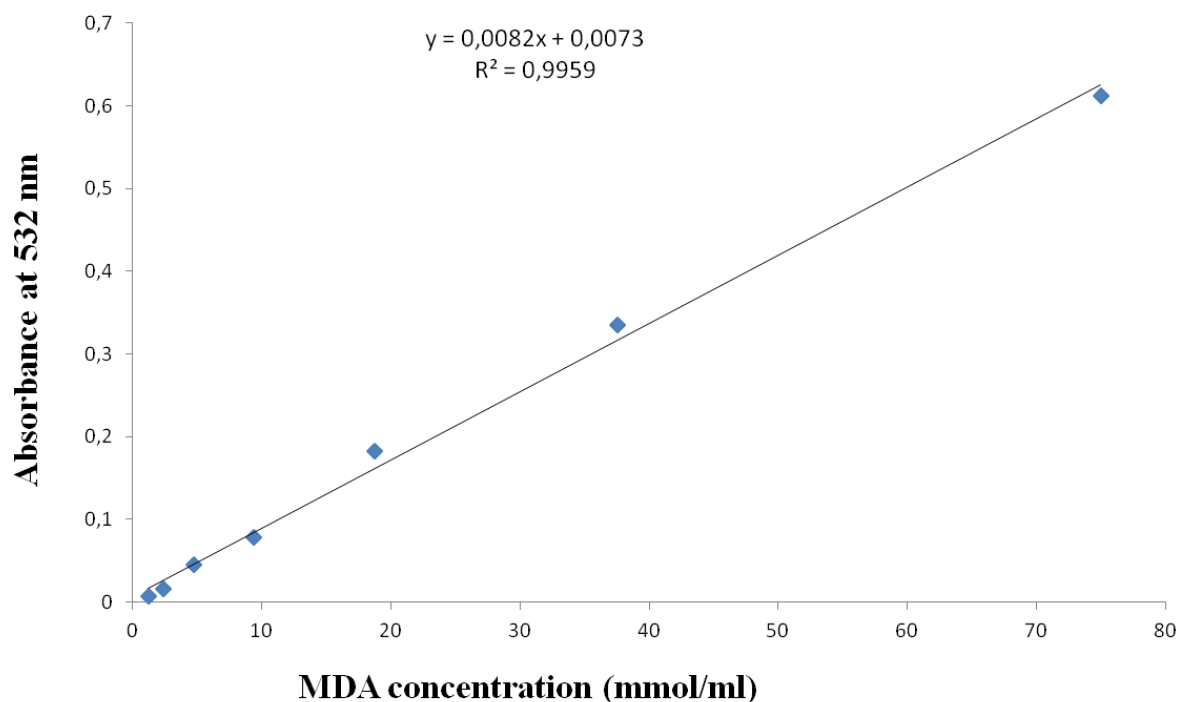


**Figure 15:** Standard curve of reduced glutathione (GSH). Each value represents mean $\pm$ SD (n=3).

#### 2.11.5 Lipid peroxidation (LPO) estimation

Stomach tissue lipid peroxidation was assessed by measuring malondialdehyde (MDA) formation following the method of Ohkawa *et al.* (1979). The principle of this method consists of the reaction of MDA with thiobarbituric acid (TBA) in acid conditions and a higher temperature (100°C) to form a pink MDA-(TBA)<sub>2</sub> complex. Briefly, 0.5 ml of TCA (20 % w/v) was added to 0.5 ml of tissue homogenate, then 1 ml of TBA (0.67 % w/v) was added. The mixture was incubated at 100 °C for 15 min, cooled immediately in ice and mixed with 4 ml of n-butanol and centrifuged at 3000 rpm for 15 min. The absorbance of the clear pink supernatant was measured spectrophotometrically at 532 nm against a blank. The concentration of MDA was determined from a standard curve of 1,1,3,3 tetraethoxypropane

(serial dilutions of the stock 10 mM) (Figure 16). The results were expressed as nmol TBA / g of gastric tissue.



**Figure 16:** Standard curve of 1,1,3,3 tetraethoxypropane for estimation of MDA.

#### 2.11.6 Determination of superoxide dismutase (SOD) activity

SOD activity was measured based on the ability of the enzyme to inhibit the process of pyrogallol autoxidation, according to Marklund and Marklund (1974) and Gao *et al.* (1998). Supernatant aliquots (5  $\mu$ l) were added to 1 ml of buffer solution (50 mM Tris HCl, pH 8.2) and 10  $\mu$ l pyrogallol (20 mM). An increase in absorbance was measured at 420 nm every 30 s for 1 min against a blank. The inhibition % =  $\frac{\text{abs control} - \text{abs sample}}{\text{abs control}} \times 100$ . The amount of SOD that inhibited the oxidation of pyrogallol by 50%, relative to the control, was defined as one unit of SOD activity which was calculated as follows:

Enzyme activity =  $\frac{\text{Inhibition}}{50} \times 100$ . The results were expressed as U/mg of protein.

## 2.12 Evaluation of *in vitro* antioxidant activity

### 2.12.1 ABTS radical cation decolorization assay

The radical scavenging assay against ABTS was measured using the method of Re *et al.* (1999) with slight modification. The ABTS radical stock solution (7 mM in water) was mixed with 2.45 mM potassium persulfate and kept for 12-16 h in the dark at room temperature. The solution was then diluted with methanol to give an absorbance of ~0.7 at 734 nm. Then 50 µl of sample was mixed with 1 ml of ABTS mixture and kept for 30 min at room temperature in the dark. The absorbance of reaction mixture was measured at 734 nm. Trolox was used as positive control. All determinations were performed in replicates. Scavenging capability of test compounds was calculated from the following equation:

$$\% \text{ inhibition} = [(\text{Absorbance of control} - \text{Absorbance of test sample}) / \text{Absorbance of control}] \times 100.$$

To determine the IC<sub>50</sub> values, a dose response curve was plotted. IC<sub>50</sub> is defined as the concentration sufficient to obtain 50% of a maximum scavenging capacity.

### 2.12.2 DPPH scavenging activity

The DPPH assay was based on the measurement of altering the purple color to yellow of DPPH radical at 517 nm after reaction with antioxidant compound. The effect of antioxidants on DPPH radical was thought to be due to their hydrogen donating ability. DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule (Shekhar and Anju, 2014)

The 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity of the extracts was determined spectrophotometrically in an MRXe tc (DYNEX Technologies GmbH, Denkendorf, Germany), by monitoring the disappearance of DPPH at 515 nm, according to the method described by Ribeiro *et al.* (2007). Briefly, 20 µl of *M. communis* L. extracts or standard solution (ascorbic acid) in absolute methanol was added to 180 µl of DPPH reagent (0.004%) in 96 well plates. Absolute ethanol was used for reagent blank. All reagents were mixed and incubated for 30 minutes at room temperature and protected from light. Experiments were done in triplicates. The percentages of the DPPH free radical scavenging activity were calculated as follows:

$$\% \text{ inhibition} = [(\text{Absorbance of control} - \text{Absorbance of test sample}) / \text{Absorbance of control}] \times 100.$$

To determine the IC<sub>50</sub> values, a dose response curve was plotted. IC<sub>50</sub> is defined as the concentration sufficient to obtain 50% of a maximum scavenging capacity.

### 2.12.3 Hydroxyl radical scavenging activity

The hydroxyl radical scavenging activity of *M. communis* extracts was measured by the salicylic method described by Smirnoff and Cumbes (1989). The reaction mixture 3.0 ml contained 1.0 ml of 1.5 mM FeSO<sub>4</sub>, 0.7 ml of 6 mM hydrogen peroxide, 0.3 ml of 20 mM sodium salicylate and varied concentrations of the extract. After incubation for 1 hour at 37°C in water bath, the absorbance of the hydroxylated salicylate complex was measured at 562 nm. The scavenging activity of hydroxyl radical effect was calculated according to the following equation:  $[1 - (A_1 - A_2) / A_0] \times 100$ .

Where A<sub>0</sub> is absorbance of the control (without extract) and A<sub>1</sub> is the absorbance in the presence of the extract, A<sub>2</sub> is the absorbance without sodium salicylate.

### 2.12.4 Hydrogen peroxide-scavenging activity

The ability of *M. communis* extracts to scavenge H<sub>2</sub>O<sub>2</sub> was determined according to the method of Ruch *et al.* (1989). A solution of H<sub>2</sub>O<sub>2</sub> (40 mM) was prepared in Na<sub>2</sub>HPO<sub>4</sub>–NaH<sub>2</sub>PO<sub>4</sub> buffer solution (pH = 7.4, 0.1 mol/L). H<sub>2</sub>O<sub>2</sub> concentration was determined spectrophotometrically from absorption at 230 nm. Different concentrations of samples in distilled water were added to a H<sub>2</sub>O<sub>2</sub> solution (0.6 mL). Absorbance of H<sub>2</sub>O<sub>2</sub> at 230 nm was determined after 10 min against a blank solution containing phosphate buffer without H<sub>2</sub>O<sub>2</sub>. The activity of all samples to scavenge H<sub>2</sub>O<sub>2</sub> was calculated using the following equation: Scavenging effect (%) =  $(1 - \text{Abs of sample 230 nm} / \text{Abs of control 230 nm}) \times 100$ .

### 2.12.5 Ferrous ion chelating activity

The chelating effect of the extracts was determined according to the method of Decker and Welch (1990) which is based on the inhibition of the formation of Fe<sup>2+</sup>-ferrozine complex after treatment of samples with Fe<sup>2+</sup> ions. Briefly, 250 µl of test material or EDTA at different concentration were added to 50 µl of FeCl<sub>2</sub> (0.6 mM in distilled water) and 450 µl of methanol. After 5 min of incubation, the reaction was initiated by the addition of 5 mM ferrozine (50 µl), the mixture was stirred and allowed to react at room temperature for 10 min. The control contained all the reaction reagents except the extract and EDTA. The absorbance of the Fe<sup>2+</sup>-ferrozine complex was measured at 562 nm. The chelating activity was expressed as a percentage using the following equation:

Chelating activity (%) = [(Abs of control – Abs of test sample)/Abs of control] x100.

To determine the IC<sub>50</sub> values, a dose response curve was plotted. IC<sub>50</sub> is defined as the effective concentration of the test material that is required to chelate 50% of iron ions.

#### 2.12.6 Reducing power

The reducing powers of the extracts from *M. communis* L. and BHT were determined according to the method described by Chung *et al.* (2005). A 0.1 ml aliquot of each extract BHT were mixed with an equal volume of 0.2 M phosphate buffer (pH 6.6) and 1% potassium ferricyanide, and then incubated at 50 °C for 20 min. 0.25 ml of 1% trichloroacetic acid was added to the mixture to stop the reaction, and then the mixture was centrifuged at 2790 g for 10 min. The supernatant (0.25 ml) was mixed with 0.25 ml distilled water and 0.1% FeCl<sub>3</sub> (0.5 ml) and then the absorbance was measured at 700 nm. The reducing powers of the tested samples increased with the absorbance values.

#### 2.12.7 Antioxidant activity determined by β-carotene bleaching method

This assay is based on the capacity of antioxidant molecules to inhibit β-carotene oxidative degradation that is caused by oxidative compounds of linoleic acid according to the method of Kartal *et al.* (2007). β-carotene/linoleic acid emulsion was prepared by mixing 0.5 mg of β-carotene in 1 ml of chloroform, 25 µl of linoleic acid and 200 mg of Tween 40. Chloroform was completely evaporated 40 °C using a vacuum evaporator. Then 100 ml of oxygenated distilled water was added with vigorous shaking. To an aliquot of 2.5 ml of this emulsion, 350 µl of *M. communis* or the reference antioxidant (BHT) were added and well mixed. The absorbance was recorded after 0, 1, 2, 4, 6 and 24 hours at 490 nm. A negative control consisted of 2.5 ml distilled water or solvent instead of extract or reference antioxidant. All samples were assayed in triplicate. The antioxidant activity (AA) was measured in terms of successful bleaching of β-carotene by using the following equation:

$$AA = \left[ 1 - \frac{A_0 - A_t}{A_0^0 - A_t^0} \right] \times 100$$

Where,  $A_0$  and  $A_0^0$  were the absorbance values measured at zero time of the incubation for test sample and control, respectively.  $A_t$  and  $A_t^0$  were the absorbance values measured in the test sample and control, respectively after incubation for 24 hours.



### 2.12.8 Ferric thiocyanate (FTC) assay

The FTC method was used to determine the amount of peroxide at the initial stage of lipid peroxidation using the method described by Yen *et al.* (2003) with slight modifications. Linoleic acid emulsion (0.02 M) was prepared with linoleic acid (155  $\mu$ L) and Tween 20 (155  $\mu$ L) in phosphate buffer (50 ml, 0.02 M, pH 7.4). A reaction solution, containing extracts with different concentrations (0.5 ml), linoleic acid emulsion (2.5 ml), and phosphate buffer (2 ml, 0.02 M, pH 7.0) was placed in a glass vial with a screw cap and mixed with a vortex mixer. The reaction mixture was incubated at 40°C in the dark. To 0.1 ml of reaction mixture, 4.7 ml of 75% ethanol and 0.1 ml of 30% ammonium thiocyanate were added. Exactly, 3 min after the addition of 0.1 ml of 0.02 M  $\text{FeCl}_2$  in 3.5% HCl, the peroxide value was determined by recording the absorbance at 500 nm every 24 hours until the absorbance of the control reached a maximum. The positive and negative controls were subjected to the same procedures as the sample, except for the negative control, in which only the solvent was added, and for the positive control in which the sample was replaced with BHT and Vitamin C. The inhibition percentage of linoleic acid peroxidation was calculated as:

$$\text{Inhibition\%} = (1 - \text{Absorbance of sample at 500 nm} / \text{Absorbance of control at 500 nm}) \times 100.$$

### 2.12.9 Thiobarbituric acid (TBA) assay

The TBA test was conducted on the final day of FTC according to the method described by Kikuzaki and Nakatani (1993) to determine the malonaldehyde (MDA) formation from linoleic acid peroxidation. The same sample preparation method as described in the FTC method was used. To 1 ml of sample solution, 20% trichloroacetic acid (2 ml) and thiobarbituric acid solution (2 ml) were added. The mixture was placed in a boiling water bath for 10 minutes. After cooling, it was then centrifuged at 3000 rpm for 20 minutes. Absorbance of the supernatant was measured at 532 nm. Antioxidant activity was recorded based on the absorbance of the final day of the FTC assay using the following equation:

$$\% \text{ inhibition} = 100 - [(\text{Abs sample} / \text{Abs control}) \times 100],$$

Where Abs control and Abs sample are the absorbances of the control (without sample) and the experimental (with sample) reactions, respectively.

### 2.13 Gastric emptying and small intestine transit measurements

A test meal made up of 0.1% phenol red (a non-absorbable and easily detectable marker dissolved in 1.5% carboxymethyl cellulose (CMC) was used in this study. Gastric emptying was measured according to the method described by Amira *et al.* (2005) with slight modifications. After 18-20 h of fasting, mice (n=6) were orally pretreated with ME, CHE, EAE or EO (50, 250 and 500 mg/kg) and atropine 1 mg/kg as positive control. After one hour of the treatment, each animal received orally 0.2 ml of the test meal and were sacrificed 20 min later. Under a laparotomy, the stomach and the small intestine were excised after ligation of the pylorus and the cardia. The stomach was homogenized with its contents in 25 ml 0.1 N NaOH. The homogenate was allowed to settle for 1 h at room temperature and 8 ml of the supernatant were added to 1 ml of 33% trichloroacetic acid to precipitate proteins. After centrifugation (1600 g for 30 min), 2 ml of 2N NaOH were added to the supernatant. The mixture was homogenized and its absorbance (abs) was read at 560 nm. On the day of each experiment, 4 animals were sacrificed just after the administration of the test meal and were considered as standards (0% of emptying). The gastric emptying (GE) rate in the 20-min period was calculated according to the following formula:

$$\text{GE (\%)} = (\text{Abs}_{\text{control}} - \text{Abs}_{\text{test}} / \text{Abs}_{\text{control}}) * 100.$$

Immediately after the excision of the stomach of the same mice used for gastric emptying model, the whole small intestine was removed for the evaluation of the intestinal transit). The intestine was grossly freed from its mesenteric attachments and its length was measured using a ruler. The intestine was opened at the level of the front of the test meal, which was then exactly localized by a drop of 0.1 N NaOH. The rate of intestinal transit was expressed as the ratio between the distance travelled by the test meal and the total length of the small intestine.

### 2.14 Evaluation of the antidiarrhoeal activity

The method described by Awe *et al* (2011) using castor oil as a method with small modifications (Figure 17) was followed for this investigation. Mice randomly divided into groups of 6 mice each were treated orally as outlined below:

Group 1: CMC (1.5%), negative control.

Group 2: Loperamide hydrochloride (5 mg/kg), positive control

Group 3: ME<sub>50</sub> mg/kg, Group 4: ME<sub>250</sub> mg/kg, Group 5: ME<sub>500</sub> mg/kg.

Group 6: CHE<sub>50</sub> mg/kg, Group 7: CHE<sub>250</sub> mg/kg, Group 8: CHE<sub>500</sub> mg/kg.

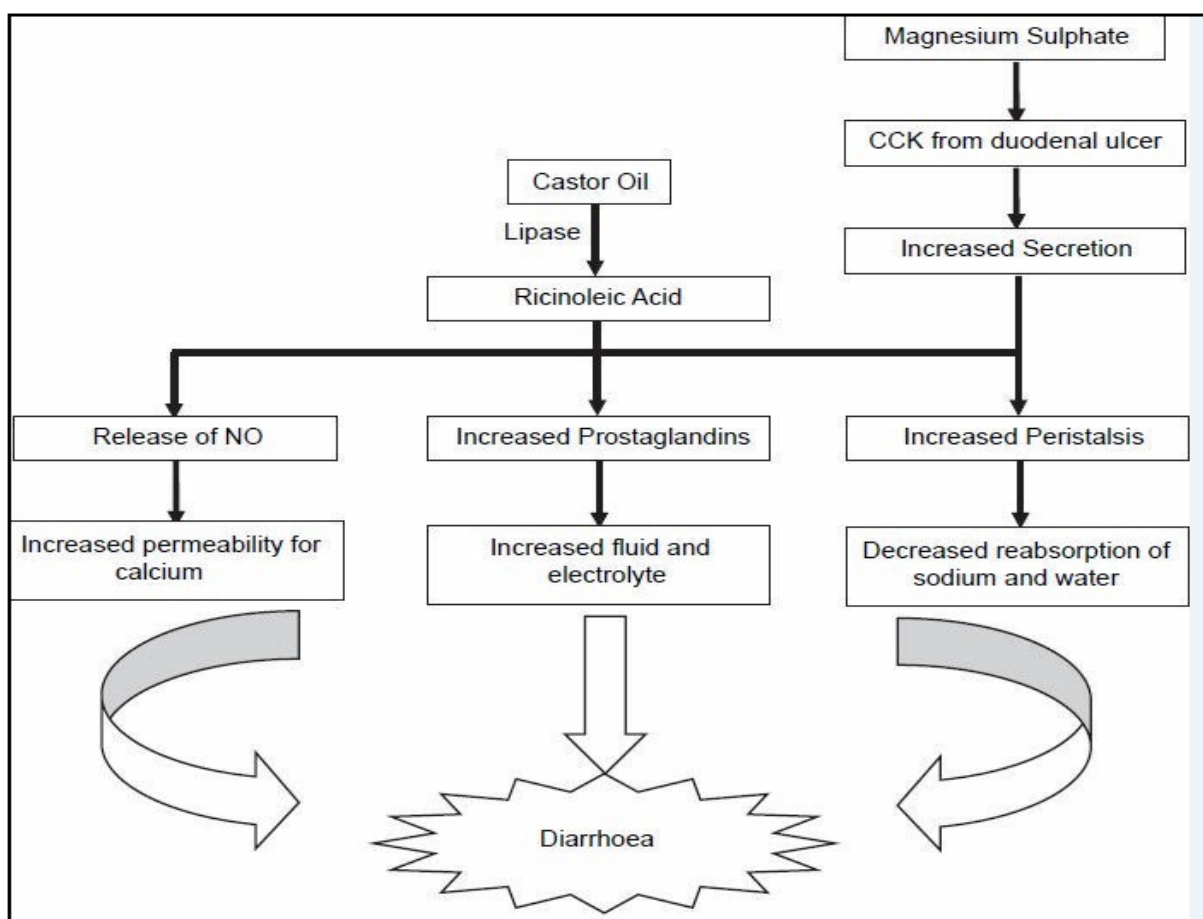
Group 9: EAE<sub>50</sub> mg/kg, Group 10: EAE<sub>250</sub> mg/kg, Group 11: EAE<sub>500</sub> mg/kg.

Group 12: EO<sub>50</sub> mg/kg, Group 13: EO<sub>250</sub> mg/kg, Group 14: EO<sub>500</sub> mg/kg.

One hour after the oral respective treatments (5 ml/kg), acute diarrhoea was induced by oral administration of castor oil (10 ml/kg)

Following the delivery of castor oil, the animals were placed in separate cages over clean white paper that was replaced every hour and inspected for 4 hours for the presence of the typical signs of diarrhoea. The time elapsed between the administration of the cathartic agent (castor oil), and the excretion of the first diarrhoetic faeces and the total number of wet faeces excreted by the animals in 4 hours were recorded. The percentage of defecation inhibition score was calculated as follows:

$$\% \text{ inhibition of diarrhoea} = \frac{[\text{Mean number of wet defecation (control-test)}]}{\text{Mean wet defecation of control}} \times 100$$



**Figure 17:** Mechanisms of castor oil-induced diarrhoea (adapted from Kaur *et al.*, 2014).

### 2.15 Intestinal fluid accumulation (Enteropooling test)

The effect of *M. communis* L. extracts on castor oil-induced fluid secretion in intestine was studied according to the method described by Awe *et al.* (2011). Animals were randomly divided into 14 groups of six mice per group. Each mouse in each group was subsequently separately placed in a cage. Group 1 mice were treated with CMC (1.5%) as negative control. Group 2 received 5 mg/kg loperamide (positive control). Groups 3, 4 and 5 were treated with ME at doses 50; 250 and 500 mg/kg. Groups 6, 7 and 8 received 50, 250 and 500 mg/kg of CHE extract. Groups 9, 10 and 11 received 50, 250 and 500 mg/kg of EAE extract Groups 12, 13 and 14 were treated with EO at doses 20, 250 and 500 mg/kg. Drugs were suspended in CMC and administered orally (5 ml/kg). One hour later, all mice received castor oil (10 ml/kg). The animals were sacrificed 30 min afterwards and the whole length of the small intestine was legated from the pylorus to the caecum. The weight of the full intestine was determined. The contents of the intestine were then expelled into a graduated measuring cylinder and its volume was determined. The weight of the empty intestine was taken, and the difference between the full and empty intestine was calculated. Percentage reduction of the intestinal secretion (volume) was calculated.

### 2.16 Trinitrobenzene sulphonic acid (TNBS) model of rat colitis

This study was carried out in accordance with the ‘Guide for the Care and Use of Laboratory Animals’ as promulgated by the National Institute of Health. Female Wistar rats (180–200 g) obtained from Janvier (St Berthevin Cedex, France) were housed in makrolon cages and maintained in an air-conditioned atmosphere with a 12 h light-dark cycle, and were provided with free access to tap water and food. The rats were randomly distributed in 5 experimental groups (6 rats per group): a non-colitic group and 4 TNBS colitic groups including an untreated group, and three that received *M. communis* L. extract (25, 50 and 100 mg/kg).

#### 2.16.1 Induction of colitis

The evaluation of anticolitic activity was performed in the Department of Pharmacology, Granada University, Spain. This work was conducted based on the anti-inflammatory activity of *Myrtus communis* L. berries methanolic extract (Amira *et al.*, 2012).

Colitis was induced by the method described by Morris *et al.* (1989) with slight modifications. Animals were fasted overnight and anaesthetized with halothane. Under light

anaesthesia, they were given 10 mg of TNBS dissolved in 0.25 ml 50% EtOH by means of a Teflon cannula inserted 8 cm through the anus. During and after TNBS administration, the rats were kept in a head-down position until they recovered from the anaesthetic and were then returned to their cages. Rats from the non-colitic (normal) group received intracolonic 0.25 ml of phosphate buffered saline instead of TNBS solution. Plant extracts were administered from the day of the colitis induction until the day before of the sacrifice of the rats, which took place seven days after the induction of the colonic damage.

#### **2.16.2 Assessment of colonic damage**

The animals were killed with an overdose of halothane, the colonic segments were obtained after laparotomy and the eventual occurrence of adhesions between the colon and adjacent organs was noted. They were placed on an ice-cold plate, cleaned of fat and mesentery and blotted on filter paper, and then they were longitudinally opened and cleaned from their luminal contents with cold saline. Afterwards, the colonic segment was weighed and its length measured. Each colon was scored for macroscopically visible damage on a 0–10 scale by two observers unaware of the treatment, according to the criteria described by Bell *et al.* (1995) (Table 1). The colon was subsequently minced, aliquoted and kept frozen at -80 °C until biochemical determinations and RNA extraction was performed.

**Table 1:** Criteria for assessment of macroscopic colonic damage (Bell *et al.*, 1995).

Score	Criteria
0	No damage
1	Hyperemia, no ulcers
2	Linear ulcer with no significant inflammation
3	Linear ulcer with inflammation at one site
4	Two or more sites of ulceration/inflammation
5	Two or more major sites of ulceration and inflammation or one site of ulceration/inflammation extending >1 cm along the length of the colon
6-10	If damage covers > 2 cm of the length of the colon, the score increased by 1 for each additional centimeter of involvement.

### 2.16.3 Myeloperoxidase (MPO) activity

Myeloperoxidase (MPO) activity was analysed in colon tissue samples to evaluate neutrophil accumulation according to the method described by Krawisz *et al.* (1984). Briefly, weighed colon strips stored at -80°C were thawed, homogenized in a 0.5% hexadecyl trimethyl ammonium bromide (HTAB) in 50 mM phosphate buffer (pH 6.0) (HTAB at a dilution of 1:20 (w/v) in ratio 1 ml per 50 mg tissue). HTAB buffer, functioning as detergent, facilitates the release of the enzyme myeloperoxidase from the intracellular granules of neutrophils, where it is stored. The samples are subjected to three freeze-thaw processes, which also contributes to the release of the enzyme, since it causes the rupture of cellular structures. The samples were then centrifuged for 10 minutes at 10,000 rpm at 4 °C. An aliquot of the supernatant was allowed to react with the chromogen (O-dianisidine dihydrochloride) in the presence of potassium phosphate buffer (monobasic potassium buffer, dibasic potassium phosphate buffer, and 1% H<sub>2</sub>O<sub>2</sub>). The absorbance was measured immediately by spectrophotometry at 450 nm. Different dilutions of human MPO enzyme of known

concentration were used to obtain a standard curve. The results were expressed as MPO units per gram of wet tissue; one unit of MPO activity was defined as that degrading 1  $\mu$ mol hydrogen peroxide/minute at 25 °C.

#### 2.16.4 Total glutathione (GSH) content estimation

The total glutathione content from colonic tissue was determined using the recycling assay described by Anderson *et al.* (1985) based on the principle that GSH can be measured by an enzymatic recycling procedure in which it is sequentially oxidised by DTNB and reduced by NADPH in the presence of glutathione reductase. Samples were thawed, minced, diluted 1:20 (w/v) in ice-cold 5% (w/v) trichloroacetic acid and homogenised. The homogenates were centrifuged at 2000xg for 5 min at 4 °C, then the supernatants were centrifuged at 9000xg for other 5 min and the obtained supernatants were used to quantify GSH levels.

A stock buffer of 143 mM sodium phosphate and 6.3 mM sodium-EDTA (pH 7.5) was made up in distilled water, and used to prepare separate solutions of 0.3 mM NADPH, 6 mM DTNB and 66.6 UI/ml GSH reductase. Using 96 wells microplate, an aliquot (20  $\mu$ l) of the colon supernatant was allowed to react with 15  $\mu$ l of PBS-EDTA-Na, 20  $\mu$ l of DTNB and 140  $\mu$ l of NADPH. After incubation of the microplate at 30 °C for 5 minutes, 15  $\mu$ l of glutathione reductase was added and the absorbance was read spectrophotometrically at 412 nm and compared with glutathione standard. The activity of GSH was expressed as nmol/g wet tissue.

#### 2.16.5 Analysis of gene expression in colonic samples by RT-qPCR

Total RNA from colonic samples was isolated using Trizol<sup>®</sup> following the manufacturer's protocol. All RNA samples were quantified with the Thermo Scientific Nano Drop<sup>™</sup> 2000 Spectrophotometer and 2  $\mu$ g of RNA were reverse transcribed using oligo (dT) primers (Promega, Southampton, UK). Real time quantitative PCR amplification and detection was performed on optical-grade 48 well plates in Eco<sup>™</sup> Real Time PCR System (Illumina, CA, USA) with 20 ng of cDNA, the KAPA SYBR<sup>®</sup> FAST qPCR Master Mix (Illumina, CA, USA) and specific primers at their annealing temperature (Ta) (Table 2). To normalize mRNA expression, the expression of the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was measured. The mRNA relative quantitation was calculated using the  $\Delta\Delta$  Ct method.

**Table 2:** Primer sequences used in real-time PCR assays in rat colonic tissue

<i>Gene</i>	Sequence (5' - 3')	Annealing T (°C)
<i>IL-1 <math>\beta</math></i>	FW -TGATGAGAATGACCTCTTCT RV -CTTCTTCAAAGATGAAGGAAA	59
<i>TNF <math>\alpha</math></i>	FW -AACTAGTGGTGCCAGCCGAT RV -CTTCACAGAGCAATGACTCC	57
<i>IL-17</i>	FW -CCTGGGTGAGCCGACAGAAGC RV -CCACTCCTGGAACCTAAGCAC	60
<i>iNOS</i>	FW -GTTGAAGACTGAGACTCTGG RV -GACTAGGCTACTCCGTGGA	60
<i>ICAM-1</i>	FW -GAGGAGGTGAATGTATAAGTTATG RV -GGATGTGGAGGAGCAGAG	60
<i>CINC-1</i>	FW: CCGAAGTCATAGCCCACTCAAG RV: TCACCAGACAGACGCCATCG	60
<i>TFF-3</i>	FW: ATGGAGACCAGAGCCTTCTG RV: ACAGCCTTGTGCTGACTGTA	60
<i>MUC-2</i>	FW -GCAGTCCTCAGTGGCACCTC RV -CACCGTGGGGCTACTGGAGAG	60
<i>GADPH</i>	FW -CCATCACCATCTTCCAGGAG RV -CCTGCTTCACCACCTTCTTG	60

#### 2.16.6 Nitrite determination assay

##### \*RAW 264.7 Cell Culture

The murine macrophage cell line RAW 264.7 was purchased from the Cell Culture Unit of the University of Granada (Granada, Spain)) and cultured in plastic bottles in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 mg/L streptomycin and 2 mM L-Glutamine, in a humidified 5%



CO<sub>2</sub> atmosphere and 95% air at 37 °C. When cultures formed a confluent monolayer, cells were scrapped and put to adhere in 96 wells plate with DMEM at a density of  $2 \times 10^6$  cell/ml (Raschke *et al.*, 1978).

### **\*\*Nitrite determination**

RAW 264.7 cells previously cultered (as described above) were seeded onto 96-well plates. The cells were pre-incubated with or without the plant extract treatment in various concentrations (12.5, 25, 50, 100 and 200 µg/mL) for 24 hours followed by an additional 24-hour incubation with LPS (Sigma, Escherichia coli 0111:B4) (100 ng/mL). After 24 hours, the supernatants were collected and nitrite release were determined by mixing 100 µl of culture supernatant with an equal volume of Griess reagents (0.1% N-naphthyl ethylenediamine solution and 1% sulphanilamide in 5% (v/v) phosphoric solution) (Green *et al.*, 1982). Briefly, equal volume of cell-free culture medium (100 µl) was reacted with Griess reagent (100 µl) and the absorbance at 550 nm was measured. Results are expressed as µM of nitrite calculated in comparison with the sodium nitrite (Na NO<sub>2</sub>) standard curve.

### **2.17 Statistical data analysis**

Results were expressed as means  $\pm$  standard error of mean (SEM). Comparison between treatment groups were performed by one way analysis of variance (ANOVA) followed by Tukey's test. Student's t-test was used were necessary The *P* Values of  $P < 0.05$  were considered significantly different using Graph Pad Prism Version 6.0 (GraphPad Software, Inc, La Jolla, CA, USA).

# Results

# 1 Pharmacological effects of *Myrtus communis* L. leaves essential oil (MLEO)

## 1.1 MLEO chemical composition

The essential oil volatile compounds, the linear retention index and their percentage are listed in table 3. Beside traces, 30 constituents were identified in MLEO and represented 99.8% of the total essential oil of the plant. The essential oil was characterized by a high percentage of monoterpene hydrocarbons (58.8 %) followed by oxygenated monoterpenes (35.6%), phenylpropanoids (1.8%) and non-terpene derivatives (1.5%). Sesquiterpene hydrocarbons and oxygenated sesquiterpene were found in smaller proportions (0.7 and 0.4%) (Table3). The major components identified were  $\alpha$ -pinene (54.1%), 1,8-cineole (26.5%), linalool (2.4%),  $\alpha$ -terpineol and geranyl acetate (2.3%), limonene (2%) and methyl eugenol (1.4%).

**Table 3:** MLEO constituents

Constituents	LRI	Percentage (%)
( <i>E</i> )-3-Hexen-1-ol	854	Tr
Isopentyl acetate	876	Tr
Propyl butyrate	896	0.3
$\alpha$ -Thujene	931	0.4
$\alpha$ -Pinene	939	54.1
Camphene	954	Tr
Thuja-2,4(10)-diene	958	Tr
Sabinene	977	Tr
$\beta$ -Pinene	980	0.6
Myrcene	991	0.2
$\alpha$ -Phellandrene	1005	Tr
$\delta$ -3-Carene	1011	0.4
Propanoic acid 2-methyl-pentyl ester	1012	0.3
<i>p</i> -Cymene	1026	0.7
Limonene	1031	2.0
1,8-Cineole	1034	26.5

( <i>E</i> )- $\beta$ -Ocimene	1051	0.5
$\gamma$ -Terpinene	1062	0.4
Terpinolene	1089	0.5
Linalool	1099	2.4
Isopentyl isovalerate	1104	0.9
Exo-fenchol	1117	Tr
<i>cis-p</i> -Menth-2-en-1-ol	1122	Tr
$\alpha$ -Campholenal	1126	Tr
<i>cis-p</i> -Mentha-2,8-dien-1-ol	1138	Tr
<i>trans</i> -Pinocarveol	1140	0.2
<i>cis</i> -verbenol	1141	0.1
<i>trans</i> -Pinocamphone	1161	Tr
Pinocarvone	1164	Tr
$\delta$ -Terpineol	1170	Tr
4-Terpineol	1178	0.4
<i>p</i> -Cymene-8-ol	1184	Tr
<i>trans-p</i> -Mentha-1(7),8-dien-2-ol	1190	Tr
$\alpha$ -Terpineol	1192	2.3
Myrtenol	1194	Tr
Estragole	1196	0.4
Verbenone	1205	Tr
<i>trans</i> -Carveol	1218	Tr
3-Methyl-3-hexen-1-yl butanoate	1237	Tr
Geraniol	1256	0.6
Linalyl acetate	1258	0.4
Geranial	1272	Tr
Isobornyl acetate	1286	Tr
<i>trans</i> -Pinocarvyl acetate	1296	Tr
$\alpha$ -Terpinyl acetate	1350	0.4
$\alpha$ -Copaene	1376	Tr
Geranyl acetate	1384	2.3
$\beta$ -Elemene	1391	Tr
Methyl eugenol	1401	1.4

$\beta$ -Caryophyllene	1418	0.4
$\alpha$ -Humulene	1455	0.2
$\beta$ -Chamigrene	1475	Tr
$\beta$ -Selinene	1485	Tr
$\alpha$ -Selinene	1494	0.1
Germacrene A	1503	Tr
$\beta$ -Bisabolene	1509	Tr
Lavandulyl isovalerate	1511	Tr
Germacrene B	1556	Tr
Spathulenol	1576	Tr
Caryophyllene oxide	1581	0.2
Geranyl 2-methyl-butyrates	1606	Tr
Humulene oxide II	1608	Tr
Caryophylla-4(14),8(15)-dien-5-ol	1636	Tr
Selin-11-en-4- $\alpha$ -ol	1653	0.2
Monoterpene hydrocarbons		<b>58.8</b>
Oxygenated monoterpenes		<b>35.6</b>
Sesquiterpene hydrocarbons		<b>0.7</b>
Oxygenated sesquiterpenes		<b>0.4</b>
Phenylpropanoids		<b>1.8</b>
Non-terpene derivatives		<b>1.5</b>
Total identified		<b>99.8</b>

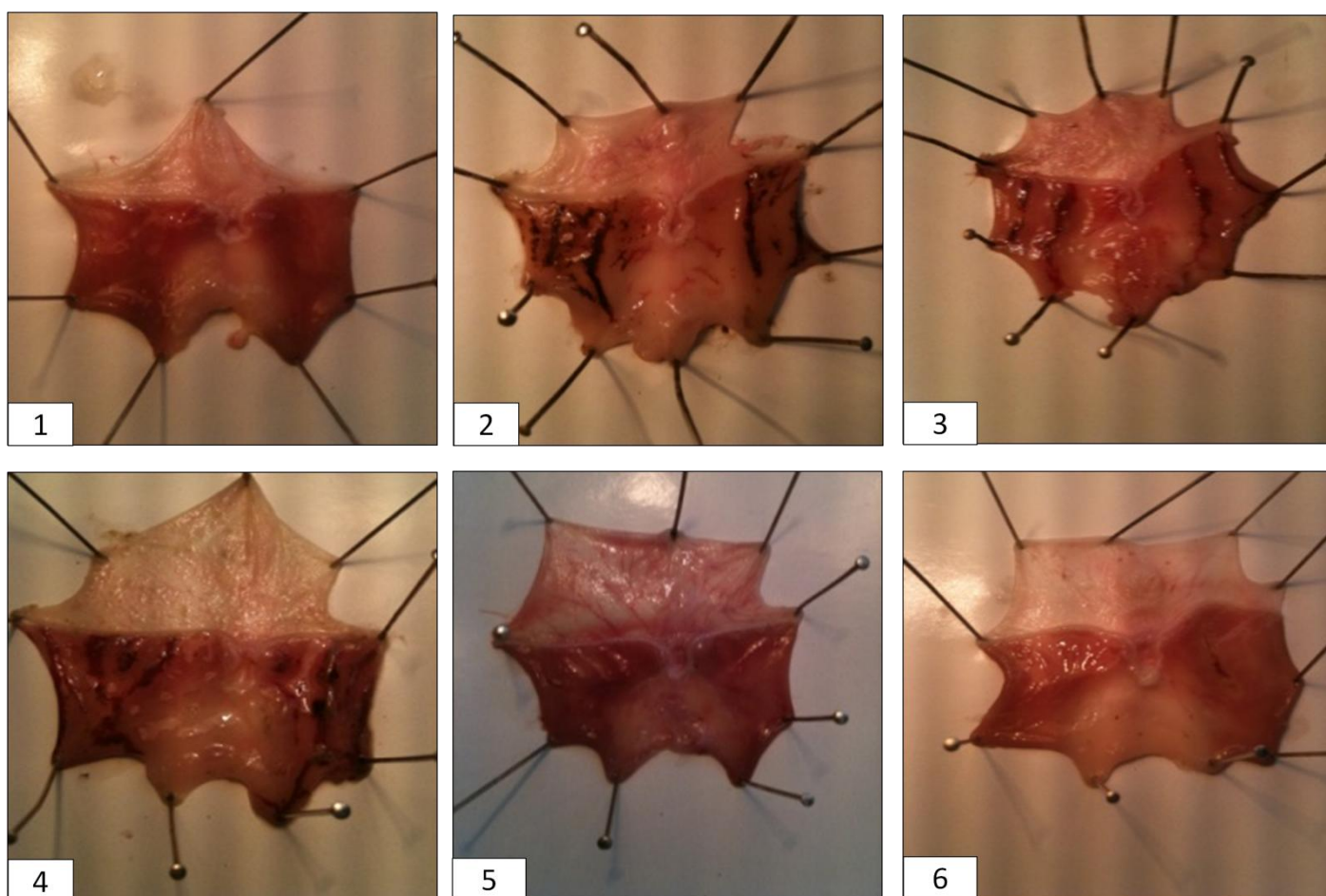
tr: Trace amounts

## **1.2 Acute oral toxicity**

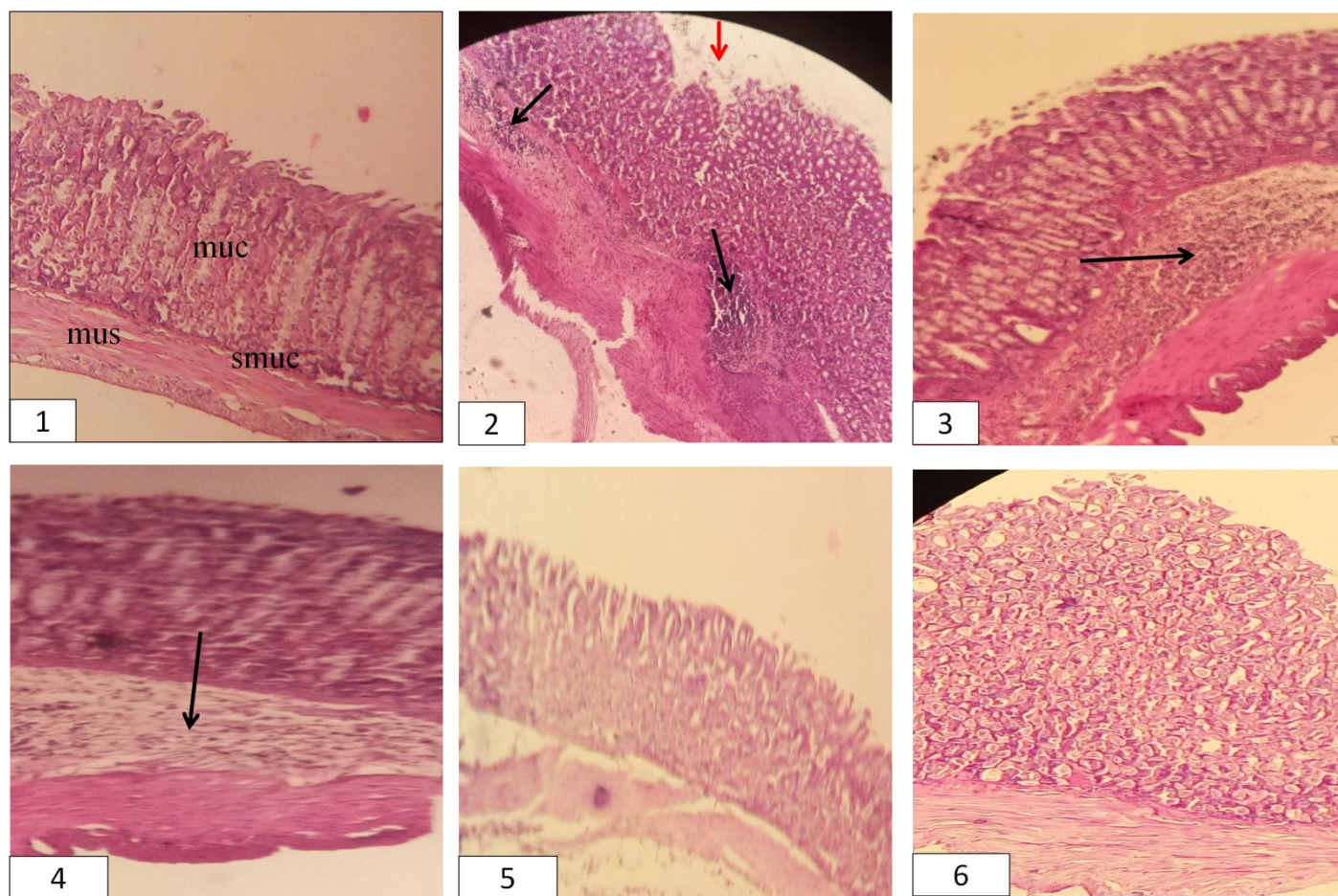
In acute toxicity test, no mortality was observed at the test dose (2000 mg/kg) within the 14 days of observation and none of the animals showed any behavioral, neurological or physical changes.

## **1.3 Effect of MLEO on macroscopic and histopathological examination**

The assay revealed a significant effect of ethanol on gastric tissue and the results are shown in Figures 18 and 19. The animals that received 70% ethanol developed a consistent macroscopic damage which was evidenced by a loss of normal colour and mucus along with the presence of petechiae, haemorrhage and oedema. This damage is attenuated by the administration of MLEO at different degrees (Figure 18). Pre-treatment of rats with MLEO at the doses employed (50, 250 and 500 mg/kg) ameliorated injuries caused by ethanol at different degrees (Figures 18). These results were demonstrated by the histopathological analysis. There is less evidence of hemorrhage lesions, less infiltration and oedema in the gastric mucosa of rats treated with MLEO at the dose of 50 mg/kg. The animals which received MLEO at the highest doses (250 and 500 mg/kg) were completely protected against the ethanol action, preserving all histological aspects when compared to control animal group (Figure 19).



**Figure 18:** Effect of MLEO on the macroscopic appearance of the gastric mucosa in ethanol-induced gastric mucosal lesions in rats. (1) Normal control group. (2) Ethanol ulcer control group. (3) The group pre-treated with ranitidine (5mg/kg). (4, 5, 6): MLEO (50, 250 and 500 mg/kg, respectively). MLEO: *Myrtus communis* L. leaves essential oil.

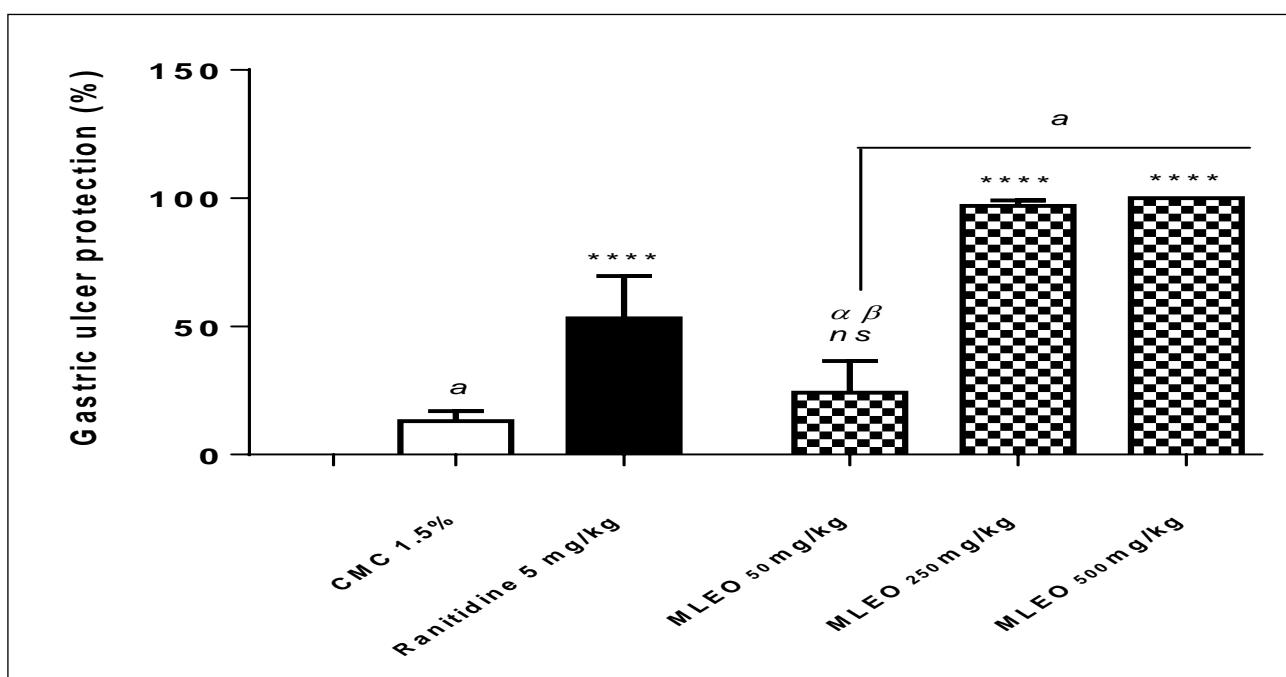


**Figure 19:** Histological evaluations for the protective effect of graded doses of MLEO 50, 250 and 500 mg/kg) on ethanol-induced gastric damage in rat stomach tissues (H&E staining; magnification 100x). (1): Section of gastric mucosa obtained from normal rats, (2): Section of gastric mucosa obtained from 70 % ethanol-induced rats, (3): Section of gastric mucosa obtained from standard drug pre-treated by ranitidine 5 mg/kg, *p.o.* and (4, 5 and 6): Sections of gastric mucosa obtained from MLEO pre-treated doses at 50, 250 and 500 mg/kg, respectively. Red arrow: surface epithelium damage; Black arrows: inflammatory infiltrate composed of polynuclear eosinophyles; MLEO: *Myrtus communis* L. leaves essential oil.



#### 1.4 Effect of MLEO on ethanol-induced gastric ulcer in rats

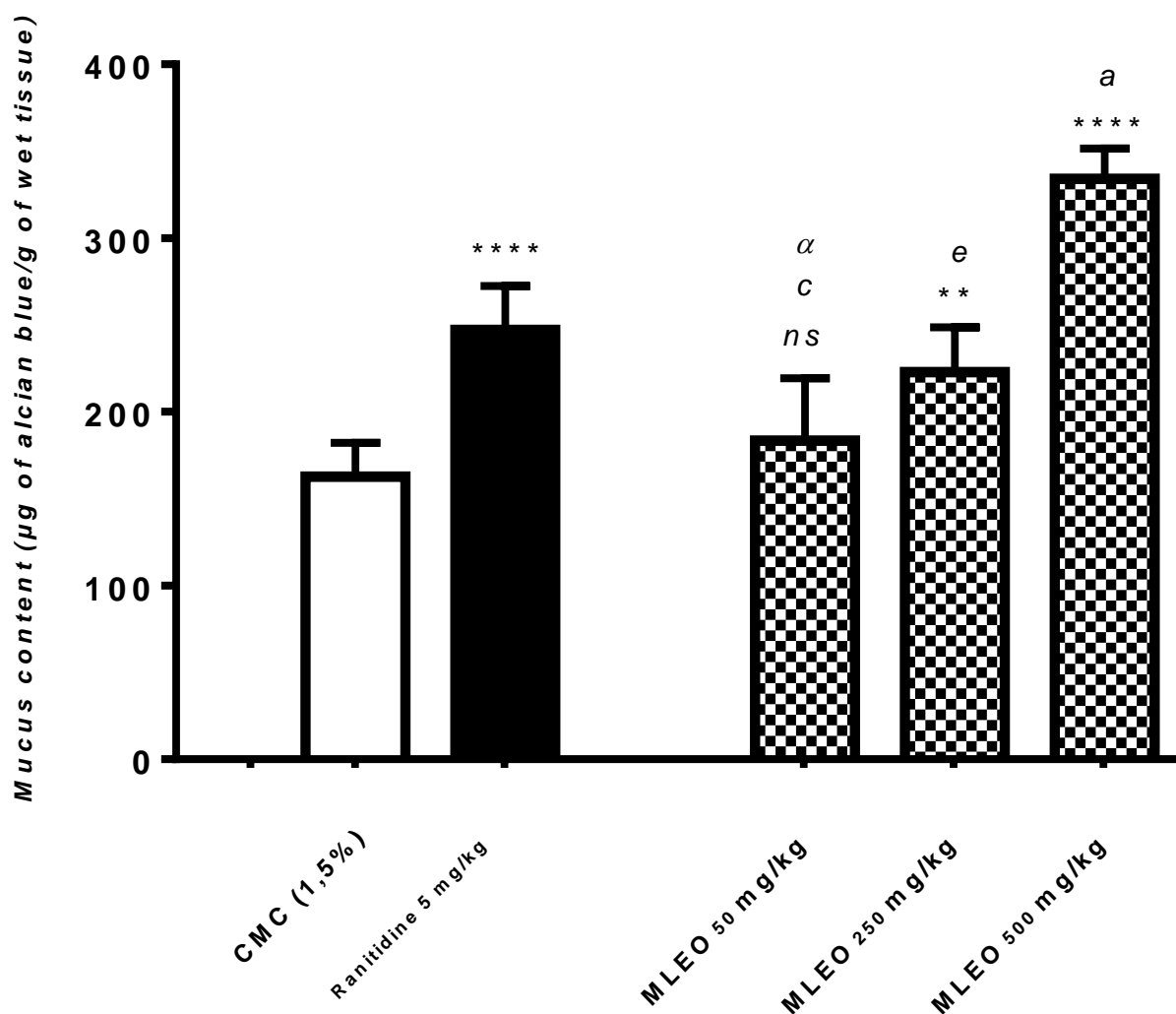
Acute oral exposure of rats to ethanol caused mucosal oedema and congestion along with inflammatory process characterized by neutrophils infiltration in the stomach (Figure 20). Post-treatment with MLEO (50, 250 and 500 mg/kg) ameliorated injuries caused by ethanol. Compared with the vehicle treated animals (CMC 1.5%), the highest doses of MLEO (250 and 500 mg/kg) significantly ( $P \leq 0.0001$ ) increased the ulcer gastric protection, whereas, the dose (50 mg/kg) did not show any significant difference *versus* the vehicle treated animals. The highest doses (250 and 500 mg/kg) of MLEO showed higher significant protection (96.98 and 100%, respectively) than the positive control (53.17 %).



**Figure 20:** Effect of MLEO on gastric ulcer protection in rats. MLEO: *Myrtus communis* L. leaves essential oil. The values of the bars chart are expressed as means  $\pm$  SEM (n=6). (\*\*\*\* $P \leq 0.0001$ ; ns: not significant) vs vehicle (CMC 1.5% *p.o.*). (<sup>a</sup> $P \leq 0.0001$  vs positive control group (Ranitidine 5 mg/kg *p.o.*). (<sup>a</sup> $P \leq 0.0001$  vs MLEO<sub>250</sub>; <sup>β</sup> $P \leq 0.0001$  vs MLEO<sub>500</sub> mg/kg).

### 1.5 Effect of MLEO on determination of gastric mucus content

The effects of MLEO on gastric mucus content are shown in figure 21. Compared with the vehicle treated animals (CMC 1.5%), MLEO significantly ( $P \leq 0.05$ -  $P \leq 0.0001$ ) increased the gastric mucus content at the higher doses (250 and 500 mg/kg). At the highest dose (500 mg/kg), MLEO showed higher effect than the positive control.

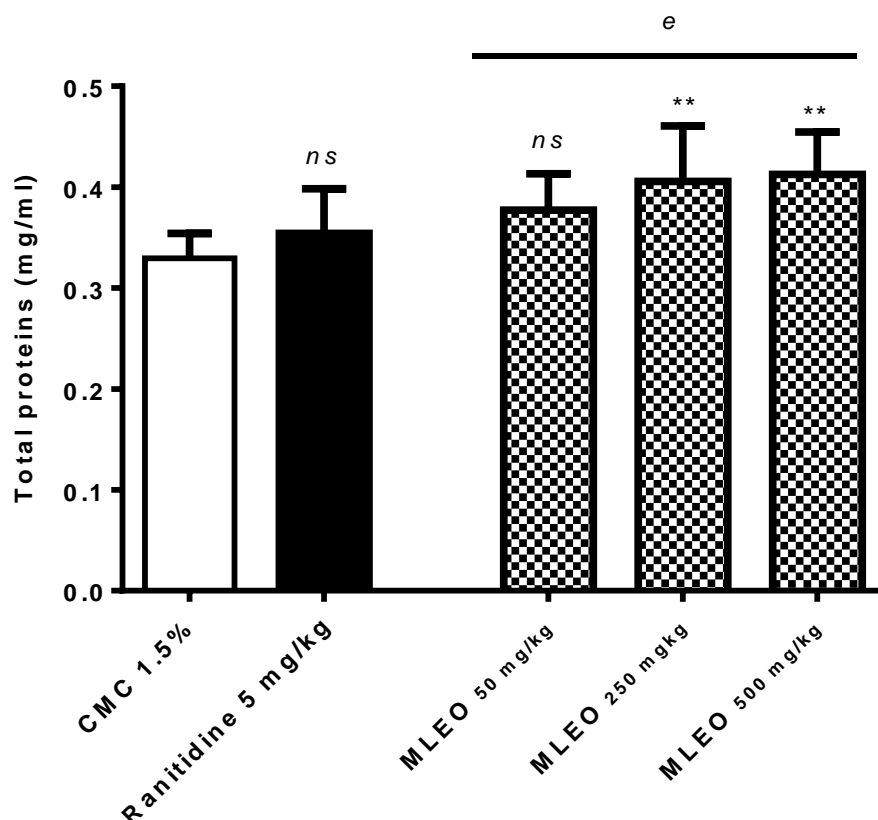


**Figure 21:** Effect of MLEO on gastric mucus content in rats. MLEO: *Myrtus communis* L. leaves essential oil. The values of the bars chart are expressed as means  $\pm$  SEM (n=6). (\*\*\*\* $P \leq 0.0001$ ; ns: not significant) vs vehicle (CMC 1.5% *p.o.*). ( $^aP \leq 0.0001$  ;  $^cP \leq 0.001$ ;  $^eP \geq 0.05$ ) vs positive control group (Ranitidine 5 mg/kg *P.O.*). ( $^aP \leq 0.0001$ ) vs EO 500 mg/kg).

## 1.6 *In vivo* antioxidant activities of MLEO in rat stomach

### 1.6.1 Effect of MLEO on total proteins level

Treatment with MLEO resulted in significant increase of total gastric protein level at the higher doses (250 and 500 mg/kg). All doses of MLEO showed similar effects ( $P \geq 0.05$ ) as ranitidine as positive control (Figure 22).

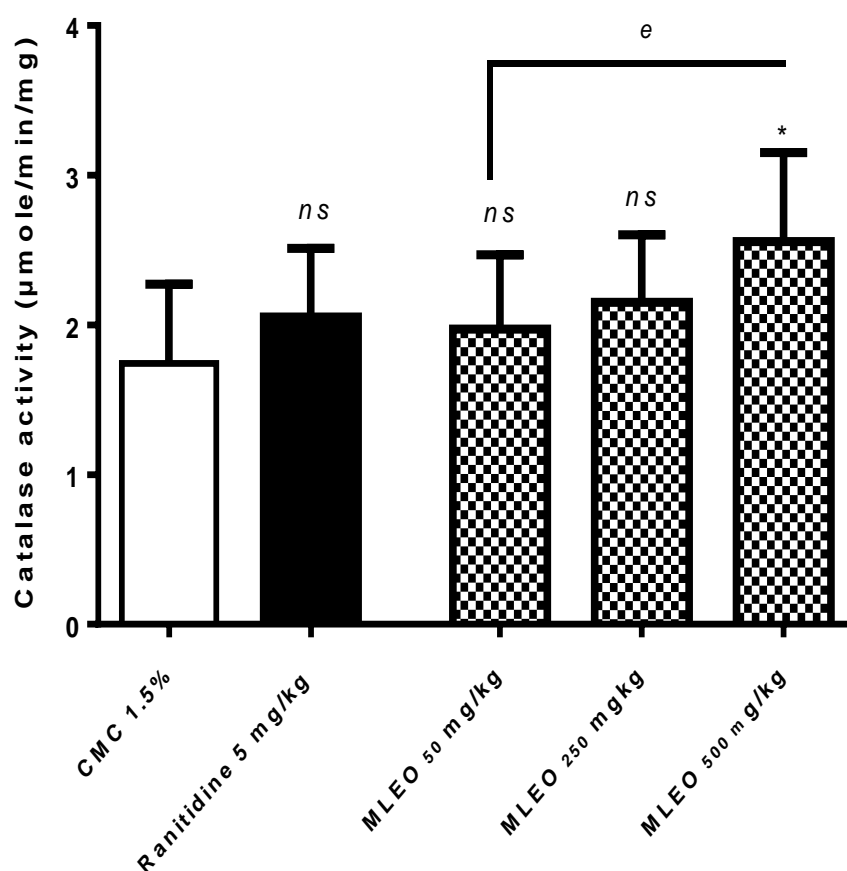


**Figure 22:** Effect of MLEO on total protein level in rat stomach tissue. MLEO: *Myrtus communis* L. leaves essential oil. The values of the bars chart are expressed as means  $\pm$  SEM

(n=6). (\*\* $P \leq 0.01$ ; ns: not significant) vs vehicle (CMC 1.5% *p.o.*). ( $^eP \geq 0.05$ ) vs positive control group (Ranitidine 5 mg/kg *p.o.*).

### 1.6.2 Effect of MLEO on catalase activity

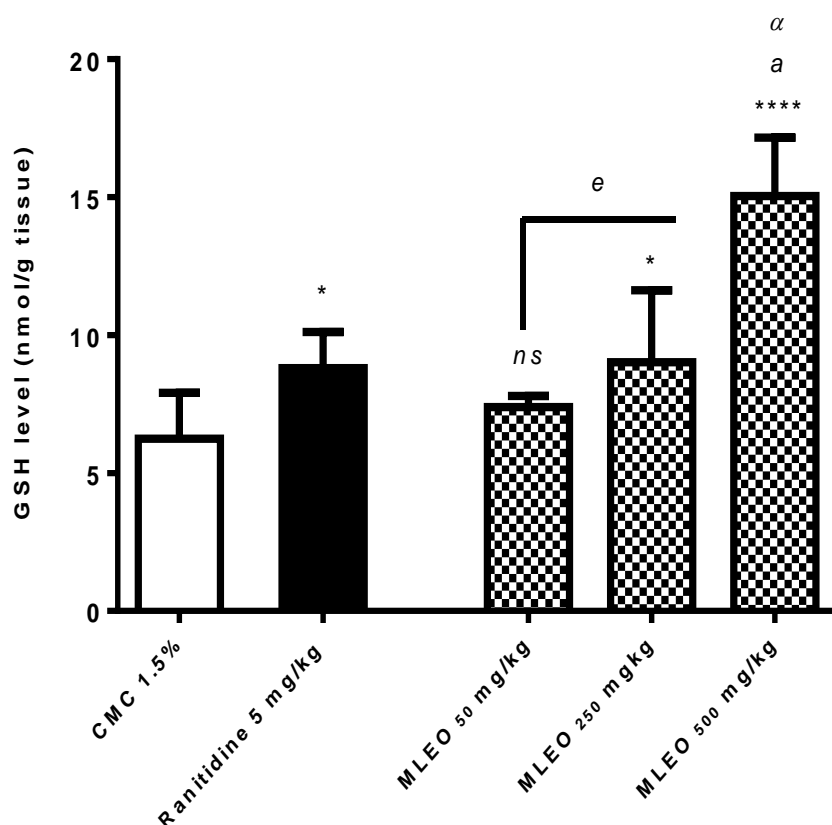
Treatment of rats with MLEO exhibited increased activity of catalase. This increase reached statistically significant difference ( $P \leq 0.05$ ) at the highest dose (500 mg/kg). At this dose, the value of catalase activity was  $2.55 \pm 0.59$ , a value higher than the ranitidine as positive control ( $2.05 \pm 0.45$ )(Figure 23).



**Figure 23:** Effect of MLEO on catalase activity in rat stomach tissue. MLEO: *Myrtus communis* L. leaves essential oil. The values of the bars chart are expressed as means  $\pm$  SEM (n=6). ( $^*P \leq 0.05$ ; ns: not significant) vs vehicle (CMC 1.5% *p.o.*). ( $^eP \geq 0.05$ ) vs positive control group (Ranitidine 5 mg/kg *P.O.*).

### 1.6.3 Effect of MLEO on GSH level

Treatment of rats with MLEO showed significant increase of GSH level at the doses of 250 and 500 mg/kg. At these doses, GSH level was  $9.02 \pm 2.6$  and  $14.85 \pm 2.25$  mmol/g tissues respectively, compared with the vehicle animal group ( $6.24 \pm 1.66$ ). The highest dose showed higher and significant values ( $****P \leq 0.0001$ ) than ranitidine as a positive control ( $8.79 \pm 1.31$ ) (Figure 24).

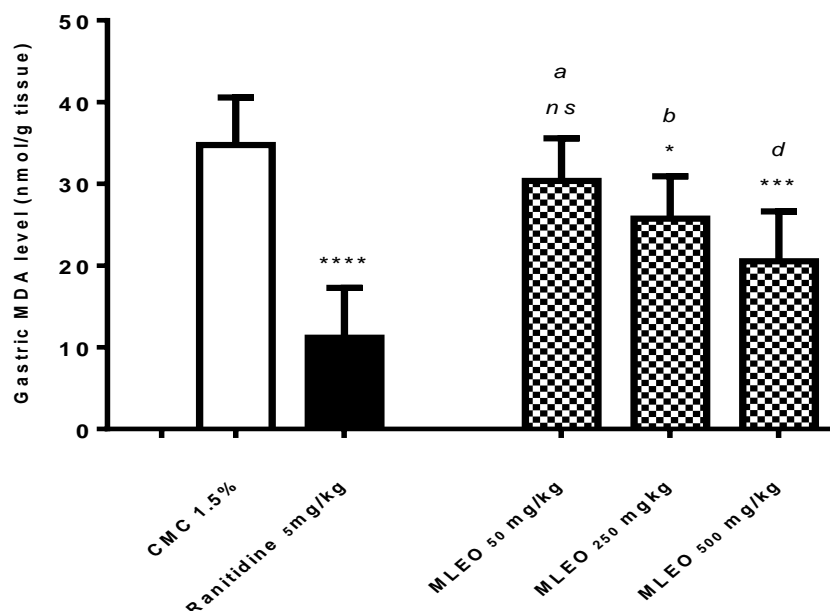


**Figure 24:** Effect of MLEO on GSH level in rat stomach tissue. MLEO: *Myrtus communis* L. leaves essential oil. The values of the bars chart are expressed as means  $\pm$  SEM (n=6). (\* $P \leq 0.05$ ;  $****P \leq 0.0001$ ; ns: not significant) vs vehicle (CMC 1.5% *p.o.*). (<sup>a</sup> $P \leq 0.0001$ ; <sup>e</sup> $P \geq 0.05$ ) vs positive control group (Ranitidine 5 mg/kg *P.O.*).

### 1.6.4 Effect of MLEO on lipid peroxidation

Lipid peroxidation and hence MDA gastric content was markedly elevated following ethanol administration in normal rats ( $34.75 \pm 5.8$ ). However, MLEO dose dependently and significantly attenuated the damage induced by ethanol at the highest doses 250 mg/kg and 500 mg/kg (\* $P \leq 0.05$  and  $***P \leq 0.001$ , respectively). At these doses, MLEO lowered the

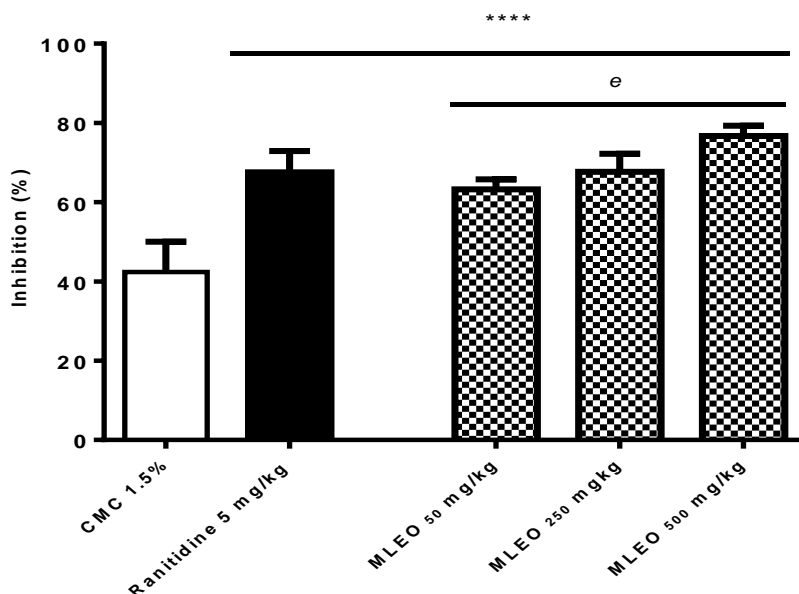
amount of MDA to values ( $25.72 \pm 5.19$  and  $20.51 \pm 6.12$ ) which were higher than that of ranitidine as positive control ( $11.16 \pm 6.19$ ) (Figure 25).



**Figure 25:** Effect of MLEO on MDA level in gastric tissues of rats. MLEO: *Myrtus communis* L. leaves essential oil. The values of the bars chart are expressed as means  $\pm$  SEM (n=6). (\*\*\*\* $P \leq 0.0001$ ; \*\*\* $P \leq 0.001$ ; \* $P \leq 0.05$ ; ns: not significant) vs vehicle (CMC 1.5% p.o.). (<sup>a</sup> $P \leq 0.0001$ ; <sup>b</sup> $P \leq 0.001$ ; <sup>d</sup> $P \leq 0.05$ ) vs positive control group (Ranitidine 5 mg/kg P.O.).

#### 1.6.5 Effect of MLEO on superoxide dismutase (SOD) activity

Statistical analysis revealed a significant decrease ( $P \leq 0.0001$ ) in SOD activity in gastric tissue after ethanol administration. Oral treatment with MLEO significantly ( $P \leq 0.0001$ ) and dose dependently inhibited the pyrogallol autoxidation when compared to control vehicle treated animals. All MLEO doses were close (not significant) to ranitidine. At the highest dose, the inhibition capacity of MLEO was higher ( $76.71 \pm 1.22$ ) than the Positive control ( $67.55 \pm 1.79$ ) (Figure 26).



**Figure 26:** Effect of MLEO on SOD activity in gastric tissues of rats. MLEO: *Myrtus communis* L. leaves essential oil. The values of the bars chart are expressed as means  $\pm$  SEM (n=6), (\*\*\*\* $P \leq 0.0001$ ) vs vehicle (CMC 1.5% *p.o.*); (<sup>e</sup> $P \geq 0.05$ ) vs positive control group (Ranitidine 5 mg/kg *P.O.*).

### 1.7 *In vitro* antioxidant activities of MLEO

Different *in vitro* antioxidant assays were performed for MLEO: ABTS, DPPH, reducing power,  $\beta$ -carotene bleaching assay, FTC and TBA. The results are shown in (Table 4). Lower  $IC_{50}$  values indicated higher antioxidant activity.

The ability of MLEO to scavenge the radical ABTS is shown in table 4. MLEO showed a significant difference ( $P \leq 0.0001$ ) with an  $IC_{50}$  of 0.36 mg/ml when compared to the standard trolox ( $IC_{50} = 0.0031$  mg/ml).

The scavenging ability of essential oil was expressed as  $IC_{50}$  value (the concentration of substrate that causes 50 % loss of DPPH activity). Low  $IC_{50}$  values indicate strong ability of the extracts to act as DPPH scavenger. MLEO showed a significant difference ( $P \leq 0.0001$ ) with  $IC_{50}$  of  $0.74 \pm 0.012$  mg/ml when compared to the standard Vit C ( $IC_{50} = 0.003$  mg/ml)(Table 4 ).

From the results in (Table 4), it is deduced that the reducing power (the effective concentration at which the absorbance was 0.5) of the MLEO was weak ( $IC_{50} = 0.7 \pm 0.05$  mg/ml) compared to the standard BHT ( $EC_{50} = 0.074 \pm 00$  mg/ml).

Table 4 shows the effect of MLEO on the changes in the percentage of the inhibition ratio of linoleic acid oxidation compared to BHT as positive control during 24 h. The addition of MLEO at 2 mg showed weak activity ( $39.07 \pm 1.35$ ) in inhibiting the oxidation of linoleic acid and subsequent bleaching of  $\beta$ -carotene, in comparison with BHT ( $94.9 \pm 1.52$ ) as positive control.

As shown in table 4, MLEO had a moderate antioxidant potential with percent inhibition of 52.63% as compared with BHT (90.56%) as positive control.

As shown in table 4, MLEO had considerably inhibited of MDA formation ( $55.12 \pm 0.09$ ) when compared with BHT ( $96.51 \pm 1.7$ ) as positive control.

Chelating ability assay was also performed in the present study to evaluate the antioxidant activity of essential oil from myrtle leaves MLEO. The essential oil showed no metal chelating activity. The absence of chelating activity in MLEO could be due to richness in monohydroxylated compounds such as 1,8-cineole which are unable to chelate ferrous ions.

**Table 4:** *In vitro* antioxidant activities of MLEO and standards: Trolox, Vit C and BHT.

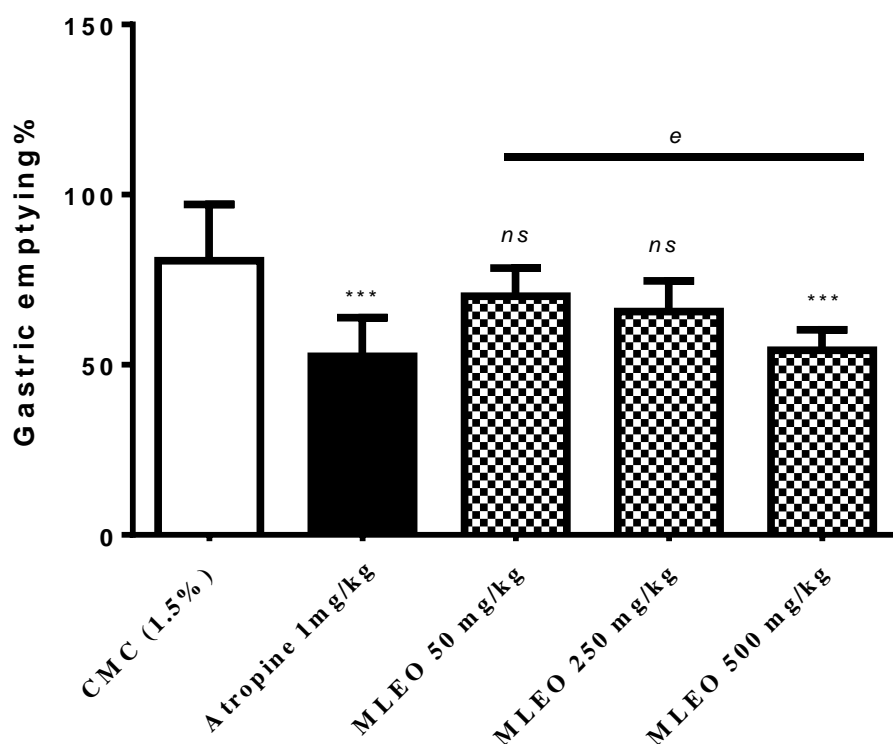
Extract	ABTS IC <sub>50</sub> (mg/ml)	DPPH IC <sub>50</sub> (mg/ml)	Reducing Power EC <sub>50</sub> (mg/ml)	$\beta$ -Carotene Inhibition (%)	FTC Inhibition (%)	TBA Inhibition (%)	Chelating ability IC <sub>50</sub> (mg/ml)
MLEO	$0.36 \pm 0$ ****	$0.74 \pm 0.012$ ** **	$0.7 \pm 0.05$ ****	$39.07 \pm 1.35$ ****	$52.6 \pm 1.6$ ****	$55.12 \pm 0.09$ * ***	/
Trolox	$0.0031 \pm 00$	/	/	/	/	/	
Vit C	/	$0.003 \pm 00$	/	/	/	/	
BHT	/	/	$0.074 \pm 00$	$94.9 \pm 1.5$	$90.56 \pm 1.7$	$96.51 \pm 0.29$	
EDTA							0.02



Data were presented IC<sub>50</sub> or Inhibition percent means  $\pm$  SD.  $P \leq 0.0005$  vs Trolox, Vit C or BHT.

### 1.8 Effect of MLEO on gastric emptying in mice

As shown in figure 27, at the lowest doses (50 and 250 mg/kg), MLEO did not show any significant decrease in gastric emptying compared to the vehicle treated animals. However, the highest dose (500 mg/kg) showed a very significant ( $P \leq 0.001$ ) effect. The tested doses did not show any significant difference ( $P \leq 0.05$ ) against the positive control.

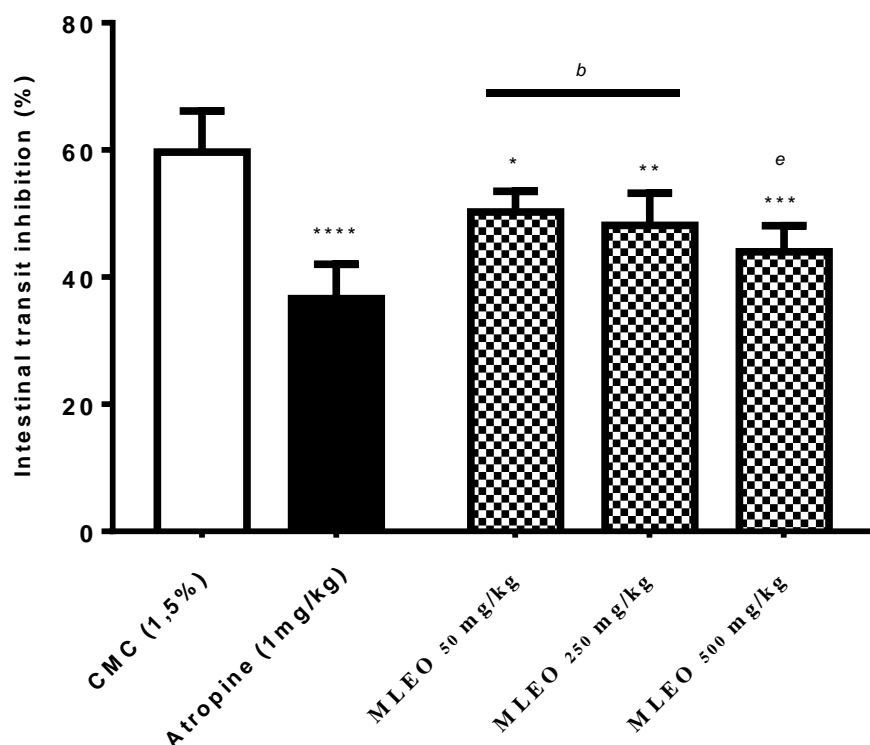


**Figure 27:** Effect of MLEO on gastric emptying in mice. MLEO: *Myrtus communis* L. leaves essential oil. The values of the bars chart are expressed as means  $\pm$  SEM (n=6). (\*\*\* $P \leq 0.001$ ; ns: no significant difference) vs vehicle treated group (CMC 1.5% *p.o.*); <sup>e</sup> $P \geq 0.05$  vs Atropine (1mg/kg *i.p.*).

### 1.9 Effect of MLEO on intestinal transit in mice

The effect of MLEO on intestinal transit is shown in figure 28. Compared with the vehicle treated animals, the MLEO dose dependently and significantly ( $*P \leq 0.05$ ,  $***P \leq$

0.001) decreased the propulsive movement and transit of red phenol through the small intestine. The intestinal transit decreased from  $59.67 \pm 2.3$  as shown in control group to  $43.97 \pm 1.85$  at the highest dose (500 mg/kg).



**Figure 28:** Effect of MLEO on intestinal transit in mice. MLEO: *Myrtus communis* L. leaves essential oil. The values of the bars chart are expressed as means  $\pm$  SEM (n=6). (\* $P \leq 0.05$ ; \*\* $P \leq 0.01$ , \*\*\* $P \leq 0.001$ , \*\*\*\* $P \leq 0.0001$ ) vs negative control group (CMC 1.5% p.o.), <sup>b</sup> $P \leq 0.001$ , <sup>e</sup> $P \geq 0.05$  vs positive control (Atropine, 1 mg/kg i.p.).

### 1.10 Effect of MLEO on castor oil-induced diarrhoea in mice

Within the observation period of 4 hours, after castor oil administration, all mice in control group (CMC 1.5%) produced copious diarrhoea. Pretreatment of mice with MLEO (50, 250 and 500 mg/kg) caused a dose dependent and significant ( $P \leq 0.0001$ ) delay of onset of diarrhoea. At the highest dose (500 mg/kg) the onset time increased from  $59.29 \pm 5.54$  in the control group to  $164.28 \pm 3.52$  %, a value less than the positive control drug loperamide ( $214.38 \pm 8.7$ ). In addition, the total number of stool and the total number of wet stool were

significantly and dose dependently reduced. Furthermore, the computed inhibition of defecation increased in a dose dependent manner (with the most remarkable percentage of inhibition at the highest dose ( $68.86 \pm 3.7$ ). At the later dose, no statistical difference was observed versus the positive control, implying that MLEO produced close effect to the positive control group (Table 5).

**Table 5:** Effect of MLEO on castor oil-induced diarrhoea in mice.

Treatment Group	Dose (mg/kg) or (ml/kg)	Onset of diarrhoea (min)	Total number of stool	Number of wet stool	Percentage of wet stool I%	Protection %
Vehicle CMC 1.5%)	10	$59.29 \pm 5.54$	$10.94 \pm 1.08$	$9.17 \pm 0.89$	$85.36 \pm 3.08$	00
MLEO	50	$120.87 \pm 3.71^{****a\lambda}$	$7.5 \pm 0.7^{***b}$	$4.87 \pm 0.44^{****d}$	$67.07 \pm 5.15$	$46.88 \pm 4.8^b$
	250	$130 \pm 3.27^{****a}$	$7.71 \pm 0.68^{**b}$	$5 \pm 0.57^{***d}$	$64.81 \pm 4.22$	$50.96 \pm 3.72^c$
	500	$164.28 \pm 3.52^{****a}$	$6 \pm 0.75^{****}$	$2.85 \pm 0.34^{****}$	$50.68 \pm 6.2^*$	$68.86 \pm 3.7$
Loperamide	5	$214.38 \pm 8.73^{****}$	$2.63 \pm 0.7^{****}$	$1.42 \pm 0.45^{****}$	$41.77 \pm 09.78^{****}$	$81.59 \pm 5.14^{****}$

Animals were pre-treated with various doses of MLEO (50, 250 and 500 mg/kg, p.o.), reference drug (Loperamide, 5 mg/kg, p.o.) or vehicle (CMC 1.5%). One hour later, animals received castor oil (10 mg/kg p.o.). MLEO: *Myrtus communis* L. leaves essential oil; CMC: carboxy methyl cellulose. \* $P \leq 0.05$ ; \*\* $P \leq 0.01$ ; \*\*\* $P \leq 0.001$ ; \*\*\*\* $P \leq 0.0001$ ; vs negative control group (CMC). <sup>d</sup> $P \leq 0.05$ ; <sup>c</sup> $P \leq 0.01$ ; <sup>b</sup> $P \leq 0.001$ , <sup>a</sup> $P \leq 0.0001$  vs positive control group. <sup>λ</sup> $P \leq 0.01$  vs MLEO<sub>500</sub> mg/kg. (One way ANOVA followed by Tukey's multiple comparison tests.

### 1.11 Effect of MLEO on castor oil-induced intestinal fluid accumulation in mice

The effects of MLEO on castor oil-induced fluid accumulation are presented in Table 6. Pre-treatment of the test groups of mice with MLEO (50, 250 and 500 mg/kg, p.o.) dose dependently and significantly inhibited the volume and the mass of intestinal content compared to the vehicle treated group. At the highest dose (500 mg/kg), the volume and the mass of intestinal content significantly ( $P \leq 0.0001$ ) decreased from  $0.72 \pm 0.04$  and  $0.86 \pm 0.1$ , respectively (as observed in control group) to  $0.37 \pm 0.034$  and  $0.37 \pm 0.021$ , respectively. Moreover, the inhibition % of mass intestinal content at the highest dose (500 mg/kg) showed no statistical difference ( $P \geq 0.05$ ) when compared to loperamide group, indicating that MLEO produced similar effect as the positive control group (Table 6).

**Table 6:** Effect of MLEO on castor oil-induced intestinal enteropooling in mice.

Treatment group	Dose (mg/kg) or (ml/kg)	Volume of Intestinal fluid (ml)	Mass of intestinal fluid (g)	Inhibition of intestinal fluid volume (ml) %	Inhibition of intestinal mass (g) %
Vehicle (CMC1.5%)	10	0.72±0.04 <sup>a</sup>	0.86±0.1 <sup>a</sup>	00	00
MLEO	50	0.48±0.03 <sup>***e</sup>	0.53±0.028 <sup>****c</sup>	32.87±4.26 <sup>a</sup>	38.04±3.3 <sup>b</sup>
	250	0.46±0.04 <sup>***e</sup>	0.49±0.036 <sup>****e</sup>	36.11±5.55 <sup>b</sup>	42.71±4.26 <sup>c</sup>
	500	0.37±0.034 <sup>****e</sup>	0.37±0.02 <sup>****e</sup>	47.91±4.74 <sup>e</sup>	56.58±2.52 <sup>e</sup>
Loperamide	5	0.41±0.03 <sup>****</sup>	0.3±0.030 <sup>****</sup>	40.97±4.29	61.98±2.76

Animals were pre-treated with various doses of MLEO (50, 250 and 500 mg/kg, *p.o.*), reference drug (loperamide, 5 mg/kg, *p.o.*) or vehicle (CMC 1.5%). One hour later, animals received castor oil (10 mg/kg). MLEO: *Myrtus communis* L. leaves essential oil; CMC: Carboxymethyl cellulose. \*\*\* $P \leq 0.001$ , \*\*\*\* $P \leq 0.0001$  vs vehicle control group (CMC). <sup>e</sup> $P \geq 0.05$ , <sup>c</sup> $P \leq 0.01$ , <sup>b</sup> $P \leq 0.001$ , <sup>a</sup> $P \leq 0.0001$  vs positive control group (Loperamide). (One way ANOVA followed by Tukey's multiple comparison tests).

## 2 Pharmacological effects of *M. communis* L. leaves extracts (MLE)

### 2.1 Total phenolics, flavonoids and tannins contents

The total phenolics, flavonoids and tannins contents among the different extracts of MLE are presented in Table 7. The total phenolic content in terms of mg GAE/g of dry weight of extract decreased in the following order: MLME > MLEE > MLCE, whereas the highest total flavonoids were found in MLEE (38.4 ± 0.9 mg QE/g DW) and tannins contents in MLME (83.35 ± 0.36 mg TAE/g DW).

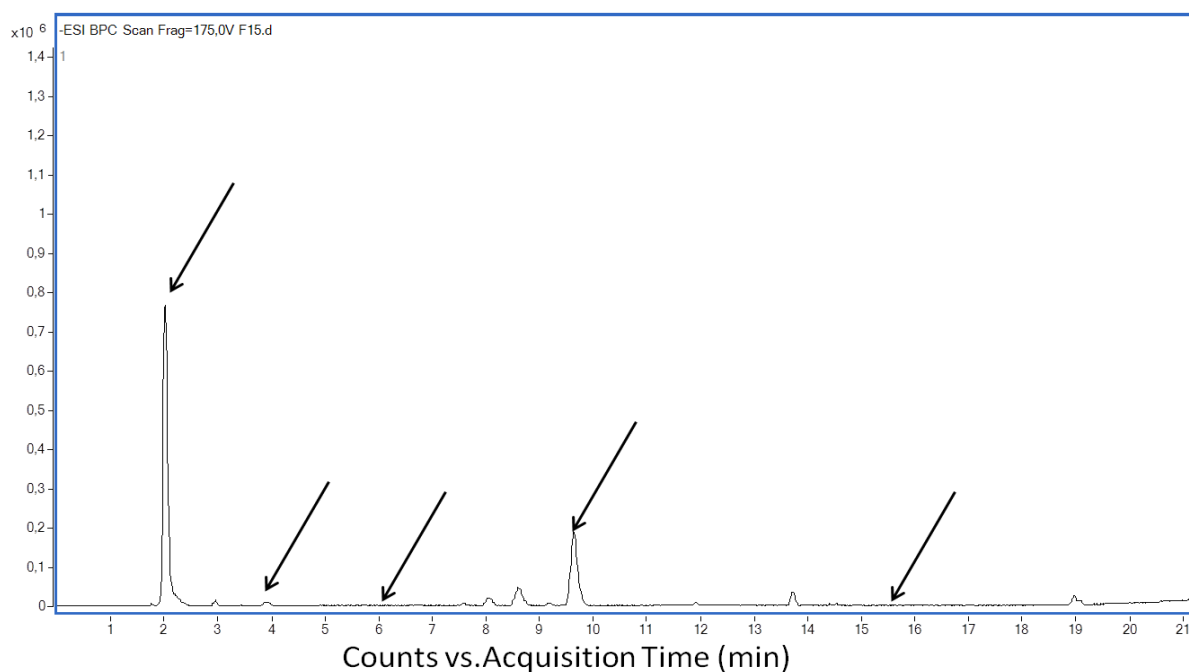
**Table 7:** Total phenolics, flavonoids and tannins contents of MLE

Extracts	Total phenolics (mg GAE/g Dw)	Total flavonoids (mg QE/g DW)	Total tannins (mg TAE/g DW)
MLME	149.25 ± 3.11	26.38 ± 0.13	83.35 ± 0.36
MLCE	81.0 ± 1.53	28.05 ± 0.15	52.3 ± 0.25
MLEE	101.88 ± 1.73	38.4 ± 0.9	49.7 ± 0.98

MLME: *Myrtus communis* leaves methanol extract, MLCE: *Myrtus communis* leaves chloroform extract, MLEE: *Myrtus communis* leaves ethyl acetate extract, DW: Dry weight. Results are expressed as means ± SEM (n=3).

## 2.2 HPLC analysis

Based on the dry powdered plant material, the yield of MLME was 16 %. The data (Table 8) show the presence of 3 compounds (1-5): Gallic acid, gentisic acid, 4-hydroxybenzoic acid, ellagic acid and quercetin. For peaks identification, see table 8.

**Figure 29:** HPLC representative chromatogram of MLME

**Table 8:** HPLC analysis of MLME

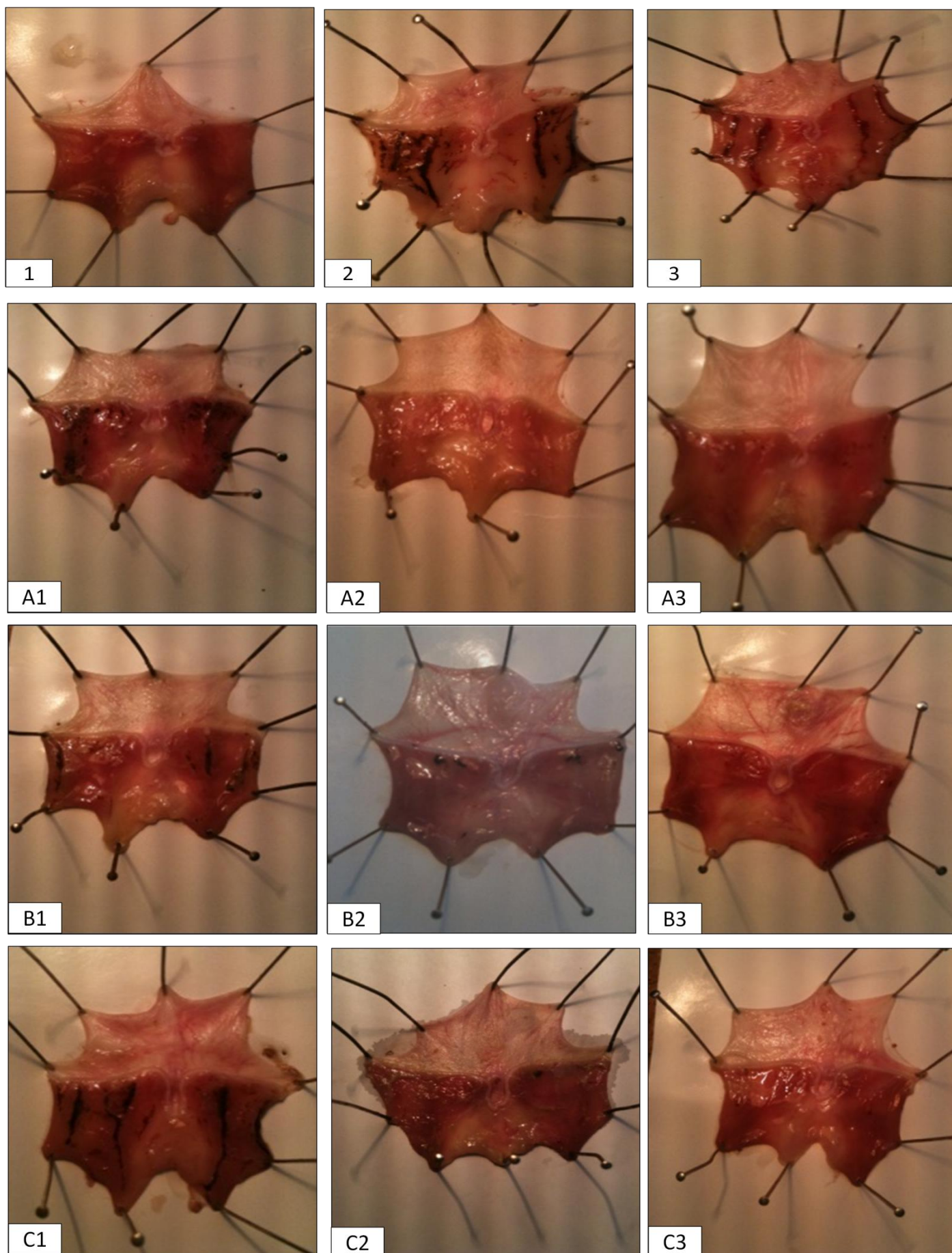
Peak N°	RT (min)	Proposed metabolite	Concentration (mg/kg)	Mass error (ppb)	Molecular formula
1	2.15	Gallic acid	59.26	179.58	C <sub>7</sub> H <sub>6</sub> O <sub>5</sub>
2	4.4	Gentisic acid	5.88	17.81	C <sub>7</sub> H <sub>6</sub> O <sub>4</sub>
3	6.42	4-hydroxy benzoic acid	5.43	16.45	C <sub>7</sub> H <sub>6</sub> O <sub>3</sub>
4	9.8	Ellagic acid	33.48	101.47	C <sub>14</sub> H <sub>6</sub> O <sub>8</sub>
5	15.6	Quercetin	1.92	5.84	C <sub>15</sub> H <sub>10</sub> O <sub>7</sub>

### 2.3 Acute oral toxicity of MLE

In acute toxicity test, no mortality was observed at the test doses for the following 14 days of observation and none of the animals showed any changes in behavioral, neurological or physical activities at the doses of 2 and 5 g/kg.

### 2.4 Effect of MLE on macroscopic and histopathological appearance in rat gastric mucosa

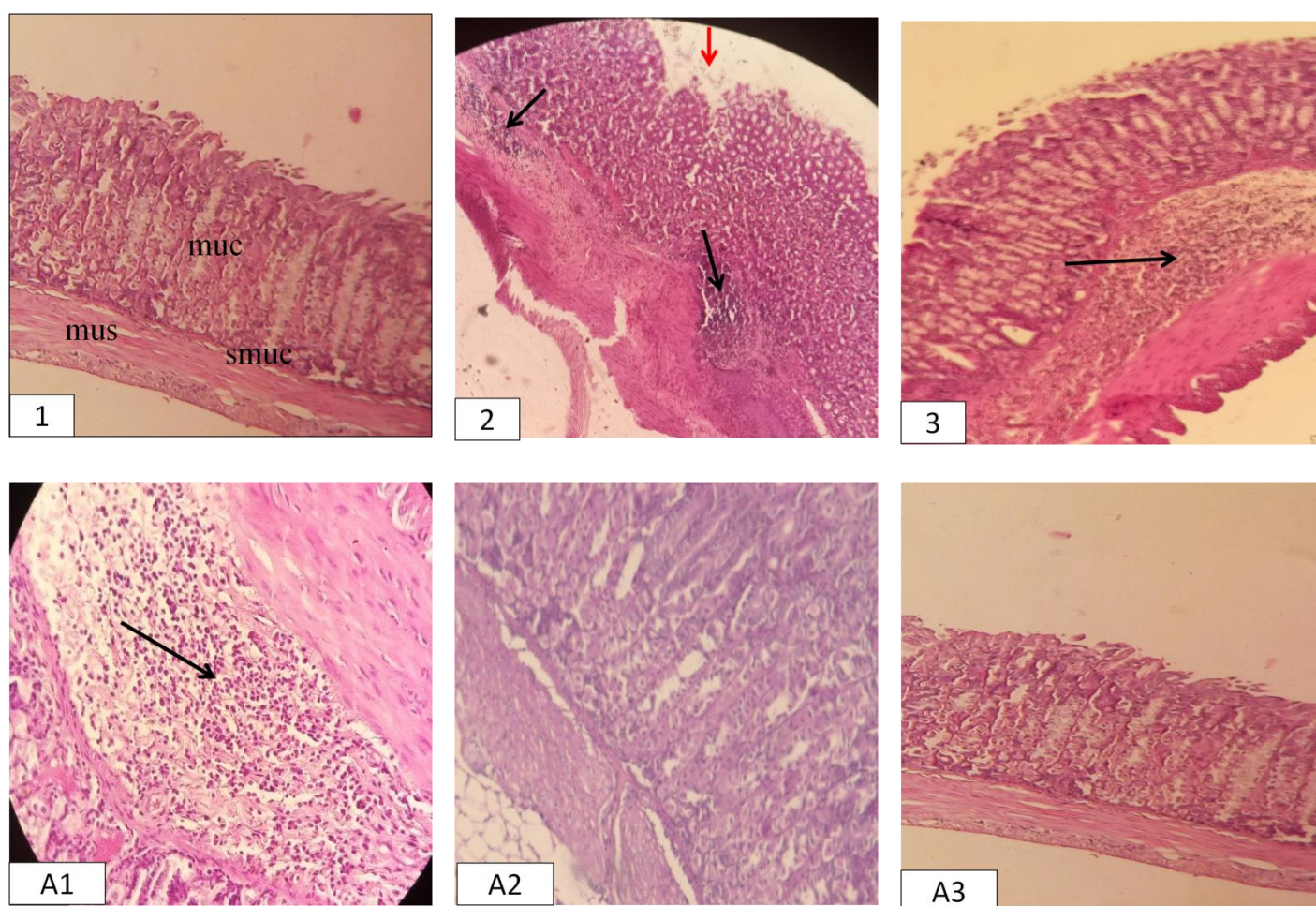
The animals that received 70% ethanol developed a consistent macroscopic damage which was evidenced by a loss of normal colour along with the presence of petechiae, haemorrhage and oedema. This damage is attenuated by the administration of the employed doses of myrtus extracts at different degrees (Figure 30). Acute exposure of rats to 70% ethanol depicted histopathological lesions leading to mucosal necrosis, congestion, erosion and hemorrhage along with inflammatory process characterized by neutrophils infiltration, thus altering the normal architecture of the stomach mucosa. Pre-treatment with MLE (50, 250 and 500 mg/kg) ameliorated injuries caused by ethanol at different degrees (Figures 31). There is less evidence of hemorrhage lesions, less infiltration and oedema in the gastric mucosa of rats treated with MLME and MLEE at the dose of 50 mg/kg and MLCE at the doses of 50 and 250 mg/kg. The animals which received MLME and MLEE at the doses (250 and 500 mg/kg) and MLCE at the dose of 500 mg/kg were completely protected against the ethanol action, preserving all histological aspects when compared to control animal group (Figure 31).



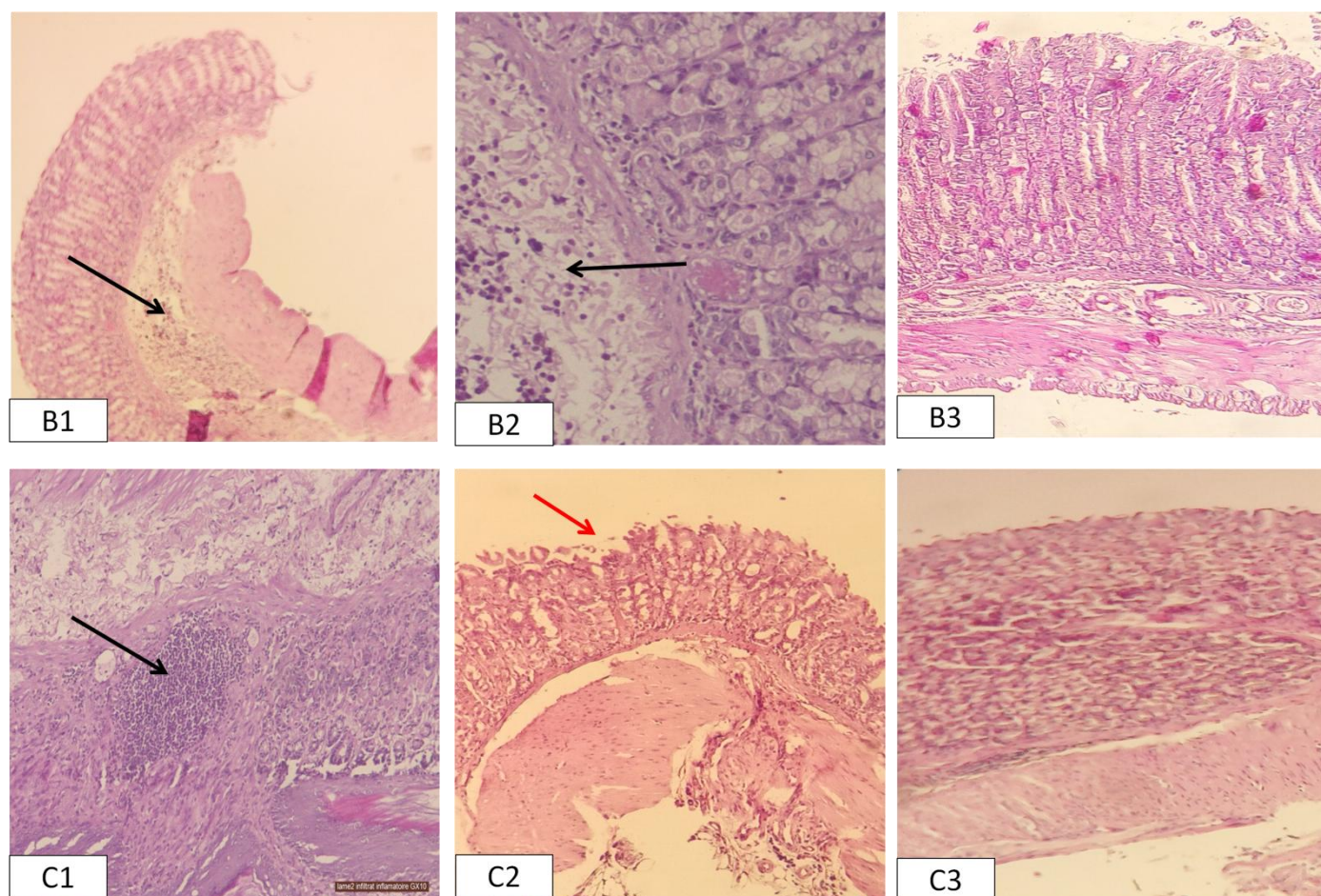


**Figure 30:** Effect of MLE on the macroscopic appearance of the gastric mucosa in ethanol-induced gastric mucosal lesions in rats.

(1): No gastric ulceration was observed in normal control group. (2): Severe hemorrhagic lesions in gastric mucosa were observed in ulcer control group. (3): The group pre-treated with ranitidine (5mg/kg) showed moderate protection against ethanol-induced gastric lesions. (A1–3): MLME (50, 250 and 500 mg/kg, respectively). (B1-3): MLCE (50, 250 and 500 mg/kg, respectively). (C1-3): MLEE (50, 250 and 500 mg/kg, respectively). MLME: *Myrtus communis* leaves methanol extract, MLCE: *Myrtus communis* leaves chloroform extract, MLEE: *Myrtus communis* leaves ethyl acetate extract.





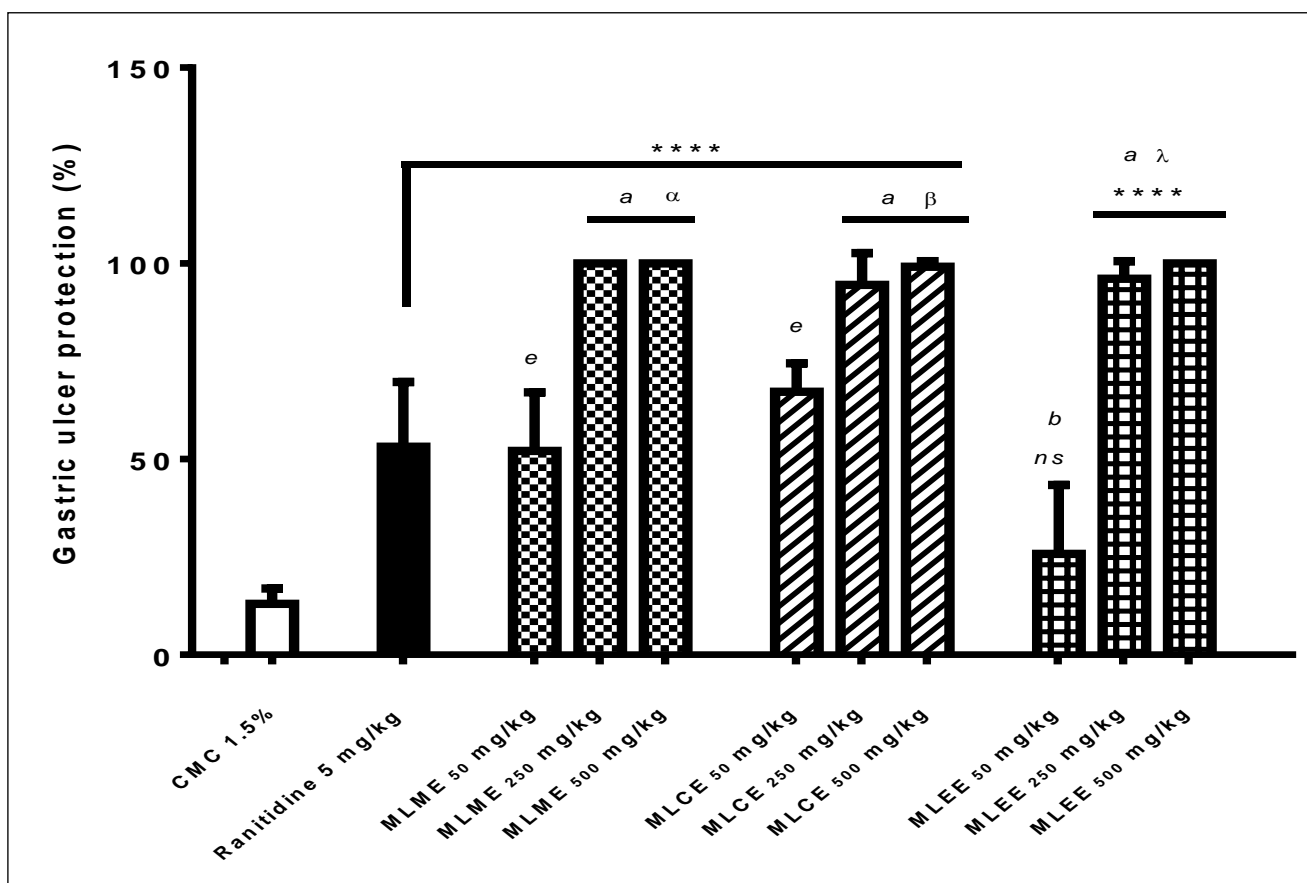


**Figure 31:** Histological evaluations for the protective effect of graded doses of MLE (50, 250 and 500 mg/kg) on ethanol-induced gastric damage in rat stomach tissues (H&E staining; magnification 100x and 400x) (1): Section of gastric mucosa obtained from normal rats, (2): Section of gastric mucosa obtained from 70 % ethanol-induced rats, (3): Section of gastric mucosa obtained from standard drug pre-treated by ranitidine 5 mg/kg, *p.o.* (B1–3): MLME (50, 250 and 500 mg/kg, respectively). (C1-3): MLCE (50, 250 and 500 mg/kg, respectively). (D1-3): MLEE (50, 250 and 500 mg/kg, respectively). MLME: *Myrtus communis* leaves methanol extract, MLCE: *Myrtus communis* leaves chloroform extract, MLEE: *Myrtus communis* leaves ethyl acetate extract. Red arrow: surface epithelium damage; Black arrow: inflammatory infiltrate composed of polynuclear eosinophiles;

## 2.5 Effect of MLE on ethanol-induced gastric ulcer in rats

Intragastric administration of ethanol (70%) to the control group of rats treated with vehicle (CMC 1.5%) produced large bandlike hemorrhagic erosions in the glandular stomach. Pre- treatment with MLME, MLCE and MLEE at the tested doses (50, 250 and 500 mg/kg, p.o.) and ranitidine (5 mg/kg) offered different degrees of protection to the mucosa against all such damages caused by ethanol (Figure 32).

Compared with the vehicle treated animals (CMC 1.5%), All tested doses of MLME and MLCE extracts dose dependently and significantly ( $P \leq 0.0001$ ) increased the ulcer gastric protection. Whereas, the dose (50 mg/kg) of MLEE did not show any significant difference *versus* the vehicle treated animals. However the highest doses (250 and 500 mg/kg) had a highly significant effect ( $P \leq 0.0001$ ). The highest dose of all extracts exhibited better protection ( $P \leq 0.0001$ ) (100%) than the positive control (Ranitidine).

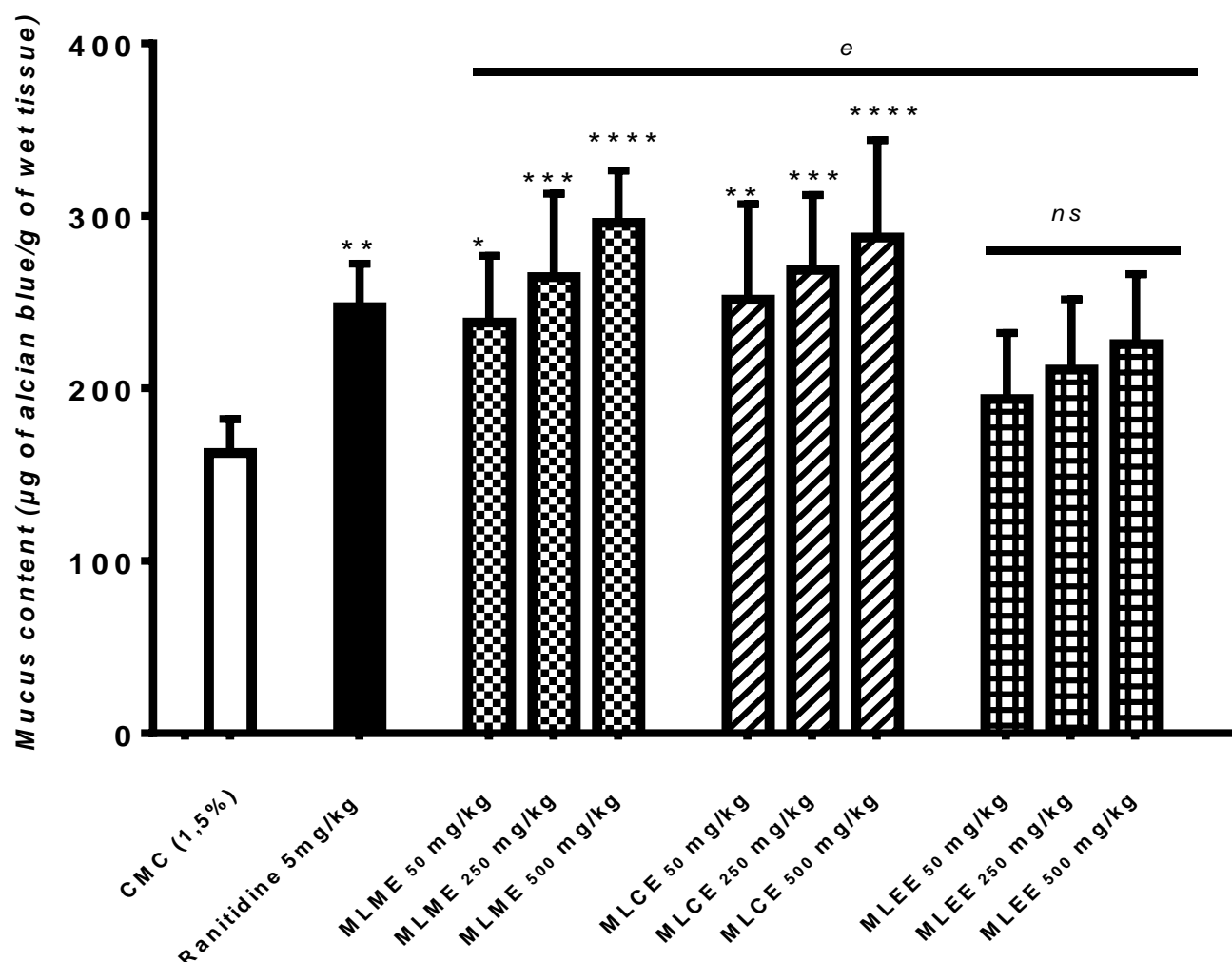


**Figure 32:** Effect of MLE on gastric ulcer in rats. MLME: *M. communis* L. leaves methanol extract, MLCE: *M. communis* L. leaves chloroform extract, MLEE: *M. communis* L. leaves ethyl acetate extract. The values of the bars chart are expressed as means  $\pm$  SEM (n=6). (\*\*\*\* $P \leq 0.0001$ ; ns: not significant) *vs* vehicle (CMC 1.5% p.o.). (<sup>e</sup> $P \geq 0.05$ , <sup>b</sup> $P \leq 0.001$ , <sup>a</sup> $P$

$\leq 0.0001$  vs positive control group (Ranitidine 5 mg/kg *P.O.*). ( $^{\alpha}P \leq 0.0001$ ) vs MLME<sub>250</sub> and MLME<sub>500</sub> mg/kg). ( $^{\beta}P \leq 0.0001$ ) vs MLCE<sub>250</sub> and MLCE<sub>500</sub> mg/kg, ( $^{\lambda}P \leq 0.0001$ ) vs MLEE<sub>250</sub> and MLEE<sub>500</sub> mg/kg.

## 2.6 Effect of MLE on gastric mucus content in rats

The effects of MLE on gastric mucus content are shown in figure 33. Compared with the vehicle treated animals (CMC 1.5%), MLME and MLCE extracts dose dependently and significantly ( $P \leq 0.01$ -  $P \leq 0.0001$ ) increased the gastric mucus content, while the effect of MLEE extract was not significantly altered for all the tested doses. All extracts showed no significant difference compared to ranitidine as positive control.



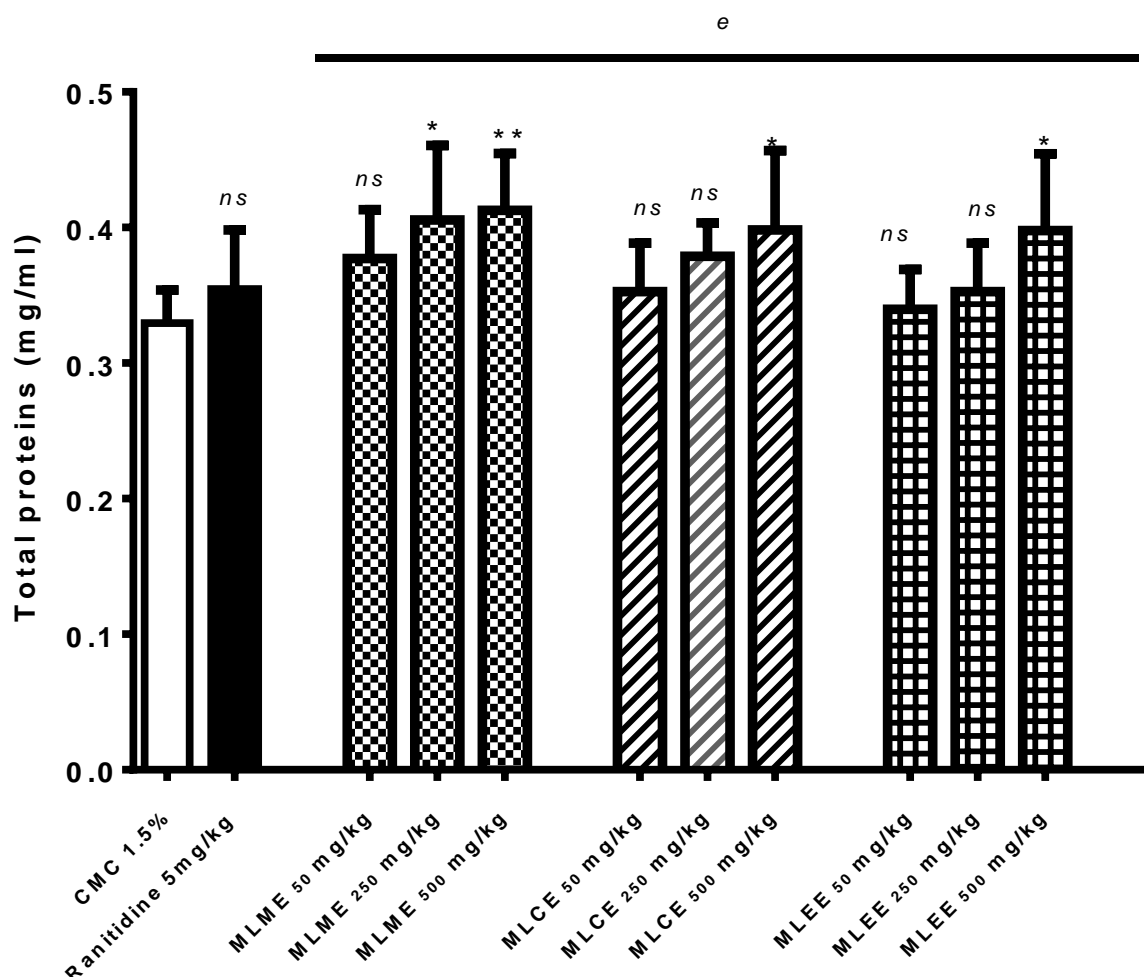
**Figure 33:** Effect of MLE on gastric mucus content in rats. MLME: *M. communis* L. leaves methanol extract, MLCE: *M. communis* L. leaves chloroform extract, MLEE: *M. communis* L. leaves ethyl acetate extract. The values of the bars chart are expressed as means  $\pm$  SEM (n=6).

(\*\*\*\* $P \leq 0.0001$ ; \*\*\* $P \leq 0.001$ ; \*\* $P \leq 0.01$ , \* $P \leq 0.05$ ; ns: not significant) vs vehicle (CMC 1.5% *p.o.*). ( $P \geq 0.05$ ) vs positive control group (Ranitidine 5 mg/kg *P.O.*).

## 2.7 *In vivo* antioxidant activities of MLE in rat stomach

### 2.7.1 Effect of MLE on total protein level

Treatment with ME resulted in significant and dose dependent increase of total gastric protein level. The increase in total protein level of MLCE and MLEE extracts was only significantly different at the highest dose ( $P \leq 0.05$ ). All extracts showed comparable effects ( $P \geq 0.05$ ) as ranitidine as positive control (Figure 34).

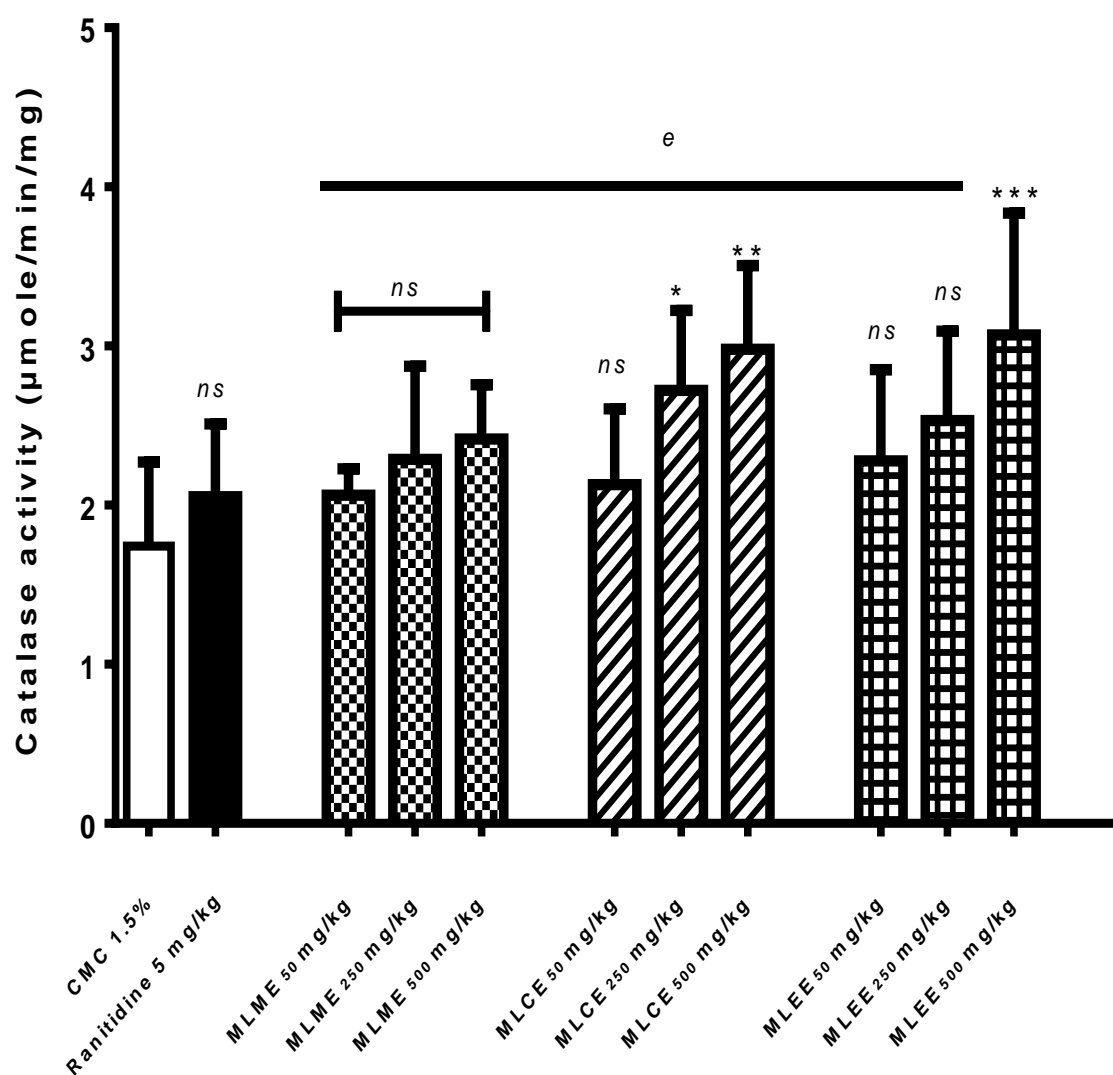


**Figure 34:** Effect of MLE on total protein level in rat stomach tissue. MLME: *M. communis* L. leaves methanol extract, MLCE: *M. communis* L. leaves chloroform extract, MLEE: *M.*

*communis* L. leaves ethyl acetate extract. The values of the bars chart are expressed as means  $\pm$  SEM (n=6). (\*\* $P \leq 0.01$ ; \* $P \leq 0.05$ ; ns: not significant) vs vehicle (CMC 1.5% *p.o.*). ( $P \geq 0.05$ ) vs positive control group (Ranitidine 5 mg/kg *P.O.*).

### 2.7.2 Effect of MLE on catalase activity

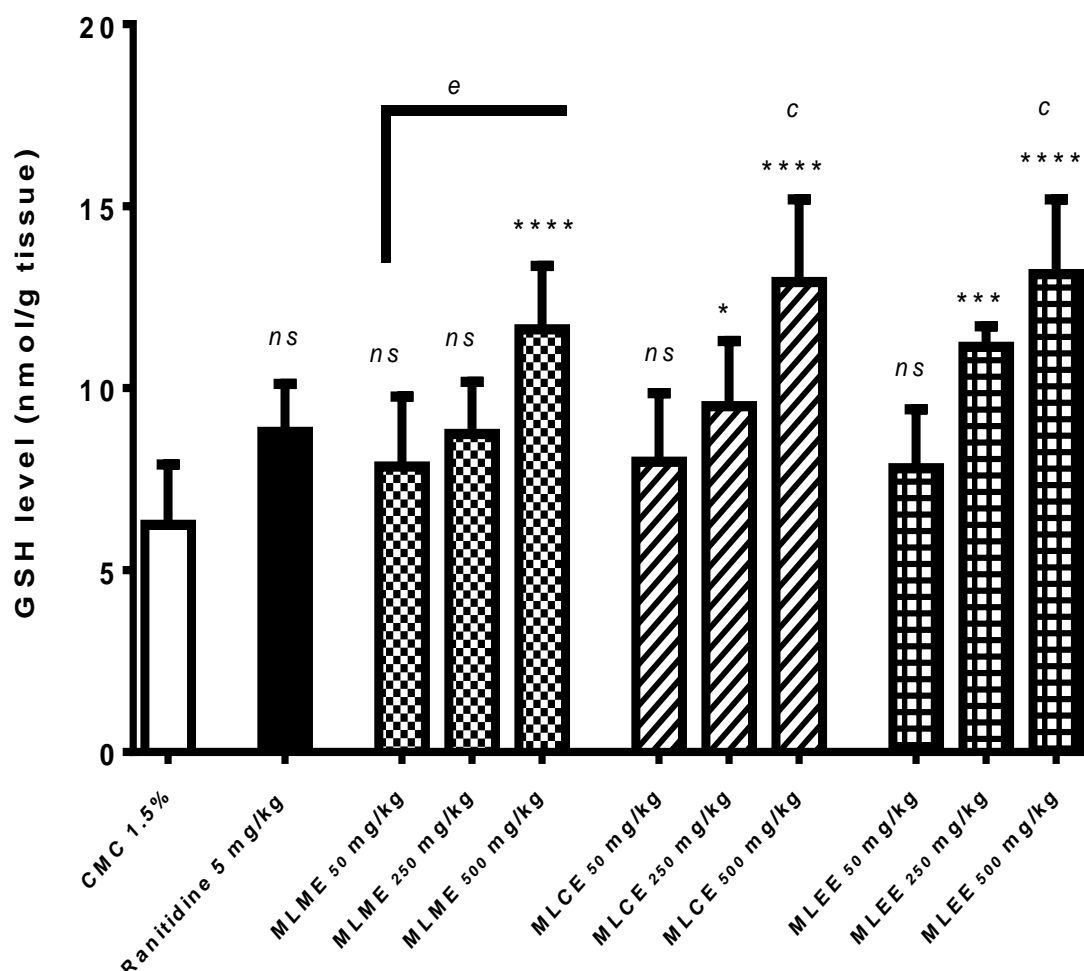
Treatment of rats with ME exhibited increased activity of catalase, but this increase was not significantly different from the negative control. MLCE and MLEE extract showed highest and significant effects at the highest dose ( $2.98 \pm 0.52$  and  $2.95 \pm 0.76$ , respectively) compared to the negative control ( $1.74 \pm 0.52$ ). All extracts showed higher effects than the ranitidine as a positive control (Figure 35).



**Figure 35:** Effect of MLE on catalase activity in rat stomach tissue. MLME: *M. communis* L. leaves methanol extract, MLCE: *M. communis* L. leaves chloroform extract, MLEE: *M. communis* L. leaves ethyl acetate extract. The values of the bars chart are expressed as means  $\pm$  SEM (n=6). (\*\* $P \leq 0.01$ ; \* $P \leq 0.05$ ; ns: not significant) vs vehicle (CMC 1.5% *p.o.*). ( $P \geq 0.05$ ) vs positive control group (Ranitidine 5 mg/kg *P.O.*).

### 2.7.3 Effect of MLE on GSH level

Treatment of rats with both MLCE and MLEE extracts showed significant increase at the doses of (250 and 500) compared with the vehicle treated animal group. Whereas, MLME exhibited significant increase ( $P \leq 0.0001$ ) only at the highest dose (500 mg/kg) (Figure 36). These values were higher than the positive control.

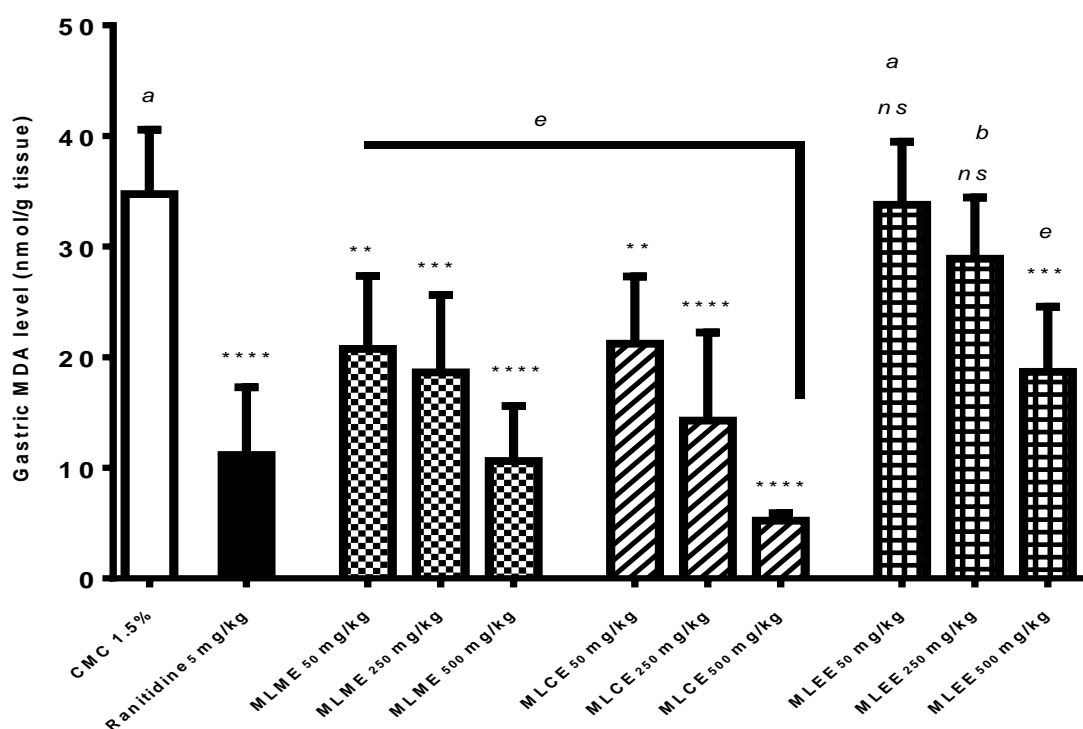


**Figure 36:** Effect of MLE on GSH level in rat stomach tissue. MLME: *M. communis* L. leaves methanol extract, MLCE: *M. communis* L. leaves chloroform extract, MLEE: *M.*

*communis* L. leaves ethyl acetate extract. The values of the bars chart are expressed as means  $\pm$  SEM (n=6). (\*\*\*\* $P \leq 0.0001$ ; \*\*\* $P \leq 0.001$ ; \* $P \leq 0.05$ ; ns: not significant) vs vehicle (CMC 1.5% *p.o.*). (<sup>c</sup> $P \leq 0.01$ ; <sup>e</sup> $P \geq 0.05$ ) vs positive control group (Ranitidine 5 mg/kg *P.O.*).

#### 2.7.4 Effect of MLE on lipid peroxidation

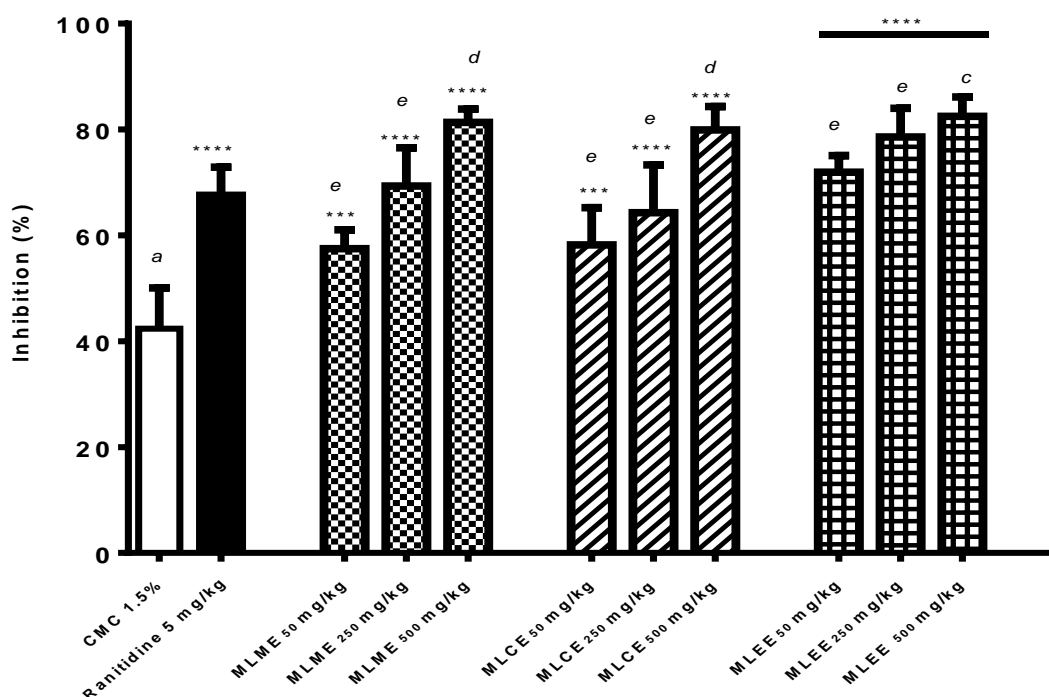
Lipid peroxidation and hence MDA gastric content was markedly elevated following ethanol administration in normal rats ( $34.75 \pm 5.8$ ). However, MLME and MLCE extracts dose dependently and significantly ( $P \leq 0.0001$ ) attenuated the ethanol effect. At the highest dose (500 mg/kg), both extracts decreased the amount of MDA to values lower than that of ranitidine as positive control. MLEE extract exerted significant effect only at the highest dose (Figure 37).



**Figure 37:** Effect of MLE on MDA level on gastric tissues of rats. MLME: *M. communis* L. leaves methanol extract, MLCE: *M. communis* L. leaves chloroform extract, MLEE: *M. communis* L. leaves ethyl acetate extract. The values of the bars chart are expressed as means  $\pm$  SEM (n=6). (\*\*\*\* $P \leq 0.0001$ ; \*\*\* $P \leq 0.001$ ; \*\* $P \leq 0.01$ ; ns: not significant) vs vehicle (CMC 1.5% *p.o.*). (<sup>a</sup> $P \leq 0.0001$ ; <sup>b</sup> $P \leq 0.001$ ; <sup>e</sup> $P \geq 0.05$ ) vs positive control group (Ranitidine 5 mg/kg *P.O.*).

### 2.7.5 Effect of MLE on SOD activity

Oral treatment with all plant extracts significantly ( $P \leq 0.0001$ ) and dose dependently inhibited the pyrogallol autoxidation when compared to control vehicle treated animals ( $42.37 \pm 0.74\%$ ). At the highest dose, the inhibition capacity of the different extracts was in the following order: MLEE ( $82.48 \pm 0.98$ ) > MLME ( $81.33 \pm 2.23$ ) > MLCE ( $79.91 \pm 0.88$ ) > Positive control ( $67.55 \pm 1.79$ ). All extracts were close (not significant) or higher (at highest dose) than ranitidine as positive control (Figure 38).



**Figure 38:** Effect of MLE on SOD activity in gastric tissues of rats. MLME: *M. communis* L. leaves methanol extract, MLCE: *M. communis* L. leaves chloroform extract, MLEE: *M. communis* L. leaves ethyl acetate extract. The values of the bars chart are expressed as means  $\pm$  SEM (n=6), (\*\*\*\* $P \leq 0.0001$ ; \*\*\* $P \leq 0.001$ ) vs vehicle (CMC 1.5% *p.o.*), (<sup>a</sup> $P \leq 0.0001$ ; <sup>c</sup> $P \leq 0.01$ ; <sup>d</sup> $P \leq 0.05$ ; <sup>e</sup> $P \geq 0.05$ ) vs positive control group (Ranitidine 5 mg/kg *P.O.*).

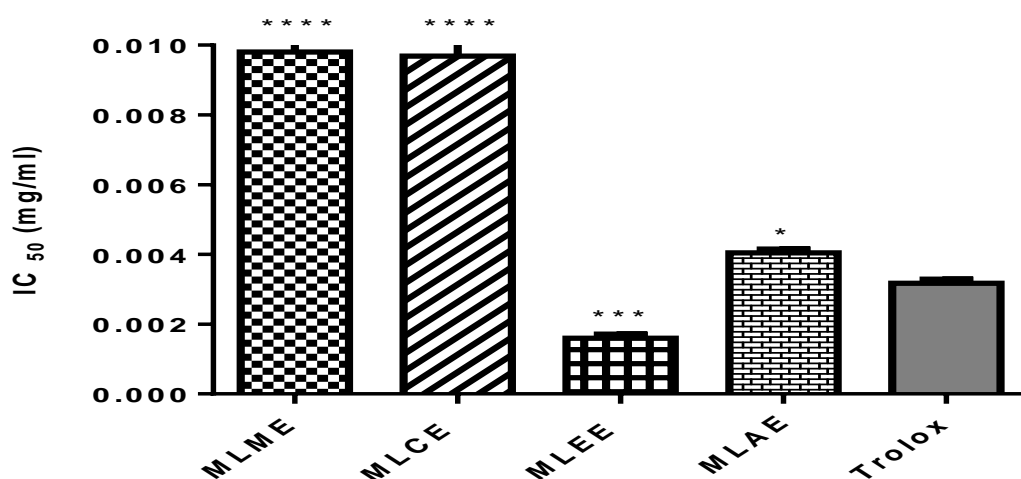
## 2.8 In vitro antioxidant activities of MLE

### 2.8.1 ABTS radical scavenging activity of MLE

The ability of MLE to scavenge the radical ABTS are shown in figure 39. All extracts exhibited high antioxidant activity and in the following order: MLEE ( $IC_{50} = 0.0015$  mg/ml) >



MLAE ( $IC_{50} = 0.004$  mg/ml) > MLCE ( $IC_{50} = 0.0096$  mg/ml) > MLME ( $IC_{50} = 0.0098$  mg/ml).

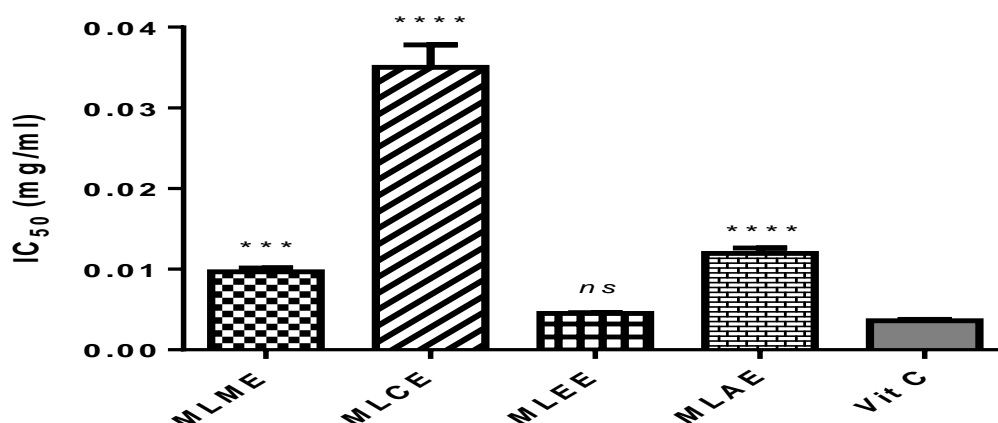


**Figure 39:** ABTS radical scavenging activity of MLE. MLME: *M. communis* L. leaves methanol extract, MLCE: *M. communis* L. leaves chloroform extract, MLEE: *M. communis* L. leaves ethyl acetate extract; MLAE: *M. communis* L. leaves aqueous extract. Data were presented as  $IC_{50}$  means  $\pm$  SD (n=3) (\*\*\*\* $P \leq 0.0001$ ; \*\*\* $P \leq 0.001$ ; \* $P \leq 0.01$ ; ns: not significant) vs Trolox as standard.

### 2.8.2 DPPH radical scavenging activity of MLE

The scavenging ability of the extracts was expressed as  $IC_{50}$  value (the concentration of substrate that causes 50 % loss of DPPH activity). Low  $IC_{50}$  values indicate strong ability of the extracts to act as DPPH scavenger.

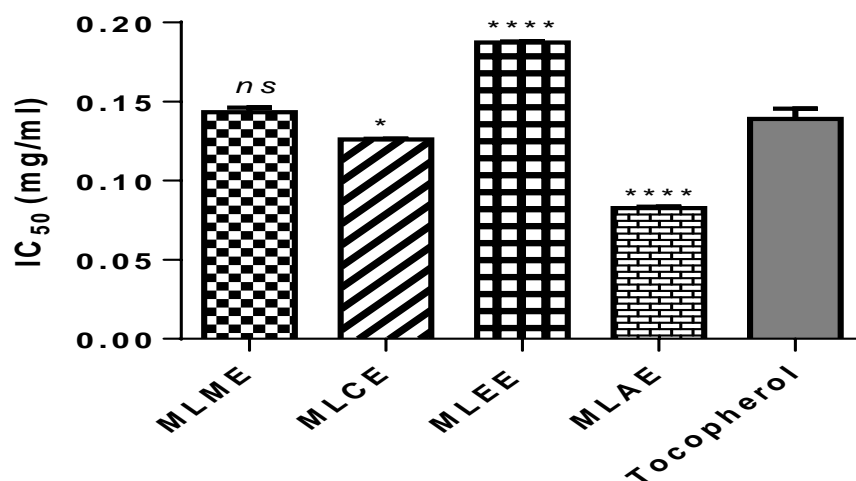
The results show that MLEE extracts exhibited the highest antioxidant activity (close to vit C as standard), followed by MLME ( $IC_{50} = 0.009$  mg/ml), MLAE ( $IC_{50} = 0.011$  mg/ml) then MLCE ( $IC_{50} = 0.035$  mg/ml). The  $IC_{50}$  for Vit C was 0.003 mg/ml (Figure 40).



**Figure 40:** DPPH radical scavenging activity of MLE. MLME: *M. communis* L. leaves methanol extract, MLCE: *M. communis* L. leaves chloroform extract, MLEE: *M. communis* L. leaves ethyl acetate extract; MLAE: *M. communis* L. leaves aqueous extract. Data were presented as IC<sub>50</sub> means  $\pm$  SD (n=3). (\*\*\*\* $P \leq 0.0001$ ; \*\*\* $P \leq 0.001$ ; ns: not significant) vs vitamin C as standard.

### 2.8.3 Hydroxyl radical scavenging activity of MLE

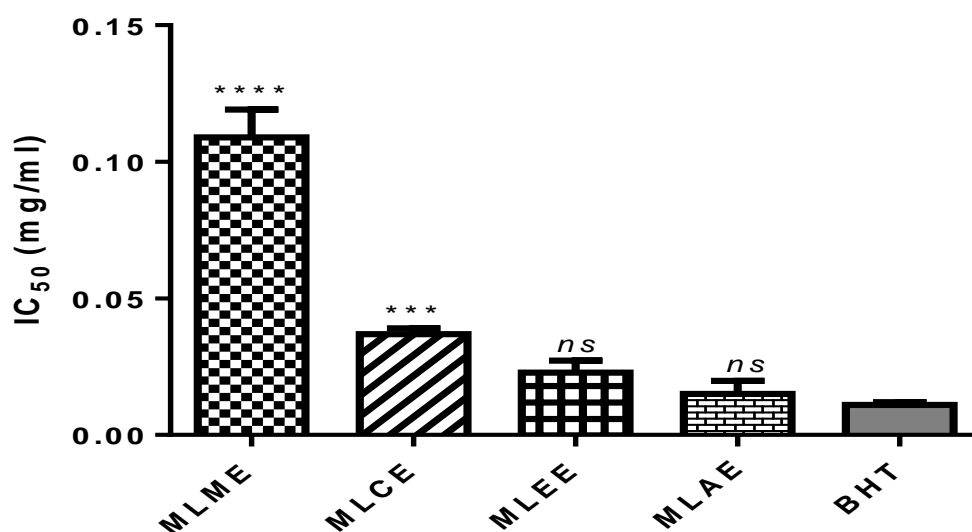
Among the oxygen radicals, hydroxyl radical is the most reactive and induces severe damage to adjacent biomolecules (Sakanaka *et al.*, 2005). The results showed that hydroxyl radical activity of MLAE showed better activity (IC<sub>50</sub> =  $0.08 \pm 0.01$  mg/ml) than the standard tocopherol (IC<sub>50</sub> =  $0.13 \pm 0.02$  mg/ml), followed by MLCE (IC<sub>50</sub> =  $0.12$ ,  $P \leq 0.01$  mg/ml) and MLME (IC<sub>50</sub> =  $0.14$  mg/ml,  $P \leq 0.05$ ). However MLEE showed the lowest activity (IC<sub>50</sub> =  $0.18$ ,  $P \leq 0.0001$ ) compared with tocopherol (Figure 41).



**Figure 41:** Hydroxyl radical scavenging activity of MLE. MLME: *M. communis* L. leaves methanol extract, MLCE: *M. communis* L. leaves chloroform extract, MLEE: *M. communis* L. leaves ethyl acetate extract MLAE: *M. communis* L. leaves aqueous extract. Data were presented as IC<sub>50</sub> means  $\pm$  SD (n=3). (\*\*\*\* $P \leq 0.0001$ ; (\* $P \leq 0.01$ ; ns: not significant) vs Tocopherol as standard.

#### 2.8.4 Hydrogen peroxide scavenging activity of MLE

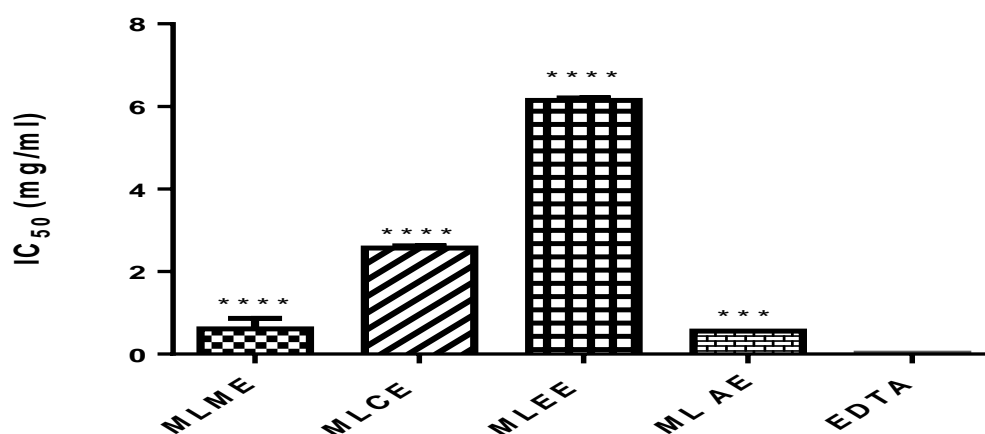
The scavenging effect of the extracts on hydrogen peroxide decreased in the following order: BHT > MLAE > MLEE > MLCE > MLME and their IC<sub>50</sub> values were found to be: 0.011; 0.015; 0.023; 0.037 and 0.109 mg/ml, respectively (Figure 42).



**Figure 42:** Hydrogen peroxide scavenging activity of MLE. MLME: *M. communis* L. leaves methanol extract, MLCE: *M. communis* L. leaves chloroform extract, MLEE: *M. communis* L. leaves ethyl acetate extract, MLAE: *M. communis* L. leaves aqueous extract. Data were presented as IC<sub>50</sub> means  $\pm$  SD (n=3). (\*\*\*\* $P \leq 0.0001$ ; \*\*\* $P \leq 0.001$ ; ns: not significant) vs BHT as standard.

### 2.8.5 Ferrous ion chelating activity of MLE

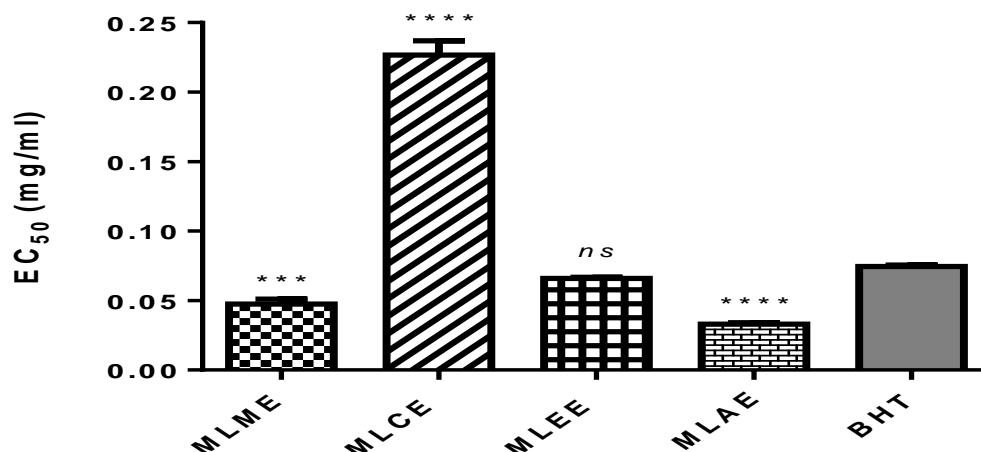
All the extracts demonstrated an ability to chelate ferric iron (II) ions. The chelating abilities on ferrous ions were in descending order: MLAE (IC<sub>50</sub> = 0.5  $\pm$  00 mg/ml) > MLME (IC<sub>50</sub> = 0.61  $\pm$  0.25 mg/ml) > MLCE (IC<sub>50</sub> = 2.56  $\pm$  0.05 mg/ml) > MLEE (IC<sub>50</sub> = 6.14  $\pm$  0.058 mg/ml). None of the extracts appeared to be better chelators of ferric iron (II) ions than the positive control EDTA (IC<sub>50</sub> = 0.02  $\pm$  00 mg/ml) in this assay system (Figure 43).



**Figure 43:** Ferrous ion chelating activity of MLE. MLME: *M. communis* L. leaves methanol extract, MLCE: *M. communis* L. leaves chloroform extract, MLEE: *M. communis* L. leaves ethyl acetate extract, MLAE: *M. communis* L. leaves aqueous extract. Data were presented as IC<sub>50</sub> means  $\pm$  SD (n=3) (\*\*\*\* $P \leq 0.0001$ ; (\*\*\*) $P \leq 0.001$ ; ns: not significant) vs EDTA as standard.

#### 2.8.6 Reducing power capacity of MLE

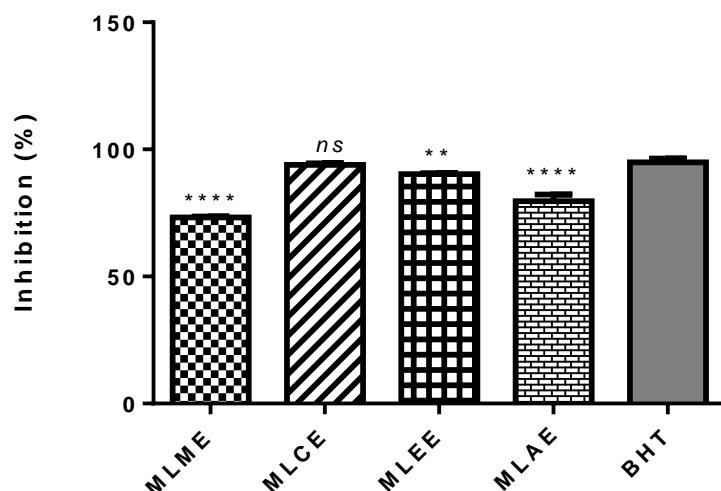
From the results (Figure 44), we can see that the best reducing power (the effective concentration at which the absorbance was 0.5) was for MLAE ( $EC_{50} = 0.033 \pm 00$  mg/ml) and MLME ( $EC_{50} = 0.047 \pm 00$  mg/ml) which were stronger than BHT ( $EC_{50} = 0.074 \pm 00$  mg/ml) as positive standard. MLEE showed comparable effect ( $EC_{50} = 0.065 \pm 00$  mg/ml) as BHT, whereas, the reducing power of MLCE was significantly ( $P \leq 0.0001$ ) lower ( $EC_{50} = 0.226 \pm 0, 01$  mg/ml) than BHT.



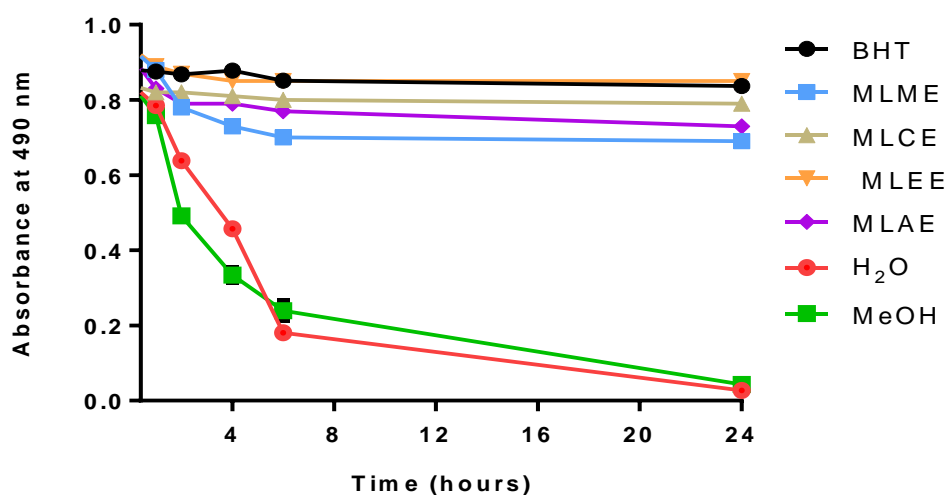
**Figure 44:** Reducing power activity of MLE. MLME: *M. communis* L. leaves methanol extract, MLCE: *M. communis* L. leaves chloroform extract, MLEE: *M. communis* L. leaves ethyl acetate extract, MLAE: *M. communis* L. leaves aqueous extract. Data were presented as IC<sub>50</sub> means  $\pm$  SD (n=3) (\*\*\*\* $P \leq 0.0001$ ; ns: not significant) vs BHT as standard.

#### 2.8.7 Antioxidant activity of MLE determined by $\beta$ -carotene /linoleic acid bleaching assay

Figure (45) shows the effect of MLE on the changes in the percentage of the inhibition ratio of linoleic acid oxidation compared to BHT as positive control during 24 h. The addition of the plant extracts and BHT at 2 mg/ml was markedly effective in inhibiting the oxidation of linoleic acid and subsequent bleaching of  $\beta$ -carotene, in comparison with the negative control which contained no antioxidant component. MLCE showed the highest antioxidant activity ( $93.95 \pm 0.53$  %) compared to BHT ( $94.9 \pm 1.52$  %) followed by MLEE ( $90.29 \pm 0.42$  %), MLAE ( $79.58 \pm 2.67$  %) and MLME ( $73.24 \pm 1.52$  %). Figure 46 shows the decrease in absorbance of  $\beta$ -carotene in the presence of 2 mg/ml extract or reference antioxidant (BHT) compared with the negative controls (MeOH and H<sub>2</sub>O).



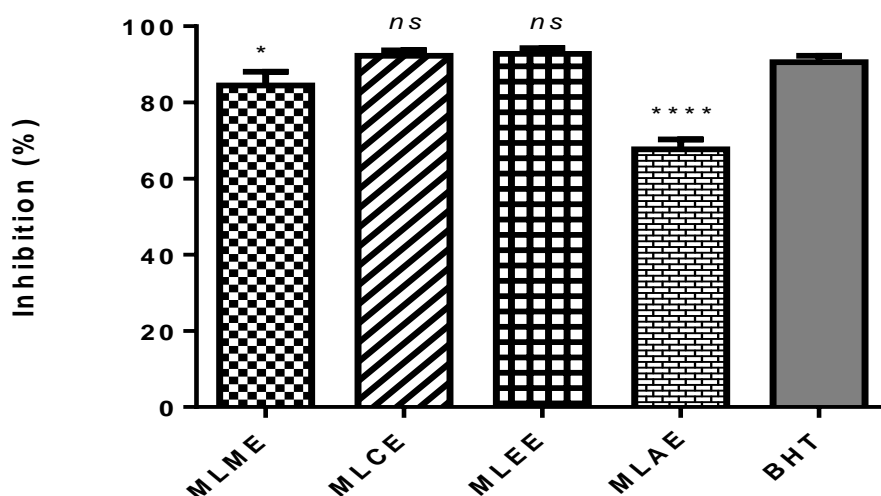
**Figure 45:** Antioxidant activity of MLE (2 mg/ml) using  $\beta$ -carotene /linoleic acid bleaching assay after 24 h. MLME: *M. communis* L. leaves methanol extract, MLCE: *M. communis* L. leaves chloroform extract, MLEE: *M. communis* L. leaves ethyl acetate extract, MLAE: *M. communis* L. leaves aqueous extract. Data were presented as means  $\pm$  SD (n=3) (\*\*\*\* $P \leq 0.0001$ ; \*\* $P \leq 0.01$ ; ns: not significant) vs BHT as standard.



**Figure 46:** shows the decrease in absorbance of  $\beta$ -carotene in the presence of 2 mg/ml of different MLE extracts or reference antioxidant (BHT) compared with MeOH and H<sub>2</sub>O as negative controls.

### 2.8.8 Antioxidant activity of MLE determined by FTC assay

As shown in figure 47, the different extracts showed good antioxidant potential with percent inhibition ranging from ( $67.65 \pm 2.59$  %) to ( $92.77 \pm 1.44$  %) as compared with BHT as positive control. The results indicated that both MLEE and MLCE exerted marked effects on inhibition of linoleic acid oxidation, which were as strong ( $92.77 \pm 1.77$  and  $92.22 \pm 1.42$ , respectively) as BHT ( $90.56 \pm 1.7$  %) ( $P \geq 0.05$ ).

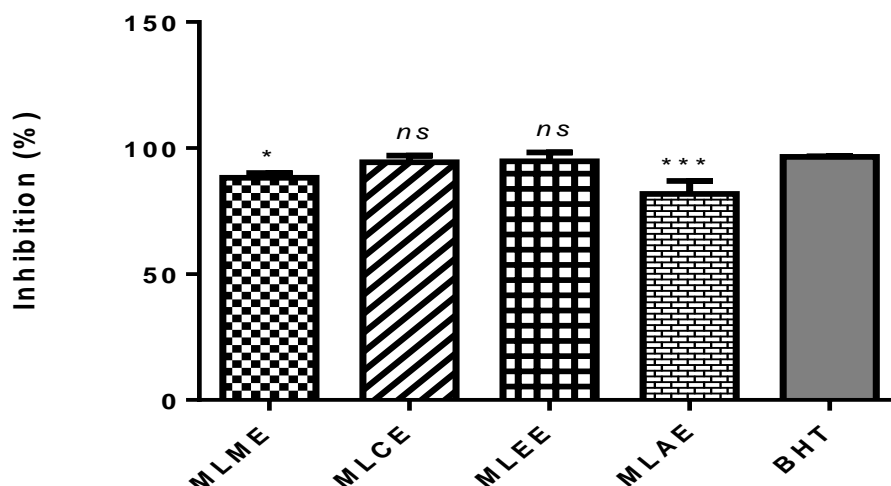


**Figure 47:** Antioxidant activity of MLE (2 mg/ml at 96 h of incubation) measured by FTC method. MLME: *M. communis* L. leaves methanol extract, MLCE: *M. communis* L. leaves chloroform extract, MLEE: *M. communis* L. leaves ethyl acetate extract, MLAE: *M. communis* L. leaves aqueous extract. Data were presented as means  $\pm$  SD (n=3). (\*\*\*\* $P \leq 0.0001$ ; \* $P \leq 0.05$ ; ns: not significant) vs BHT as standard.

### 2.8.9 Antioxidant activity of MLE determined by thiobarbutiric acid assay (TBA)

As shown in figure 48, the plant extracts inhibited MDA formation in the following order: BHT ( $96.51 \pm 1.7$  %) > MLEE ( $94.77 \pm 3.52$  %) > MLCE ( $94.31 \pm 2.73$  %) > MLME ( $88.18 \pm 1.86$  %) > MLAE ( $81.81 \pm 5.08$  %). The percentage of inhibition exhibited by MLEE and MLCE was comparable to the positive control (BHT).

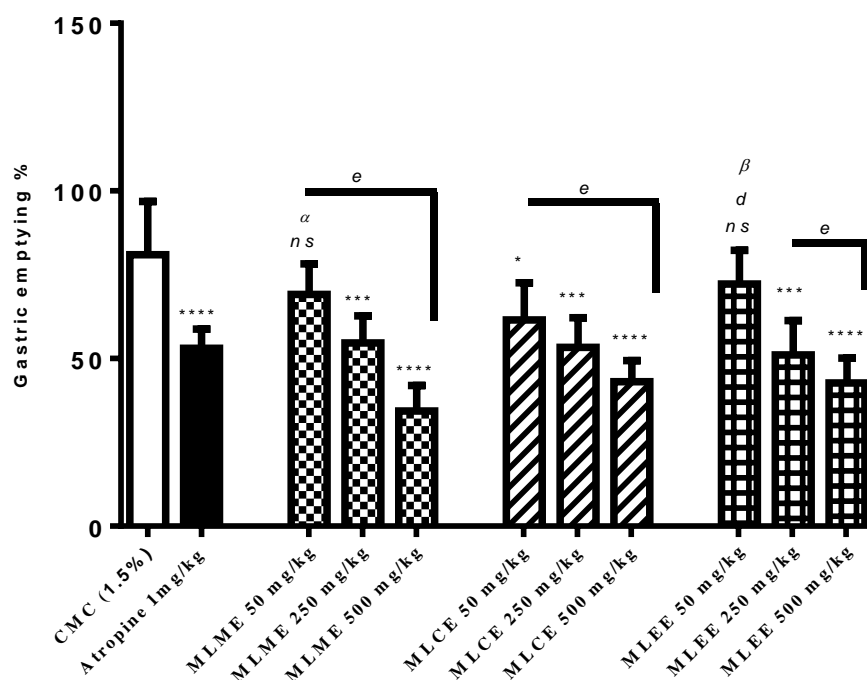




**Figure 48:** Antioxidant activity of MLE (2 mg/ml at 96 h of incubation) measured by TBA assay. MLME: *M. communis* L. leaves methanol extract, MLCE: *M. communis* L. leaves chloroform extract, MLEE: *M. communis* L. leaves ethyl acetate extract, MLAE: *M. communis* L. leaves aqueous extract. Data were presented as means  $\pm$  SD (n=3). (\*\*\* $P \leq 0.001$ ; \* $P \leq 0.05$ ; ns: not significant) vs BHT as standard.

## 2.9 Effect of MLE on gastric emptying in mice

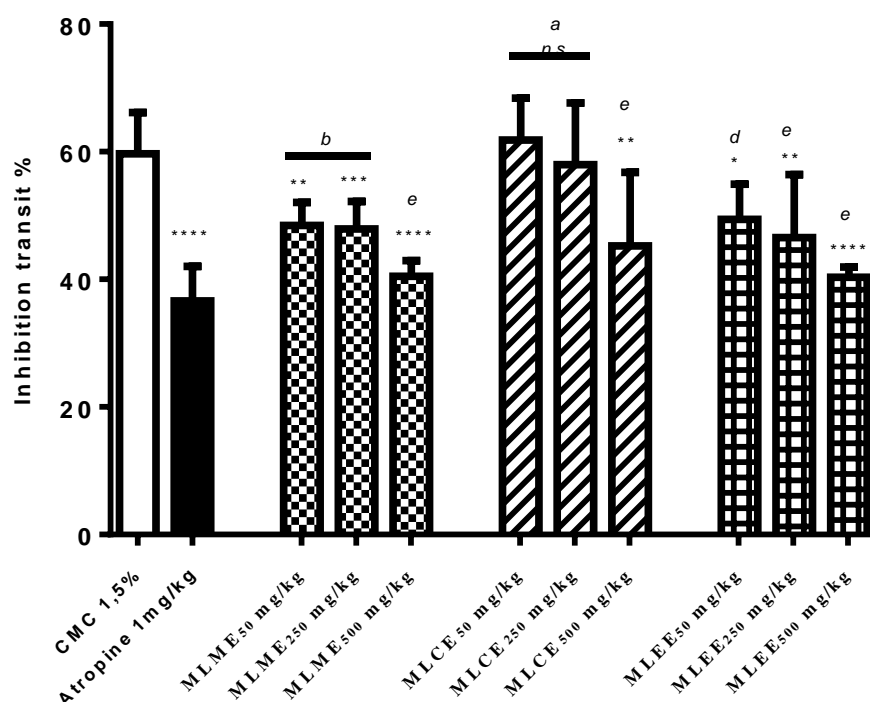
All extracts exerted dose dependent reduction in the emptied quantity of the test meal compared to the vehicle (negative control). This effect was significant ( $P \leq 0.01$  and  $P \leq 0.001$ ) for the highest doses (250 and 500 mg/kg) and the effect of these doses was not significantly different from that of atropine (Figure 49).



**Figure 49:** Effect of MLE on gastric emptying in mice. MLME: *M. communis* L. leaves methanol extract, MLCE: *M. communis* L. leaves chloroform extract, MLEE: *M. communis* L. leaves ethyl acetate extract. The values of the bars chart are expressed as means  $\pm$  SEM (n=6). (\*\*\*\* $P \leq 0.0001$ ; \*\*\* $P \leq 0.001$ ; \*\* $P \leq 0.01$ ) vs vehicle (CMC 1.5% *p.o.*). (<sup>a</sup> $P \leq 0.0001$ , <sup>b</sup> $P \leq 0.001$ , <sup>e</sup> $P \geq 0.05$ ) vs positive control group (Atropine sulphate, 1 mg/kg *i.p.*). <sup>a</sup> $P \leq 0.0001$  vs MLME<sub>500</sub> mg/kg *p.o.*;  <sup>$\beta$</sup>  $P \leq 0.05$  vs MLCE<sub>500</sub> mg/kg *p.o.*

## 2.10 Effect of MLE on intestinal transit in mice

The effects of MLE on intestinal transit are shown in figure 50. Compared with the vehicle, all tested extracts lowered the transit of phenol red through the small intestine. This decrease was significant at the highest dose for all extracts. At this dose, all extracts showed no significant difference in intestinal transit compared to the positive control.



**Figure 50:** Effect of MLE on intestinal transit in mice. MLME: *M. communis* L. leaves methanol extract, MLCE: *M. L.* leaves chloroform extract, MLEE: *M. communis* L. leaves ethyl acetate extract. The values of the bars chart are expressed as means  $\pm$  SEM (n=6). (\*\*\*\* $P \leq 0.0001$ ; \*\*\* $P \leq 0.001$ ; \*\* $P \leq 0.01$ , \* $P \leq 0.05$ ) vs vehicle (CMC 1.5% p.o.). (<sup>a</sup> $P \leq 0.0001$ ; <sup>b</sup> $P \leq 0.001$ <sup>d</sup>,  $P \leq 0.05$ , <sup>e</sup> $P \geq 0.05$ ) vs positive control group (Atropine sulphate, 1 mg/kg i.p.).

### 2.11 Effect of MLE on castor oil-induced diarrhoea in mice

Within the observation period of 4 hours, after castor oil administration, all the mice in control group produced copious diarrhoea. Oral pretreatment of mice with the extracts caused dose dependent and significant delay of the onset of diarrhoea. This effect decreased in the following order: MLME > MLCE > MLEE. The most powerful delay was observed for ME extract at 500 mg/kg. At this dose, the onset of diarrhoea increased from  $59.29 \pm 5.54$  min (vehicle group) to  $215.71 \pm 8.95$  min, a value not significantly different from the positive control ( $214.38 \pm 8.7$  min). In addition, the total number of stool and the total number of wet stool were reduced significantly and dose dependently. The most significant decrease was noted with the highest dose (Table 9). Furthermore, the inhibition of defecation increased in a dose dependent manner with the most remarkable percentage of inhibition at the highest dose for all extracts: MLME ( $73.53 \pm 5.75$ ), MLCE ( $72.53 \pm 3.24$ ) and MLEE ( $71.98 \pm 4.67$ ).

These values were not significantly different from the value of the positive control ( $81.59 \pm 5.14$ ).

**Table 9:** Effect of MLE on castor oil-induced diarrhoea in mice.

Treatment Group	Dose (mg/kg) or (ml/kg)	Onset of diarrhoea (min)	Total number of stool	Number of wet stool	Percentage of wet stool (%)	Protection(%)
Vehicule (CMC 1.5%)	10	$59.29 \pm 5.54$	$10.94 \pm 1.08$	$9.17 \pm 0.89$	$85.36 \pm 3.08$	00
MLME	50	$98.25 \pm 9.03^{***a\alpha}$	$7.25 \pm 0.86^{***b}$	$5.5 \pm 0.86^{**b}$	$73.15 \pm 7.86^d$	$33.06 \pm 7.3^{a\pi}$
	250	$119.28 \pm 8.13^{****a}$	$7 \pm 1.1^{***c}$	$4.14 \pm 0.45^{****}$	$65.08 \pm 9.04$	$54.85 \pm 5^d$
	500	$215.71 \pm 8.95^{****}$	$5.42 \pm 0.68^{****}$	$2.42 \pm 0.52^{****}$	$46.33 \pm 8.7^{**}$	$73.53 \pm 5.75$
MLCE	50	$100.5 \pm 8.36^{****a\beta\delta}$	$8.12 \pm 0.95^{**a}$	$5.87 \pm 0.58^{**b\mu}$	$74.19 \pm 4.91^d$	$35.98 \pm 6.32^{a\#f}$
	250	$143.28 \pm 8.93^{****a}$	$4.85 \pm 0.45^{****}$	$3 \pm 0.43^{****}$	$62.11 \pm 6.65$	$67.31 \pm 4.75$
	500	$195 \pm 3.61^{****}$	$4.71 \pm 0.56^{****}$	$2.42 \pm 0.29^{****}$	$51.84 \pm 4.31^*$	$72.53 \pm 3.24$
MLEE	50	$110 \pm 3.13^{****a\epsilon}$	$8.12 \pm 0.51^{**a}$	$5.75 \pm 0.52^{**b}$	$70.56 \pm 5.73$	$37.34 \pm 5.73^a$
	250	$137.14 \pm 2.64^{***a}$	$5.42 \pm 1.39^{****}$	$3.57 \pm 0.89^{****}$	$64.46 \pm 4.96$	$67.31 \pm 8.89^{s\&}$
	500	$179.28 \pm 6.21^{****d}$	$4.83 \pm 0.57^{****}$	$2.57 \pm 0.42^{****}$	$53.27 \pm 6.87$	$71.98 \pm 4.67$
Loperamide	5	$214.38 \pm 8.73^{****}$	$2.63 \pm 0.7^{****}$	$1.42 \pm 0.45^{****}$	$41.77 \pm 09.78^{****}$	$81.59 \pm 5.14$

Animals were pre-treated with various doses of MLME, MLCE and MLEE (50, 250 and 500 mg/kg, p.o.), reference drug (loperamide, 5 mg/kg, p.o.) or vehicle (CMC 1.5%). One hour later, animals received castor oil (10 ml/kg p.o.). \* $P \leq 0.05$ ; \*\* $P \leq 0.01$ ; \*\*\* $P \leq 0.001$ ; \*\*\*\* $P \leq 0.0001$ ; vs negative control group. <sup>d</sup>  $P \leq 0.05$ ; <sup>c</sup>  $P \leq 0.01$ ; <sup>b</sup>  $P \leq 0.001$ ; <sup>a</sup>  $P \leq 0.0001$  vs positive control group; <sup>a</sup>  $P \leq 0.0001$  vs MLME<sub>500</sub> mg/kg; <sup>β</sup>  $P \leq 0.01$  vs MLCE<sub>250</sub> mg/kg; <sup>δ</sup>  $P \leq 0.0001$  vs MLCE<sub>500</sub> mg/kg; <sup>ε</sup>  $P \leq 0.0001$  vs MLEE<sub>500</sub> mg/kg; <sup>μ</sup>  $P \leq 0.05$  vs MLCE<sub>500</sub> mg/kg; <sup>π</sup>  $P \leq 0.001$  vs MLME<sub>500</sub> mg/kg; <sup>#</sup>  $P \leq 0.05$  vs MLCE<sub>250</sub> mg/kg; <sup>£</sup>  $P \leq 0.01$  vs MLCE<sub>500</sub> mg/kg; <sup>\$</sup>  $P \leq 0.05$  vs MLEE<sub>250</sub> mg/kg; (One way ANOVA followed by Tukey's multiple comparison test). MLME: *M. communis* L. leaves methanol extract, MLCE: *M. communis* L. leaves chloroform extract, MLEE: *M. communis* L. leaves ethyl acetate extract.

## 2.12 Effect of MLE on castor oil-induced intestinal fluid accumulation in mice

The intestinal fluid accumulation test was induced by castor oil. Pre-treatment of the test group's animals dose dependently and significantly inhibited the volume and the mass of

intestinal content compared to the vehicle (Table 10). The percent inhibition of mass intestinal content with both MLME and MLCE extracts was not significantly different from Loperamide group at the highest dose, indicating the efficacy of these extracts.

**Table 10:** Effect of MLE on castor oil-induced intestinal fluid accumulation in mice

Treatment group	Dose (mg/kg) or (ml/kg)	Volume of intestinal fluid (ml)	Mass of intestinal fluid (g)	Inhibition of intestinal fluid volume (ml) %	Inhibition of intestinal mass (g) %
Vehicle (CMC 1.5%)	5	0.72±0.04 <sup>a</sup>	0.86±0.1 <sup>a</sup>	00	00
MLME	50	0.56±0.024 <sup>e</sup>	0.62±0.03 <sup>*a</sup>	22.22±3.4 <sup>a</sup>	27.95±3.8 <sup>a</sup>
	250	0.48±0.02 <sup>***e</sup>	0.56±0.027 <sup>****a</sup>	32.53±3.62 <sup>a</sup>	34.63±3.19 <sup>a</sup>
	500	0.42±0.02 <sup>****e</sup>	0.49±0.06 <sup>****d</sup>	41.66±2.77 <sup>d</sup>	47.71±5.17 <sup>e</sup>
MLCE	50	0.55±0.034 <sup>*e</sup>	0.6±0.03 <sup>**a</sup>	23.61±4.74 <sup>a</sup>	29.25±3.5 <sup>a</sup>
	250	0.51±0.03 <sup>d**e</sup>	0.58±0.03 <sup>**a</sup>	25.24±4.26 <sup>a</sup>	32.65±4.39 <sup>a</sup>
	500	0.45±0.03 <sup>****e</sup>	0.45±0.026 <sup>****e</sup>	37.5±4.26 <sup>c</sup>	45.91±2.49 <sup>e</sup>
MLEE	50	0.56±0.033 <sup>e</sup>	0.59±0.034 <sup>**a</sup>	21.29±4.62 <sup>a α</sup>	31.18±4 <sup>a</sup>
	250	0.48±0.016 <sup>***e</sup>	0.49±0.03 <sup>****d</sup>	32.5±2.31 <sup>a</sup>	42.71±4.26 <sup>c</sup>
	500	0.4±0.016 <sup>****e</sup>	0.43±0.02 <sup>****e</sup>	44.44±2.31 <sup>c</sup>	46.91±2.49 <sup>d</sup>
Loperamide	5	0.41±0.03 <sup>****</sup>	0.3±0.030 <sup>****</sup>	40.97±4.29	61.98±2.76

Animals were pre-treated with various doses of MLME, MLCE and MLEE (50, 250 and 500 mg/kg, *p.o.*), reference drug (loperamide, 5 mg/kg, *p.o.*) or vehicle (CMC 1.5%). One hour later, animals received castor oil (10 ml/kg). \* $P \leq 0.05$ , \*\* $P \leq 0.01$ , \*\*\* $P \leq 0.001$ , \*\*\*\* $P \leq 0.0001$  vs vehicle group (CMC). <sup>e</sup> $P \geq 0.05$ , <sup>d</sup> $P \leq 0.05$ , <sup>c</sup> $P \leq 0.01$  vs, <sup>b</sup> $P \leq 0.001$ , <sup>a</sup> $P \leq 0.0001$  vs positive group (Loperamide); <sup>α</sup> $P \leq 0.05$  vs MLEE<sub>500</sub> (One way ANOVA followed by Tukey's multiple comparison test). MLME: *M. communis* L. leaves methanolic extract, MLCE: *M. communis* L. leaves chloroform extract, MLEE: *M. communis* L. leaves ethyl acetate extract.

### 3 Anti-inflammatory and antioxidant activities of *M. communis* L. berries methanolic extract (MBME).

#### 3.1 Total phenolics, flavonoids and tannins contents in MBME

The total phenolics, flavonoids and tannins contents of MBME extract are presented in Table 11. The total phenolic content expressed in mg gallic acid equivalents (GAE) / g of dry extract was  $65.55 \pm 3$ . Total flavonoids content in terms of mg quercetin equivalents (QE) / g of dry extract was  $30.13 \pm 1.4$ , and the total tannins content expressed in mg tannic acid equivalents (TAE) / g of dry extract was  $55.91 \pm 3.6$ .

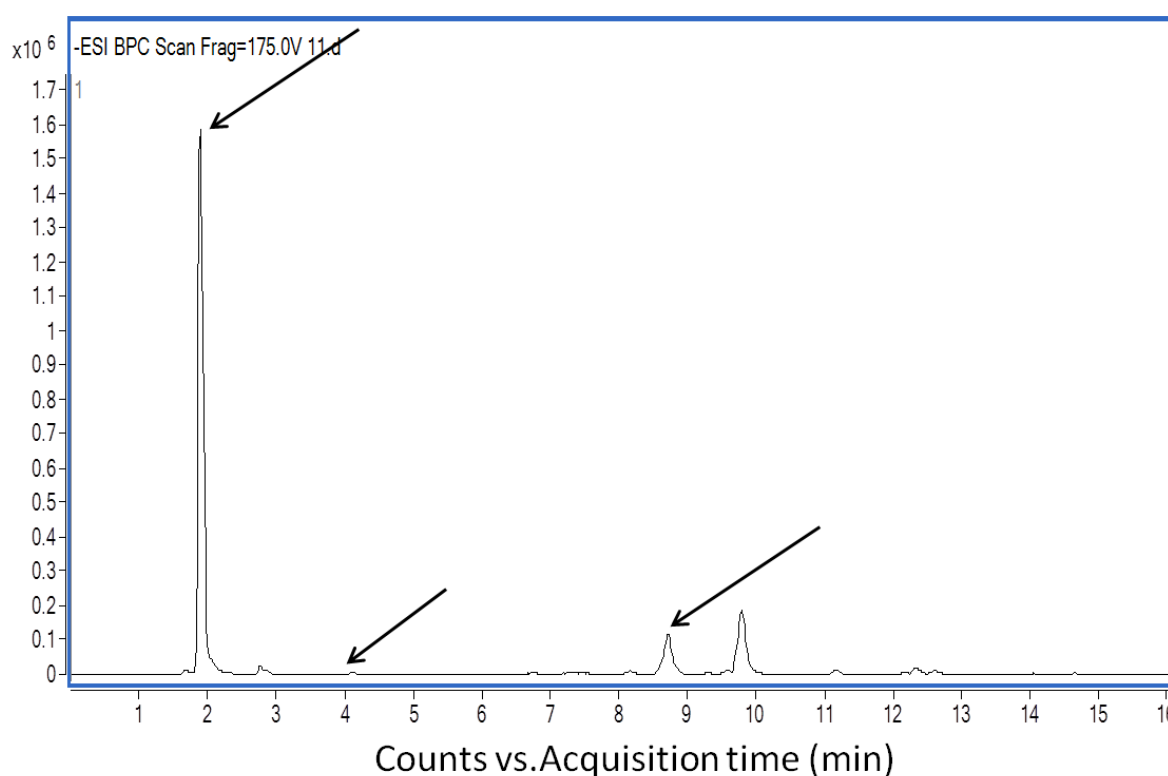
**Table 11:** Total phenolics, flavonoids and tannins contents of MBME

Extracts	Total phenolics (mg GAE/g Dw)	Total flavonoids (mg QE/g DW)	Total tannins (mg TAE/g DW)
MBME	$65.55 \pm 3.75$	$30.13 \pm 1.4$	$55.91 \pm 3.6$

MBME: *M. communis* L. berries methanolic extract, DW: Dry weight. Results are expressed as means  $\pm$  SEM (n=3).

#### 3.2 HPLC-MS analysis of MBME

Based on the dry powdered plant material, the yield of the methanolic extract from *M. communis* L. berries was 26 %. The results of HPLC analysis are shown in table 12. A total of 3 compounds (1-3) were identified as: Gallic acid, gentisic acid and vanilic acid, the peaks numbers correspond to the names of compounds in table 12.



**Figure 51:** HPLC-MS representative chromatogram of phenolics from MBME.

**Table 12:** HPLC-MS analysis of MBME.

Peak N <sup>o</sup>	RT (min)	Proposed metabolite	Concentration (mg/kg)	Mass error (ppb)	Molecular formula
1	2.84	Gallic acid	167.54	322.21	C <sub>7</sub> H <sub>6</sub> O <sub>5</sub>
2	4.39	Gentisic acid	5.62	10.81	C <sub>7</sub> H <sub>6</sub> O <sub>4</sub>
3	8.01	Vanilic acid	2.89	5.57	C <sub>8</sub> H <sub>8</sub> O <sub>4</sub>

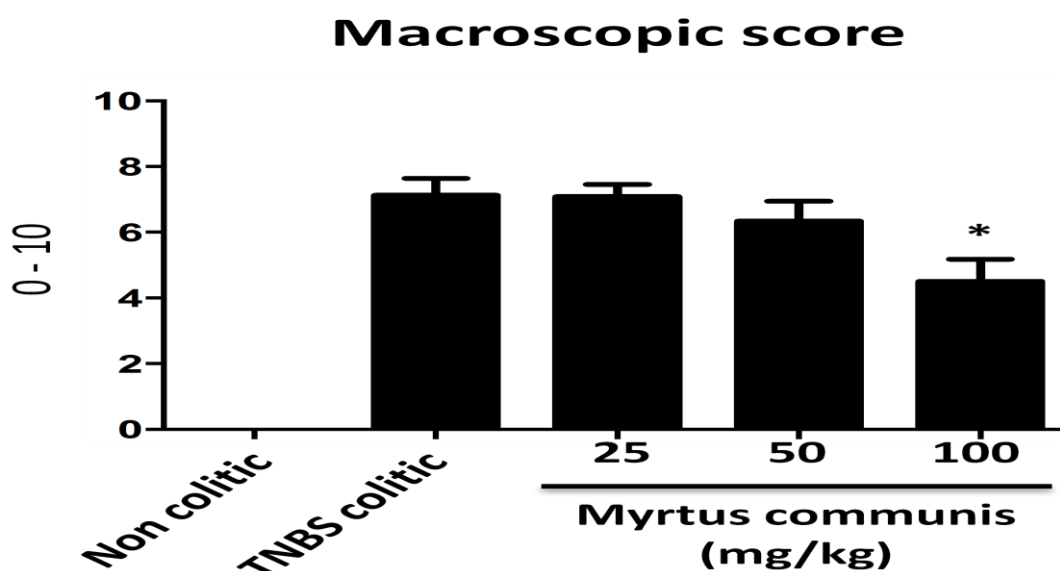
### 3.3 Acute oral toxicity of MBME

In acute toxicity test, the oral administration to the rats of a single dose 2000 or 5000 mg/kg prepared from MBME did not cause any mortality for the following 14 days of observation and none of the animals showed any changes in their behavioral, neurological or physical activities.

### 3.4 Intestinal anti-inflammatory effect of MBME on TNBS-induced colitis in rats

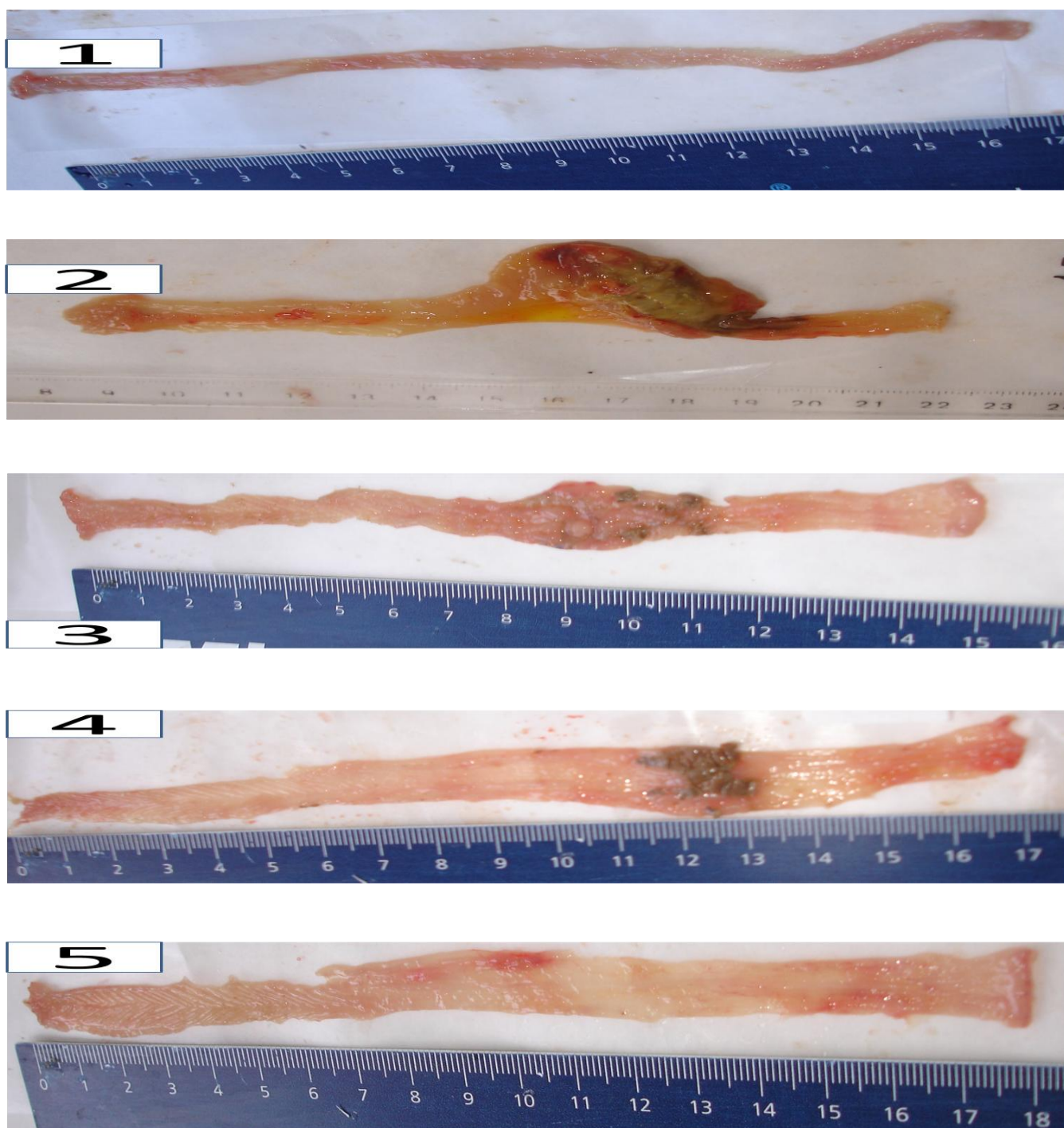
#### 3.4.1 Effect of MBME on macroscopic score

Rats treated with TNBS showed prostration, pilo erection and hypo motility. After sacrifice, macroscopic inspection of the colon showed evidence of severe macroscopic colonic mucosal necrosis, with oedema, deep ulcerations and haemorrhage extending along the colon accompanied by bowel wall thickening, hyperaemia and focal adhesions to adjacent organs, as well as high macroscopic score when compared to control group that showed no mucosal injuries (Figure 52 and 53). In addition, a significant increase in the weight/length of the rat colon (as an indicator of inflammation), was noted (Figure 54). After oral administration of MBME at the doses of 25, 50 mg/kg reduced the previous macroscopic manifestations, but the macroscopic damage score did not reach significant value when compared to the TNBS group (Figures 52 and 53). However, the highest dose (100 mg/kg) markedly attenuated the macroscopic mucosal damage (Figure 53) and significantly ( $P < 0.05$ ) decreased the macroscopic damage score (Figure 52). The inflammatory changes of the intestinal tract were associated with a dose dependent decrease of weight/length of the colon rats (Figure 54). This effect was significant ( $P < 0.05$  vs TNBS colitic group) at the highest dose (100 mg/kg).

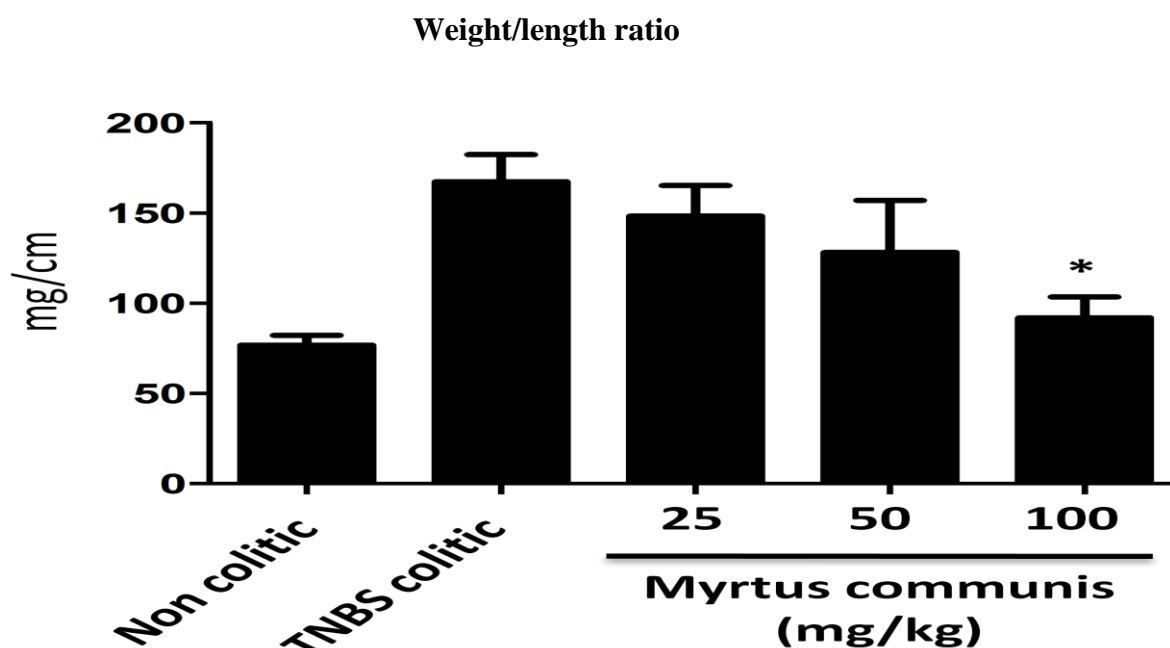


**Figure 52:** Effect of MBME (25, 50 and 100 mg/kg) on colonic macroscopic score in TNBS-induced colitis in rats. Data were expressed as means  $\pm$  SEM (n=6). \* $P < 0.05$  vs TNBS colitic group.





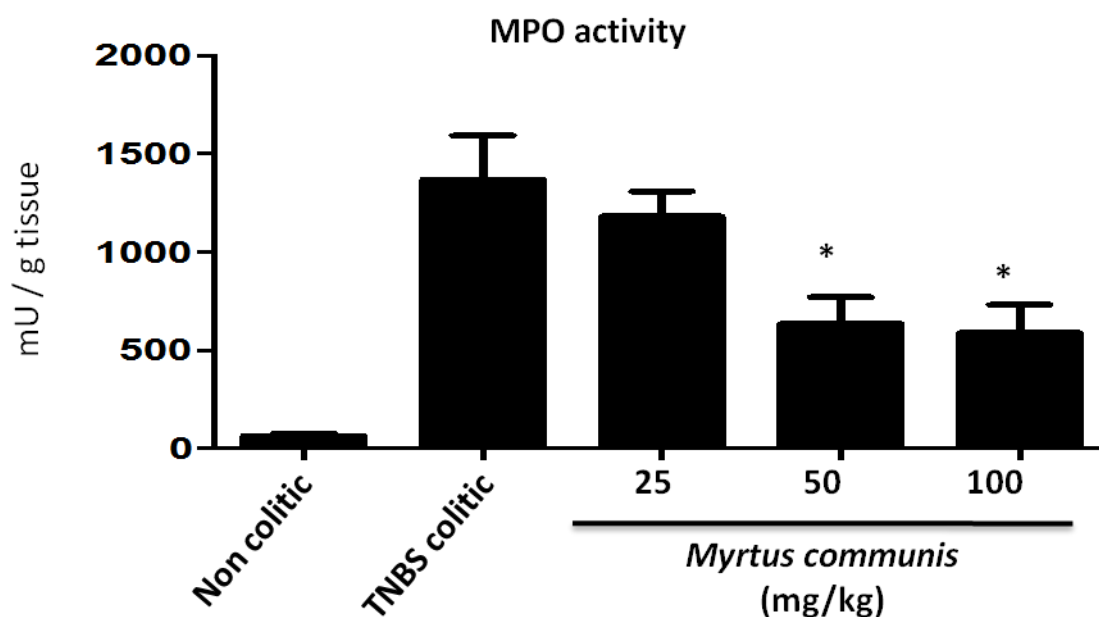
**Figure 53:** Effect of MBME (25, 50 and 100 mg/kg)(3,4,5) on colonic macroscopic appearance in TNBS-induced colitis in rats compared with normal control (1) and TNBS colitic untreated group (2). MBME: *M. communis* L. berries methanolic extract.



**Figure 54:** Effect of MBME (25, 50 and 100 mg/kg) on colonic weight/length ratio in TNBS-induced colitis in rats. Data were expressed as means  $\pm$  SEM (n=6). \* $P < 0.05$  vs TNBS colitic group. MBME: *M. communis* L. berries methanolic extract.

### 3.4.2 Effect of MBME on myeloperoxidase activity (MPO) activity

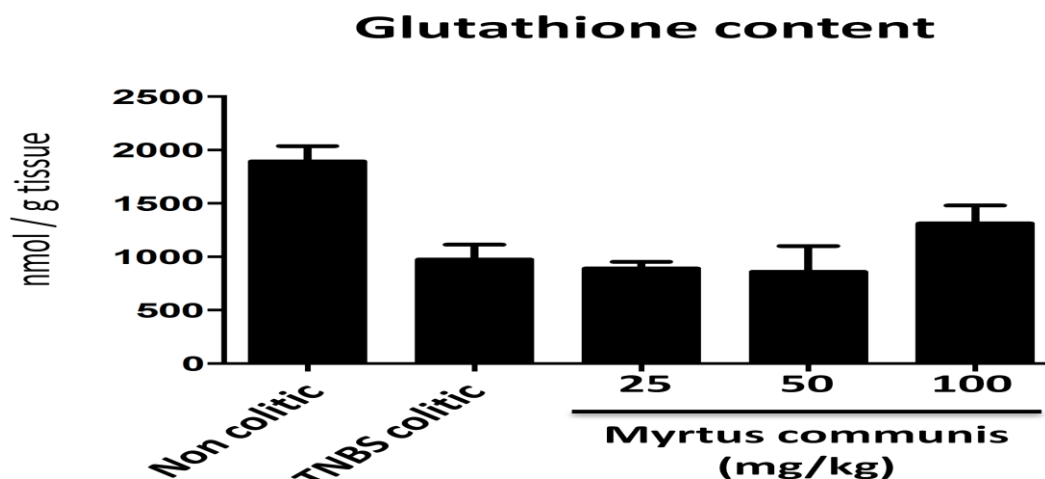
MPO activity is considered to be an index of neutrophil infiltration (MPO is predominantly found in these cells) and it is largely used to quantify intestinal inflammation (Roncucci, *et al.*, 2008). TNBS-induced colitis was associated with significantly increased neutrophil infiltration, as evaluated by MPO (Figure 55). Administration of MBME could significantly (\* $P < 0.05$ ) suppress MPO accumulation in colonic tissue in dose-dependent manner at the highest doses (50 and 100 mg/ kg) compared to TNBS colitic control animals.



**Figure 55:** Effect of MBME (25, 50 and 100 mg/kg) on colonic MPO activity in TNBS-induced colitis in rats. Data were expressed as means  $\pm$  SEM (n=6). \*  $P < 0.05$  vs TNBS colitic group. MBME: *M. communis* L. berries methanolic extract.

### 3.4.3 Effect of MBME on glutathione (GSH) content

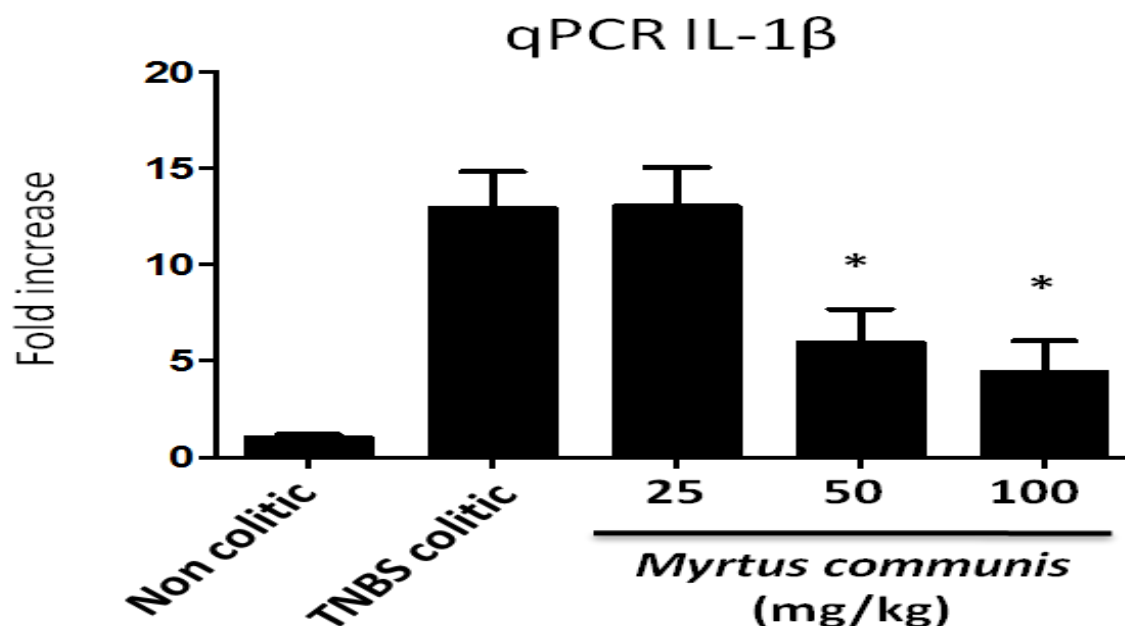
The oral administration of MBME at the doses of 25, 50 mg/kg did not show any important variation in GSH content compared to the TNBS-colitic group, whereas the highest dose (100 mg/kg) brought about an increase in GSH content although it did not reach a significant value (Figure 56).



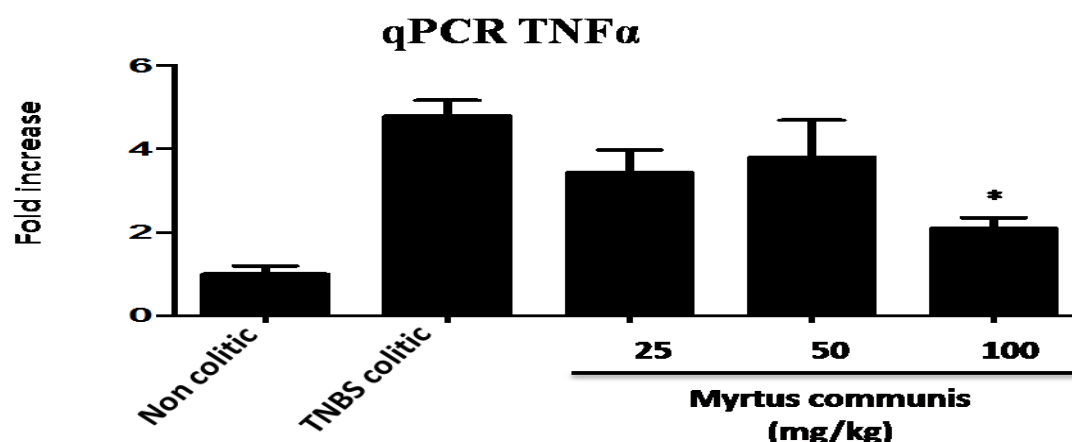
**Figure 56:** Effect of MBME (25, 50 and 100 mg/kg) on colonic GSH content in TNBS-induced colitis in rats. Data were expressed as means  $\pm$  SEM (n=6). \* $P < 0.05$  vs TNBS colitic group. MBME: *M. communis* L. berries methanolic extract.

#### 3.4.4 Effect of MBME on colonic inflammatory biomarkers

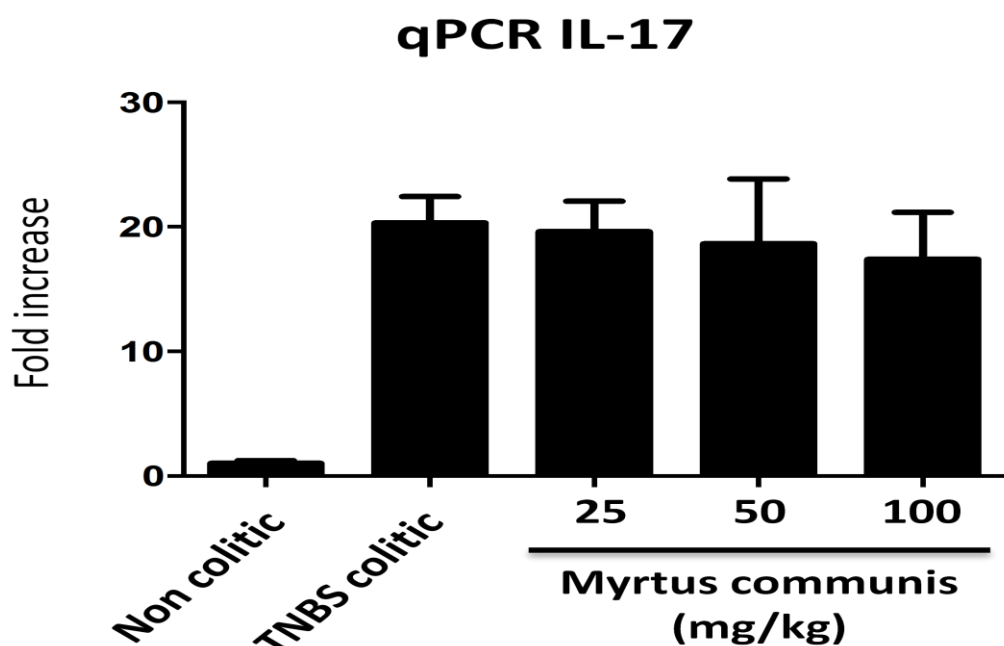
The levels of pro-inflammatory markers (IL-1 $\beta$ , TNF  $\alpha$ , IL-17, iNOS), ICAM-1 and CINC-1 were significantly increased by TNBS (Figure 57-64). Treatment with MBME at the doses of 50 and 100 mg/kg significantly (\* $P < 0.05$  vs TNBS colitic group) decreased the level of IL-1 $\beta$ , iNOS and ICAM-1 (Figures 57, 60 and 61). In addition, the plant extract also significantly (\* $P < 0.05$  vs TNBS colitic group) decreased the level of TNF  $\alpha$  (Figure 58) and CINC-1 (Figure 62) but only at the highest dose (100 mg/kg). IL-17 (Figure 59) and both the mucosal barrier TFF-3 and MUC-2 (Figure 63 and 64, respectively) were not significantly altered.



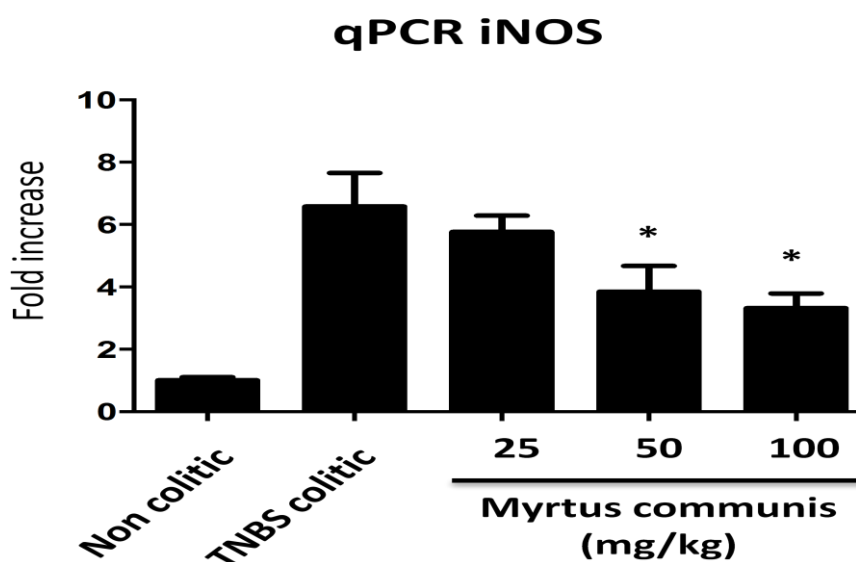
**Figure 57:** Effect of MBME (25, 50 and 100 mg/kg) on colonic inflammatory gene expression of IL-1 $\beta$ , in TNBS-induced colitis in rats. Data were expressed as means  $\pm$  SEM (n=6). \*  $P < 0.05$  vs TNBS colitic group. MBME: *M. communis* L. berries methanolic extract.



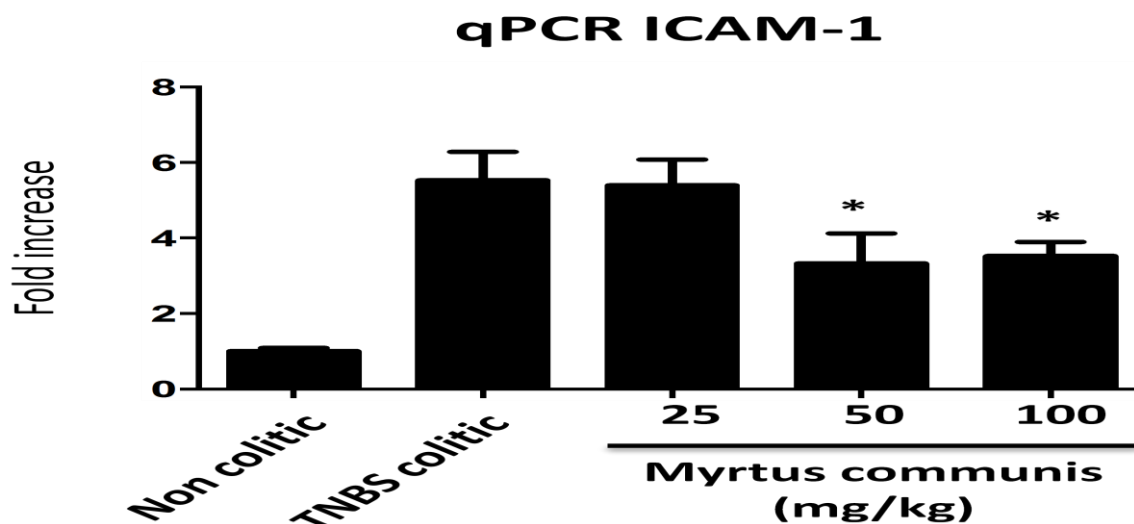
**Figure 58:** Effect of MBME (25, 50 and 100 mg/kg) on colonic inflammatory gene expression of TNF $\alpha$  in TNBS-induced colitis in rats. Data were expressed as means  $\pm$  SEM (n=6). \*  $P < 0.05$  vs TNBS colitic group. MBME: *M. communis* L. berries methanolic extract.



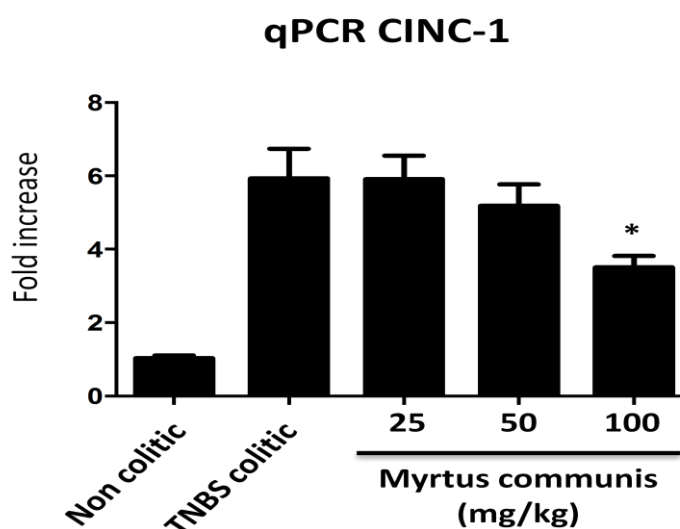
**Figure 59:** Effect of MBME (25, 5 and 100 mg/kg) on colonic inflammatory gene expression of IL-17 in TNBS-induced colitis in rats. Data were expressed as means  $\pm$  SEM (n=6). \*  $P < 0.05$  vs TNBS colitic group. MBME: *M. communis* L. berries methanolic extract.



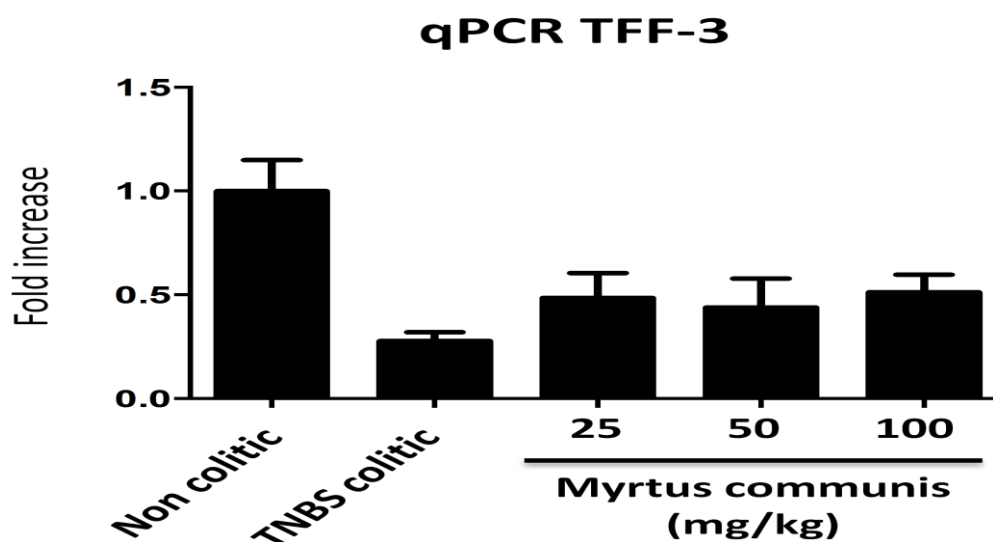
**Figure 60:** Effect of MBME (25, 50 and 100 mg/kg) on colonic inflammatory gene expression of iNOS in TNBS-induced colitis in rats. Data were expressed as means  $\pm$  SEM (n=6). \*  $P < 0.05$  vs TNBS colitic group. MBME: *M. communis* L. berries methanolic extract.



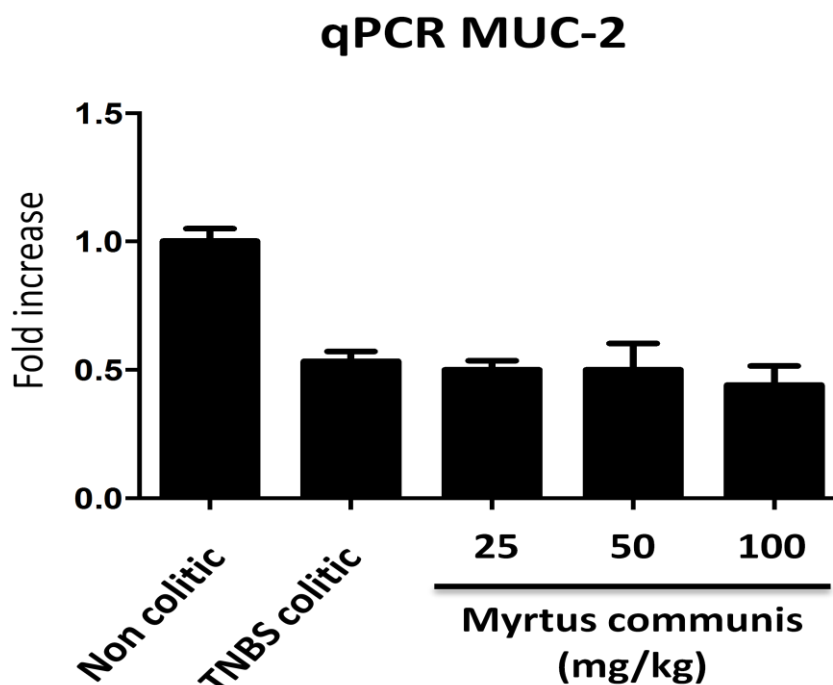
**Figure 61:** Effect of MBME (25, 50 and 100 mg/kg) on colonic inflammatory gene expression of ICAM-1 in TNBS rat colitis. Data were expressed as means  $\pm$  SEM (n=6). \*  $P < 0.05$  vs TNBS colitic group. MBME: *M. communis* L. berries methanolic extract.



**Figure 62:** Effect of MBME (25, 50 and 100 mg/kg) on colonic inflammatory gene expression of CINC-1 in TNBS-induced colitis in rats. Data were expressed as means  $\pm$  SEM (n=6). \*  $P < 0.05$  vs TNBS colitic group. MBME: *M. communis* L. berries methanolic extract.



**Figure 63:** Effect of MBME (25, 50 and 100 mg/kg) on colonic inflammatory gene expression of TFF-3 in TNBS-induced colitis in rats. Data were expressed as means  $\pm$  SEM (n=6). \*  $P < 0.05$  vs TNBS colitic group. MBME: *M. communis* L. berries methanolic extract.

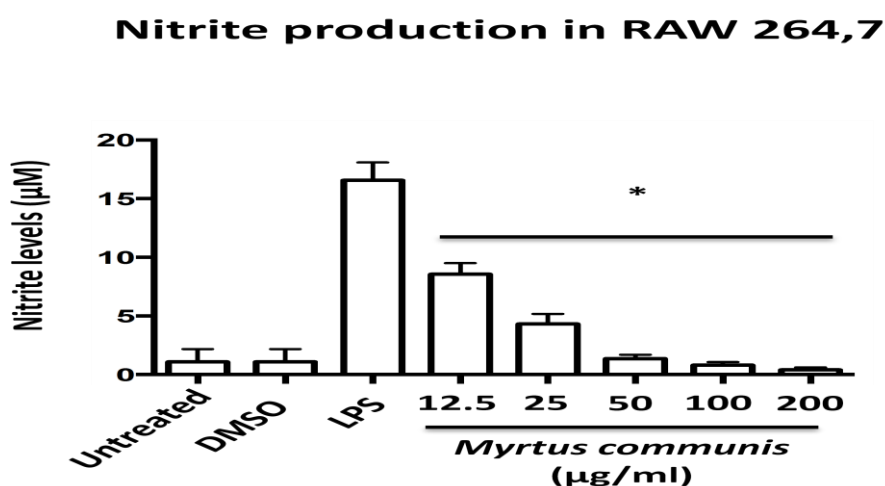




**Figure 64:** Effect of MBME (25, 50 and 100 mg/kg) on colonic inflammatory gene expression of MUC-2 in TNBS-induced colitis in rats. Data were expressed as means  $\pm$  SEM (n=6). \*  $P < 0.05$  vs TNBS colitic group. MBME: *M. communis* L. berries methanolic extract.

### 3.4.5 Effect of MBME on nitrite production in RAW 264.7 cells

Incubation of RAW 264.3 cells with increasing concentrations of MBME (12.5, 25, 50, 100 and 200  $\mu$ g /ml) for 24 h exerted dose dependent and significant decrease ( $P \leq 0.05$ ) in nitrite production (Figure 65).



**Figure 65:** Effect of MBME (12.5, 25, 50, 100 and 200  $\mu$ g/kg) on colonic nitrite production (Griess reaction) in RAW 264.7 cells treated with LPS (100 ng/ml). Data were expressed as means  $\pm$  SEM (n=6). \*  $P < 0.05$  vs TNBS colitis. LPS: Lypopolysaccharide, TNBS: Trinitrobenzene sulfonic acid. MBME: *M. communis* L. berries methanolic extract.

### 3.5 In vitro antioxidant activities of MBME

Different *in vitro* antioxidant assays were performed for MBME: ABTS, DPPH, iron chelation, reducing power,  $\beta$ -carotene, FTC and TBA. The results are shown in (Table 13). Lower  $IC_{50}$  values indicated higher antioxidant activity. MBME showed high antioxidant activity in all the different used assays (Table 13).

MBME was able to scavenge both the radical ABTS and DPPH with  $IC_{50}$  of 0.013 mg/ml and 0.016 mg/ml respectively. However, MBME could not reach the activity of the used positive standars in both tests: Trolox ( $IC_{50} = 0.0031$  mg/ml) and Vit C ( $IC_{50} = 0.0036$  mg/ml).

MBME also showed a promissing result in the reducing power capacity with an  $EC_{50}$  of  $0.52 \pm 0.02$  mg/ml. This value was significantly ( $P \leq 0.0001$ ) different from BHT as positive

control ( $EC_{50} = 0.07$  mg/ml). Moreover, MBME exhibited a good ability to chelate ferrous irons ( $IC_{50} = 0.82$  mg/ml). This value was significantly lower than the standard EDTA ( $IC_{50} = 0.02$  mg/ml).

The addition of the plant extract and BHT at 2 mg/ml was markedly effective in inhibiting the oxidation of linoleic acid and subsequent bleaching of  $\beta$ -carotene, in comparison with the negative control which contained no antioxidant component. MBME showed high antioxidant activity ( $86.96 \pm 1.91$  %) compared to BHT ( $94.9 \pm 1.52$  (Table 13)

As shown in table 13, MBME had a powerful effect on inhibition of linoleic acid oxidation with percent inhibition of  $84.57 \pm 0.42$  compared to BHT ( $90.92 \pm 1.05$ ). In addition, MBME strongly inhibited MDA formation ( $94.28 \pm 0.76$  %), a value close to BHT as positive control ( $98.47 \pm 0.55$  %).

**Table 13:** *In vitro* antioxidant activities of MBME and standards.

Extract	ABTS $IC_{50}$ (mg/ml)	DPPH $IC_{50}$ (mg/ml)	Reducing Power $EC_{50}$ (mg/ml)	Iron Chelation $IC_{50}$ (mg/ml)	$\beta$ -Carotene Inhibition (%)	FTC Inhibition (%)	TBA Inhibition (%)
MBME	0.013 <sup>****</sup>	0.016 <sup>****</sup>	$0.52 \pm 0.02$ <sup>****</sup>	$0.82 \pm 0.03$ <sup>****</sup>	$86.96 \pm 1.91$	$84.57 \pm 0.42$	$94.28 \pm 0.76$
Trolox	0.0031						
Vit C		0.0036					
BHT			$0.07 \pm 0.0$		$94.90 \pm 1.52$	$90.92 \pm 1.05$	$98.47 \pm 0.55$
EDTA				$0.02 \pm 0.0$			

Data ( $IC_{50}$  or as inhibition percent) were presented as means  $\pm$  SD. <sup>\*\*\*\*</sup> $P \leq 0.0001$ ; *ns*: not significant vs Trolox, Vit C, BHT or EDTA. MBME: *Myrtus communis* L. berries methanolic extract.

# Discussion

## 1 Pharmacological effects of *M. communis* L. leaves essential oil (MLEO)

### 1.1 Phytochemical analysis of MLEO

The MLEO yield in the examined myrtle dried leaves was (0.36%). Compared with oil yields of the same plants originating from the surrounding Mediterranean areas, like north west of Algeria (0.32%)( Berka-Zougali *et al.*, 2010), Tunisia (0.61%)(Aidi Wannes *et al.*, 2010), Greece (0.4) (Chryssavgi *et al.*, 2008), Spain (0.5)( Boelens and Jimenez, 1992). The myrtle in the present study could be characterized as being quite rich in essential oil. Strong variability of the MLEO composition could be observed depending on the geographic region of production, the season of harvest and the length of distillation (Tuberoso *et al.*, 2006; Sumbul *et al.*, 2011). Gardeli *et al.* (2008) studied the effect of the seasonal variation on the essential oil content of *M. communis* L. from a Greek island. The authors observed that, the monoterpenes fraction was the main chemical group in all periods. As reported in the results section, the major components identified in the EO in this study were  $\alpha$ -pinene (54.1%), 1,8-cineole (26.5%), linalool (2.4%),  $\alpha$ -terpineol (2.3%), geranyl acetate (2.3%), limonene (2%) and methyl eugenol (1.4%). The myrtle EO of the present study is characterized by its richness in  $\alpha$ -pinene (54.1 %) and 1,8-cineole (26.5%). Close amounts of  $\alpha$ -pinene were reported for myrtle EO from northern west of Algeria (50.8%) (Berka-Zougali *et al.*, 2010), Corsica (53.5-56.7) (Chalchat *et al.*, 1998) and Tunisia (51.1-52.9) (Chalchat *et al.*, 1998; Aidi Wannes *et al.*, 2010), but different from that reported from Morocco and Spain (Boelens and Jimenez, 1992).

### 1.2 Effect of MLEO on ethanol-induced gastric ulceration in rats

Santos *et al.*, (2004) found that cineole which is one of the main constituents of MLEO (26.5 %) exerts anti-inflammatory, analgesic, gastroprotective and hepatoprotective effects. In addition, it had a moderate antioxidant activity and did not show genotoxic effects (Mitic'-C'ulafic' *et al.*, 2009).

Gastric mucus is the first line of defense against acid and other ulcerogens. It adheres to the epithelium together with bicarbonate secreted by the epithelium to serve as a barrier against self-digestion (Allen and Flemstron, 2005). The results obtained in the present work show a significant increase in the amount of adherent mucus in the animals treated with MLEO thus justifying the previously observed gastroprotective action.

The present study shows for the first time that MLEO exhibits a significant antiulcerogenic activity by reducing the gastric mucosa lesion induced by ethanol. gastroprotective effect of this oil was observed as it significantly increased mucus, GSH, SOD and total proteins at higher doses (250 and 500 mg/kg) and also significantly decreased lipid peroxidation. Similarly, Caldas *et al.* (2015) reported that 1,8-cineole which is one of the main constituents of MLEO significantly increased levels of mucus, prevented depletion of –SH groups and reduced the level of lipid peroxidation and protected the gastric mucosa. These findings suggest a potential role of MLEO in gastric protection via the involvement of antioxidant and cytoprotective mechanisms.

Finally, the present results also showed that MLEO exhibited good *in vitro* antioxidant activities in the different tested assays, which may confirms the *in vivo* antioxidant results and the gastroprotective effects, since antioxidants have been implicated in gastroprotection (Ben Ali *et al.*, 2015; Liju *et al.*, 2015).

It is known that the biological activity of essential oils is associated with the composition and concentration of monoterpenes and sesquiterpenes present in their chemical composition, being often related to a synergism among these terpenoids (Rodríguez *et al.*, 2006; Moraes *et al.*, 2009). The major components identified in the essential oil of MLEO in the present study are alpha-pinene (54.1%) and cineole (26.5%). It is believed that these two components are most likely involved in the observed effects of the essential oil. Alpha-pinene is the most widely encountered monoterpene in nature and among that, essential oils with gastroprotective activity obtained from diverse medicinal plants (Santin *et al.*, 2011; Rozza and Pellizzon, 2013). Pinheiro *et al.* (2015) showed that  $\alpha$ -pinene which is one of the major components of Hyptis essential oil exhibited significant antiulcerogenic activity with a great correlation between the concentration of  $\alpha$ -pinene and the gastroprotective effect of *Hyptis* species

### 1.3 Antidiarrhoeal and ant motility effects of MLEO

As shown in results, MLEO did not show any mortality nor toxic effects at the single tested doses (2 g/kg), indicating that the oil is safe at 2 g/kg in mice.

Impaired gastrointestinal motility has a well-recognized contribution to some of the pathophysiological conditions of the gastrointestinal tract. The present study reveals that MLEO dose dependently decreased gastric emptying, an effect that was significantly different

at the highest dose. The inhibitory effect was very close to that of atropine as a positive control (1 mg/kg). The MLEO also dose dependently and significantly decreased the intestinal transit when compared to control group. However, the percentage of inhibition was lower than that of atropine. The control of gastric emptying is complex and involves the coordination of motor activity of the proximal stomach, the antrum, the pylorus and the duodenum, as well as the passive forces created by intragastric volume and gravity (Morran *et al.*, 1999). The tonus of the pyloric sphincter plays a crucial role in the rate of gastric emptying (Ishigushi *et al.*, 2000).

Intestinal transit is controlled by neural and myogenic mechanisms that are governed by several neurotransmitters and mediators. The principal excitatory neurotransmitter in the enteric nervous system is acetylcholine, while nitric oxide is the main inhibitory mediator (Waterman and Costa, 1994). The delaying effect of the essential oil on gastric emptying can result from the reinforcement of the contraction of the pyloric sphincter, whereas its effect on intestinal transit may be due to its inhibitory effect on the intestinal muscle contraction and /or consolidation of the inhibitory component of the musculature. These effects may be attributed to the peculiar actions of its single chemical constituents and / or to their synergism. Alpha-pinene and 1, 8 -cineole are the two main phytochemical constituents of the MLEO. It is believed that these two components are most likely involved in the observed effects of the essential oil. Cămara *et al.* (2003) found that the essential oil of *Plectranthus barbatus* which contains alpha-pinene as a major constituent as well as pure alpha-pinene in the ratio and amount in the oil had intestinal relaxant and antispasmodic effects in guinea-pig. Similarly, Jalilzadeh-Amin and Mahan (2015a) found that 1,8-cineole a major terpene in myrtle essential oil showed a significant decrease in intestinal transit at its highest dose (120 mg/kg). Moreover, the same authors (2015b) showed that *Mentha longifolia* L. which contains 1,8-Cineole as its major constituent dose-dependently decreased gastrointestinal transit in rats.

Experimental diarrhoea in this study was induced using castor oil. The latter provokes diarrhoea through the inflammatory effect of ricinoleic acid, a hydroxylated fatty acid released by lipases in the intestine. Once in the lumen, it induces the secretion of fluid and electrolytes, decreased the absorption and alters the intestinal motility leading to a watery content that is quickly evacuated (Capasso *et al.*, 1994; Dash *et al.*, 2014). Ricinoleic acid seems to cause its effect by promoting prostaglandins (Galvez *et al.*, 1993), platelet-activating factor and nitric oxide formation (Mascolo *et al.*, 1996).

The MLEO dose- dependently delayed the onset of diarrhoea, reduced the number of wet stools and thus inhibiting diarrhoea. In the enteropooling test, the essential oil significantly decreases the volume and weight of the intestinal content. Therefore, the antidiarrhoeal effect of MLEO seems to occur via the inhibition of gastrointestinal motility, inhibition of intestinal secretion and/or activation of reabsorption. Pure 1, 8 -cineole (Jalilzadeh-Amin and Maham, 2015a; Plamen *et al.*, 2015), as well as *Mentha longifolia* L. essential oil containing 1, 8 -cineole as a major constituent (Jalilzadeh-Amin and Maham, 2015b) delayed intestinal transit and had antidiarrhoeal activity. Moreover, 1, 8 -cineole and alpha-pinene present as main components in different essential oils had spasmolytic actions in the intestine (Pedro *et al.*, 1998; Sadraei *et al.*, 2001, 2015; Gilani *et al.*, 2009).

Another mechanism by which monoterpenes can induce the antidiarrhoeal effect is through their anti-inflammatory activity by inhibiting the release of prostaglandins and other autacoids from the intestinal wall. In fact, several studies have demonstrated the anti-inflammatory effects of monoterpenes and among these, the two major constituents of MLEO whether as pure compounds or as the main components in other essential oils (Santos *et al.*, 2004; de Cássia da Silveira e Sá *et al.*, 2013; Rufino *et al.*, 2014; Jalilzadeh-Amin and Maham, 2015 b; Kim *et al.*, 2015). The precise mechanism (s) of action of MLEO and its active components deserve further investigations.

## **2 Pharmacological effects of *M. communis* L. leaves extracts (MLE)**

### **2.1 Phytochemical analysis of MLE**

As shown in the results section, methanolic extract (MLME) and its fractions (MLCE and MLEE) did not show any sign of toxicity at 2 and 5 g/kg doses, concluding that these extracts are safe up to these doses.

Several reports have described *M. communis* L. as being rich in phenolic acids, flavonoids, tannins, essential oils and fatty acids (Nassar *et al.*, 2010; Sumbul *et al.*, 2011; Alipour *et al.*, 2014). The results of the present study revealed that MLE are rich in polyphenols. The highest levels of polyphenols were identified in ME extract. These results were quite close to that found by Kanoun *et al.* (2014) and Dahmoune *et al.* (2015), but lower than that found by Gardeli *et al.* (2008) and Nassar *et al.* (2010). The flavonoids content of this study were in line with those of Romani *et al.* (2004), but lower than that of Nassar *et al.*

(2010) and Tumen *et al.* (2012) and higher than those of Aidi Wannes *et al.* (2010) and Kanoun *et al.* (2014) and Dahmoune *et al.* (2015). The highest levels of tannins were detected in the ME extract. These values were lower than those of Amessis-Ouchemoukh *et al.* (2014) and higher than those of Dahmoune *et al.* (2015). These discrepancies are probably due to different degrees of polarity of the solvents used for the extraction procedure, methods of quantification, geographic region, and the season of harvest (Gardeli *et al.*, 2008).

## 2.2 Effect of MLE on ethanol-induced gastric ulceration in rats

It is widely accepted that gastric ulcer results from an imbalance between the aggressive and defensive factors. The main aggressive factors include acid, pepsin, *Helicobacter pylori*, NSAIDS, ethanol, ROS, whereas the major defensive factors are mucin, bicarbonate, nitric oxide and growth factors. Excessive alcohol consumption may induce gastritis, hemorrhagic gastric erosions, mucosal oedema, cellular exfoliation and inflammatory cell infiltration (Hussain *et al.*, 2015).

Ethanol is well known as a potent necrotizing agent that destroys the defensive factors of the mucosa, leading to the depletion of gastric wall mucus (Wallace, 2001). It is also reported that acute exposure of the gastric mucosa of rats to ethanol can result in gastric lesions similar to those occurring in gastric ulcer; hence, ethanol-induced gastric ulcers have been widely used for the evaluation of gastro protective activity (Boligon *et al.*, 2014). Accordingly, in the present study, it was observed that ethanol administration to rats caused macroscopic lesions to gastric tissue, such as the loss of normal colour and mucus along with the presence of petechiae, haemorrhage and oedema. These lesions are most likely related to mucus depletion and a constrictive effect on veins and arteries of the gastric mucosal, producing congestion, inflammation and tissue injury. In order to confirm the results of antiulcer effect; the stomachs were also evaluated using histopathological examination. The histological observation of the stomachs of the healthy animals (Non-treated animals) was not affected, whereas rats exposed to ethanol presented damage to gastric tissue at the microscopic level. Histopathological injury caused by ethanol administration is characterized by severe detachment of surface epithelium, oedema, formation of gastric pit lesions and haemorrhage, as well as inflammatory process characterized by neutrophils infiltration. Treatment with MLE extracts reversed the damaging effect of ethanol by preserving the epithelium and vasculature. This effect is reflected as a decrease in the ulcer index and a high percentage of protection.



Ethanol-inducing gastric ulceration is closely related to increased ROS level. The ROS ulcerative effect is antagonized by the endogenous antioxidant system composed of enzymatic (SOD, CAT, GST, GPx) and non enzymatic components such as GSH, SH groups, Vit C and Vit E (Bonamin *et al.*, 2014).

GSH and CAT are the first line of defense against ROS. GSH is an important intracellular antioxidant that protects the mucosa from ROS-inducing injury (Sidahmed *et al.*, 2013). CAT converts the peroxy radical ( $H_2O_2$ ) into a safe substance like water (Kwiecien *et al.*, 2002).

To assess the oxidative stress in the ethanol-inducing gastric ulcer in rats, several oxidants-antioxidant parameters were evaluated. The results of the present study showed that the administration of ethanol induced a marked oxidative stress, as evidenced by lipid peroxidation and reduced antioxidant capacity due to GSH level, CAT and SOD activities attenuation.

Treatment with MLE significantly decreased MDA and increased GSH, CAT and SOD. The extracts were also found to possess good antioxidant activity as evidenced by the *in vitro* antioxidant tests, such as, DPPH, ABTS and reducing power. The *in vitro* results were in line with those of Aidi Waness *et al.* (2010) and Amessis-Ouchemoukh *et al.* (2014).

This suggests that the gastroprotective effects of *M. communis* L. is at least partly due to its antioxidant activity. In fact several phytochemical constituents such as those ones found in *M. communis* L. leaves (phenolics, tannins and flavonoids) have been reported to possess antioxidant actions and to protect the stomach from ulcerogeny (Borrelli and Izzo, 2000; Zakaria *et al.*, 2014, 2016).

The gastric mucus barrier has a crucial role in gastric ulcer protection. The ulcerogenic substances induce dissipation of the mucus gel layer and thus causing ulceration (Al-Batran *et al.*, 2013; Adzu *et al.*, 2015). In the present study, pretreatment with MLME and MLCE extracts, in the ethanol-induced ulcer model, significantly increased the gastric mucus content of the adherent gel layer, indicating that gastroprotection of *M. communis* L. is also mediated by preservation of the gastric mucus layer. Other possible mechanisms of gastric protection may include the cyclooxygenase pathway, nitric pathway, inhibition of gastric secretion and reinforcement of blood flow to the mucosa. The protective effect of *M. communis* L. is

most likely mediated via its phytochemical constituents such as tannins, flavonoids, phenolic acids, terpenoids and so forth.....

In fact, numerous studies have ascribed the plant gastroprotective activity to these phytoconstituents and the mechanism(s) of action seems to vary according to the type of these compounds. Flavonoids are one of the well studied phytochemicals. Quercetin, a widely distributed flavonoid in plants and among that *M. communis* L. extracts as well as other plant extracts containing other flavonoids have been studied for their gastroprotective effects. Several mechanisms have been proposed to explain these effects. Quercetin has been found to protect the stomach in several ulcer models. It does so by enhancing neutral glycoproteins production in the gastric mucosa (Di Carlo *et al.*, 1999) and decreasing acid secretion through the inhibition of the histamine pathway and  $H^+/K^+$ -ATPase (Beil *et al.*, 1995). Another possible mechanism of quercetin gastric protective action is via stimulation of prostaglandins secretion in the mucosa (Moroney *et al.*, 1988), but probably the best known antiulcerogenic effects of quercetin as well as other plant extracts containing flavonoids are via their antioxidant properties via different enzymatic and non-enzymatic antioxidant systems (Martin *et al.*, 1998; Coskun *et al.*, 2004; Kahraman *et al.*, 2012; da Silva Junior *et al.*, 2016; Dos Reis Livero *et al.*, 2016; Ribeiro *et al.*, 2016). These findings are in line with the results of the present study.

Similarly, different plant extract containing tannins as their major phytochemical constituents like *M. communis* L. have been reported to afford gastroprotection in different animal gastric ulcer models and with different mechanisms including mucus layer enhancement and also by preserving the antioxidant system of the mucosa, which further corroborate the findings of the present study ( De Jesus *et al.*, 2012; Arun *et al.*, 2014; Prado *et al.*, 2014; Zakaria *et al.*, 2014; Al-Sayed and El-Naga, 2015).

### **2.3 Antidiarrhoeal and ant motility effects of MLE**

Diarrhoea is a real health problem particularly among people in developing countries representing a prominent cause of morbidity and mortality of millions each year. Although diarrhoea is caused by different factors, at least four major mechanisms are usually involved in its pathophysiology, namely: increased intestinal osmolarity, increased electrolytes secretion, decreased electrolytes absorption and disturbed intestinal motility (Palombo, 2006). Many people use medicinal plants against gastrointestinal disorders without any scientific relevance to this use, thus one of the aims of this study is to provide the scientific bases for

the traditional utilization of one of these plants namely *Myrtus communis* L. in the treatment of diarrhoea.

The present study reveals that MLE dose dependently decreased gastric emptying and intestinal transit. This effect is highly significant especially with the highest dose of all extracts (500 mg/kg). At this dose, the extracts showed the most powerful effect; an effect that was close to that of atropine. The control of gastric emptying and intestinal transit is a complex process and involves both neural and myogenic mechanisms that are governed by numerous neurotransmitters and mediators. The main excitatory transmitter is acetylcholine, whereas nitric oxide is the major inhibitory mediator (Waterman and Costa, 1994). The delaying effects of the extracts on gastric emptying may result from the relaxation of the stomach musculature and/or from the constriction of the pyloric sphincter, while the delay of intestinal transit may involve the inhibition of muscle contraction and/or consolidation of the inhibitory component of the intestinal muscle.

This inhibitory action of the extracts on gastrointestinal motility will delay the passage of gastrointestinal contents allowing more time for intestinal absorption in a manner similar to atropine (Nwinyi *et al.*, 2004) and the faeces to become desiccated, thus further retarding movement through the colon (Akindele and Adeyemi, 2006).

The inhibitory effect of *M. communis* L. extracts on gastrointestinal motility and their antidiarrhoeal activity could be attributed to the polyphenolic compounds; mainly tannins and flavonoids as well as other phytochemicals contained in the extract. The HPLC analysis of polyphenols in the plant methanolic extract revealed the presence of gallic and ellagic acids as major components as well as gentisic acid, hydroxybenzoic acid and quercetin. It is believed that the biological activities of the plants may result from their single chemical constituents or from the synergistic effects of their constituents. According to literature, flavonoids and Tannins have different antidiarrhoeal mechanisms of action and one effect is via the inhibition of the gastrointestinal motility at both gastric and intestinal levels. Indeed several studies have pointed out to the inhibitory effect of the flavonoids whether as pure compounds or as major components of different plant extracts on the motility of the gastrointestinal tract (Galvez *et al.*, 1993; Morales *et al.*, 1994; Akah *et al.*, 1999; Amira *et al.*, 2008; Rajan *et al.*, 2012; Santos *et al.*, 2012; Janbaz *et al.*, 2013).

Castor oil from the plant *Recinus communis* is a well known diarrhoea inducer in rodents. It does so via the release of ricinoleic acid (a hydroxylated fatty acid) in the intestinal

lumen under the effect of lipases. Once liberated, it provokes irritation and inflammation of the mucosa leading in this way to increased secretion of fluid and electrolytes, decreased of mucosal absorption, stimulation of intestinal motility and thus inducing a rapid evacuation of the intestinal content (Capasso *et al.*, 1994; Croci *et al.*, 1997). The effect of ricinoleic acid is mediated through several mediators including prostaglandin (Pierce *et al.*, 1971; Galvez *et al.*, 1993) platelet-activating factor and nitric oxide formation (Mascolo *et al.*, 1996).

The plant extracts in the present study dose- dependently delayed the onset time of diarrhoea, reduced the number of wet stools, and decreased the volume and the weight of the intestinal content in the castor oil-treated groups. Thus, the antidiarrhoeal activity of the extracts appears to occur through the inhibition of gastrointestinal motility, inhibition of intestinal water and electrolytes secretion and/or stimulation of reabsorption. These effects are most likely due to the main phytochemicals in the plant. According to previous studies, the antidiarrhoeal activity of many plants has been attributed to the presence of tannins (Mukherjee *et al.*, 1998; Liu *et al.*, 2014; Benbarka *et al.*, 2016; Sheng *et al.*, 2016). They act mainly through the formation of a precipitated protein coat (protein tannate) that covers the intestinal mucosa and thus reducing hydroelectrolytic secretion (Palombo, 2006). Flavonoids may also inhibit diarrhoea by diminishing the secretion of water and electrolytes from the intestinal mucosa (Di Carlo *et al.*, 1993; Venkatesan *et al.*, 2005; Yakubu *et al.*, 2015 Benbarka *et al.*, 2016) or by enhancing their mucosal reabsorption (Osadebe *et al.*, 2012). The induction of secretory diarrhoea by castor oil involves the liberation of several inflammatory mediators including prostaglandins and other autacoids that increase inflammation and motility. The phytochemicals of the plant extracts may exert their antidiarrhoeal effects via the blockade of these mediators, since many studies have pointed out to the anti-inflammatory of these compounds (Amira *et al.*, 2012; Lipińska *et al.*, 2014; Yakubu *et al.*, 2015; Abiodun *et al.*, 2016; Azevedo *et al.*, 2016).

### **3 Anti-inflammatory and antioxidant activities of *M. communis* L. berries methanolic extract (MBME)**

No mortality was observed in mice treated with MBME extract even at the dose of 5 g/kg, signifying that this plant extract is safe at this dose.

The pathogenesis of IBD originates from a combination of inputs: a genetic predisposition, an environmental trigger, and an abnormal immune response. Dysregulation of either immunity or intestinal barrier function allows the initiation of IBD resulting in chronic active inflammation with the production of pro-inflammatory mediators including eicosanoids, platelet-activating factor, cytokines and reactive oxygen and nitrogen metabolites (Cestari *et al.*, 2011).

According to Ardizzone and Bianchi Porro (2005), inflammation in IBD results mainly from a desequilibrium between pro-inflammatory cytokines, such as TNF- $\alpha$  and IL-1, IL-6, IFN- $\gamma$  and anti-inflammatory cytokines (IL-4, IL-10 and IL-11). IL-1 $\beta$  appears to be a primary stimulator of diarrhoea, the main symptom of intestinal inflammation (Siegmund *et al.*, 2001). In addition, TNF- $\alpha$  and IL-1 $\beta$  are the key immunoregulatory cytokines that amplify the inflammatory response by activating a cascade of immune cells, such as neutrophils and others immune cells (Jainu and Devi, 2006).

The pharmacotherapy of ulcerative colitis is principally aimed at inhibiting the production of inflammatory mediators and at modulating the immune system. The ideal aim of treatment of ulcerative colitis is to relieve pain, heal the ulcer and delay ulcer recurrence. To date, no drug meets all the goals of therapy, besides existing drugs are relatively expensive. Thus, phytotherapy could be presented as an alternative in dealing with IBD.

In the present study, TNBS induced extensive colonic damage in rats. The gavage of MBME extract brought about a reduction in the damaged macroscopic score and the weight/length ratio of the colon, indicating a net recovery of the injured tissue. These histological changes were consolidated by the biochemical findings, in which several inflammatory markers (MPO, TNF  $\alpha$ , IL-1 $\beta$ , iNOS, ICAM-1 and CIN-1) were reduced at varying degrees following the treatment with the extract. The lowering effect on MPO activity (a well known marker of leukocyte infiltration, oxidative stress and inflammatory severity) in the colitic rat may indicate a recovery and healing of the ulcerative tissue owing to a decrease

in neutrophil and macrophage infiltration that occur in the beginning of inflammation (Algieri *et al.*, 2014).

The colonic expression of several cytokines involved in ulcerative colitis, including TNF  $\alpha$ , IL-17, IL-1 $\beta$ , the chemokines CIN-1 and the adhesion molecule ICAM-1 were also increased in TNBS treated rats. The curative treatment with the plant extract in the current study counteracted the changes in TNF $\alpha$ , IL-1 $\beta$ , CIN-1, ICAM-1 but not IL-17. Thus, the attenuation of the colonic inflammation is most likely due at least in part to the down-regulation of these mediators that plays key roles in colitic inflammation (Hur *et al.*, 2012; Debnath *et al.*, 2013).

TNF  $\alpha$  is released from macrophages in the start of inflammation and it has numerous implications in the pathogenesis of IBD and in the TNBS model of colitis (Liu and Wang, 2011). It causes disruption of the colonic barrier and induces chemokine secretion from epithelial cells (Cho *et al.*, 2014). TNF $\alpha$  also provokes its pro-inflammatory activity via increased IL-1 $\beta$  and IL-6 production, expression of adhesion molecules and initiation of cytotoxic, apoptotic responses (Begue *et al.*, 2006), leukocyte activation and tissue infiltration (He *et al.*, 2011a).

IL-1 is released in the beginning of the inflammatory process and has wide pro-inflammatory actions. It is produced from different cells, such as macrophages, monocytes, endothelial cells and fibroblasts (Beck and Wallace, 1997; Munoz *et al.*, 2008). IL-1 promotes T cells and NK activation, B cells proliferation and increases adhesion molecule expression and eicosanoid and NO production (Beck and Wallace, 1997). IL-1 $\beta$  promotes Th 17 cells (Acosta-Rodriguez *et al.*, 2007).

The IL-17 family include several members (IL-17 A, IL-17 B, IL-17 C, IL-17 D, IL-17E or IL-25 and IL-17 F. This group induce pro-inflammatory effects both *in vivo* and *in vitro* (Kolls and Linden, 2004). IL-17 is one of the key factors in colitis by acting on different cell types in the intestinal wall (Vezza *et al.*, 2016). It is increased in inflamed colitic tissue (Camuesco *et al.*, 2012; Algieri *et al.*, 2013). It increases the expression of several pro-inflammatory cytokines, chemokines, chemoattractant and metalloproteinases (Park *et al.*, 2005). By contrast, some studies have claimed a protective role of IL-17 in IBD (O'Eonnor *et al.*, 2009; Palla *et al.*, 2016).

Increased adhesion molecules, such as ICAM-1 (Vainer *et al.*, 2010), and the chemoattractant CINC-1 (Algieri *et al.*, 2013; Abiodun *et al.*, 2016) were also associated with colitis.

The down-regulating activity of the above discussed inflammatory mediators by the extract in this study is in agreement with several previous reports on the plant anti-colitic effects (Algieri *et al.*, 2013; Abiodun *et al.*, 2016; Cazarin *et al.*, 2016; Choi *et al.*, 2016; Pervin *et al.*, 2016). However, the upregulation of IL-17 in colitic rats was not altered following extract treatment, suggesting that the later is not acting through this pathway or that the mediator itself may has behaved as a protector rather than an efector (Palla *et al.*, 2016).

It is well known that oxidative stress is increased in IBD patients and animal models and that both ROS and RNS play an important role in IBD (Rezaie *et al.*, 2007; Piechota-Polanczyk and Fichna, 2014; Rana *et al.*, 2014; Rogler, 2015). Following neutrophils infiltration into the colon, MPO is released and enhances the formation of several oxidants that overwhelms the tissue, such as hypochlorous which is an important factor in neutrophils inducing-inflammation (Eiserich *et al.*, 1998; Mariani *et al.*, 2014).

In fact, the colonic damage is usually associated with alteration in several enzymatic and non-enzymatic antioxidants (Rezaie *et al.*, 2007; Algieri *et al.*, 2013; Piechota-Polanczyk and Fichna, 2014; Abiodun *et al.*, 2016). In the present study, glutathione tissue content (an index of oxidative stress) was reduced in TNBS-treated animals. However, treatment with the extract did not conteract this reduction, although an increase in GSH content was noticed with the highest dose, but without reaching significant level, implying that the effect is not strong enough to overcome the reduction. Although other oxidative stress markers were not evaluated *in vivo* in the presence of the extract, other mechnisms could not be ruled out since a good *in vitro* antioxidant activity was observed in the present study.

Nitric oxide is a free-radical gaseous molecule that plays important roles in several physiological and pathological states (Moncada and Higgs, 1991). Inducible nitric oxide synthase (iNOS) is one of three enzymes producing NO from L-arginine (Aktan, 2004) and its activation produces high levels of NO that interact with the superoxide anion to form peroxynitrite, leading to several types of inflammation, including IBD (Kleinert *et al.*, 2004; Kolios *et al.*, 2004; Martin *et al.*, 2007). Thus, compounds that cause the inhibition of NO over-production may be of interest in inflammatory conditions such as colitis.

In the present study, the expression of iNOS was increased in both inflamed colitic tissues as well as in the *in vitro* LPS-induced leukemic monocyte macrophages nitrite production model. The curative treatment with MBME extract in the first model and pretreatment with the extract in the second model significantly reduced iNOS over expression and nitrite overproduction, respectively. These findings are in line with the previous which showed that the inhibition of the overactivity of the nitrenergic system may help in the recovery of the colitic tissues (Algieri *et al.*, 2013; Qian *et al.*, 2015; Xu *et al.*, 2015).

The colonic mucosal barrier plays a crucial role in the protection of the colon (Su *et al.*, 2009). Treatment of the rats with TNBS brought about a decrease in the expression of both MUC-2, an important constituent of the mucus layer of the colon and TFF-3, a key peptide that helps in maintaining the colonic mucosa integrity (Algieri *et al.*, 2013). The curative treatment of the extract was not able to significantly reverse the reduction in the expression of both elements, although an increase was noticed with the TFF-3 expression with all doses.

The results show for the first time the beneficial effect of MBME extract in attenuating the damaging effect of TNBS on the colon of the rat, a model that mimics the ulcerative colitis in humans, it also confirms its early anti-inflammatory findings (Amira *et al.*, 2012).

Different pathways seem to be involved in its action, among which the immunomodulatory and the anti-inflammatory activities. The presence of phenolics, tannins, alkaloids, saponins, flavonoids, steroids, and terpenoids in the extracts of *M. communis* L. (Sumbul *et al.*, 2011) and the present study may have contributed to the effects of this plant on TNBS induced-colitis model. Indeed, many studies have pointed out to the beneficial effects of plant phytochemicals (including those which are present in *M. communis* L.) on IBD. Molecular studies have revealed that phenolic compounds (flavonoids, tannins) can exert modulatory actions in cell by interacting with a wide spectrum of molecular targets central to the cell signalling machinery. These include down-regulation of pro-inflammatory enzymes and also by modulating the expression of different cytokines. Many studies have shown that inducible and pro-inflammatory cytokines (Soobrattee *et al.*, 2005; Martin *et al.*, 2007; Sánchez-Fidalgo *et al.*, 2007; Hur *et al.*, 2012; Algieri *et al.*, 2014; Li *et al.*, 2014; Pandurangan *et al.*, 2015; Somani *et al.*, 2015; Abiodun *et al.*, 2016; Talhaoui *et al.*, 2016; Vezza *et al.*, 2016).



# **Conclusion and future prospects**

The present study showed that the plant in study is a rich source of phenolic compounds and essential oil; and was devoid of serious toxicological effects. These characteristics have prompted us to study some pharmacological effect on the gastrointestinal tract. *Myrtus communis* L. leaves essential oil and extracts have shown antimotility, antisecretory and antidiarrhoeal activity. These effects may be attributed to the presence of several classes of chemicals in the leaves of this plant. The same components have also produced important antiulcer activity, an effect that may result from their efficacy in scavenging different free radicals that result from the ethanol-inducing ulcer model. The stimulatory effects of the phytochemicals on the mucus secretion is another patch that may help in attenuation of ulceration. The last part of the study has demonstrated the beneficial effect of the plant on experimental colitis in rats. The mechanisms of action seem to be related to the antioxidant and anti-inflammatory activities of the plant as evidenced by the inhibition of some markers that are involved in induction of these reactions.

As a general conclusion, it is widely accepted that the available modern medicine for the treatment of gastrointestinal disorders have several limitations due mainly to its cost and intolerance, which may explain the promotion of the use of natural compounds in the relief and medication of the gastrointestinal tract disorders.

The results of this study show promising health promoting effects in the gastrointestinal tract. However, and although it seems that plant chemical constituents are devoid of any serious side effects, it will be of interest in the future to further examine the probable side effects of these constituents with higher doses and with large groups of animals and in acute and chronic states, especially with essential oils, since few studies have mentioned the possible intoxication by high doses of ingested essential oil.

To further clarify the mechanisms involved in the *in vivo* studies, it will be interesting to extend the studies to other models of acute and chronic diarrhoea and ulceration and motility.

Profound chemical analysis is also needed, and the isolation and the study of single phytochemical effects on the studied models may shade light on the exact mechanisms involved in the effect of the plant.

Finally, the extension of the study of the plant to other physiopathological alterations in the body may be of great importance



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