ACUTE TOXICITY AND ANTI-ULCER MECHANISMS OF TEUCRIUM ZANONII EXTRACT AGAINST ETHANOL-INDUCED GASTRIC MUCOSAL INJURIES IN RATS

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ABSTRAK

ABSTRACT

The study was performed to assess the anti-ulcer activity of *Teucrium zanonii* leaves ethanolic extract against ethanol induced gastric ulcer in rat model. Apart from that, acute toxicity, antibacterial and antioxidant activities of *T. zanonii* ethanolic extract were evaluated. Experimental groups were orally administered with different concentrations of *T. zanonii* ethanolic extract (50, 100, and 200 mg/kg) diluted with 10% Tween 20 solution. Negative control group (ulcer control) was pretreated with vehicle only (10% Tween 20). Positive control (reference group) was orally administered with 20 mg/kg Omeprazole. One hour after administration, animals in all groups received absolute ethanol to generate gastric mucosal injuries. An hour later, all rats were sacrificed and the ulcerous areas of the gastric wall were ascertained. The ulcer control group showed intense mucosal injuries while pretreatment with *T. zanonii* indicated high protection level against gastric mucosal injuries, enhancing the production of mucus and flattening of gastric mucosa. Histological evaluation of gastric wall in ulcer control group revealed acute damage of gastric mucosa along with edema and leucocytes infiltration in submucosal layer compared to pretreated groups with *T. zanonii*. Acute toxicity test up to 5 g/kg of this extract did not display any toxicological symptoms in rats. The antibacterial study of *T. zanonii* did not demonstrate any antibacterial activity against *B. subtilis*, *E. coli*, *Staph. aureus*, *P. aeruginosa*, *Klebsiella pneumonia* and Methicillin-resistant *Staphylococcus Aureus* (MRSA). In addition, *T. zanonii* showed low antioxidant activity compare to quercetin, ascorbic acid, gallic acid and butylated hydroxytoluene. In brief, the findings of this study proposed that protective effect of *T. zanonii* against gastric ulcer was obviously tangible with comparative diminished ulcerative area, declining of edema and leucocytes infiltration in submucosal layer.
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CHAPTER ONE: INTRODUCTION

1.1 Peptic ulcer

1.1.1 History of peptic ulcer

Peptic ulcer is characterized by an area of gastrointestinal tract (GI) which has been damaged by gastric acid or pepsin. A peptic ulcer can refer to gastric ulcer, duodenal ulcer or esophageal ulcer. Symptoms of peptic ulcer are various such as nausea, vomiting, bloating, changing of appetite, burning or gnawing pain. These symptoms usually will be quelled after eating and will return back again. Duodenal ulcer is more popular than gastric ulcer; fifteen percentages is gastric ulcer while eighty five percentages is duodenal ulcer. Although peptic ulcer is low in prevalence but increasing in virulence can be seen, which might be caused by the age of world population. Peptic ulcers most often happen as a single wound but sometimes can occur in multiple patterns (Escott-Stump, 2007; Smeltzer et al., 2009).

Exploding occurrence of peptic ulcer in the first half of 20th century in the medical scene led to recommend it as an epidemiological illness, which was followed by a reducing range of incidence and finally vanished as a surgical medicinal condition. The reason of this decline is due to more effective acid suppression drugs such as H2 receptor blockers, PPIs and recognition of H. pylori. (Harbison et al., 2005). For years, the belief that peptic ulcer was prompted by acid was strongly accepted among surgeons and the famous saying “no acid, no ulcer” was very common; this dictum was expressed by Karl Schwards in 1910. For a long time, operation was the preferred curative procedure among surgeons to deal with symptomatic ulcers. Partial gastrectomies, gastroenterostomies and vagotomies, in addition to pharmaceuticals were common approach to control and cope with peptic ulcer. Between 1970s and 1980s, there were
development of diverse drugs in which each class of them has impression on the responsible chemical agent of peptic ulcer such as Cimitidine as histamine receptors blockers and Timoprazole which was the first drug of PPI class (Gustafson et al., 2010).

1.1.2 Causes of peptic ulcer

There are some significant pathophysiological agents which play a remarkable role in contributing to peptic ulcer disease.

1.1.2.1 H. pylori

In some aspects, the importance of *H. pylori* infection in ulcer formation will be more tangible by paying attention to history where epidemic data and outcome of peptic ulcer disease after its elimination and animal model of *H. pylori* infection. Some studies have reported that 70% to 90% of patients with gastric ulcer possess *H. pylori* infection, so by eradicating that, peptic ulcer recurrence will be diminished considerably. Although *H. pylori* is a critical factor in promoting peptic ulcer, only around 15% to 20% of people with *H. pylori* infection in their GI tract show peptic ulcer disease during their life time. (Harbison, et al., 2005). *H. pylori* was explained for the first time by Barry Marshall and Robin Warren in 1983 as a gram negative microaerophilic spiral bacillus which possess urease enzyme. It has the ability for conversion of urea to ammonia and carbon dioxide, confirming their survival in acidic stomach environment. Various mechanisms are involved in pathogenesis of *H. pylori* in ulcer formation, namely by declining the thickness of mucosal layer, reduction of mucosal blood flow, raising the production of gastric acid, impressing the inflammatory cell and emancipating the inflammatory mediators and toxin production such as CagA and the chance to be infected by *H. pylori* will be raised noticeably through aging process (Goroll et al., 2009; Gustafson, et al., 2010; Majumdar et al., 2011; Yuan et al., 2006).
1.1.2.2 NSAIDs

Nonsteroidal anti-inflammatory drugs such as aspirin are indivisibly related to peptic ulcer by interrupting the essential mechanisms of mucosal protection in a way that they prevent Cyclooxygenases (COX1 and COX2) and their transformation from arachidonic acid to prostaglandin, which is a vital molecule in ulcer healing mechanism. NSAIDs can prompt harmful effects like bleeding and perforation. Patients with inflammatory illnesses like rheumatoid arthritis who is consistently taking NSAIDs are 15 to 20% more prone to peptic ulcer than others. Seriousness of peptic ulcer (hemorrhage and perforation) is more prevalent in patients who take NSAIDs. The risk of peptic ulcer formation in NSAIDs users can be even higher at the age of above 60 and in individuals with prior GI problems (Goroll et al., 2009; Harbison et al., 2005; James M. Scheiman, 2005).

1.1.2.3 Smoking, stress, alcohol, coffee and heredity

Epidemiological studies have shown that smoking is one of the significant risk factors for peptic ulcer. The development of that is two times more often in smokers compared to non-smokers. The relatedness of gastric ulcer with the number of smoked cigarettes exists and the deterioration rate is higher while the healing process is lower. The slow, harmful effect of smoking is due to enhancement of free radicals in tissues from the smoke, changing normal blood flow, diminishing effect on circulation, epidermal growth factor and suppression of cell multiplication, where the combination of all of these cause delayed ulcer healing. Stress is another factor which can be considered as a factor to prompt ulcer by impressing physiological situation. Behavioral conditions may boost personal susceptibility to suffer from ulcer via various physiological changes like hormonal changes, reducing effect on wound healing and advancing acid and pepsin secretion in addition to behavioral changes such as cigarettes smoking and alcohol consumption. Alcohol and coffee are involved in ulcer formation.
via different mechanisms. Alcohol compromises the mucus barrier and coffee provokes the acid secretion. Heredity is the other risk factor of incurring peptic ulcers which play a role in prevalence and occurrence of ulcer among the people who have family members with peptic ulcer, by promoted gastrin and pepsin secretion as heritable agents (Goroll, et al., 2009; Jones, 2006; Ko et al., 2000; Rolfes et al., 2008).

1.1.3 Symptoms and signs of peptic ulcer diseases (PUD)

Symptoms of PUD are diverse including burning pain, gnawing pain, post-prandial epigastric pain, bloating, nausea, vomiting and abdominal pain which happens in almost 100% patients with PUD condition. These symptoms improve after taking food in cases of duodenal ulcer. However, in patients with gastric ulcer, taking food is not beneficial. The range of symptoms in duodenal cases is two third and in cases with gastric ulcer is one third. Lack of specificity and sensitivity are disadvantages in distinguishing PUD based on classic signs. The absence of symptoms has been reported in some patients; prevalence of silent ulcers is higher in elder people and in NSAIDs users. Silent ulcers can be discriminated through endoscopy. Alarming signs like weight loss is useful in diagnosis of peptic ulcer disease (Flynn, 2007; Gillen et al., 2011).

1.1.4 Epidemiology of Peptic Ulcer Disease

In the last thirty years, the number of hospitalized patients with PUD condition has diminished continuously and a tangible decline in elective operation was seen in Sweden and other places since half a century ago. The reason of this tendency can be ascribable to factors like lower ubiquity of *H. pylori* infection, better therapeutic strategies and also ulcer preventive schemes in NSAIDs users (Christensen et al., 1988; Gustavsson et al., 1988). Susser and Stein determined the difference in prevalence of duodenal ulcer and gastric ulcer in people born in different decades (Susser et al., 2002).
1.2 *Teucrium zanonii*

*Teucrium zanonii* belongs to the family Lamiaceae (Labiatae). This plant is common and endemic in Libya, where it usually grows around Benghazi city (Abdelshafeek et al., 2009; Abdelshafeek et al., 2010). The family is named mint or aromatic family; this is because the existence of some oils with aromatic fragrance. Species in this family are significant in food industry, pharmacology and production of perfumes (Abdelshafeek, et al., 2009).

This family contains 252 genera and 6700 species. (K. Abdelshafeek, et al., 2009). The Labiatae family has been known as one of the enormous flowering plant families (Naghibi et al., 2005). *Teucrium* genus has 300 species around the world (Abdelshafeek, et al., 2009), 13 species of them can be found in Libya which 5 of them are endemic (Abdelshafeek, et al., 2009; Abdelshafeek, et al., 2010) and 49 species can be grown in Europe (Galati et al., 2000). *Teucrium* genus is also called Germanders although this genus has been considered as a valuable source of neo-clerodances diterpenoid but hepatotoxicity is the important repercussion. It can be due to furano neoclerodance diterpenoids, most probably teucrin A which is found in *T. vicidum*, *T. chamaedrys*, *T. puliom* and *T. capitatus* (Sarkhail, 2011). The species of this genus are scattered universally all over the world especially in the Mediterranean where it has been used in traditional medicine since long time ago. Phenolic compounds are tangibly present in this genus (Stankovic et al., 2011). Regarding the global distribution of this family, its existence in Iran can be mentioned as an example with 46 genera and 410 species in which *T. zanonii* is one of the 12 species of *Teucrium* were available in Iran. Species in this family are significantly used for three purposes; therapeutic, culinary and decorative. Firstly, it is used to treat medical conditions associated with digestive tract like indigestion and flatulence, and also infection. Secondly, members of this family are popular for the essential oils that add flavors to the food. Lastly, in order to beautify,
different genera of this family can be used as ornamentals, some of them are *Nepeta*, *Stachys* and also *Teucrium* (Naghibi, et al., 2005). In addition, some research data suggested that some species of *Teucrium* can be considered as weight losing complement (Sarkhail, 2011). *Teucrium zanonii* is a source of sesquiterpens, triterpens, sterol, flavonoids and phenolic acids (Abdelshafeek, et al., 2009; Abdelshafeek, et al., 2010).

Many of *Teucrium* species have been used as medicinal plants since more than 2000 years ago (Abdelshafeek, et al., 2009; Galati, et al., 2000). An example of medicinal usage of this species among the people of Cyprus is using the decoction of this flowering plant as an anti spasmodic agent in gastric ulcer and inflammatory of intestine and notably making use of the infusion of *Teucrium divaricatum* as external cicatrisant (Galati, et al., 2000). They have shown some biological activities such as antiseptic, antispasmodic, antipyretic, diuretic, hypoglycemic and antifeedant (Abdelshafeek, et al., 2009).

In addition to those biological activities, *Teucrium* extracts has been utilized in traditional folk medicine to cure some medical conditions such as gastrointestinal problems, as vermifuge and renal inflammatory (Abdelshafeek, et al., 2009). On the other hand, mixture of leaves and flowers is used as a replacement of hop in beer flavoring (Abdelshafeek, et al., 2010).

Flavonoidal compounds isolated from this plant are cirsiliol, luteolin, cherysoeiriol and xanthomicrol from ethyl acetate fraction and also separation of other compounds such as apigenin 6, 8-di-O-glucoside and luteolin-7-O-rutinaside from butanol fraction. (Abdelshafeek, et al., 2009).

Insecticidal activity of various types of extraction methods for this plant has been studied against *Phloeotribus oleae*, a beetle that can infest olive trees. The highest
insecticidal activity was showed by aqueous extract of the plant (86.67%). (Abdelshafeek, et al., 2009).

Anticancer activity of *Teucrium* species has been studied, with results showing that methanolic extract of some plants in this genus having the ability to prevent the multiplication of HCT-116 cell line and also stimulate the apoptosis of these cells (Stankovic, et al., 2011).

### 1.3 Omeprazole

In this effort, Omeprazole was used as a positive control. This drug is one of the proton pump inhibitor (PPI) medications which have been accepted as treatment of acid peptic disorders such as PUD and gastroesophageal reflux. PPI drugs has been introduced in late 1980s with better acid suppression ability compare to H2 receptor blockers (Vanderhoff et al., 2002). It has helped the medical community to help so many people who are suffering from this class of diseases.

PPI drug is administered as capsules or enteric coated tablets which should be swallowed whole without chewing. The absorption process will be done in small intestine, despite their short half life; the period of their action is longer due to special mechanism of action. After entering the acidic parietal cell canaliculus by crossing parietal cell membrane, they will become protonated and activated sulphenamide form will be made which join covalently with H/K ATPase and by the aegis of that, acid productive enzyme of stomach wall will be inhibited from acid production by Omeprazole, therefore enabling the gastric ulcer to heal (Mahmood et al., 2011; Olbe, 1999). Diarrhea, nausea, headache and abdominal pain are the most significant and prevalent side effects of this drug. Among these side effects, diarrhea shows signs of being correlated with production of acid which has been deeply declined, consequently changing the bacterial content of intestine (Reilly, 1999). Usage contraindication should be considered in patients with hypersensitivity and hepatic disorders. Moreover, this
drug can display drug interaction and prevent the absorption of some drugs such as vitamin B12 and ketoconazole because of pH rising effect. Omeprazole also seems to have antimicrobial effect on *H. pylori* which perhaps is because of the ability to obstruct urease enzyme produced by *H. pylori* (Vanderhoff, et al., 2002). Bioavailability of Omeprazole will be decreased by the presence of food, so it is better to take on an empty stomach, and also the reason why all rats were kept in fasting condition for 24 hours in this study before conducting the experiment.

1.4 Ethanol

Ethanol in this study was used to generate gastric ulcer. Considerable damage in gastric mucosa caused by absolute ethanol can prompt neutrophil infiltration into the area. As a result, free radicals originated from these neutrophils will obstruct the healing of gastric ulcer (Suzuki et al., 1998). Production of necrotic gastric lesion in the gastric mucosa by ethanol is due to the poisonous impact, declining secretion of bicarbonate and production of mucus (Marhuenda et al., 1993).

Cellular damage will be extended by exposing to ethanol increasingly in a dose dependent manner (Abdulla et al., 2010). Gastric ulcers induced by ethanol appear as multiple hemorrhagic red stripes which differ in length along the glandular portion of the stomach. Ulcer induction by ethanol is a common method for inducing ulcer in experimental rats, with the ability to produce ulcer immediately via damaging the gastrointestinal mucosa. Damaging begins with micro vascular injuries; it is especially devastating to vascular epithelium because of increased permeability, edema and epithelium lifting follows. Severe damage in gastric mucosa by ethanol will leads to higher degree of neutrophil infiltration into the gastric mucosa, producing reactive oxygen species free radicals in ulcer area and inhibit healing (Suzuki, et al., 1998). Intervention effect of neutrophils on lipid peroxidation is to generate superoxide anions (Abdulla, et al., 2010).
Reactive oxygen species can be distributed by neutrophils as they are the main origin of inflammatory mediators. Reactive oxygen species such as superoxide, hydrogen peroxide and myeloperoxidase derived oxidants have poisonous effect on cells and cause motivated injuries of tissue (Cheng et al., 2000). Gastric ulcer healing will be increased by repressing the penetration of neutrophils (Abdulla, et al., 2010). The association between alcohol and peptic ulcer has lead to consider alcohol as an etiologic factor of peptic ulcer. However, the relationship between inflammatory damages and the concurrence of \textit{H. pylori} infection in alcoholic has been observed. The impacts of alcohol are definite on histology and mucosal barrier which play vital role in modifying the mechanisms of mucosal defense (Ko, et al., 2000).

1.5 Antioxidant

The word antioxidant originated from chemistry which can be described as any molecule which is able to donate electron to free radicals and by that stabilize them and prevents their oxidation before assaulting body cells (Bourassa et al., 2005; Lane, 2002). Some antioxidants are known as dietary antioxidants, which can be characterized by these characters: present in human diet, measurable and capable of diminishing the adverse effects of reactive species (Antioxidants et al., 2000). In human body, there are complicated antioxidant systems, namely enzymic antioxidants and non-enzymic antioxidants which work together. Glutathione peroxidase is one of the enzymic antioxidants in body, the other one is catalase which exists in peroxosome of aerobic cells and is very important in stimulating the alteration of hydrogen peroxide to water and oxygen molecule. In addition, SOD is another intracellular enzymic antioxidant which plays a significant role in catalyzing the alteration of superoxide anions to dioxygen and hydrogen peroxide. On the other hand, vitamin E (can be dissolved in fat which is vital as a protective agent against lipid peroxidation), vitamin C (water soluble
vitamin), thiol antioxidants, melatonin, colored pigments (Carotenoids) and flavonoids are known as non-enzymic antioxidants (Rahman, 2007).

Free radicals are molecules (reactive chemical species) with one or more unpaired electrons, which can generate energy and emancipate it in reaction with other molecules such as lipid, carbohydrate, protein and nucleic acids. The most important class of free radicals is reactive oxygen species (ROS) such as superoxide radical, hydroxyl radicals and peroxyl radicals which considerably able to impair biological systems through oxidative damage. Various mechanisms are involved in generation of ROS like radiation (X-ray and gamma-ray), leucocytes and macrophage, toxic chemicals (tobacco and pesticide) and environmental contaminations (Olinescu et al., 2002; Rahman, 2007; Wong et al., 2006). Free radicals are involved in several medical conditions including cardiovascular disease, stroke, cancer, Alzheimer’s disease and Parkinson’s disease which the last two are considered as neurodegenerative ailments (Fusco et al., 2007; Rahman, 2007).

1.6 Objectives

To determine the acute toxicity of *Teucrium zanonii* extract in experimental rats.

To determine the antimicrobial and antioxidant activities of *T. zanonii* extract *in vitro*.

To evaluate the anti-ulcer mechanisms of *T. zanonii* extract in experimental rats microscopically and macroscopically.
CHAPTER TWO: REVIEW OF RELATED LITERATURE

2.1 Peptic ulcer (Gastric Ulcer)

One of the most prevalent medical conditions is peptic ulcer disease (PUD) which implicates the mucosal thickness and pierces the muscles of gastric. Occurrence of peptic ulcer is caused by unevenness among the protective (mucin, prostaglandin and bicarbonate) and aggressive factors (H. pylori, acid, pepsin and NSAIDs), in addition to some other factors (Wasman et al., 2010). Gastric ulcer is a medical problem which has affected a large number of people all over the world. Different agents can cause gastric ulcer such as stress, dietary habits, smoking, drinking alcohol and some medications like NSAIDs that are known as aggressive factors, while some other parameters will protect gastric versus ulcer namely: mucosal barrier, proliferation of cells, blood flow and internal defensive factors like PG (Mahmood et al., 2010).

2.2 Ulcerogenic factors

2.2.1 NSAIDs

As investigated by innumerable studies, non-steroidal anti-inflammatory drugs (NSAIDs) are deemed as a prominent causative factor impacting gastrointestinal (GI) tract. NSAIDs can trigger harmful effects on GI tract such as dyspepsia and other medicinal conditions like bleeding and perforation. Even though the prevalence of symptomatic ulcer is not too high, more than 100,000 hospitalization and 10,000 deaths has been correlated to the usage of NSAIDs. However, the level of microscopic inflammatory is low in ulcer formation by NSAIDs. The mechanisms of NSAIDs induced GI damage encompasses their effects on (COX1, 2) enzymes which play notable roles by supplying prostaglandin. Prostaglandin produced by COX1 is crucial in several ways, such as maintaining the unity and integrity of gastric and duodenal, implicating the protection of GI mucosa and also as a stimulator of platelet
agglomeration, meanwhile prostaglandin secreted by COX2 is significant due to its curative and healing effect on ulcer. Moreover, other beneficial effects of that has been clarified in different aspects such as ovulation, implantation and renal growth. The presence of COX2 in undamaged gastric is less compared to damaged gastric tissue. Prostaglandin is an important molecule which plays critical roles in promoting the ulcer healing mechanisms produced in mucosal cells by COX enzymes, a constellation of enzymes responsible for secretion of prostaglandins as they play different roles in numerous physiological and pathological situations. COX enzyme has two isomer forms (COX1, 2) with high range of similarity in structure. Some of the COX2 selective inhibitors such as celecoxib can obstruct the ulcer genesis. On the other hand, they also reinforce the previous ulcers through postponing the ulcer healing procedure. It has been speculated that the secreted prostaglandins by COX family enzyme possess a noteworthy influence on ulcer healing via various mechanisms like simulative effect on mucosal bicarbonate production, increasing blood flow, obstructive impact on mucosal barrier interruption, increasing angiogenesis and intensify the cell multiplication. In addition, secretion of acid plays a considerable role in NSAIDs induced gastric ulcer in accordance with supportive evidence which shows that acid suppressive drugs like PPI drugs are efficient as an alleviative and protective agent against NSAIDs induced gastric ulcer. In order to prevent and control NSAIDs induced ulcers because of their impact on prostaglandin production, some drugs are used, namely misoprostol and omeprazole, but the usage of misoprostol has been depleted immensely due to its adverse side effects such as diarrhea and abdominal cramping (James M. Scheiman, 2005; Poonam et al., 2005).

Vast application of NSAIDs has led this group of medicine to take a place among widely used medications. Aspirin is one of the members of this drug’s group with prophylactic effects on cardiovascular disease as well as Alzheimer’s but the side
effects of these drugs should be kept in mind as it mostly affect gastrointestinal tract and kidney. Continual usage of NSAIDs causes corrosion in approximately 35 to 60% of the patients, ulceration in 10 to 25% and complications like bleeding and perforation in less than one percent of NSAIDs users. In general, incidence of ulcer complications specifically hospitalization, bleeding, perforation and death can be increased 3 to 10 times by consuming NSAIDs. Inhibiting COX either by aspirin or non-aspirin NSAIDs is the fundamental stage of anti-inflammatory effect of this drug constellation, which prevents the production of prostaglandin along with renal problems and retaining of platelet agglomeration. COX prevention causes decline in production of mucus and bicarbonate while diminishing mucosal blood flow. Besides, leucocytes accumulation and vascular injuries would occur. Two kinds of prostaglandins (PGE, PGI) are momentous as vasodilator where their production takes place in vascular epithelium. Vasoconstriction is the result of obstructing their production. Furthermore, noticeable intensification in neutrophils happens along with their adhesion to gastric venules. Although repressive effect of NSAIDs on PG is the central mechanism of ulcer formation, is not the only route (Halter et al., 2001). NSAIDs increases gastrointestinal penetrability remarkably where offensive agents can enter the gastric mucosal epithelial cells. Ionization will take place, free radical consolidates in the cell and the development of local damages will occur (Hogben et al., 1957). Morphological changes in mitochondria caused by NSAIDs leads to reduction of ATP, the capability of managing cell functionality and retainment of pH (Mahmud et al., 1996; Somasundaram et al., 2000). New generation NSAIDs possess antioxidant activity or Nitric Oxide (NO)-coupled NSAIDs such as flurbiprofen. This class of NSAIDs has vasodilating property due to the existence of NO which contributes in constant mucosal blood flow in resting situation and boosts the denseness of gastric mucosal gel (Halter, et al., 2001).
2.2.2  *H. pylori*

*H. pylori* is a significant causative factor of gastric ulcer. In some previous studies presence of *H. pylori* have been mentioned in 70% to 90% of patients’ GI tracts but just 15% to 20% of them revealed peptic ulcer in their life time (Harbison, et al., 2005). In 1983 *H. pylori* was reported by Barry Marshall and Robin Warren for the first time where it was described as Gram negative microaerophilic spiral bacillus which acts through altering urea to ammonia and carbon dioxide by urease enzyme which allows the microorganism to live in acidic condition. Ulcer formation potentiality of *H. pylori* is due to its effect on different factors like declining effect on the thickness of mucosal layer, decreasing mucosal blood flow, releasing inflammatory mediators and some toxicological agents such as CagA (Goroll, et al., 2009; Gustafson, et al., 2010; Majumdar, et al., 2011; Yuan, et al., 2006).

2.2.3  **Smoking, stress, alcohol, coffee and heredity**

Smoking can be considered as a risk factor of peptic ulcer, where by being a smoker, it increases the prevalence by two folds. Lower rate of ulcer healing in smokers is related to several parameters like existence of free radicals in tissues at greater level, alteration in blood flow and reduction in circulation. Moreover, stress is another agent that play a role in ulcer formation by influencing the behavioral and psychological conditions in a way that induce personal potentiality to be affected by gastric ulcer through modifying of hormones, increasing gastric acid secretion, tendency of drinking alcohol and smoking cigarette. Consuming alcohol and coffee are implicated in ulcer production by effecting mucus barrier and induction of acid secretion respectively. Ulcer formation can be provoked through heredity via genes especially among people who have family members affected by peptic ulcer (Goroll, et al., 2009; Jones, 2006; Ko, et al., 2000; Rolfes, et al., 2008).
2.3 Various drugs to deal with peptic ulcer

Different drugs have been used to cope with peptic ulcer diseases especially gastric ulcer. Some of them are sucralfate, misoprostol and PPI. Sucralfate is a salt of sucrose sulfate and aluminum hydroxide which in the stomach produces a paste with high viscosity that will be attached to the ulcerated area and acts as an obstacle same like mucus and by that it will affect the ulcer healing. Various mechanisms have been suggested for its ameliorative effect such as acting like antacids or barrier action. Gastric acid is important for activation of this drug and it is better not to take Sucralfate with food. Presence of food will impact the gastric pH and the binding of drug will be diminished on ulcerated area. Taking Sucralfate during pregnancy and breast feeding should be considered cautiously because the secretion of it is vague in breast milk. Misoprostol is another remedy with anti-ulcer activity which is the only synthesized PG that acts by increasing the mucosal protection and prohibition of acid secretion (Anthony J. DiMarino et al., 2002; Aschenbrenner et al., 2008). In addition, PPI is a group of drug to treat PUD such as gastric ulcer. Omeprazole and esomeprazole are two examples of PPI remedies. Different properties are involved in mechanism of omeprazole action, where some of them possess lipophilic activity that facilitates the penetration to cell membrane. Additionally, omeprazole is a weak base so it can be concentrated in acidic environment. It is not stable in acidic condition and its half-life is short in acidic surrounding. Omeprazole will affect gastric H+/K+ ATPase at secretory membrane of stomach parietal cells where it prompts the last stage of acid production. H+/K+ATP stoppage will result in acid suppression (Olbe et al., 2003).
2.4 Traditional medicine and the usage of medicinal plants

2.4.1 Medicinal plants and traditional medicine

Looking for natural based products with therapeutic properties has become an important field of interest. Many studies have been done for the purpose of finding natural based new drugs all over the world, even in industrialized countries. Medicinal plants are used in traditional medicine in different parts of the world to treat a wide range of ailments remarkably with various parts of plants such as leaf, stem, seeds, fruit or root, in countries like China, India and Arab regions. Remedies derived from herbs are considered a prominent part of modern medicinal care ever since it was acknowledged by WHO in 2005. In Africa, most of the people (80%) still turned to local indigenous ways of curing. Meanwhile in the U.S., about 50% at least once tried to find a suitable traditional health care. HIV and cancer are two major diseases which is beneficial for medicinal plants to treat as therapeutic agents for incurable diseases (De Vos, 2010).

2.4.2 Usage of medicinal plants in different aspects of medicine

2.4.2.1 Medicinal plants for gastroprotection and anti-ulcer activity

Many plants have been evaluated to determine their anti-ulcer activity. *Polygonum minus* is one of the medicinal plants with curative properties to treat various medical conditions such as gastric problems. This plant is called Kesum in Malay language. Pharmacological activity of the mentioned plant versus Hela (human cervical carcinoma) cell lines and antimicrobial activity were carried out. In addition to that, this plant was deemed as a natural antioxidant because it possess high level of Gallic acid and phenolic content. Moreover, anti-ulcer activity of plant was reported by Qader et al. (2012) and S. Wasman et al. (2010) to be in a dose dependent order due to notable
increase in pH, high protection level of gastric mucus, lesser edema of submucosal layer and absence of leucocytes infiltration has been observed.

Other medicinal plant which has been investigated to possess anti-ulcer activity is *Gynura procumbens* which is famous as Sambung nyawa in Malaysia and vastly dispersed in Southeast Asian countries like Malaysia, Indonesia and Thailand. Usefulness of the plant to cure different diseases such as constipation, renal problems and inflammatory ailments in traditional medicine was proved previously meanwhile its anti-ulcer property was studied by Mahmood, et al. (2010). Flavonoids, saponins, terpenoids and tannins are some active chemical elements of (GPELE) which were ascertained by previous studies (Akowuah et al., 2002).

*Ficus deltoidea* is another plant in different countries of Southeast Asia which is known as *Mas cotek* or *serapat angin* and some other names in Malaysia. Various parts of this plant are useful to cure some medicinal conditions. Fruits are effective to alleviate headache, toothache and cold while powdered root and leaves are helpful for wound amelioration and also to reduce the pain in rheumatism patient; its herbal drink is commonly used to fortify the uterus after child birth (Sulaiman et al., 2008). The anti-ulcerogenic property as examined by Anuar (2009), demonstrated high level of gastric protection against induced ulcer by absolute ethanol by the way of preventing edema and inhibiting leucocytes infiltration in submucosal layer.

Other example of traditional medicinal plant which possesses gastroprotective activity is *Andrographis paniculata*. This plant has been utilized for different medicinal purposes such as antimicrobial, antiviral, antidiabetic and anti inflammatory. Its gastroprotective capability was ascertained by Wasma and colleagues (2011) which showed obvious gastroprotection level against gastric lesions of both ethanolic and aqueous obtained extracts of the plant. *Andrographis paniculata* ethanolic extract and
aqueous extract diminished gastric ulcers by increasing gastric pH level and mucus content in stomach.

*Strobianthes crispusis* is another illustration of medicinal plant that has gastroprotective activity which has shown tangible reduction in gastric lesions dose dependently due to raising gastric mucus production, boosting and elevating of gastric pH value. This plant is local in tropical countries like Malaysia. Boiled leaves of the plant in water are used as antidiabetic, antilytic and laxative. Moreover, anti-AIDS, anti-leukemia, anticarcinogenic, anti-hyperglycemic and hypolipidomic effects are some other medicinal activities of *S. crispus*. High and noticeable amount of mineral has been detected in leaves of this plant like iron, potassium and phosphorous and so are high extent of water soluble vitamins such as vitamin C, B1 and B2 (Mahmood, et al., 2011).

Dates (*Phoenix dactylifera* L.) is a separate example of plants which is beneficial for mankind all over the world, especially in the Middle East where it has wide range of usage since 6000 years ago. During the fasting month, consumption of dates escalates among Muslim people which can be due to its protective effect against peptic ulceration via gastric acid. Evaluation of its ability to protect gastric ulcer was investigated by Al-Qarawi et al. (2005) which showed that the ethanolic extract of date fruit and in a lower degree date pits extract, are capable to mitigate the gastric ulcer in a rat model experiment by alleviating the raised histamine and gastrin level (Al-Qarawi, et al., 2005).

Other example of medicinal herbs which showed anti-ulcer potential is *Centella asiatica* which is used in various parts of the world in many countries. Several therapeutic applications were reported for this plant namely, tonic in diseases related to skin and leprosy, memory enhancing, wound healing and also curative effect on bronchitis, asthma, renal problems, dysentery and anti-cancer property which was announced by Abdulla, et al. (2010). Moreover, estimation of gastroprotective activity
of *Centella asiatica* indicated ameliorative effect of the plant on induced ulcers in experimental animals dose dependently (Abdulla, et al., 2010).

*Terminalia pallid* Brandis is another precedent of plants which was investigated for its remedial effect on gastric ulcer. Dispersal of this plant is predominantly in India. Different parts of the plant have been applied to cure diverse medical problems. For instance, the fruits of this evergreen tree is effective for mitigating ulcer, venereal diseases, diarrhea and diabetes which was posited by Gupta and colleagues (2005). Meanwhile, its bark has diuretic effect which was speculated by Gupta, et al. (2005). Anti-ulcer investigation of this plant was carried out and its effective gastroprotection ability was seen in high dose obviously (Gupta, et al., 2005).

Other illustration of medical plants with gastroprotective effect and antiulcer activity is *Pongamia pinnata* which *Karanja* is the common name of that in Hindi. Constituents of kernel of this plant are mostly oil, protein and some other components like flavonoids, which has reductive effect on cardiovascular disease and its incidence, antioxidant and analgesic properties. Also gastroprotective activity via antioxidative effect along with inhibitory effect on H+/K+ ATPase has been indicated. Furthermore, *Karanja* oil is beneficial in leather softening and Ayurvedic preparation. Some other therapeutic applications have been reported to cure leucoderma, lumbago and leprosy. The anti-ulcer ability of this plant extract is more substantial in swim induced ulcers and at a lesser extent in ethanol induced ulcers (Vismaya et al., 2011).

Spices are another example of natural based alternative options for inhibiting gastric ulcer and its therapy. They have different applications in diverse areas. Various usages as food additives, carminatives and also their medical applications in managing many medical conditions such as antibacterial, vermicidal, antifungal, anti-inflammatory agent has been proved. Additionally, anticarcinogenic ability, anti-hypercholesterolemic capability and anti-mutagenic potentiality has been reported.
Achieving a pivotal treatment for gastric ulcer is possible by neutralizing offensive factors such as acid-pepsin, platelet aggravating factor (PAF) and NSAIDs along with boosting mucosal defense like mucus, blood flow, PG, and NO. Despite the availability of several anti-secretory drugs, they are just effective as acid suppressor without any effects on other factors which interfere in ulcer formation, regardless of their high price, harmful effects and potential deterioration of ulcers. Interest in finding new drugs from natural medical plants is necessary due to affordability and availability. *Cissus quadrangularis*, *Maytenus ilicifolia*, *phytosphingosine*, *Cecropia glaziovii* Sneth (*Cecropiaceae*) are some examples with great inhibitory impression on acid production and pepsin content consequently resulted in remarkable decrease in ulcerative index (Al Mofleh, 2011).

In other study which was done to find newer and more effective plant based drugs to deal with gastric ulcer, methanolic extract of *Cayratia trifolia* was evaluated for its gastroprotective qualification. *Cayratia trifolia* belongs to Vitaceae family, and is called by different names such as Fox grape in English, Amlabel, Ramchana in Hindi and in Sanskrit is known as Amlavetash. This plant is local to India, Australia and Asia with greenish white flowers. The entire plant has constituents like steroids, flavonoids, tannins and terpenoids meanwhile leaves possess stillbene. Usage of leaves as anti-ulcer is common while grounded roots with black pepper will be mixed and utilized as poultice. Its tuber paste is useful to ameliorate snake bite (Gupta et al., 2012).

Based on increasing tendency to find natural based drugs to treat gastric ulcer, other effort was done in Turkey on a group of medicinal plants to prove their anti-ulcer influence scientifically, which they have been used as effective agents to deal with some common symptoms of gastric ulcer such as stomach ache and heart burn. Different parts of six conventional plants were screened namely aerial parts of *Malva neglecta* Wallr, leaves of *Potentilla reptans* L. (Rosaceae), fruits of *Rumex patientia* L. (Polygonaceae),
aerial parts of *Sanguisorba minor* Scop. ssp. *muricata* (Spach) Briq. (Rosaceae), aerial parts of *Sideritis caesarea* Duman, (Lamiacea) and also flowers of *Verbascum cheiranthifolium* Boiss var. *cheiranthifolium* (Scrophulariaceae). The study reported the greatest anti-ulcerogenic effect correlated to *Potentilla reptans* with almost hundred percent protection whereas lowest preservation belonged to *Sanguisorba minor* ssp. *Muricata* with 62% protective effect are effective against gastric ulcer (Gürbüz et al., 2005).

Continuous seeking to find more effective plant based remedies to cure gastric ulcer is in progress all over the world. In other study which was carried out in Pakistan, gastroprotective effect of methanolic extract of several parts of four medicinal herbs was considered confirm in their usage in folkloric medicine as gastroprotective agents. Considered plants namely were flower buds of *Bauhinia racemosa* and *Moringa plerygosperma*, entire parts of *Thrianthema pentandra* in addition to fruits of *Cordia Latifolia*. Declining impression of *Bauhinia racemosa* and *Thianthema pentandra* extracts were more substantial in decreasing ulcerative index compare to *Cardia Latifolia* with no diminishing effect on ulcerative index and *Moringa plerygospera* with lower effectiveness on decreasing ulcerative index (Akhtar et al., 1995).

To confirm the usage of medical herbs as curative agents against gastric ulcer, some investigation was performed to find out gastric protective effect of *Verbena hastata* leaf extract in Nigeria. *Verbena hastate* belongs to Verbenaceae family which usually present in Southern Nigeria. Stem, root and leaves are three parts that are beneficial in folk medicine. Aqueous and alcoholic extract of leaves are considered as treatments to cure dysentery and diarrhea in addition to treat cold, lung congestions and malaria. Supplementary medical applications can be characterized as expectorant, antiperiodic and eradicative effect on intestinal worms. Moreover, this study signified the anti-ulcerogenic property of this plant (Akuodor et al., 2012).
*Ficus religiosa* is another medicinal herb which cannot be left out. It belongs to Moraceae family. In Malaysia and India, it is famous as *Pokok are suci* and *Peepal* tree. *Ficus religiosa* normally will be planted around temples. This plant has been applied as natural remedy to cure and cope with several medical conditions such as diarrhea, dysentery, leucorrhoea and menorrhagia. Furthermore, wound healing effect of ethanolic bark extract has been declared. In addition, aqueous bark extract of *Ficus religiosa* possess some constituents like carbohydrates, polyphenolic compounds, flavonoids and tannins. Anticonvulsant activity of ethanolic extract of its figs has been claimed. Regarding the investigation of its anti-ulcer activity, gastroprotective activity of *Ficus religiosa* is due to enhancing gastric pH and also simultaneous depletion of gastric juice amount (Khan et al., 2011).

According to increasing interest in finding novel and more effective drugs for treating gastric ulcer, other research was performed to evaluate antiulcer activity of ethanolic extract of *Buchanania lanzan* Spreg roots in India. *Buchanania lanzan* Spreg is a subdeciduous tree, its dimension is up to 1.3m and 13 to 17m in high with dark grey bark and greenish white flowers. This plant in Hindi is called Char and among the people of Jharkhand and Chhattisgarh who live in tribes, the common applications are substantially due its medicinal values as wound healer, anti-diarrheal agent and painkiller. Additionally, assessment of *Buchanania lanzan* Spreg root for its anti-ulcer property revealed high gastroprotective efficacy by reducing ulcerative index remarkably (Kodati et al., 2010).

*Raphinus sativus* Linn (Radish) is another plant which has been monitored to determine its anti-ulcer activity against gastric ulcer. Radish is planted in various parts of the earth. This plant has many uses and can be used for different purposes. In some studies performed on gastric ulcer, the prevalence among South Asian people showed a lower rate. Slighter scale of gastric ulcer in this area can be correlated to dietary habits.
Leaf of *Raphinus sativus Linn* is one of the foods that have been declared to have anti-ulcer activity. Some other therapeutical effects have been suggested for root and leaves of Radish such as gut stimulatory, hepatoprotective potentiality, cardioprotective ability and antioxidant activity. Ethanolic extract of Radish leaves showed obvious anti-ulcer capability with 80% ulcer prevention (Devaraj et al., 2011).

For the purpose of finding better naturally derived drugs to deal with gastric ulcer, the assessment of *Nigella sativa* seeds were carried out. *Nigella sativa* seeds have been utilized as a natural based treatment for more than forty centuries. The therapeutic effects of NSO against ethanol induced gastric ulcer and gastric secretion was evaluated which clarified evident increase in mucin and glutathione level along with tangible decline in mucosal histamine. Glutathione play an important role as cofactor in producing PGs. High level of gastric protection using *Nigella sativa* seeds were recommended as gastric ulcer therapeutic agent habitually (Khalil et al., 2010).

*Jasminum sambac* is another illustration of medicinal plants which has been used to cope with several ailments. Different parts of *J. sambac* have various medical uses to cure wide range of diseases like rheumatism, gallstones and diabetes mellitus. Flowers of *J. sambac* are applied for reducing fever and bee stings meanwhile, leaves of *J. sambac* are employed as anti-acne. Additionally, *Jasmine* root will mitigate headache and intensify fracture healing. Moreover, essential oil of *Jasmine* is used in skin care products as redolence. Anti cancer and anti fungal activity of *Jasmine* were ascertained in some studies and anti-ulcer activity of this plant was also investigated which showed gastroprotective effect through diminishing the acidity of stomach and notable increase in gastric mucus (AlRashdi et al., 2012).

Other instance of medicinal plants with anti-ulcer potentiality is *Pithecellobium Jiringa* (djengkol bean) which belongs to Leguminosae family. Southeast Asia is the origin of this plant and is called by different names in that area; for instance, in
Cambodia it is called krakos, in Indonesia it is called djengkol and among Malay people it is known as jering. Djengkol bean has pesticidal activity as an organic pesticide which is because of possessing djengkolic acid and sulphur due to their inhibitory effect on pests. On the other hand, antiviral effect of *P. jiringa* on EBV has been demonstrated. Moreover, *P. jiringa* embraces dietary fibers, proteins and unsaturated fatty acids and also is rich in polyphenolic compounds along with influential antioxidant activity (Aziz Ibrahim et al., 2012).

In light of finding newer gastroprotective agents and intensive interest of searching for more effectual natural based drugs nettle (*Urtica dioica* L.) is other instance of plants which has been considered to investigate its antiulcer potentiality. It has been declared that nettle has hepatoprotective effect on rats. In folk medicine of Turkey this plant has been used as a curative agent to ameliorate stomachache. Other therapeutic activities of nettle have been announced such as painkiller to treat rheumatism, applying as anti-hepatic deficiency agent and against cold and cough. Gastroprotective evaluation of nettle (WEN) has revealed a noticeable protective effect on gastric ulcer dose dependently which can be regarded as natural based antiulcer remedy (Gülçin et al., 2004).

2.4.2.2 Medicinal plants and antioxidant activity

Some researches claimed that two-thirds of herbs in the world have therapeutic values which some of them have prominent potentiality as natural antioxidant and by possession of that, they are able to dwindle oxidative stress in body’s cells, consequently being advantageous in treating several human ailments like cardiovascular diseases, cancer and inflammatory medical conditions. Besides, some synthetic antioxidants are being produced as well which BHT and BHA are some examples (Krishnaiah et al., 2011). Oxidative stress has harmful effects on mankind health. An investigation by WHO was done to find out the popularity of traditional medicine
among world population. The results represented that 80% of people use traditional medicine as first step of health care which includes plant extract and related active constituents (Craig, 1999).

Nowadays, the request and seeking for medicinal plants is rising increasingly. Different plants have antioxidant activity which is pondered as a significant parameter with therapeutic effects. For example, antioxidant based drugs are used to restrain and cure ailments like stroke, cancer, diabetes, Alzheimer’s and atherosclerosis. In a study which was done by Khalaf et al. (2008) on some plants to evaluate their antioxidant capability by performing DPPH assay, the alcoholic extract of green tea and black tea (Camellia sinensis Linn.) reportedly had high antioxidant activity where free radical scavenging of green tea was even better than ascorbic acid which was used as standard. It has lowest IC$_{50}$ comparing to other plant in that study because of possession of polyphenols such as flavonols, flavandiols, flavonoid and phenolic acids like gallic acid. In addition, antioxidant assessment of black pepper (Piper nigrum Linn.) was studied which was affluent in glutathione peroxidase and glucose-6-phosphate dehydrogenase a powerful antioxidant (Khalaf, et al., 2008).

According to adverse effects of ROS such as super oxide radicals, hydrogen peroxide and hydroperoxyl radicals and their causative on OS they should be highly deactivated. Despite the existence of essential AODS like SOD, CAT, GPx and GRed, unevenness in ROS producing and counteracting will lead to oxidative damage and through that it causes several habitual and degenerative ailments such as diabetes mellitus, cancer and Parkinson’s. Finding natural antioxidant obtained from natural sources is a point of interest all over the world. In an effort which was done on different parts of four Indian medicinal herbs namely fresh leaves of *Trichosanthes dioica*, fruits of *Moringa oleifera*, fruits of *Emblica officinalis* and aerial roots of *Ficus bengalensis* total antioxidant activity was evaluated by performing FRAP assay. This study
demonstrated the high antioxidant capability and flavonoids contents of *Emblica officinalis* fruit’s seed comparing to other tree plants which can be deemed as a natural based antioxidant (Sharma et al., 2009).

Continuous studies have been performed universally to assess the antioxidant activity of different plants all over the world. For this purpose nettle (*Urtica dioica* L.) has been evaluated for its antioxidant power by doing various antioxidant assessing methods like reducing power assay and DPPH assay which indicated high antioxidant impact. In accordance to its ability as antioxidant it can be consumed as herbal antioxidant widely (Gülçin, et al., 2004).

2.4.2.3 Medicinal plants and antimicrobial activity

Healing power of plants has been determined since long time ago. Their usage as anti-infectious agent to cure some common infectious diseases is an important therapeutic ability of plants. Still usage of some plants is popular as antimicrobial agent to cope with various infectious problems for instance, bearberry and cranberry extract are useful to deal with urinary tract infectious diseases meanwhile, lemon balm, tee tree and garlic have been recognized as wide range natural antimicrobial agent. According to previous studies, phenolic compounds considered as dramatic active chemicals and Gram positive bacteria showed the highest degree of sensibility. Researches on finding out the antimicrobial activity of medicinal herbs has become increasingly popular in the world despite the presence of some factors that can affect the antimicrobial assessing process, like the selection of microorganisms, high dosage of plant and vague definition of positive control. At this moment, many efforts are in progress to specify the area of antimicrobial evaluation of medicinal plants as antimicrobial agents (Rios et al., 2005).

*Sida acuta burm.f. (malvaceae)* is an example of medicinal plants which can be characterized as erect small branched herb that have the ability to be cultivated and grown on roadsides and waste land of Nigeria. This plant has been applied in medicine
traditionally to deal with different medical conditions in some countries. For instance, Indian people use the entire plant hot water extract as febrifuge, abortifacient and diuretic, meanwhile in Nicaragua, whole plant decoction is used to treat ache, ulcers, asthma, fever, anti-worm remedy and also to cope with venereal ailments. Antibacterial activity of *sida acuta burm.f. (malvaceae)* dried leaf and its seed chloroform extract have been investigated which revealed antimicrobial effect against *Mycobacterium smegmatis*, *E. coli*, *Pseudomonas circhorii* and *Salmonella typhimurium* respectively. Additionally, ethanolic extract of the aerial parts of this herb has been considered for its antimicrobial potentiality versus some bacterial species like Standard strain of *Staphylococcus aureus*, clinical isolates of *Staphylococcus aureus*, *B. subtilis*, *Streptococcus faecalis*, *E. coli*, *P. aeruginosa*, *K. pneumonia* and *Candida albicans* as a fungus. No antimicrobial activity was observed against *E. coli*, *K. pneumonia*, *P. aeruginosa* and *Candida albicans* despite the growth inhibitory effect on Gram positive aerobic organisms in this effort (Obah et al., 2007).

*Ziziphora clinopodioides* is another instance of therapeutic plants which is eatable. This plant has been scattered in Torkey-Anatolia, various parts of the plant namely leaves, flowers and stem are often consumed as vegetable and food additive. *Ziziphora clinopodioides* is well known as Karinanesi among local people. Aromatic tea obtained from the plant has several curative effects such as carminative, wound healer and also antiseptic. Methanolic extract and essential oil of the plant shown antimicrobial activity while essential oil was more efficacious in a greater extend which can be because of bacterial tolerance and the constituents of essential oil (Ozturk et al., 2007).

Regarding the prevalence of novel infectious diseases and resistant microorganisms to common antibiotics, continual researches are done in different parts of the world to find out new antimicrobial agents. Staphylococcus species are significant groups of microorganisms to be controlled which can cause various medical problems. An effort
was done to evaluate the antimicrobial activity of 34 Indian herbs belonging to 28 different families. These plants with therapeutic effects were assessed against three species of Staphylococcus namely *Staphylococcus aureus, Staphylococcus epidermidis* and *Staphylococcus subflava*. These species are involved in several medical conditions, for instance *Staphylococcus aureus* play an important role to prompt toxic shock syndrome, wound infection and abscesses meanwhile, *Staphylococcus epidermidis* interferes in infection of prosthetic therapeutic instruments such as artificial joints and heart valves. Varied medical problems like infectious ailments can be managed by natural products derived from plants. Assessing antimicrobial activity of 34 Indian plants revealed that the effectiveness of alcoholic extracts were higher compare to aqueous extracts and among the tested species *S. aureus* was much more vulnerable and the alcoholic extract of *Woodfordia fruticosa* indicated the greatest antimicrobial capability (Parekh et al., 2008).

According to the urgent need to find newer and more efficient antimicrobial agents, nettle (*Urtica dioica* L.) extract was assessed against ten different microorganisms including five G- such as *E. coli, P. aeruginosa, Proteus mirabilis, Citrobacter koseri* and *Enterobacter aerogenes* in addition to four G+ namely, *S. aureus, Streptococcus pneumonia, Micrococcus luteus* and *Staphylococcus epidermidis* and also *Candida albicans* as a fungus. Nettle extract showed various range of inhibitory effect on all tested organisms except *P. aeruginosa* (Gülçin, et al., 2004).

In light of continuous studies on discovering novel antimicrobial agents with greater efficacy, the assessment of water and ethanolic extracts of *Hydnora johannis* root was done to investigate the antibacterial activity. Roots of *Hydnora johannis* have been employed in folk medicine of Sudan to deal with different health problems such as diarrhea, cholera, dysentery and swelling tonsillitis. Determination of antibacterial activity of the plant indicated that the water extract revealed antimicrobial potentiality
against *B. subtilis*, *B. cereus*, *S. aureus* and *Enterococcus faecalis*, meanwhile the ethanolic extract exhibited remarkable antimicrobial activity on *Enterococcus faecalis* and *S. aureus* strains (Yagi et al., 2012).

Other investigation of antimicrobial activity of plants was performed on South African medicinal plants. Regarding the diversity of botanical inheritance, South Africa possess 30,000 kinds of herbs in which 3000 species of them have ameliorative effects on broad range of diseases such as TB, gastrointestinal problems, skin diseases and also some of them have been used as wound healer in addition to antimicrobial agent as well. Surveying these plants revealed different ranges of antimicrobial potentiality on various microbial species. For example, ethanolic extract of *Felicia arigeroides* roots showed antimicrobial capability against *Candida albicans* meanwhile the methanolic bark extract of *Warburgia salutaris* indicated antimicrobial effect against *B. subtilis* and *S. aureus*. Moreover, *Hermannia depressa* roots ethanolic extract signified antibacterial ability versus *B. subtilis*. Thus, plants derived antimicrobial agent can be helpful to cure infectious diseases more effectively along with lesser side effects (Van Vuuren, 2008).

2.5 Acute Toxicity Test of Plants

Tendency of using complementary and alternative medicines has been increased among the people of the world, for instance in America to gain better health. In spite of this express proliferation there is not enough data about the efficacy and toxicity of alternative remedies such as plant derived drugs. Researches on medicinal herbs are in progress due to the usefulness for human being, thereby, hoping to diminish the reliance on imported drugs. An effort was done to demonstrate the acute toxicity of *Aegle marmelos Corr* which is a plant with therapeutic property known as bael tree in English and vilvam in Tamil. Leaves and fruits of this plant are two parts which are used in folk medicine significantly. Leaves and fruits decoctions are consumed to treat dysentery, diarrhea, upper respiratory infections and heart ailments. In accordance to the result, A.
M leaves are not toxic up to 1000mg/kg body weight meanwhile in some studies, hepatic lesions have been observed in fruit extract treated rats (Veerappan et al., 2007).

Fruit of *Schinus mollether* is another example of plants which has been investigated for its acute toxicity. This plant is an indigenous plant of South America, meanwhile few other species can be found in different places like mild North and Eurasia locally. Whole parts of the plant have been traditionally used in folk medicine as antibacterial, astringent, antiviral, wound healer, antidepressant and also to overcome urinary and respiratory tract infections. Additionally, in some studies its insecticidal activity has been mentioned. Regarding information obtained from acute toxicity investigation, ethanolic extract showed an increase in daily food intake of the experimental subject while body weight data analysis signified no tangible difference between the groups in addition to the absence of any clinical abnormalities (Ferrero et al., 2007).

Constant effort still is in progress in different parts of the world on assessing the acute toxicity of medicinal plants. For this purpose, another experiment was done to evaluate the acute toxicity of *Orthosiphon stamineus* Benth extract on SD rats. *Orthosiphon stamineus* Benth is called cat whiskers among the local people of Malaysia. Leaves of the plant are useful due to diuretic property to make diuretic tea which can be helpful versus kidney and bladder inflammation. Leaves of OS are rich in some active chemical components like sterols and polyphenols. Acute toxicity assessment of this plant showed no mortality and no behavioral changes in rats up to 5000 mg/kg body weight which revealed LD50 at higher doses (Abdullah et al., 2009).

Further studies have been performed to evaluate the acute toxicity of a large number of herbs up to now. *Jasminum sambac* is another instance of medicinal plants which was considered for its toxicity to know if it can be used as a safe natural based drug. Studying of kidney parameters namely Sodium, Potassium, Chloride, CO2, Anion gap, Urea and Creatinine in addition to liver parameters such as total protein, albumin,
globulin, TB, CB, AP, ALT, AST and GGT did not manifest any oblivious differences compared to the control group at two different doses (2g/kg and 5g/kg) of *Jasmine* ethanolic leaves extract and all animals were animated during the observation period till the end of the experiment (AlRashdi, et al., 2012).

*Pithecellobium Jiringa* is another plant with therapeutic potentiality which was assessed for its toxicity to discover safer and more effectual drugs to deal with gastric ulcer. Evaluation of acute toxicity of this plant was done at two different doses (0.5mg/kg and 2mg/kg). During the observation period all experimental animals stayed alive and no toxicological symptoms were seen. Moreover, evaluation of serum biochemistry and data obtained from histological observation did not indicate any considerable difference between control and experimental groups and no mortality was observed within the two weeks of acute toxicity test. It can be speculated that *Pithecellobium Jiringa* is safe with no detection of drug related toxicity and can be used as plant derived drug (Aziz Ibrahim, et al., 2012).

Other survey of plant acute toxicity was performed on leaves of *Raphinus sativus* Linn. No death was recorder at whole period of the observation time. The data gained from this study *Raphinus sativus* Linn (Radish) did not manifest any conspicuous toxicity and its safety has been clarified (Devaraj, et al., 2011).

According to the increasing interest in working on the area of herbal medicine to find safer and more effective herbal remedies with lesser side effects, toxicity studies are important to achieve this goal. *Lagodium flexuosum* which is a climbing fern was evaluated for its toxicological effects. This plant is found in India and Western Ghat area. Different parts of the plant such as leaf paste, root and rhizome are useful to cure jaundice. *Lagodium flexuosum* is rich in saponins. Acute toxicity study of this plant up to 5g/kg revealed no behavioral changes and also no alterations in biochemical parameters such as AST, ALT, urea, triglycerides, cholesterol, glucose and Creatinine.
in comparison with normal control group. Regarding the absence of toxicity safety evidence, *Lagodium flexuosum* can be declared safe to be used as natural drug (Wills et al., 2012).

Toxicity studies have been done on numerous plants in different parts of the world. As an illustration, methanolic extract of *Pteleopsis hylodendron* stem bark has been evaluated for its toxicity. This plant is common among Cameroonian people because of its therapeutic values to treat chickenpox, measles, renal and hepatic ailments, sexually transmitted diseases as well as dropsy. This tree belongs to Combretaceae family. In some reports, the antioxidant and antimicrobial activities of ethyl acetate and methanolic extracts of its stem bark have been mentioned. It possess antimicrobial activity against microorganisms such as *B. cereus*, *Corynebacterium diphtheriae*, *K. pneumoniae*, *Proteus mirabilis* and *P. aeruginosa*. The acute toxicity evaluation of this plant was performed by oral administration of different doses of plant extract 2, 4, 6, 8 g/kg and all groups were observed for 3 hours to monitor toxicological symptoms. Some behavioral changes were seen in all experimental groups namely: breathing quickly, lack of movement, slower respond to exterior stimulants which were present for 5 days. In accordance to the result of this study, all animals pretreated with 6 and 8g/kg of plant extract died while mortality rates in treated groups with 2 and 4g/kg were forty and eighty during the first two days. The assessment of LD50 demonstrated 3.8 and 3 g/kg bw for male and female animal groups. Assessing the food and water intakes along with body weight indicated a perceptible decline in all three parameters dose dependently especially within the first week of the experiment. Increasing effect of stem bark methanolic extract of *Pteleopsis hylodendron* on some biochemical parameters such as ALT, AST and Creatinine has been recorded in a dose dependent manner. This study suggested that the extract of this plant can be consumed along with
being aware of its possible side effects due to effects on liver and kidney (Nana et al., 2011).
CHAPTER THREE: DESIGN, METHODS AND EXPERIMENTAL PROCEDURE

3.1 Collection of plant

Fresh *Teucrium zanonii* leaves were obtained from Ethno Resources Sdn Bhd, Selangor, Malaysia, and identified by comparison with the Voucher specimen deposited at the Herbarium of Rimba Ilmu, Institute of Science Biology, University of Malaya, Kuala Lumpur by the supervisor Professor Dr Mahmood Ameen Abdulla, Department of Molecular Medicine, Faculty of Medicine, University of Malaya, Kuala Lumpur. Next, fresh leaves were air dried and after that, were ground to get powder of *Teucrium zanonii* leaves for the extraction part of the experiment.

3.2 Preparation of plant extract

The leaves of *Teucrium zanonii* were ground into powder to ease the extraction process. In this afford, three concentrations of ethanol extraction was prepared. The dried and ground leaves of *T. zanonii* were weighted for 100 g and were placed into a 1L Schott bottle. One liter ethanol 95% was added in the ratio of (1:10) where 1g of dried and ground *T. zanonii* is to 10ml ethanol 95%. The Schott bottle was left in room temperature for approximately 3 to 7 days. After that, a rough pieces strainer was applied to remove the powder. Then the extract was filtered again using filter paper (chm) by chm lab group (filter paper circle size 185mm) to remove the crude part. To separate the ethanol from the extract, Buchi rotary evaporator Rota vapor (R-215) was used under reduced pressure. The extract was kept in a falcon tube and put in the oven (43°C) to make sure the ethanol was completely evaporated and dispersed, and lastly it was kept at -20 °C until the usage time. The amount of final extract was 9.44 g.
3.3 Acute toxicity experiment

3.3.1 Animal grouping and acute toxicity test

Acute toxicity test was done to determine the safe dose of plant extract. Thirty-six healthy *Sprague Dawley* rats (18 male and 18 female) 8 to 10 weeks old were needed in this study. Their body weight ranged between 225 to 250 g and obtained from the Animal House, Faculty of Medicine, University of Malaya, Kuala Lumpur (Ethics No. ISB/30/05/2012/RA (R)). They were assigned into 3 groups equally labeled as vehicle (10% tween 20) 5 ml/kg, 2 g/kg and 5 g/kg body weight of plant extract in vehicle. All animals were housed separately (one rat in each cage) with a period of 12 hours light and 12 hours dark in a condition of 50% to 60% humidity to retain a normal circadian rhythm. All rats were fasted overnight (food but not water) prior dosing. The reason for fasting was to eradicate food inside the gastrointestinal tract that may complicate observation of the test material. Food withholding was done for further 3 to 4 hours after dosing. Observation of animals for the onset of clinical or toxicological symptoms was done for 30 min and 2, 4, 24 and 48 hour after the administration. The animals were sacrificed on 15th day as mentioned by AlRashdi et al. (2012) with sight modification. Histological (liver and kidney) parameters were determined. Animal handling was according to experimental protocols approved by the Committee for the Supervision of Animal Experimentation in University of Malaya. There were no abnormalities and alterations in behavior or body weight during the period of experimental observation.
Table 3.1: Acute toxicity test of *Teucrium zanonii* extract using 10% Tween 20.

<table>
<thead>
<tr>
<th>Species of the animal</th>
<th>Sprague Dawley rats</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>8 to 10 weeks</td>
</tr>
<tr>
<td>Number of animal</td>
<td>12 rats in each group (6 rats of each sex per dose level)</td>
</tr>
<tr>
<td>Dose</td>
<td>Two dose level (2 g/kg and 5 g/kg body weight of prepared plant extract plus a control group 5 ml/kg of 10% Tween 20)</td>
</tr>
<tr>
<td>Observation period</td>
<td>14 days</td>
</tr>
</tbody>
</table>

3.4 Preparation of control studies for anti-ulcer experiment

3.4.1 Preparation of omeprazole as positive control

Omeprazole is a proton pump inhibitor (PPI) which works by blocking the secretion of gastric acid and in this effort, was used as a positive control. This drug is widely used for treating gastric ulcer. Omeprazole was utilized as a reference for the action of anti-ulcerogenic effect of *Teucrium zanonii* extract. In this study Omeprazole 20 mg capsule was supplied by Immunology Laboratory, Department of Molecular Medicine, Faculty of Medicine, University of Malaya, Kuala Lumpur.

3.4.2 Preparation of 10% Tween 20

Tween 20 acts as solvent in this study and it was used as a negative control for the formation of ulcer in rat’s stomach. Ten percent of Tween 20 was prepared by adding 10 ml of absolute Tween 20 to 90 ml of distilled water. The mixture was mixed using vortex.
3.5 Preparation of oral application of *T. zanonii*

Oral applications of *T. zanonii* were prepared in three concentrations, 50 mg/kg as low dose (LD), 100 mg/kg as medium dose (MD) and 200 mg/kg as high dose (HD). Standard weight for each rat was considered as 200 g. After weighing them one by one, the feeding was done based on the weight and the group of rats.

3.6 Experimental animals for anti ulcer experiment

For this study, thirty male *Sprague Dawley* (SD) rats which were obtained from the Animal House, Faculty of Medicine, University of Malaya were used. The estimated age of animals was around 6-8 weeks and the approximate weight was about 225-250 g. All animals have been kept under controlled condition at room temperature (22-24°C), 50-60% humidity in a standard light-dark cycle (12 h light, 12 h dark) and free access to tap water and standard diet. They were adapted to standard laboratory conditions for around two weeks.

3.7 Steps of the anti-ulcer experiment

3.7.1 Experimental procedure

Thirty male SD rats were divided into five groups. Six rats in each group, all groups were under fasting state for 24 hours but they were allowed to drink water only before the day of the experiment. The purpose for fasting was to inhibit the cross reaction of gastric content with administered treatments. All rats were orally given particular pretreatment based on their groups and their weight.
3.7.2 Animals pretreatment

Table 3.2: Groups of rats regarding to various pretreatment

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of rats</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (LD)</td>
<td>6</td>
<td>All rats in this group will be treated by 5 ml/kg of 50 mg/kg of T.z ethanol extract</td>
</tr>
<tr>
<td>2 (MD)</td>
<td>6</td>
<td>All rats in this group will be treated by 5 ml/kg of 100 mg/kg of T.z ethanol extract</td>
</tr>
<tr>
<td>3 (HD)</td>
<td>6</td>
<td>All rats in this group will be treated by 5 ml/kg of 200 mg/kg of T.z ethanol extract</td>
</tr>
<tr>
<td>4 Positive Control</td>
<td>6</td>
<td>All rats in this group will be treated by 5 ml/kg of omeprazole 20 mg/kg</td>
</tr>
<tr>
<td>5 Negative Control</td>
<td>6</td>
<td>All rats in this group will be treated by 5 ml/kg of tween 20 10%</td>
</tr>
</tbody>
</table>

3.7.3 Induction of ulcer

One hour after exposing all animals to special treatment, ulcer induction was done by using ethanol 95% (absolute ethanol) and all rats were left again for one hour more. After that, all animals were sacrificed by over dose injection of ketamine and xylazine and their stomachs were instantly taken out according to the illustrated procedure by Al-Bayaty et al. (2011) with some modifications.
3.8 Experimental evaluations

3.8.1 Ulcer area estimation

Performance of ulcer area estimation was to see if the ulcer lesions are present or not. All stomachs were removed and the intact stomachs were put in sample container in normal saline 0.9% to keep them undamaged. The stomach was then observed for ulcer lesion and stomach content was removed into centrifuge tube. All samples were cut along the greater curvature. Content of stomachs will be used in mucosa weight examination. The stomachs were washed with normal saline to clean the surface.

Gastric lesions were counted and the existence of them was proved by utilizing dissecting microscope (1.8x) with a square-grid eyepiece (2mm). Ulcerated gastric mucosa appeared as hemorrhagic elongated bands parallel to the long axis of gastric.

The ulcerated area was calculated and determined as ulcerative index (UA) and the inhibition percentage was estimate by the following formula.

\[
\text{Inhibition \%} = \left( \frac{\text{UA control} - \text{UA treated}}{\text{UA control}} \right) \times 100
\]

3.8.2 Examination of mucosa weight

The purpose of performing this part of the experiment was to know the weight of the mucosa layer which inhibits the lesions from forming. During the stomach dissection, their content was taken and put into centrifuge tube and the tubes were centrifuged at 1000 rpm for 10 minutes. The aim of centrifuging was to separate the mucosa and stomach’s acid. The supernatant part was used in pH examination before being removed and the sediment (mucosa) was retain in falcon tube and weighted by weight balance for determination of the mucosa.
3.8.3 **pH examination**

The pH examination was done to know the acidity of the stomach’s content by collecting the supernatant from mucosa weight experiment and putting in falcon tube. Supernatant in the same group was put in the same falcon tube because of limited volume. At last, acidity of collected supernatant was determined using a pH meter.

3.8.4 **Histological examination of gastric mucosa**

Solution of 10% buffered formalin was used to fix the stomachs for 1 to 4 days. Then, stomachs were trimmed and wrapped with cold paper and kept in the cassette. After that, the cassettes were drenched in fresh 10% buffered formalin for at least another 2 days to make sure that fixation has been done properly. Processing the stomachs was done using automated tissue processing machine. The next step was to fix the biopsied stomachs in paraffin wax to obtain 5 micrometer tissue sections by using rotary microtome. At last, obtained tissue sections were stained with haematoxylin and eosin (H&E) and observed under light microscope to see the existence of gastric lesion and mucosa layer to recognize the presence of any histological changes such as edema, necrosis, hemorrhage and congestion.

3.8.5 **In vitro antioxidant activity of plant extract**

In the present study, two methods were used to test and measure antioxidant activity of plant extract.

3.8.5.1 **Ferric-reducing Antioxidant power (FRAP) assay**

This method is a popular method with cheap and reasonable procedure to measure the ferric reducing ability (antioxidant power) of a sample. The basis of this assay is the reduction of ferric tripyridyltriazine (FeIII-TPTZ) complex and generating a
blue color ferrous tripyridyltriazine by antioxidant. This reaction will be done in acidic condition (Antolovich et al., 2002). The maximum absorption was measured at 593 nm. Generation and progression of blue color demonstrate the presence of antioxidant in plant extract sample. Thus, the correlation between FRAP assay and molar concentration of antioxidant is deemed in a linearly pattern. The assay was done in 37°C in 96 holes micro titer plate. The assay mixture consist of 10 µl of sample (plant extract) and 300 µl of FRAP reagent.

FRAP reagent contained of 300 mmol/L Acetate buffer pH 3.6, 10 mmol TPTZ in 40 mmol/L HCL and 20 mmol/L FeCl3.6H2O (Benzie et al., 1996) which the required amount of them was 25ml, 2.5ml and 2.5 ml respectively. FeSO4.H2O was used as standard. In addition, A.C (Ascorbic Acid), BHT (Butylated Hydroxytoluene), G.A (Gallic Acid) and Q (Quercetin) were used as control.

FRAP values were obtained spectrophotometrically under a kinetic mode by applying a microplate reader (KCjunior) for four minutes at 593 nm. The comparison between the absorbance of sample and calculation with the standard curve obtained from iron (II) sulfate-heptahydrate (FeSO4.7H2O) were conducted.

3.8.5.2 DPPH Free Radical Scavenging Activity Test

Capability of the plant extract to remove 1, 1-diphenyl-2-picryl-hydrazyl free radical was ascertained by DPPH assay to ascertain the free radical scavenging activity. DPPH free radical possesses a delocalized electron, thus the molecule cannot be dimerised. This delocalization prompts dark purple color. By mixing DPPH with a hydrogen atom donor, it will be reduced and turns to light yellow color. This method
has been established by Marsden Blois apparently half a century ago (Molyneux, 2004).

Efficient concentration, EC$_{50}$ also called IC$_{50}$ is a significant parameter to elucidate the result of the method which has been expressed as substrate concentration that can quench 50% of DPPH activity (Molyneux, 2004). In 96 holes well plate, 1ml of plant extract and 5 ml of freshly prepared 0.1 mM DPPH ethanolic solution were mixed and maintained in the dark for 30 minutes. Reaction mixture’s absorbance was quantified at 515nm using a spectrophotometer. The plant extract was substituted with 1ml ethanol for the blank.

The percentage of free radical scavenging activity was calculated by the formula below:

\[
\text{Scavenging activity percentage (%) = } \frac{\text{Blank Abs mean at 515 nm} - \text{Sample Abs mean at 515nm}}{\text{blank Abs mean at 515nm}} \times 100
\]

The standard for DPPH assay was Vit.C (Ascorbic acid), and the controls of the experiment were GA (Gallic acid), Q (Quercetin) and BHT (Butylated Hydroxytoluene).

### 3.8.6 Antimicrobial activity of *T. zanonii* extract

#### 3.8.6.1 Disk Diffusion Susceptibility Test

This test was done in two steps, preparing of Mueller-Hinton agar plate and the preparation of inoculums. For preparation of Mueller-Hinton agar medium, the powder was obtained from BBL, BD Company, USA-France (Ref No: 211438, 500 g). Thirty eight g of powder was suspended in one liter of purified water and reconstituted. Then, the solution was heated with recurrent agitation and boiled for 1 minute to get perfectly dissolved powder. The obtained liquid was autoclaved in 121°C for 15 minutes and after
that, was poured in sterile plates to be solidified at room temperature and kept at 4°C to be used. The second step of Disk Diffusion Susceptibility Test (preparation of inoculums) was performed by a sterile inoculating loop. Few isolated colonies of organisms was touched with the sterile inoculating loop, dispersed into Broth medium and incubated overnight to grow perfectly. Previously, Broth medium was prepared by dispersing 38 g of Brain Heart Infusion Broth LAB 049, UK in 1L of deionized water, stocked for 10 minutes, agitated to mix and warmed calmly to be dissolved. After that, it was poured into final container and sterilized by autoclaving for 15 minutes at 121°C. After incubating the organisms, 50µl of each Broth medium was taken and scattered on the surface of Mueller-Hinton agar plate. One agar plate for each bacterial species was needed, totally 6 plates; for B. subtilis, E. coli, S. aureus, P. aeruginosa, Klebsiella pneumonia and Methicillin-resistant Staph.aureus (MRSA). After inoculation of Mueller-Hinton agar plates by streaking the plate via swap, impregnated disks with different concentration of plant extract (50 mg/ml, 100 mg/ml, 150 mg/ml, and 200 mg/ml) which were allowed to be evaporated were placed on the surface of the agar plate along with positive control and negative control. Negative control disk was impregnated with 20 µl of ethanol for all tested bacterial species but for positive control disks, various antibiotic disks were used depending on the bacterial species. Vancomycin for (MRSA), Staphylococcus aureus and B. subtilis, Gentamicin for Klebsiella pneumonia and P. aeruginosa. After that, the plates were been incubated overnight (18h) at 37°C. At last, antibacterial activity was demonstrated at the end of the incubation time by measuring the diameter of inhibition zone. Inhibition zone of 14mm or more were considered as high and remarkable antimicrobial activity. Disk Diffusion Susceptibility test was performed according to described method by Hudzicki (2012) which has been modified slightly.
(MIC) Minimum Inhibitory Concentration was determined by micro-dilution method. One hundred mg of plant extract was dissolved in 1ml of 10% DMSO. Then, different dilutions of plant extract were prepared in 96-well plate.

Hundred µl of plant extract was added to the first well of each bacteria and the rest of 5 wells in each row for each bacteria which have been loaded with 50 µl of broth medium. Next, 50 µl of stock from the first well was transferred to the next well. From the second well, another 50 µl was transferred to the third and so on and so forth until the 6\textsuperscript{th} well of each row for tested species. At last, all wells were loaded with 10 µl of each tested species, the well plate were covered with foil and incubated overnight. Cultures of those different concentration of organisms obtained from micro-dilution method on Mueller-Hinton agar plate was to see if any of the tested organisms has Minimal inhibitory concentration.

3.9 Statistical analysis

Student version of SPSS program was used in this study, in order to analysis the data of this research. All of the data values in each group were expressed as mean ± S.E.M. Statistical differences between treatments was performed through one way ANOVA with the level of significance set at p < 0.05.
CHAPTER FOUR: RESULTS

4.1 Acute toxicity test results

The acute toxicity test was performed by treating animals with two different doses of T. zanonii plant extract (2 g/kg and 5 g/kg). All animals were under observation for 14 days. All rats survived and in the observational duration of 2 weeks, there were no remarkable toxicological symptoms, signs of abnormalities such as alteration in body weight and behavioral changes along with macroscopic findings in mentioned treated rat groups. In this effort, the effect of plant extract on kidney and liver parameters like sodium, potassium, chloride, CO₂, anion gap, urea and creatinine as renal parameters along with total protein, albumin, globulin, TB, CB, AP, ALT, AST and GGT as liver parameters in administered rats were considered and displayed no noticeable changes in comparison with normal control rats. The obtained results from present study proved the safety of T. zanonii extract at high doses without any acute toxicity effects and lethal dose (LD₅₀) in both sexes was higher than 5 mg/kg body weight. Effect of T. zanonii on renal and liver function of male and female rats are presented in Table 4.1 (A, B) and Table 4.2 (A, B) respectively.
Table 4.1 A: Effect of *T. zononii* on renal function of male rats. LD: Low dose, HD: high dose. Values are expressed as the mean ± S.E.M. There are no significant differences between groups. Significant value at p < 0.05.

<table>
<thead>
<tr>
<th>Dose (g/kg)</th>
<th>Sodium (mmol/L)</th>
<th>Potassium (mmol/L)</th>
<th>Chloride (mmol/L)</th>
<th>CO₂ (mmol/L)</th>
<th>Anion gap (mmol/L)</th>
<th>Urea (mmol/L)</th>
<th>Creatinine (µmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicl (10% Tween 20)</td>
<td>142.08 ± 3.59</td>
<td>4.88 ± 0.08</td>
<td>104.68 ± 2.82</td>
<td>24.82 ± 0.85</td>
<td>18.57 ± 0.88</td>
<td>5.20 ± 0.55</td>
<td>34.83 ± 2.87</td>
</tr>
<tr>
<td>LD (2 g/kg)</td>
<td>143.50 ± 2.52</td>
<td>5.00 ± 0.10</td>
<td>106.33 ± 1.02</td>
<td>22.60 ± 1.38</td>
<td>18.27 ± 0.76</td>
<td>6.06 ± 0.85</td>
<td>32.67 ± 3.19</td>
</tr>
<tr>
<td>HD (5 g/kg)</td>
<td>145.15 ± 2.84</td>
<td>4.93 ± 0.07</td>
<td>105.17 ± 2.54</td>
<td>23.92 ± 1.03</td>
<td>19.25 ± 1.14</td>
<td>5.63 ± 0.49</td>
<td>35.13 ± 3.96</td>
</tr>
</tbody>
</table>
Table 4.1B: Effect of *T. zononii* on renal function of female rats. LD: Low dose, HD: high dose. Values are expressed as the mean ± S.E.M. There are no significant differences between groups. Significant value at p < 0.05.

<table>
<thead>
<tr>
<th>Dose</th>
<th>Sodium (mmol/L)</th>
<th>Potassium (mmol/L)</th>
<th>Chloride (mmol/L)</th>
<th>CO₂ (mmol/L)</th>
<th>Anion gap (mmol/L)</th>
<th>Urea (mmol/L)</th>
<th>Creatinine (µmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicl (10% Tween 20)</td>
<td>144.23 ± 1.40</td>
<td>4.82 ± 0.12</td>
<td>105.43 ± 2.67</td>
<td>24.33 ± 1.21</td>
<td>18.00 ± 0.52</td>
<td>7.73 ± 0.51</td>
<td>40.06 ± 2.79</td>
</tr>
<tr>
<td>LD (2 g/kg)</td>
<td>142.47 ± 2.89</td>
<td>4.57 ± 0.16</td>
<td>102.83 ± 1.37</td>
<td>22.95 ± 0.81</td>
<td>17.12 ± 0.67</td>
<td>7.97 ± 0.69</td>
<td>42.00 ± 3.14</td>
</tr>
<tr>
<td>HD (5 g/kg)</td>
<td>140.87 ± 2.08</td>
<td>4.51 ± 0.09</td>
<td>107.03 ± 2.93</td>
<td>21.95 ± 0.77</td>
<td>17.87 ± 0.49</td>
<td>8.38 ± 0.62</td>
<td>43.37 ± 2.52</td>
</tr>
</tbody>
</table>
Table 4.2 A: Effect of *T. zononii* on liver function of male rats. TB: Total bilirubin; CB: Conjugated bilirubin; AP: Alkaline phosphatase; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; GGT: G-Glutamyltransferase. LD: Lowdose, HD: highdose. Values are expressed as the mean ± S.E.M. There are no significant differences between groups. Significant value at p < 0.05.

<table>
<thead>
<tr>
<th>Dose</th>
<th>Totalprot</th>
<th>Album</th>
<th>Globul</th>
<th>TB</th>
<th>CB</th>
<th>AP</th>
<th>ALT</th>
<th>AST</th>
<th>GGT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(g/L)</td>
<td>(g/L)</td>
<td>(g/L)</td>
<td>(µmol/L)</td>
<td>(µmol/L)</td>
<td>(IU/L)</td>
<td>(IU/L)</td>
<td>(IU/L)</td>
<td>(IU/L)</td>
</tr>
<tr>
<td>Vehicle</td>
<td>61.54</td>
<td>9.58</td>
<td>52.12</td>
<td>2.27</td>
<td>1.00</td>
<td>153.00</td>
<td>50.80</td>
<td>173.00</td>
<td>3.17</td>
</tr>
<tr>
<td>(10% Tween 20)</td>
<td>± 2.47</td>
<td>± 0.67</td>
<td>± 2.04</td>
<td>± 0.17</td>
<td>± 0.00</td>
<td>± 48</td>
<td>± 3</td>
<td>± 56</td>
<td>± 0.27</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>LD(2 g/kg)</td>
<td>58.85</td>
<td>8.62</td>
<td>49.77</td>
<td>2.13</td>
<td>1.00</td>
<td>157.00</td>
<td>48.10</td>
<td>180.00</td>
<td>3.69</td>
</tr>
<tr>
<td></td>
<td>± 1.88</td>
<td>± 0.46</td>
<td>± 1.60</td>
<td>± 0.16</td>
<td>± 0.00</td>
<td>± 37</td>
<td>± 8</td>
<td>± 83</td>
<td>± 0.46</td>
</tr>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HD(5 g/kg)</td>
<td>60.35</td>
<td>9.37</td>
<td>50.33</td>
<td>2.05</td>
<td>1.00</td>
<td>155.00</td>
<td>46.50</td>
<td>175.00</td>
<td>3.36</td>
</tr>
<tr>
<td></td>
<td>± 2.65</td>
<td>± 0.58</td>
<td>± 2.54</td>
<td>± 0.14</td>
<td>± 0.00</td>
<td>± 12</td>
<td>± 5</td>
<td>± 66</td>
<td>± 0.35</td>
</tr>
<tr>
<td></td>
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</tr>
</tbody>
</table>
Table 4.2 B: Effect of *T. zononii* on liver function of female rats. TB: Total bilirubin; CB: Conjugated bilirubin; AP: Alkaline phosphatase; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; GGT: G-Glutamyltransferase; LD: Low dose, HD: High dose. Values are expressed as the mean ± S.E.M. There are no significant differences between groups. Significant value at p < 0.05.

<table>
<thead>
<tr>
<th>Dose</th>
<th>Total Protein (g/L)</th>
<th>Albumin (g/L)</th>
<th>Globulin (g/L)</th>
<th>TB (µmol/L)</th>
<th>CB (µmol/L)</th>
<th>AP (IU/L)</th>
<th>ALT (IU/L)</th>
<th>AST (IU/L)</th>
<th>GGT (IU/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle (10% Tween 20)</td>
<td>64.73 ± 3.16</td>
<td>11.17 ± 0.37</td>
<td>53.67 ± 1.28</td>
<td>2.00 ± 0.00</td>
<td>1.00 ± 0.00</td>
<td>108.8 ± 5</td>
<td>43.47 ± 2.96</td>
<td>171.5 ± 7.84</td>
<td>3.67 ± 0.47</td>
</tr>
<tr>
<td>LD(2 g/kg)</td>
<td>63.67 ± 2.17</td>
<td>11.04 ± 0.45</td>
<td>51.35 ± 2.16</td>
<td>2.00 ± 0.00</td>
<td>1.00 ± 0.00</td>
<td>95.83 ± 3</td>
<td>41.85 ± 2.76</td>
<td>172.4 ± 5.99</td>
<td>3.50 ± 0.50</td>
</tr>
<tr>
<td>HD(5 g/kg)</td>
<td>65.38 ± 2.68</td>
<td>11.50 ± 0.48</td>
<td>53.01 ± 2.72</td>
<td>2.00 ± 0.00</td>
<td>1.00 ± 0.00</td>
<td>101.6 ± 8</td>
<td>44.52 ± 3.88</td>
<td>176.8 ± 6.37</td>
<td>3.00 ± 0.62</td>
</tr>
</tbody>
</table>
### 4.2 Ulcer area evaluation

Table 4.3: Anti-ulcerogenic effect of *Teucrium zanonii* extract against ethanol induced gastric ulcer in experimental rats. All values have been expressed as mean ±S.E.M of 6 animals. Mean with different superscripts are significantly different at p < 0.05*

<table>
<thead>
<tr>
<th>Animal groups</th>
<th>Number of rats</th>
<th>Pretreatment (5 ml/kg dose)</th>
<th>Mucosa weight (g) ±</th>
<th>PH of gastric content</th>
<th>Ulcer area (mm2) ±</th>
<th>Inhibition percentage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6</td>
<td>Tween 20-10% (ulcer control)</td>
<td>0.512 ± 0.015</td>
<td>2.95 ± 0.4</td>
<td>850.00 ± 3.65</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>6</td>
<td>OMP (positive control 20 mg/kg)</td>
<td>0.46 ± 0.025</td>
<td>5.60 ± 0.5*</td>
<td>178 ± 3.65*</td>
<td>79.00</td>
</tr>
<tr>
<td>3</td>
<td>6</td>
<td>Tz (50mg/kg)</td>
<td>2.95 ± 0.031*</td>
<td>5.82 ± 0.6*</td>
<td>48.00 ± 23.56*</td>
<td>94.35</td>
</tr>
<tr>
<td>4</td>
<td>6</td>
<td>Tz (100mg/kg)</td>
<td>1.90 ± 0.020*</td>
<td>3.74 ± 0.4*</td>
<td>48.00 ± 21.25*</td>
<td>94.35</td>
</tr>
<tr>
<td>5</td>
<td>6</td>
<td>Tz (200mg/kg)</td>
<td>2.11 ± 0.035*</td>
<td>4.60 ± 0.58*</td>
<td>60.0000 ± 28.84*</td>
<td>92.94</td>
</tr>
</tbody>
</table>
Figure 4.1: The effect of *T. zanonii* extract on ulcer inhibition percentage in different pretreated groups (%)

The above bar chart shows the various pretreated groups versus its inhibition percentage in a comparative order. Inhibition percentage raised tangibly in LD, MD, HD groups in comparison with negative control group and positive control group. All values were expressed as mean ± S.E.M of 6 animals in each group.

Figure 4.2: The effect of *T. zanonii* extract on ulcer area in groups of pretreatments.

Ulcer area in negative control group is significantly higher compare to 4 other groups in this study. This shows ulcer formation in negative control group is
prominently more than positive control, HD, MD and LD groups. From this chart, lowest ulcer formation can be deduced in LD and MD groups which indicate better protective activity against gastric ulcer. All values have been expressed as mean ± S.E.M of 6 animals in each group.

4.3 Mucus weight examination result

As indicated in Figure 4.3 and Table 4.4, mucus production level is obviously higher in LD, HD and MD groups which were pretreated with different dose of *T. zanonii* ethanolic extract compared to positive control and negative control groups. As it is deductible, highest mucus production level was reported in LD group. All values have been expressed as mean ± S.E.M of 6 animals in each group.

Table 4.4: The effect of *Teucrium zanonii* extract on mucus weight (mucus production) in ethanol-induced gastric ulcer in rats. (Mean ± S.E.M)

<table>
<thead>
<tr>
<th>Animal group</th>
<th>Number of rats</th>
<th>Pretreatments</th>
<th>Mucus weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(5 ml/kg dose)</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>6</td>
<td>10% Tween20 (ulcer control)</td>
<td>0.51 ± 0.02</td>
</tr>
<tr>
<td>2</td>
<td>6</td>
<td>OMP (positive control 20 mg/kg)</td>
<td>0.46 ± 0.03</td>
</tr>
<tr>
<td>3</td>
<td>6</td>
<td>Tz (50mg/kg)</td>
<td>2.95 ± 0.03*</td>
</tr>
<tr>
<td>4</td>
<td>6</td>
<td>Tz (100mg/kg)</td>
<td>1.90 ± 0.02*</td>
</tr>
<tr>
<td>5</td>
<td>6</td>
<td>Tz (200 ml/kg)</td>
<td>2.11 ± 0.04*</td>
</tr>
</tbody>
</table>
The effect of *T. zanonii* extract on mucus weight in different groups of animals.

### 4.4 pH examination results

Table 4.5: The effect of *Teucrium zanonii* extract on pH level (Mean ± S.E.M) of ethanol-induced gastric ulcer in experimental rats.

<table>
<thead>
<tr>
<th>Animal groups</th>
<th>Number of rats</th>
<th>Pretreatment</th>
<th>pH level (5 ml/kg dose)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6</td>
<td>10% Tween20 (ulcer control)</td>
<td>2.95 ± 0.4</td>
</tr>
<tr>
<td>2</td>
<td>6</td>
<td>OMP (positive control 20 mg/kg)</td>
<td>5.60 ± 0.5*</td>
</tr>
<tr>
<td>3</td>
<td>6</td>
<td>Tz (50mg/kg)</td>
<td>5.82 ± 0.6*</td>
</tr>
<tr>
<td>4</td>
<td>6</td>
<td>Tz (100mg/kg)</td>
<td>3.74 ± 0.4*</td>
</tr>
<tr>
<td>5</td>
<td>6</td>
<td>Tz (200mg/kg)</td>
<td>4.60 ± 0.5*</td>
</tr>
</tbody>
</table>
The evaluation of pH reveals that, stomach pH level in low dose (LD) group of *T. zanonii* extract is remarkably higher than other animal groups of the experiment. Low dose of tested plant was more effective in enhancing gastric pH to protect against ulcer formation. All values have been expressed as mean ± S.E.M of 6 animals in each group.

### 4.5 Gross evaluation (Macroscopic examination)

![Figure 4.5: Macroscopic appearance of gastric mucosa in rats pretreated with only 10% Tween 20 (ulcer control) in group 1.](image)
All rats in ulcer control group (group 1) were pretreated with 5ml/kg of 10% Tween 20 before induction of ulcer by absolute ethanol showed the existence of thick and dark red streaks on gastric wall which indicate intensive ulceration that can be observed by naked eyes while the ulcer area of this group was 850.00 ± 3.65 mm$^2$.

![Figure 4.6: Macroscopic appearance of gastric mucosa in rats pretreated with omeprazole (20 mg/kg) positive control in group 2.](image)

Rats of this group were pretreated by 5 ml/kg of omeprazole 20 mg/kg before ulcer induction by absolute ethanol. Macroscopic appearance of gastric mucosa in rats of this group indicate less red streaks and fewer lesions with less severity in gastric wall, thus showing milder injuries compared to injuries seen in ulcer control rats. Meanwhile, the ulcerated area of this group was 178 ± 3.65 mm$^2$. 
All rats in (LD) group were pretreated with 5 ml/kg of 50 mg/kg of *T. zanonii* extract before the ulcer induction via absolute ethanol. Macroscopic appearance of gastric mucosa in rats of this group indicate pale red streaks in gastric wall mucosa that reveal milder lesions compare to lesions observed in ulcer control group. Ulcer area in this group was $48 \pm 23.56 \text{ mm}^2$. 

Figure 4.7: Macroscopic appearance of gastric mucosa in rats pretreated with (50 mg/kg) of *T. Zanonii* extract (LD). Group 3

Figure 4.8: Macroscopic appearance of gastric mucosa in rats pretreated with (100 mg/kg) of *T. zanonii* extract. (MD) in group 4.
Figure 4.9: Macroscopic appearance of gastric mucosa in rats pretreated with (200 mg/kg) of *T. zanonii* extract (HD) in group 5.

All rats in (MD) group were pretreated with 5 ml/kg of 100 mg/kg of *T. zanonii* extract before the ulcer induction via absolute ethanol. Macroscopic appearance of gastric mucosa in rats of this group shows only a few red lines on gastric wall, considerably milder lesions compare to lesions seen in ulcer control group. The ulcer area was $48.00 \pm 21.25051 \text{mm}^2$.

All rats in (HD) group were pretreated with 5ml/kg of 200 mg/kg of *T. zanonii* extract before the ulcer induction via absolute ethanol. Some lesions can be observed in gastric wall of rats in this group, which still are notably less than control group and with remarkably less severity. However, ulcer area in this group was $60 \pm 28.83997 \text{mm}^2$. 
4.6 Immunohistochemistry of gastric lesions

Figure 4.10 shows the histological study of ethanol induced gastric mucosal damage in rats pretreated with 10% Tween 20 as negative control group indicates disruption of surface epithelium and mucosal damage, edema and also leucocytes infiltration (H&E stain 10x).

Figure 4.10: Histological section of gastric mucosa in rats pretreated with 5 ml/kg of 10% Tween 20 as (ulcer control group).
Figure 4.11 shows the histological study of ethanol induced gastric mucosal damage in rats administered by omeprazole as positive control group. Mild and faint disruption of surface epithelium along with mild edema and leucocytes infiltration of submucosal layer can be observed. (H &E stain, 10x).

Figure 4.11: Histological section of gastric mucosa in rats pretreated with 5 ml/kg omeprazole (20 mg/kg) as positive control group.
Figure 4.12: Histological section of gastric mucosa in rats pretreated with 5 ml/kg of 50 mg/kg of \textit{T. zanonii} extract (LD) group.

Figure 4.12 demonstrates the histological evaluation of ethanol induced gastric injuries in experimental group pretreated with 50 mg/kg \textit{T. zanonii} extract as low dose group. High protection level of gastric mucosa along with no edema and leucocytes infiltration can be seen which is notably less than negative control group (H&E stain, 10x).
Figure 4.13: Histological section of gastric mucosa in rats pretreated with 5 ml/kg of 100 mg/kg of *T. zanonii* extract (MD) group.

Figure 4.13 indicates the histological evaluation of pretreated animal group with 100 mg/kg of *T. zanonii* extract as medium dose group, point out no disruption of surface epithelium, no edema in submucosal layer and the absence of leucocytes infiltration (H&E stain, 10x).
Figure 4.14: Histological evaluation of gastric tissue in (HD) group (200 mg/kg) of *T. zanonni* extract.

Figure 14.4 reveal mild disruption of surface epithelium, along with faint edema in submucosal layer and low leucocytes infiltration level in animal group pretreated by 200 mg/kg of *T. zanonii* extract as high dose group (H&E stain, 10x).

### 4.7 Antioxidant result

#### 4.7.1 FRAP assay

Table 4.6: Antioxidant result, FRAP value (in vitro). All values have been expressed as mean ±S.E.M of 6 animals. Mean with different superscripts are significantly different at p < 0.05

<table>
<thead>
<tr>
<th>Samples</th>
<th>FRAP (mmol/100g) ± S.E.M</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Teucrium zanonni</em></td>
<td>501.7 ± 0.00</td>
</tr>
<tr>
<td>AC (vit C)</td>
<td>745.7 ± 0.004</td>
</tr>
<tr>
<td>BHT</td>
<td>1098.7 ± 0.143</td>
</tr>
<tr>
<td>Quercetin</td>
<td>1516.3 ± 0.012</td>
</tr>
<tr>
<td>Gallic acid</td>
<td>1746.3 ± 0.002</td>
</tr>
</tbody>
</table>
Figure 4.15 indicates a direct ratio between standard concentration and its absorbance. (Higher absorbance in higher concentration)
Figure 4.16 shows the antioxidant activity of ethanolic extract of *T. zanonii* leaves, but its activity was lower than controls of this study. As a result, it can be concluded that the extract cannot be considered as a very influential antioxidant compared to the controls.
4.7.2 DPPH assay of plant extract

Table 4.7: Different concentration of *T. zanonii* extract and their related inhibition percentage

<table>
<thead>
<tr>
<th>TZ (µg/ml)</th>
<th>% inhibition percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.25</td>
<td>-5.4</td>
</tr>
<tr>
<td>12.5</td>
<td>-32</td>
</tr>
<tr>
<td>25</td>
<td>-6.4</td>
</tr>
<tr>
<td>50</td>
<td>-17</td>
</tr>
<tr>
<td>100</td>
<td>22</td>
</tr>
</tbody>
</table>

DPPH assay was done for the extract, but according to the results of this study there was not IC$_{50}$ for ethanolic extract of *T.zanonii* plant in various concentrations, so it did not have free radical scavenging ability.
Figure 4.17: DPPH standard curve versus Ascorbic acid

Figure 4.17 displays a raised DPPH inhibition percentage of Ascorbic acid in a concentration dependant order.

4.8 Antibacterial result of T. zanonii ethanolic extract

The results obtained from T. zanonii leaves ethanolic extract to evaluate its antibacterial property did not show any antibacterial activity versus all 6 tested microorganisms in this study such as B. subtilis, E. coli, S. aureus, P. aeruginosa, Klebsiella pneumonia and Methicillin-resistant staph. aureus (MRSA).

Also, micro dilution was done to determine the (MIC) for all microorganisms but there was no any Minimal Inhibitory Concentration for even one of tested microorganisms.
Figure 4.18: Antimicrobial activity of *T. zanonii* against *B. subtilis*

Figure 4.19: Antimicrobial activity of *T. zanonii* against *E. coli*
Figure 4.20 Antimicrobial activity of *T. zanonii* against *S. aureus*

Figure 4.21 Antimicrobial activity of *T. zanonii* against *P. aeruginosa*
Figure 4.22 Antimicrobial activity of *T. zanonii* against *Klebsiella pneumonia*

Figure 4.23 Antimicrobial activity of *T. zanonii* against Methicillin-resistant *Staphylococcus aureus* (MRSA).
CHAPTER FIVE: DISCUSSION AND CONCLUSION

5.1 Experimental findings

The aim of this study was to ascertain the acute toxicity, anti-ulcer, antioxidant and antimicrobial effect of *Teucrium zanonii* ethanolic extract.

5.1.1 Acute toxicity findings

Despite the broad usage of different plants in medicine as curative agents, not many scientific studies have been performed to investigate and support their safety and effectiveness. Although *Teucrium zanonii* has shown various medical properties such as antiseptic, antipyretic, diuretic, hypoglycemic along with the potentiality to treat some diseases like intestinal problems, renal inflammatory and asthma (Abdelshafeek, et al., 2009; Abdelshafeek, et al., 2010), no research was done on the toxicity of its plant extract. The results of this research indicated the safety of plant extract according to absence of any behavioral changes, clinical symptoms and mortality after 14 days of observation. In accordance to the obtained results of this effort, oral lethal dose of *Teucrium zanonii* ethanolic extract is higher than 5 g/kg. The acute toxicity result derived from this research agrees with some other studies on acute toxicity of different plants like *Aegle- marmelos Corr* (Veerappan, et al., 2007) and *Pithecellobium Jiringa* (Aziz Ibrahim, et al., 2012). On the other hand, it disagrees with a research which was done on alcoholic extract of *Pteleopsis hyloendron* by Nana, et al. (2011) due to mortality and behavioral changes along with alterations in liver and kidney parameters dose dependently.

5.1.2 Anti-ulcer finding

As it has been pointed out in (Khan, et al., 2011) by Wallace, gastric ulcer is a continuous corrosion of gastric wall which can lead to perforation and serious
hemorrhagic lesion due to preventive effect on synthesis of prostaglandin, mucus production and production of bicarbonate. Inequality between defensive factors such as bicarbonate, prostaglandin with aggressive factors like acid, *H. pylori* and pepsin is the main accepted cause of peptic ulcer as speculated by Lakshmi in (Wasman, et al., 2010).

Anti-ulcerogenic effect of *Teucrium zanonii* leaves extract was evaluated to know the efficacy of this plant in preventing ulcer formation. It was done by assessing the ulcer formation which then was used to estimate the inhibition percentage, mucus weight and the pH of stomach content. Three different concentrations of plant ethanolic extract were prepared as LD group (50 mg/kg), MD group (100 mg/kg) and HD group (200 mg/kg) in addition to negative control and positive control. Negative control was to survey the perceptible difference of plant extract in inhibiting gastric ulcer.

Omeprazole which was used as positive control showed tangible results as anti-ulcerogenic agent while it inhibited the gastric ulcer formation up to 79%, with ulcer area measured $178 \pm 3.65$ mm$^2$ ($p < 0.05$). The mucus weight of this group was $0.46 \pm 0.025$g which indicates that omeprazole is a significant anti-ulcer drug. The pH of pretreated group with omeprazole was $5.60 \pm 0.5$, whereas 10% Tween 20, which was the negative control did not reveal any protection against gastric ulcer. The measured ulcer area was $850.00 \pm 3.65$ mm$^2$ ($p < 0.05$) along with the absence of any inhibition percentage and the obvious acidic pH of stomach was seen at $2.95 \pm 0.4$ in this group compare to other groups indicating that 10% Tween 20 cannot be considered as anti-ulcer agent and the mucus weight of this group was $0.512 \pm 0.015$ g.

Anti-ulcer activity of three different doses of *Teucrium zanonii* extract against ethanol induced gastric ulcer in experimental rats indicated that LD and MD plant extract showed highest inhibition percentage, which is 94.35% for LD along with good prevention of ulcer formation by $48.0000 \pm 23.56$ mm$^2$ ($p < 0.05$), meanwhile in MD group, ulcer prevention was $48.0000 \pm 21.25$ mm$^2$ ($p < 0.05$). Mucus weight and pH of
gastric content in LD group are 2.95 ± 0.031g and 5.82 ± 0.6 respectively and in MD group are 1.90 ± 0.020g and 3.74 ± 0.4. As it can be seen, mucus weight is higher in LD and MD groups in comparison with negative control while pH and mucosal weight in LD group are higher than OMP group and on the other hand, higher mucosal weight can be observed in MD group in comparison with OMP group although the pH is still lower than OMP group. Additionally, inhibition percentage in these two groups (LD and MD), both at 94.35% is higher than inhibition percentage in treated group with OMP at 79% obviously. Even in HD group where the rats were treated with 200 mg/kg plant extract, perceptible differences can be observed in mucus weight 2.11 ± 0.035 g, better inhibition percentage 92.94% and lower ulcer rate 60.00 ± 28.84 mm\(^2\) (p < 0.05) comparing to positive control group and negative control group.

The high anti-ulcer effect of *Teucrium zanonii* might be because of the presence of chemical compounds isolated from this plant such as sesquiterpenes, Flavonoids, triterpenes and phenolic acids. Richness of *Teucrium* species (Germenders) which belongs to Labiatae family (Lamiaceae) as a natural source of mentioned chemical constituents has been postulated in many studies (Abdelshafeek, et al., 2009; Abdelshafeek, et al., 2010; Galati, et al., 2000; Naghibi, et al., 2005; Sarkhail, 2011; Stankovic, et al., 2011)

As it has been mentioned in (Wasman, et al., 2010) by Goel and Sairam, peptic ulcer is a popular gastrointestinal medical problem despite its vague etiology but the imbalance between aggressive factors like acid and pepsin and defensive factors which lead to maintenance the unity of mucus has been admitted as the main cause of peptic ulcer.

On the other hand, Baron suggested in (Mahmood, et al., 2010) that acid and pepsin are at lower level of importance as ulcerogenic agents comparing to deficiency of defending mechanisms.
Stated by Sezabo, Marhuenda and Mutoh in (Mahmood, et al., 2010) ulcer induction by ethanol in experimental rats causes severe injuries in stomach which will begin with microvascular damages and leads to enhancing in vascular permeability. Direct toxic effect of ethanol prompts necrotic damages to gastric mucosa by decreasing mucus production and diminishing effect on bicarbonates discharge. Moreover, it has been declared by Glayin and Sezabo in Gupta et al. (2005) that ethanol gastric ulceration is a well known and accepted method to for assessment of gastroprotective activity. Ulcer induction by ethanol is due influential effect on and declining the gastric mucosal blood flow and diminishing level of glutathione and prostaglandin in addition to provoking of ischemia, production of free radicals and emancipating histamine, flow of potassium and sodium along with influxing of calcium.

Li in (Mahmood, et al., 2010) has cited that Omeprazole is a PPI with high level of usage popularity which acts by inhibitory effect on gastric acid secretion. This drug is particular for proton pump and it will be subjected to conversion to its active form in acidic surrounding. Then, a reaction will happen between the active form of omeprazole with the SH group of proton pump which through that prevents the acid secretion to occur.

The obtained results of this study indicated that the ethanolic extract of *Teucrium zanonii* leaves considerably shows anti-ulcer and cytoprotective ability in rats through diminishing ulcerative area, increasing pH and mucus weight, declining submucosal edema and leukocyte infiltration. Anti-ulcer and cytoprotective effect of this plant agrees with previous studies done by Mahmood, et al. (2010) to evaluate the anti-ulcer activity of (GPELE), Gupta, et al. (2005) to estimate the ulcer protective effect of (EETP) and some other works done all over the world on different plants, such as assessing the ameliorative effect of dates against gastric ulcer by Al-Qarawi, et al. (2005), which all demonstrated reduction in ulcer area, number of produced ulcers and
severity. Although treated rats with *Teucrium zanonii* leaves extract revealed high protection against gastric mucosal injuries along with lesser leukocytes infiltration and lower edema in addition to higher inhibition percentage and lesser ulcer area with lower range of ulcer index, but these gastroprotective effects are not dose dependent as clarified by the result of present study. Highest anti-ulcer effect was manifested in LD and MD pretreated groups by showing 94.35% inhibition percentage and smaller ulcer area up to 48,000 ± 23.56 and 48,000 ± 21.25 but not in HD group.

### 5.1.3 Antioxidant findings

Despite the importance of oxidative metabolism in keeping body cells alive, producing free radicals and other ROS is the side effect of it which affect the natural antioxidative enzymes such as SOD and Peroxidase (Antolovich, et al., 2002). As stated by Davasagayam in Khalaf, et al. (2008), usefulness of antioxidant based drugs in inhibiting of some ailments like stroke, cancer, diabetes, atherosclerosis and Alzheimer’s has been proved. Anxiety on security and safety of synthetic antioxidants has led to universal interest in finding natural derived antioxidants. In order to achieve this goal, many studies have been performed to discover the antioxidant potentiality of various plants. The acquired results of this research concurred with some other studies done on antioxidant activity of diverse herbs namely, antioxidant activity of some Indian plants by (Sharma, et al., 2009), antioxidant activity of some common plants by (Khalaf, et al., 2008), investigation of antioxidant activity of nettle by (Gülçin, et al., 2004) and assessment of antioxidant property of 30 Chinese medicinal herbs by (Wong, et al., 2006). In accordance to the result of this effort *Teucrium zanonii* can be considered as a natural source with antioxidant activity.
5.1.4 Antimicrobial findings

Medicinal plants have been used to treat different ailments. Nowadays, due to global problem with antibiotic resistant microorganisms, interest in finding natural antimicrobial agents is growing increasingly (Ozturk, et al., 2007). For this purpose, many studies have been performed to evaluate the antimicrobial activity of different medical plants. Antimicrobial activity of *Teucrium zanonii* leaves ethanolic extract did not show any antimicrobial effect on all 6 tested microorganisms which consist of *B. subtilis*, *S. aureus*, MRSA, *E. coli*, *P. aeruginosa* and *K. pneumonia*. Outcome of this research agrees with a study carried out to investigate the antimicrobial potentiality of *Verbena hastate* leaf extract by (Akuodor, et al., 2012), while it differs from the end results of other performed researches namely antimicrobial evaluations of *Side acuta burm* by (Obah, et al., 2007), antimicrobial ability of South African medicinal plants by (Van Vuuren, 2008) and assessment of antimicrobial property of *Ziziphora clinopodioides* by (Ozturk, et al., 2007). Based on the antimicrobial results of this study, ethanolic extract of *Teucrium zanonii* leaves did not manifest any antimicrobial effect on tested microorganisms. The outcome was predictable due to different potentiality and activity of various parts of the same plant.

5.2 Limitation of the study

There were several limitations and problems to cope with. One of them was the prolonged delay in preparation of plant extract because of insufficient equipments like rotary evaporator which extends the time of extraction.

Other limitation was the small size of sample, only six rats in each group that can easily affect the standard error of mean (S.E.M). This prompts S.E.M to be large, therefore, it may influences the significant difference of plant extract in preventing ulcer formation. In addition, only one solvent (Ethanol) was used in preparation of *Teucrium zanonii* extract. The solvent may extract some special components and not isolates all the
components of *Teucrium zanonii* which probably affects the efficacy of the plant extract in prevention of ulcer formation in experimental animals. This can be overcome by applying different solvents like methanol or distilled water.

Limited dosage was other limitation. Only three dosages were prepared and used in present study, which were 50 mg/kg, 100 mg/kg and 200 mg/kg to find out which dose gives the highest anti-ulcerogenic effect against ethanol induced ulcers in rats. This limitation will be subdued via preparing wider range of dosages.

The evaluation tests for assessing anti-ulcerogenic effect of *Teucrium zanonii* were not adequate. There were only pH measurements, assessment of mucus weight in addition to gross and histological evaluation of gastric in this study. All of these performed tests may not give a powerful evaluation on anti-ulcer mechanisms of *Teucrium zanonii* extract.

Staining was done by one staining method, Hematoxylin and Eosin, which may indicate the basic appearance of gastric tissue sections. Time and financial situation are two factors that affect the selection of methods. Mucus sections can be observed by stains like Alcian blue.

In addition to all mentioned limitations some others can be deemed. As an illustration, existing antioxidant activity by DPPH and FRAP assays may not result in an accurate assessment of antioxidant activity of plant extract, so to increase the accuracy more related tests should be performed such as ABTS assay.

Furthermore, antimicrobial activity of plant extract against six tested bacterial species may not provide enough scientific information about its antimicrobial activity. To deal with this problem, testing *Teucrium zanonii* extract against more bacterial species will result in more accurate and clear insight about antimicrobial potentiality of plant extract.
5.3 Further study

For further studies, needed instruments should be available to speed up plant extraction, thus this will decrease the time of performance, resulted in lesser wasted time. Besides, various staining methods should be applied in histological evaluation like Alcian blue to give evident prospect of mucus on sections of gastric wall in tested animal groups. To raise the fidelity of the experiment, increasing the sample size will be helpful by declining the S.E.M and improving the quality of performed study to relay on its outcomes. Moreover, applying different solvents to prepare the plant extract such as methanol, DW or ether to know which one gives the optimum effect. Additionally, using different dosages of plant extract will be beneficial by gradually increasing the dosage to find the exact dose that gives the highest protection against ethanol induced ulcer in rats.

Other routes of administration can be applied in treating rats like intravenous or intraperitoneal to realize which one shows the best effect in a shorter time compare to oral administration only. Using variety of methods to evaluate the antioxidant ability will result in clear perspective to recognize the antioxidant capability. Furthermore, testing the plant extract on more bacterial species for antimicrobial activity of plant extract will indicate precise view about its activity.

5.4 Conclusion

Based on results of the present study, it can be concluded that the ethanol extract of *Teucrium zanonii* LD and MD possess the highest anti-ulcer activity against ethanol induced gastric ulcer in rats followed by HD. When these doses compared to the negative control, tangible differences in protection of gastric ulcer can be seen. Although LD, MD and HD indicate higher protective effect in comparison to positive control (Omeprazole), this protective potentiality against formation of gastric ulcer is not in a dose dependent manner. In other words, LD and MD reveal higher level of
protection compare to HD. Investigation of acute toxicity profile of *Teucrium zanonii* extract did not manifest any toxicological symptom and adverse side-effect which this determines the safety of plant extract to be used as anti-ulcer drug. Despite the antioxidant activity of *Teucrium zanonii* still is lower than standards, it can still be considered as a natural antioxidant. The outcome of this study clarified that *Teucrium zanonii* extract did not possess any antimicrobial effects against all tested bacterial species. In conclusion, further pharmacological tests are required to be done to confirm the safety of *Teucrium zanonii* extract on human beings.


Appendix A: *Teucrium zanonii* plant

Appendix B: Histological Technique

1. Tissue / Specimen preparation
2. Automated Tissue Processing
3. Tissue Embedding
4. Sectioning
5. Staining
6. Mounting

1. **Tissue / Specimen preparation**

   a) Tissue accessioning (received the tissue from the animal experimental Laboratory fixed in 10% buffered formalin).

   b) Gross examination by naked eyes. Then, screen for the ulcer formation.

   c) After that, fix the tissue in formalin.

   d) Trimming
• Size 1 cm × 1 cm thickness.
• Several block of the stomach
• Put in cassette and close.
• Labelled properly with pencil.
• Put into fixative again (10% buffered formalin).

2. Automated tissue processing (Leica TP1020)

<table>
<thead>
<tr>
<th>Process</th>
<th>Solution</th>
<th>Period (hour)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fixation</td>
<td>10% buffered formalin 110%</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>buffered formalin II</td>
<td>1</td>
</tr>
<tr>
<td>Dehydration</td>
<td>70% ethanol</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>95% ethanol I</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>95% ethanol II</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>95% ethanol III</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Absolute ethanol I</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Absolute ethanol II</td>
<td>1.5</td>
</tr>
<tr>
<td>Cleaning</td>
<td>Alcohol : Xylene (1:1)</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Xylene I</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td>Xylene II</td>
<td>1.5</td>
</tr>
<tr>
<td>Infiltration</td>
<td>Paraffin wax I</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td>Paraffin wax II</td>
<td>1.5</td>
</tr>
</tbody>
</table>

3. Tissue embedding (Leica HISTOEMBEDDER)

➢ Tissue must be align or oriented properly
➢ Use of paraffin wax.
➢ Do on the hot plate. Then, put on cold plate for paraffin hardening

4. Sectioning (Leica RM 2135)

➢ Sectioning with microtome and put under water bath.

➢ Picking the section from water bath with the clean slide.

➢ The glass slides – placed on hot plates (15 min) – to allow sections to adhere

onto the slides.

5. Staining (H&E staining) process

a. Bring section to water:

<table>
<thead>
<tr>
<th>Process</th>
<th>Solution</th>
<th>Period (minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dewaxing</td>
<td>Xylene I</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Xylene II</td>
<td>3</td>
</tr>
<tr>
<td>Rehydration</td>
<td>Absolute alcohol</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>95% alcohol I</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>95% alcohol II</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>70% alcohol</td>
<td>2</td>
</tr>
<tr>
<td>Bring section to water</td>
<td>Running tap water</td>
<td>3</td>
</tr>
</tbody>
</table>
**b. Staining with Haematoxylin & Eosin:**

<table>
<thead>
<tr>
<th>Process</th>
<th>Solution</th>
<th>Period</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Staining</strong></td>
<td><strong>Haematoxylin stain</strong></td>
<td><strong>10 min</strong></td>
</tr>
<tr>
<td></td>
<td>Running tap water</td>
<td>until excess colour wash off</td>
</tr>
<tr>
<td><strong>Differentiation</strong></td>
<td>0.5% acid alcohol</td>
<td><strong>2-3 dips</strong></td>
</tr>
<tr>
<td></td>
<td>Running tap water</td>
<td><strong>2-3 min</strong></td>
</tr>
<tr>
<td></td>
<td>2% sodium acetate</td>
<td><strong>2 sec</strong></td>
</tr>
<tr>
<td></td>
<td>Running tap water</td>
<td><strong>2-3 min</strong></td>
</tr>
<tr>
<td></td>
<td>Rinsein 80% alcohol  (alcoholise the slide)</td>
<td><strong>2-3 dips</strong></td>
</tr>
<tr>
<td><strong>Staining</strong></td>
<td>Eosin stain</td>
<td><strong>5 min</strong></td>
</tr>
<tr>
<td><strong>Dehydration</strong></td>
<td>95% alcohol I</td>
<td><strong>5 sec</strong></td>
</tr>
<tr>
<td></td>
<td>95% alcohol II</td>
<td><strong>2 min</strong></td>
</tr>
<tr>
<td></td>
<td>Absolute ethanol I</td>
<td><strong>2 min</strong></td>
</tr>
<tr>
<td></td>
<td>Absolute ethanol II</td>
<td><strong>2 min</strong></td>
</tr>
<tr>
<td><strong>Cleaning</strong></td>
<td>Xylene I</td>
<td><strong>2 min</strong></td>
</tr>
<tr>
<td></td>
<td>Xylene II</td>
<td><strong>2 min</strong></td>
</tr>
<tr>
<td></td>
<td>Xylene III</td>
<td><strong>1 min</strong></td>
</tr>
</tbody>
</table>
6. Mounting with DPX

- Mount slides with DPX mounting media and cover the section with cover slip
- Wipe slide to remove excess xylene. Then, observed under light microscope.

Appendix C: Experimental animal Sprague Dawley (SD) rat.

Appendix D: MIC results of *Teucrium zanonii* plant extract against 6 tested bacterial species.

MIC result against *K. pneumonia*
MIC result against *P. aeruginosa*

MIC result against *S. aureus*

MIC result against *E.coli*
MIC result against *B. subtilis*

MIC result against *(MRSA)*