GASTROPROTECTIVE EFFECT OF CRUDE ETHANOL EXTRACT OF ETHIOPIAN PROPOLIS AGAINST CHEMICAL INDUCED GASTRIC MUCOSAL LESIONS IN MICE

A Thesis submitted to the School of Graduate Studies of Addis Ababa University in Partial Fulfillment of the Requirements for the Degree of Master of Science in Medical Physiology

BY DUBERO SIME

Addis Ababa, August, 2007
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LIST OF ACRONYMS

Ach  =  acetylcholine
ANOVA =  analysis of variance
cAMP  =  cyclic adenosine monophosphate
CAPE  =  caffeic acid phenethyl ester
COX-1 =  cyclooxygenase-1
COX-2 =  cyclooxygenase-2
DNA  =  deoxyribonucleic acid
ECL =  enterochromaffin like
EEP  =  ethanol extract of propolis
EHNRI =  Ethiopian Health and Nutrition Research Institute
GERD =  gastroesophageal reflux disease
GI  =  gastrointestinal
GIT =  Gastrointestinal tract
GSH =  glutathione
i.p =  intraperitoneal
IOCCP =  Institute of Organic Chemistry with Centre of Phytochemistry
NO =  nitric oxide
NSAID =  non-steroidal anti-inflammatory drugs
OTC =  Over-The–Counter
PG =  prostaglandin
PGE$_2$ = prostaglandin E$_2$
PMN = polymorphonuclear leukocyte
PUD = peptic ulcer diseases
ROS = reactive oxygen species
SEM = standard error of the mean
SOD = superoxide dismutase
SPSS = statistical package for social sciences
TNF$\alpha$ = tumor necrosis factor alpha
%IUN = percent inhibition of ulcer number
%IUI = percent inhibition of ulcer index
%ILI = percent inhibition of lesion index

%ILN = percent inhibition of lesion number
ABSTRACT

Gastric hyperacidity and peptic ulcer are very common causes of human suffering in this era of globalization. Treatment of peptic ulcer is targeted at either counteracting aggressive factors or stimulating the mucosal defenses. Natural products from bees and plants are recently becoming the focus of attention as preventive medicine in providing protection against acute and chronic gastric lesions. In the present study, the gastroprotective effects of ethanol extract of propolis (EEP) from Ethiopian central high land was evaluated against ethanol and indomethacin-induced gastric ulcers in mice. Half kilogram of propolis was soaked in 70% ethyl alcohol for two weeks at room temperature with intermittent shaking twice a day. After evaporating the alcohol and lyophilizing the residue to dryness, a gummy consistent crude propolis extract of 35.35g yield was obtained. The phytochemical screening with thin layer chromatography (TLC) and gas chromatography-mass spectrometry (GC/MS) showed that the major phyto-constituents of the extract were amryn type triterpenic alcohols (26.2%), sugars (24.9%) and fatty acids (7.5%), with significant amount of aromatic acids, esters and other alcohols including diterpenic alcohol. Swiss albino mice of 24-32g body weight were fasted for 24hours and pretreated with varying doses of EEP or standard drugs (omeprazole or cimetidine) fifty minutes before ulcer induction either with alcohol or indomethacin. In alcohol-induced ulcers, EEP at doses of 25, 50 and 100mg/kg significantly reduced lesion index and number of the total lesions (P<0.05) in the glandular area of the stomach. In indomethacin-induced ulcers, the same dose of EEP as for alcohol- induced ulcers also significantly decreased ulcer index and number of the total lesions (P<0.05) in the glandular region of the stomach compared to the control. In both cases, the extract was shown to reduce gastric lesions in a dose-dependent manner. The intraperitoneal (i.p) pretreatment with indomethacin one hour before the extract did not affect the gastroprotective effects of the EEP on alcohol-induced ulcers. The histological observations in the glandular area of the stomach also revealed that severe hemorrhagic patchy lesions occurred covering most parts in the absence of EEP. Mucosal epithelial damage was confirmed by microscopic observation of the Hematoxylin-eosin fixed tissue taken from the same glandular area of the stomach. The gastroprotective mechanism of EEP could be due to its antioxidant effects, and/or its film forming properties. Further investigation on the chemical composition and the biological activities of Ethiopian propolis from different agro-ecological zones are recommended.
Keywords: Gastroprotective, propolis, EEP, ulcer index, alcohol, indomethacin, gastric lesion
1. LITERATURE REVIEW

1.1 General considerations

The gastrointestinal tract possesses a remarkable ability to remain intact despite being constantly bathed in acid and proteolytic enzymes. When a superficial mucosal injury occurs following direct physical trauma or ingestion of noxious agents, it is rapidly healed. This is because of its mucosal defense system and repair mechanisms which involve a high rate of cellular turnover, an efficient mucosal blood flow, a continuous adherent alkaline mucus layers, and prostaglandin E series that increase the thickness of gel mucosal layer and stimulate secretion of bicarbonate ions (Playford and Ghosh, 2005). These provide protective coating for the mucosal lining against corrosively acid gastric secretions and other irritants (Guha and Kaunitz, 2002).

Diseases of the gastrointestinal tract (GIT) are common, accounting for one out of seven complaints. Disorders of the stomach and duodenum make up a large portion of these. Peptic ulcer is a common disorder causing human suffering in today’s era of globalization and millions of people suffer from this disease in the world. The etiology of gastroduodenal ulcers is influenced by various aggressive and defensive factors such as acid-pepsin secretion, parietal cell mass, mucosal barrier, mucus secretion, blood flow, cellular regeneration, and endogenous protective agents like prostaglandins (PG), nitric oxide (NO) and epidermic growth factors (EGF) (Repetto and Llesuy, 2002). Peptic ulcer treatment is often directed at either reduction of aggressive factors, or strengthening of the defense system of gastric mucosa (Jain et al., 2007). These therapeutic strategies extend from the use of simple conventional antacids to the use of more complex and effective proton pump inhibitors (PPIs). In addition, inclusion of antibiotic in the regimen for the treatment of Helicobacter pylori (H. pylori) associated peptic ulcer is indispensable (Chang et al., 1996). However, associated side-effects with these agents are becoming a cause of concern. For instance, the prolonged use of irreversible proton pump inhibitors brings about acid suppression thus upsetting the normal physiology of the gastric mucosa. Extreme acid suppression at recommended doses some times leads to achlorohydria and predispose to enteric infections like typhoid, cholera, and dysentery (Jain et al., 2007).
Nowadays, the search for natural products with medicinal properties, particularly those from plants and honeybees with less toxic anti-ulcerogenic principles, which either supplements modern drugs or is used as an alternative is a topic of interest in different parts of the world. Propolis (bee’s glue) is a major breakthrough in the quest for a nontoxic, powerful, all-encompassing healer that can assist the body in fighting a broad spectrum of infectious agents, heal ulcers and improve the overall immune response (Literature search service, 2003).

1.2. Mechanism of gastric acid secretion

Physiological regulation of acid secretion by parietal cells is an important factor behind the rationale of use of various agents to reduce gastric acidity. Three major pathways activating parietal acid secretion include: (1) neuronal stimulation via the vagus nerve, (2) paracrine stimulation by local release of histamine from enterochromaffin-like (ECL) cells, and (3) endocrine stimulation via gastrin released from antral G cells. In neuronal pathway, acetylcholine (Ach) released by vagal nerve directly stimulates gastric acid secretion through muscarinic M$_3$ receptors located on the basolateral membrane of parietal cells. Acetylcholine indirectly stimulates release of histamine from ECL cells in the fundus and gastrin from the G cells in the gastric antrum (Jain et al., 2007). Histamine released from ECL cells activates parietal cells in paracrine fashion by binding to H$_2$ receptors. Gastrin released under regulation of central neural activation, local distension, and chemical composition of gastric content stimulate parietal cells by binding with gastrin receptors (Salena and Hunt, 2005; Jain et al., 2007).

The production of PGs by cyclooxygenases, mainly prostaglandin E$_2$ (PGE$_2$), remains a critical factor in gastric homeostasis. Prostaglandin E$_2$ inhibits acid secretion and the fluctuation of its levels as a result of NSAID therapy remains a major concern in preserving the integrity of the gastric mucosa (Salena and Hunt, 2005).

Stimulation of acid secretion typically involves an initial elevation of intracellular calcium and/or cAMP followed by activation of a cAMP-dependent protein kinase cascade that triggers the translocation and insertion of the proton pump enzyme, H$^+$- K$^+$-ATPase, into the apical plasma membrane of parietal cells. In the resting parietal cell, the proton pump resides in cytoplasmic tubulovesicles in an inactive form, presumably because of low permeability of
these membranes to K\(^+\). The H\(^+\)-K\(^+\)-ATPase catalyzes the electro-neutral exchange of intracellular protons for extracellular K\(^+\), thus generating the enormous proton gradients associated with gastric HCl secretion (Yao and Forte, 2003; Guyton and Hall, 2006).

**1.3. The gastric mucosal defense system**

Gastric mucosal layers form a barrier that limits exposure of the gastric mucosal cells to numerous injurious luminal agents and irritants of exogenous and endogenous origins (Zayachkivska et al., 2005). However, if the barrier is weakened and/or corroding challenge is increased, the epithelial layers will be overwhelmed and the underlying tissue is digested leading to formation of lesion or ulcer (Playford and Ghosh, 2005). Anything that breaches the mucosal lining results in the inflammation of underlying tissue and erosion of the stomach wall which ends in gastric ulceration (Kwiecien et al., 2002; Pocock and Richard, 2004).

The endogenous gastroprotective components of the gastrointestinal mucosa against aggressive factors mainly consist of functional, humoral and neuronal factors. Alkaline mucus secretion, mucosal microcirculation and motility act as functional factors, while prostaglandin, bicarbonate and nitric oxide act as humoral factors, all of which are known to contribute to mucosal protection against injurious luminal agents (Repetto and Llesuy, 2002).

The physiological basis of mucosal barrier function involves several factors and mechanisms. These are: 1) mucus coating of epithelial cells, 2) HCO\(_3\)\(^-\) component that neutralizes the acid, 3) epithelial cells joined by tight junction, and 4) high epithelial cell turnover rate. They could be envisioned as pre-epithelial, epithelial and sub-epithelial components of mucosal protective barrier (Zayachkivska et al., 2005) (Figure1.1).
The first line of defense is a mucus-bicarbonate layer which serves as a physicochemical barrier to multiple molecules including H⁺. The mucous-gel functions as a non-stirred water layer impeding diffusion of ions and molecules such as pepsin and H⁺. Gastric mucus consists of a viscous, elastic, adherent and transparent gel formed by 95% water and 5% glycoproteins that covers the entire gastrointestinal mucosa. Mucus is capable of acting as an antioxidant, and thus can reduce mucosal damage mediated by oxygen free radicals (Guha and Kaunitz, 2002; Valle, 2005).

The protective properties of the mucus barrier depend not only on the gel structure but also on the amount or thickness of the layers covering the mucosal surface. When cells containing mucus are damaged by extra-cellular oxygen radicals, the intracellular mucus may be released into the gastric tissue and prevent additional damage by scavenging them (Repetto and Llesuy, 2002). Thus a decrease in gastric mucus makes epithelial cells susceptible to injuries induced by acid or chemicals like aspirin (Salena and Hunt, 2005).

The surface epithelial cells, the second layer, provide the next line of defense through several factors including mucus and bicarbonate production, and formation of intercellular tight junctions. Several growth factors such as epidermal growth factor (EGF), transforming growth factor alpha (TGFα), and basic fibroblast growth factor (FGF) modulate the process of restoring the damaged regions (restitution) of the mucosa (Guha and Kaunitz, 2002; Valle, 2005).
The sub-epithelial defense/repair system is an elaborate microvascular system within the gastric sub-mucosal layer. Mucosal blood flow is also an important component of the gastroduodenal barrier function. In the stomach, the presence of luminal acid increases the delivery of vascular bicarbonate into the overlying mucous layer by the mucosal microcirculation, thereby neutralizing $H^+$ invading from the lumen. The circulatory bed in the sub-mucosa provides $HCO_3^-$, micronutrients and $O_2$ while removing toxic metabolic by-products. The endogenous PGs play an important role in the maintenance of mucosal integrity, which includes continuous secretion of $HCO_3^-$ and a mucus production in the stomach and duodenum (Kwiecien et al., 2002; Valle, 2005).

1.4. Peptic ulcer disease (PUD)

Any small portion of the skin or surface of an internal tissue that develops inflammation with shallow breaches is called lesion. Though sometimes insignificant, the small or shallow breaches may herald ulcers. They can have the appearance of a second-degree burn with reddening, blistering, or both in their early stage. If left untreated, the inflammation leads to tissue necrosis and the lesion may become infected and begin bleeding. As it deepens, the lesions become more craters like, eventually turning into festering (decaying) open types of ulcers known as peptic ulcers (Helpern, 2004; Majumdar et al., 2007).

Generally, peptic ulcer results from an imbalance between defensive mechanisms of the mucosa and aggressive factors. The development of peptic ulcer could also be due to compromised mucosal defense system because of endogenous or exogenous agents. Somatostatin, PGs, NO, bicarbonates, and mucin act as mucosal defense factors while the aggressive factors comprise of acid plus pepsin, active oxidants, leukotrienes, endothelins, bile or exogenous factors including NSAIDs, cigarette smoking, ethanol consumption and stress (Salena and Hunt, 2005; Jain et al., 2007; Majumdar et al., 2007). The defensive mechanisms (factors) of the mucosa and aggressive factors are summarized here in Table 1.1.
1.4.1. Types of peptic ulcers

The two main types of ulcer are gastric and duodenal. Though caused by the same factors, and are also diagnosed and treated the same way, they differ in some ways. Both can cause dyspepsia, pain or uncomfortable feeling in the pits of the stomach. More gastric ulcers than duodenal ulcers are caused by the use of NSAIDs (Helpern, 2004; Rao et al., 2006). Gastric ulceration occurs on a background of pangastritis, often arising at the highly inflamed transitional zone between antrum and pylorus. Identical hormonal changes do occur in both, but acid production from the inflamed corpus is reduced or is normal in gastric ulcers. Gastric ulcers are most commonly found on the lesser curvature, near the junction of acid-producing parietal cells and the antral mucosa, extending to an area 2–3 cm above the pylorus. Duodenal ulcers are usually found in the duodenal bulb or the pyloric channel area (Salena and Hunt, 2005; Majumdar et al., 2007). Figure 1.2 shows the most common sites of peptic ulcer in the gastroduodenal mucosa.

The majority of gastric ulcers and substantial number of duodenal ulcers do not have increased gastric acid secretion. In case of duodenal ulcers, there is an increase in basal acid secretion. In gastric ulcers, however, there is a weakening of mucosal defenses that can lead to injury in spite of low acid secretion. Gastric ulcers have been classified into Type I,
occurring along the lesser curve, Type II, with concurrent or historical duodenal ulcer, Type III, prepyloric and Type IV, cardiac (Salena and Hunt, 2005; Ostrow, 2006; Jain et al., 2007).

1.4.2. Complications of peptic ulcers

The majority of ulcers heal without difficulty, but an ulcer that goes untreated or fails to heal lead to serious complications. These include hemorrhaging, perforation, penetration, and obstruction. Such problems can occur without any warning, especially in the case of patients who are taking NSAIDs (Helpern, 2004). The most common peptic ulcer complications are discussed as follows:

Figure 1.2. Common sites of peptic ulcer (PUD) (Source: Jain et al., 2007).
a. Hemorrhage

Minor bleeding by erosion of small blood vessels occurs in all ulcers and can be detected by testing the stool for occult blood. If the ulcer sore occurs on an important artery, the chances of its bleeding are significant. A mild hemorrhage will leak blood slowly and cause the patient to feel dizzy and light headed while a severe hemorrhage leads to bloody vomit and /or bloody stools. Most patients who hemorrhage are above age of 60 and taking NSAIDs. Hemorrhaging occurs in 15% of ulcer patients (Helpenn, 2004; Jain et al., 2007).

b. Perforation

Perforation occurs when an ulcer sore burrows deep into the wall of the stomach or duodenum, so that gastric acid and other stomach contents are allowed to leak into the otherwise sterile peritoneum. When the peritoneum is inflamed and infected, patients experience sudden, sharp, severe pain and sometimes go into septic shock, which is a life-threatening condition that requires immediate surgery. Perforation occurs more commonly in chronic duodenal ulcers than chronic gastric ulcers, leading to the following sequelae:

(i) On perforation the contents escape into the lesser sac or into the peritoneal cavity, causing acute peritonitis.
(ii) Air escapes from the stomach and lies between the liver and the diaphragm giving the characteristic radiological appearance of air under the diaphragm and
(iii) Perforation may extend further to involve adjacent organs (liver and pancreas).

Perforation occurs in approximately 7% of patients while the mortality rate is roughly 19% in the total population world wide (Helpenn, 2004; Jain et al., 2007).

c. Penetration

Penetration occurs when an ulcer sore penetrates the muscular wall of the stomach or the duodenum, continues into a nearby organ like pancreas or liver. The patient experiences sharp, piercing pain in the organ affected (Salena and Hunt, 2005).

d. Obstruction

This is development of fibrous scar at or near the pylorus resulting in pyloric stenosis. It occurs when an ulcer scar, swelling from inflamed tissue or an ulcer sore that blocks the passage from stomach to the duodenum. The symptoms of such complication are bloating,
lack of appetite, weight loss and sometimes vomiting. Sometimes treating obstruction by surgery is necessary (Helpern, 2004; Jain et al., 2007).

1.5. Prevalence of peptic ulcer

Peptic ulcer is the most common cause of acute upper gastrointestinal bleeding, accounting for about 50% of all cases (Arlt and Leyh, 2001). Worldwide, the two most common causes of peptic ulceration are \textit{H. pylori} infection and NSAIDs including aspirin. \textit{H. pylori} infection remains the primary cause of peptic ulcer throughout the world, but in industrialized countries it is not as such infectious. Next to \textit{H. pylori}, NSAIDs are the leading causes of peptic ulcer, accounting for about 25% of the causes (Helpern, 2004). The use of NSAIDs due to old age diseases will probably surpass \textit{H. pylori} infections as the primary cause within a generation or two in these countries (Majumdar et al., 2007).

The prevalence of gastric and duodenal ulceration has decreased in Western Europe and the USA over recent decades, following a decrease in the prevalence of \textit{H. pylori} (Majumdar and Atherton, 2006). \textit{H. pylori} infects about 40% of adults in developed countries and is strongly associated with aging and with markers of overcrowding and poor hygiene during childhood (Helpern, 2004). In the developing world, however, 80% of the population shows evidence of \textit{H. pylori} infection. In Africa, the infection is present in the majority of the population and 90% of duodenal ulcers are \textit{H. pylori} positive (Ostrow, 2006; Majumdar et al., 2007). \textit{H. pylori} is usually acquired in childhood from mother or from other children. In developing countries, 80% of the population may be infected by the age of 20 years (Majumdar and Atherton, 2006).

According to a study on 300 adult patients with dyspepsia; chronic gastritis and peptic-ulcer were the most common endoscopic findings in Ethiopia and the apparent overall prevalence of \textit{H. pylori} infection varies based on the detection method ranging from 69% to 91% (Asrat et al., 2004). Ersumo et al. (2004) had reported that complicated ulcer diseases comprised of 3.8% of the total major surgery done from 1997 to 2001 and one of 10 top diseases for surgical admission in Tikur Anbessa Hospital, Addis Ababa, Ethiopia. It was pointed out that the frequency of PUD and its complication is on rise particularly perforated type though the exact incidence of complicated peptic ulcer is unknown in Ethiopia (Ersumo et al., 2004).
1.6. **Pathogenesis of peptic ulcer**

The pathogenesis of peptic ulcer is multifactorial, including *H. pylori* infection, chronic use of NSAIDs, alcohol, and reactive oxygen species (ROS) (Birdane *et al.*, 2007). Only 15% of infected people with *H. pylori* develop ulcer in their lifetime. This depends on the virulence of the strain of *H. pylori*, host genetic susceptibility to disease and environmental factors (e.g. smoking). Smoking is not a risk factor for ulceration in uninfected people, but markedly increases the risk in those infected (Majumdar and Atherton, 2006; Majumdar *et al.*, 2007). A possible cause of ulceration by *H. pylori* infection is thought to be due to its profound potentiation of polymorphic nuclear oxidative burst, leading to a considerable production of ROS, the central factor causing irreversible membrane damage, DNA strand breaks, changes in secondary and tertiary protein structures. Oxygen derived free radicals, primarily superoxide anion (O$_2^-$) and hydroxyl radical (OH$^-$), play an important role in the pathogenesis of acute experimental gastric lesions induced by stress, ethanol or NSAIDs such as indomethacin, aspirin etc (Arlt and Leyh, 2001; Ray *et al.*, 2002).

NSAIDs cause gastric and duodenal damage through inhibition of the enzyme cyclo-oxygenase1 (COX-1), which is important for the formation of protective prostaglandins in the stomach. The anti-inflammatory effects of NSAIDs are mediated through another isoform of cyclo-oxygenase, COX-2 (Laine, 2002). COX-1 derived PGs are responsible for mucosal defense and cytoprotection in the GIT, while COX-2 derived PGs mediate inflammation, pain, and fever. Most NSAIDs are nonselective, blocking both COX-1 and COX-2 isoenzymes. Selective COX-2-inhibiting NSAIDs have lower gastrotoxicity, but their cardiovascular side effects and costs limit their use (Peura, 2002; Salena and Hunt, 2005; Majumdar *et al.*, 2007; Stillman and Stillman, 2007). In addition, many intra- and extracellular phospholipases are activated from the cytoplasmic membrane phospholipids and activate cyclooxygenase (COX) and lipoxygenase (LOX) enzymes, which act on arachidonic acid and eicosanoid metabolism (de Almeida and Menezes, 2002).

Heredity also plays some role in peptic ulcer pathogenesis; especially in duodenal ulcers. About 20% to 50% of patients with duodenal ulcers have a positive family history for PUD. Studies done on identical twins indicated that in 50% of the cases, if one twin had an ulcer, so did the other. The parents, siblings, and children of people with ulcer are three times more
likely to have an ulcer; and people with blood type O are 30% to 35% more likely to get a duodenal ulcer compared with those of other blood groups (Helpern, 2004; Salena and Hunt, 2005).

1.6.1. **Oxidative stress and free radicals gastric ulceration**

Oxygen free radicals are detrimental to the integrity of biological tissues. The mechanism of damage involves lipid peroxidation, which destroys cell membranes with the release of intracellular components, such as lysosomal enzymes, leading to further tissue damage. The radicals also promote mucosal damage by causing degradation of the epithelial basement membrane components, complete alteration of the cell metabolism and DNA damage. Moreover, lipid peroxidation leads to loss of membrane fluidity and impairment of ion transport and membrane integrity on the surface of epithelial cells and helps to generate gastric lesion (Demir et al., 2003; Dokmeci et al., 2005).

The body has developed several endogenous antioxidant systems to deal with the production of ROS. Antioxidants act as radical scavengers, inhibit lipid peroxidation and other free radical-mediated processes, and thereby protect the human body from several diseases attributed to the reactions of radicals (Repetto and Llesuy, 2002; Dokmeci et al., 2005). They can be divided into enzymatic and nonenzymatic groups. The enzymatic antioxidants include superoxide dismutase (SOD) which is the major antioxidative enzyme, catalase, and glutathione peroxidase that work as a system to protect the body against the deleterious effects of free radicals. These enzymes require trace metal co-factors for maximum efficiency, including selenium for glutathione peroxidase, copper, zinc or manganese for SOD and iron for catalase (Demir et al., 2003; Nasuti et al., 2006).

The non-enzymatic antioxidants include the lipid soluble vitamins, vitamin E and A, and the water-soluble vitamin C and glutathione (GSH). Glutathione, which is synthesized intracellularly from cysteine, glycine, and glutamate, is capable of scavenging ROS either directly or enzymatically via glutathione peroxidase(Demir et al., 2003).

Oxygen handling cells have different systems, e.g. SOD, peroxidase, catalases and tissue thiol group which are able to protect them against the toxic effects of free radicals, one of the most devastating being O$_2^-$ (Repetto and Llesuy, 2002). Several mucosal defense
mechanisms protect the stomach and duodenum from noxious agents. The ROS generated by the metabolism of arachidonic acid, platelets, macrophages, and smooth muscle cells may contribute to gastric mucosal damage. Neutrophils produce O2\(^{•-}\) which reacts with cellular lipids, leading to the formation of lipid peroxides that are metabolized to malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) (Kwiecien et al., 2002; Nasuti et al., 2006).

Reactive oxygen intermediates may participate in inflammatory events, such as:

(a) Polymorphonuclear leukocyte (PMN) and monocyte/macrophage chemotaxis;
(b) specific stimulus related to respiratory burst, especially in inflammatory cells with greater free radical production; (c) low concentration of scavenger enzymes in interstitial spaces; and
(d) formation of metal immune complexes which can also produce OH\(^{-}\) (de Almeida and Menezes, 2002).

1.6.2. The effect of ethanol on gastric mucosal membrane

Acute ethanol administration increases O2\(^{•-}\) and OH\(^{-}\) production and lipid peroxidation in the gastric mucosa leading to mucosal damage while chronic administration causes additional cell proliferation in animal models. Ethanol-induced gastric damage may be due to direct action on the gastric epithelium causing lipid peroxidation or induction of intracellular oxidative stress. This damage can be prevented by prostaglandin administration or intracellular antioxidants like glutathione, indicating the protective action of these endogenous substances against the damaging effect of ethanol to gastric mucosal cells. This would suggest the involvement of superoxide free radicals in the pathogenesis of ethanol-induced gastric mucosal damage (Repetto and Llesuy, 2002).

Administration of high ethanol concentration (90–100% v/v) in animal model has been frequently used as an effective method to evaluate gastric lesions. Further examinations in rats exposed to acute intragastric ethanol plus tobacco smoke revealed a synergistic deleterious effect on the gastric mucosa due to decreased mucosal blood flow, aggravation of inflammation and increased free radical production (Siegmund et al., 2003).

Oxidative stress and physiological consequences of acute ethanol intoxication in gastric mucosa is also as the result of activation of phagocytes (because of production of O\(^{2-}\), H\(_2\)O\(_2\), NO\(^{-}\) and HOCl) which is followed by liberation of arachidonic acid and peroxide enzymatic
formation (like lipoxygenase and cyclooxygenase production). Peroxides generate alcohoxyl (RO) and peroxyl radicals (ROO), which can damage other lipids and proteins. The mitochondrial damage as well produces an increase of electron transfer, which in turn produces $O_2^-$. Increase in intracellular $Ca^{2+}$ levels and triggering of nuclease activity and $Ca^{2+}$ dependent nitric oxide synthase, generating more NO also increases the risk of oxidative stress in damaging gastric mucosal membrane (Figure 1.3) (Repetto and Llesuy, 2002; Siegmund et al., 2003).

![Diagram of ethanol effects on the stomach](source: Siegmund et al., 2003).

**Figure 1.3. Schematic representation of the acute and chronic ethanol effects on the stomach**

1.7. Treatment strategies for peptic ulcer

1.7.1. Conventional treatments for peptic ulcer

Prior to 1970, antacids and bismuth were used to relieve most peptic ulcer pain. But nowadays, there are plenty of powerful drugs and drug combinations that relieve pain, increase stomach’s defenses, eradicate *H. pylori* and even heal ulcers. However, many of these medications also cause potential side effects (Helpen, 2004). For instance, antacids cause alkalosis, belching, nausea, abdominal distension, flatulence, diarrhea, and constipation,
while the parasympathetic side effect of anti-secretary drugs such as pirenzepine brings about dry mouth, blurred vision, and constipation (Jain et al., 2007).

Remedies and drugs for ulcer treatment fall into three categories:
(1) Medications that neutralize gastric acids or inhibit the production of gastric acid. These include antacids, H$_2$ receptor antagonists, and proton pump inhibitors (PPIs).
(2) Medications and natural supplements that bolster the stomach’s mucosal defenses against harm from gastric acid fall in the second category. These include sucralfate, prostaglandin analogs, and bismuth, and
(3) Antibiotics that eradicate $H. pylori$ bacteria in the stomach and duodenum (Helpern, 2004; Jain et al., 2007).

Antacids are designed to temporarily relieve the overt symptoms of gastric distress by neutralizing HCl secreted in the stomach. They are not the best treatment for PUD. While one self-medicate with antacids to relieve the pain as ulcer comes and goes, the underlying problem remains, and in most instances worsens as time goes by (Helpern, 2004).

The current medical treatment of peptic ulcer is generally based on the inhibition of gastric acid secretion by H$_2$- antagonists, proton pump inhibitors and anti-muscarinic drugs, as well as the acid-independent therapy provided by sucralfate and bismuth. One of the major problems in gastroduodenal ulcer treatment with H$_2$-antagonists and proton pump inhibitors is that the rate of ulcer recurrence within 1 year after stopping treatment is between 40 and 80% (de Barros et al., 2007).

1.7.2. Herbal medicines as alternative for peptic ulcer treatment

For most of humankind’s history, traditional methods of healing were used to treat every sort of health disorder (Helpern, 2004). The World Health Organization estimates that around 80% of the world population in developing countries relies on traditional plant medicines for primary healthcare needs, of which a major proportion corresponds to plant extracts or their active principles (Sampson et al., 2000). Plants and herbs have been used since ancient times to treat different gastrointestinal illnesses, including peptic ulcers. In China, Traditional Chinese Medicine is practiced in hospitals in addition to western medicine. In Germany, all medical physicians are also trained in the use of herbs (Helpern, 2004).
Considering the several side effects of modern medicine, indigenous drugs possessing fewer side effects should be looked for as a better alternative for the treatment of peptic ulcer (Bafna et al., 2004). Recently, many efforts have been made in order to identify new anti-ulcer drugs from natural resources. Anti-ulcer drugs such as carbenoxolone from Glycyrrhiza glabra, solon from sophoradin and gefarnate from cabbage are some of such drugs (Rodriguez et al., 2006). Liquorice from the root and rhizome of different varieties of *Glycyrrhiza glabra* has been extensively used in medicine for its anti-ulcer activity. The principal constituent of liquorice, is a triterpenoid saponin. It is the substance responsible for its gastroprotective action against ulcers and has been extensively used in medicine (Borrelli and Izzo, 2000).

Zinc-carnosine, another natural supplement consisting of zinc and L-carnosine, strengthens the stomach’s mucosal defenses and harnesses the stomach’s natural ability to fight disease, battle infection, and heal itself. Its component L-carnosine, a dipeptide made up of L-histidine and β-alanine, demonstrates antioxidant properties that also add to its protective and healing effects (Helpern, 2004).

The medicinal properties of folk plants are mainly attributed to the presence of flavonoids, and other organic compounds such as coumarins, phenolic acids, tannins, antioxidants and inorganic micronutrients, e.g., Cu, Mn and Zn. These secondary plant metabolites have been shown to scavenge free radicals and are viewed as promising therapeutic options (Repetto and Llesuy, 2002). Therefore, by scavenging free radicals, antioxidants from plant metabolites might be useful in protecting the gastric mucosa from oxidative damage or in accelerating healing of gastric ulcers (Ray et al., 2002; Repetto and Llesuy, 2002). The potential role and basic mechanisms of plant-originated gastroprotective substances applied intragastrically (i.g.) are known to account for mucosal protection against various irritants and ulcerogens (Zayachkivska et al., 2005). These materials might possess anti-inflammatory action by suppressing the neutrophil/cytokine cascade in gastrointestinal tract, promoting tissue repair through expression of various growth factors, exhibiting antioxidant activity, scavenging ROS, showing anti-nucleolytic, anti-necrotic and anti-carcinogenic activities (Liu et al., 2002; Bankova, 2005; Zayachkivska et al., 2005).

1.8. **Propolis and its uses**

The term propolis is derived from the two Greek words: pro for ‘in front of’ or ‘at the entrance to’ and polis for ‘community’ or ‘city’ and means a substance for defense of the city.
or the beehive (Bankova et al., 2000). Propolis is a natural hive product with a complex chemical composition, consisting of mixture of balsams (resins), beeswaxes, oils, and pollen. It is a sticky resinous substance collected by honey bees (Apis mellifera) from buds and barks of different trees. Honeybees may also use material actively secreted by plants, or exuded from wounds in plants which are lipophytic material on leaves, mucilages, gums and resins (Gomez-Caravaca et al., 2006). They enrich propolis in the hive by action of salivated secretion like \( \beta \)-glucosidase, and addition of wax (Bankova et al., 2000). The gums and resins that bees gather from plants for propolis are the very substances exuded by plants for their own protection and healing (Bradbear, 2003).

Propolis is used by worker bees to line the inside of nest cavities and all brood combs, seal small cracks in the hive and for making the entrance of the hive weather tight or easier to defend (Bankova et al., 2000). Propolis is also used as an “embalming” substance to cover hive invaders which bees have killed but cannot transport out of the hive and to seal any dead bodies or insects so as to keep the inside of their hives a sterile environment (Krell, 1996; Zayachkivska et. al, 2005). Propolis is the most important ‘chemical weapon’ of bees against pathogenic microorganisms because of its antimicrobial properties (Bankova, 2005). It is because of this later property of propolis that humans make use of it as folk medicine.

1.8.1. Production and the chemical make up of propolis

Depending on the bees, climate, forest resources and the trapping mechanism, the average production of propolis ranges from 10 to 300g per colony per year (Krell, 1996). As observed in Brazilian (from Africanized honeybees) and Egyptian propolis samples, African honeybees produce a significant amount of propolis with different chemical constituents compared to that of the Europeans because of different plant sources (Bankova, 2005; Bruce, 2005). All honeybees in Brazil are now Africanized and presumably more productive than European bees with regard to propolis (Salatino et al., 2005).

Propolis contains a large number of biologically active components including different flavonoids, polyphenolic esters, terpenoids, steroids, amino acids, caffeic acids and their esters (Kumazawa et al., 2004; Bruce, 2005). The flavonoids and polyphenolic compounds are the major constituents of propolis making 45-55% in most samples from different
countries (Burdock, 1998). In addition, propolis contains a significant amount of waxes and fatty acids (25-35%), volatile oil (about 10%), pollen (about 5%) and over 16 different vitamins (Krell, 1996). Chemical studies conducted with propolis extracts revealed the existence of a very complex mixture of different, naturally occurring compounds with more than 300 constituents identified to date (Banskota et al., 2001; Paulino et al., 2003). Upon analysis of propolis sample from England, about 150 compounds were identified from a single sample (Krell, 1996).

Diterpenic acids and triterpenic alcohols seem to be another important class of Brazilian propolis constituents for which new valuable biological activities have been identified. Some triterpenic alcohols (amyrin type and cycloartenol) were found in propolis from Brazil and Egypt (Bankova et al., 2000; Kumazawa et al., 2004).

1.8.2. The medicinal values of Propolis

Propolis has been used as a remedy by humans since ancient times dating back to the times of ancient Greece and Rome, for treating wide spectrum of disorders and diseases (Burdock, 1998; Bankova, 2005; Ahn et al., 2007). In addition to its use in the treatment of various diseases, propolis is also incorporated in products like ‘health foods’ and ‘bio-cosmetics’, because of its versatile biological activities (Trusheva et al., 2006). Propolis is used in foods and beverages to improve health and prevent diseases such as inflammation, diabetes, heart disease, and cancer (Banskota et al., 2001). Use of products containing propolis by humans has a long history because of its beneficial effects in many pathological processes (Burdock, 1998; Ahn et al., 2007). It has been reported to possess antibacterial, antiviral, anti-inflammatory, anticancer, antifungal, and anti-tumoral properties (Padmavathi et al., 2006). Propolis is one of the most frequently used remedies in the Balkan states and in Africa, applied for treatment of wounds and burns, sore throat and stomach ulcer (Krell, 1996; Suzuki, 2002; Literature search service, 2003). It is one of the few natural remedies that maintained its popularity over a long period of time as folk medicine. Modern herbalists recommend it for its anti-bacterial, anti-fungal, anti-viral, hepatoprotective and anti-inflammatory properties, to increase the body’s natural resistance to infections and to treat gastroduodenal ulcers among others (Castaldo and Capasso, 2002).
Propolis is generally considered to be safe in low doses although reports of allergic reactions are commonly observed at doses over 15 g/day (Castaldo and Capasso, 2002). It has a low order of acute oral toxicity with reported LD₅₀ ranging from 2000 to 7300 mg/kg in mice (Burdock, 1998). Propolis, administered orally to mice at levels up to 4000 mg/kg/day for 2 weeks had no effect. Ninety days of administration to mice in drinking water at 1400 mg/kg/day was declared to be a no-effect level (NOEL) (Burdock, 1998).

Recently, Propolis has gained popularity as an alternative medicine or food for health amelioration and disease prevention in various parts of the world, including the USA, the European Union and Japan (Teixeira et al., 2004). Furthermore, substances identified from Brazilian propolis, mainly phenolic components were found to have hepatoprotective and neuroprotective activities, and activities against H. pylori (Bankova, 2005; Shimazawa et al., 2005), while red propolis from Brazil and Cuba was found to possess cytotoxic activity against several tumor cell lines and to have radical scavenging action (Bruce, 2005; Trusheva et al., 2006).

The anti-tumour and anti-hepatotoxic activities of propolis could be through scavenging ROS that are thought to be associated with tumour promotion and hepatotoxicity. The antioxidant property of propolis seems to be responsible for its anti-carcinogenesis and hepatoprotective activities (Banskota et al., 2001; Padmavathi et al., 2006). Propolis also exhibits immunostimulatory and immunomodulatory effects on macrophages in vitro; while in vivo it increases the ratio of CD₄/CD₈ T cells in mice (Castaldo and Capasso, 2002). Moreover, propolis has been shown to have activity against many of the opportunistic pathogens associated with the acquired immunodeficiency syndrome (AIDS) (Burdock, 1998; Banskota et al., 2001). Propolis samples from several geographic regions was found to potently inhibit HIV-1 expression in the primary cell targets of HIV-1, i.e. CD₄⁺ lymphocytes and microglial cell cultures (Gekker et al., 2005).

1.8.3. Geographical variations in propolis samples

The composition of propolis depends upon the local flora of the area from which it is collected and the season of its collection. That is geographic and climatic characteristic of an
area determines the chemical makeup of the propolis sample (Krell, 1996; Bankova et al., 2000; Kumazawa et al., 2004; Lahouel et al., 2004).

In the temperate zone, including Europe, Asia and North America, the bud exudates of *Populus* species and their hybrids are the main source of propolis. It is generally accepted and chemically demonstrated that samples originating from these regions are characterized by similar chemical composition, the main constituents being phenolics, flavonoid aglycones, aromatic acids and their esters (Bankova et al., 2000).

The constituents of propolis from tropical zones appear to be different from those of temperate zones because of the difference in vegetation. The resins exuded by *Clusia minor*, *Clusia major* (Guttiferae), *Araucaria heterophylla* (Compositae) and different *Baccharis* spp. (Compositae) were reported to be the dominant sources of components found in tropical propolis from Venezuela and Brazil (Burdock, 1998). These plants are rich in polyprenylated benzophenones and various diterpenes, which are reported from tropical propolis (Annex 3). A clerodane and several labdane-type diterpenoids, which are virtually absent in propolis from temperate zones, were reported to be present in propolis from tropical regions (Bankova et al., 2000). Flavonoids are also reported from tropical propolis because of their wide distribution in the plant kingdom. Interestingly, in spite of the difference in their constituents, propolis from all regions, including the temperate and tropical zones, exhibit similar biological properties (Burdock, 1998).

1.8.4. **Ethiopian Propolis**

Ethiopia's wide climatic and edaphic variability have endowed the country with diverse and unique flowering plants, thus making it highly suitable for sustaining a large number of honeybee colonies and the long established practice of beekeeping (Deffar, 1998). According to Fichth and Adi (1994), there are about 500 plant species in Ethiopia (400 herbs and shrubs, and 100 trees) that have been chosen to be important to honeybees. Ethiopia with about 10 millions bee colonies is the largest honey and beeswax producer in Africa, the 10th largest
honey producer and 4th wax producer in the world (Hartmann, 2004). Other honeybee products such as propolis, bee pollen, bee venom, and royal jelly are not assessed yet. Of these products, propolis can be easily accessed without affecting the production of honey and beeswax. However, due to lack of knowledge and/or awareness about its economical, nutritional and medicinal values, propolis is regarded as an unwanted hive by-product by Ethiopian beekeepers.

Honey is well known for its traditional medicine, as food supplements and in beverages, and currently it is used in modern medicine in wound dressing in different parts of the world (Bradbear, 2003) as well as in Ethiopia. But, there is no recorded data or information whether propolis has been used as traditional medicine in the Ethiopian community. Only one study has been reported on Ethiopian propolis so far by Nuru et al., (2002) on the production and potentiality of Ethiopian honeybees. In this study they reported that the Ethiopian honeybees have the potential to produce significant amount of propolis without significantly affecting honey yield. It is indicated that simple induction of colonies for more propolis production in both traditional basket and modern Langstroth hives is possible.

The photograph in Figure 1.4 below shows a sample of Ethiopian propolis collected from Gedo highland areas in Oromia regional state, West Showa Zone. It is dark brown in color. It is the sample that was used in the present study.
In developing countries like Ethiopia where 80% of the population is dependent on the traditional medicine and where malnutrition is common, the popularization of plant-derived, multipurpose honeybee product like propolis is of paramount importance. Ethiopian propolis could have a remarkable medicinal and nutritional value due to high plant diversity and high bee population of the country. However, the medicinal use of Ethiopian propolis has not yet been investigated. Therefore, the present study was an attempt to evaluate the gastroprotective effects of crude ethanol extracts of propolis collected from honeybees (*Apis melifera*) hives against chemical induced gastric lesions and ulceration in mice.

2. **OBJECTIVES**

2.1 **General objective**

To investigate the gastroprotective activity of ethanol extract of Ethiopian propolis.

2.2. **Specific objectives**

1. To find out the major constituent of local ethanol extract of propolis through preliminary phytochemical screening using TLC and GC/MS
2. To evaluate the effects of ethanol extract of propolis on absolute ethanol-and indomethacin-induced gastric mucosal damage in mice.

3. To suggest the possible gastroprotective mechanism of propolis against mucosal damage by exogenous corrosive substances.

3. **MATERIALS AND METHODS**

3.1. **Study design**: Laboratory based experiment (quantitative and descriptive)

3.2. **Study setting**: AAU, FOM, Core laboratory

3.3. **Chemicals and drugs**
The following drugs and chemicals were used: Absolute alcohol (Alpha Chemika, India); Cimetidine (Kwang Myung Pharm Co.Ltd, Korea); Formalin (Alpha Laboratory Reagent, India); Indomethacin (Lagap. Vezia, Switzerland); Omeprazole (Cadila Pharm. Ltd, India); Sodium bicarbonate (BDH Chemicals Ltd Poole, England); Tween 80 (BDH Laboratory Supplies, Poole, England)

3.4. Propolis collection and extract preparation

Lumps of propolis (500g) were collected from honeybee hives during the months of December, 2005 to January, 2006 from Gedo apiary site of Holeta Honeybee Research Center which is about 200km West of Addis Ababa, and were kept in the refrigerator until processed. They were crushed into pieces and the debris was removed by shaking in glass jar containing warm water. The cleaned propolis was then dried, weighed and mixed vigorously with 70% ethyl alcohol in a ratio of 1gram: 5ml (w/v) and then sealed in a container with intermittent shaking twice a day for two weeks as suggested by Krell (1996). After two weeks, the supernatant liquid was filtered with Whatman filter paper No.1. The alcohol was evaporated with a Rota vapor under vacuum and then lyophilized with lyophilizer (Vacaubrad, GMGH, Germany). A consistent gummy material was obtained with a yield of 35.35g (7.07%). It was kept in a clean dark, airtight bottle in a refrigerator at 4°C until used. The sample of EEP was used for both phytochemical screening and evaluation of gastroprotective effects.

3.5. Animal preparation

Eighty Swiss albino mice weighing 24-32g of either sex were used for the present study. Some of the mice (20 mice) were obtained from Addis Ababa University, Science Faculty, Department of Biology and the rest (60 mice) were purchased from Ethiopian Health and Nutrition Research Institute (EHNRI). The mice were given two weeks of acclimatization period in the animal house, Faculty of Medicine, AAU. They were fed with standard pellet diet and water *ad libitum*, and were handled as per the international guidelines for handling experimental animals. The mice were randomly divided into experimental and control groups and were housed in groups of five in standard cages at room temperature with 12hours dark/12hours light cycles. They were deprived of food and were kept in cages with grating floors to prevent coprophagy for 24hours before the experiment but were allowed free access to water. Fifty minutes before the induction of ulcer, the experimental groups were pretreated
with different doses of ethanol extract of propolis (EEP) (25, 50 and 100mg/kg) dissolved in a vehicle (1% Tween 80 aqueous solution); while the positive control groups were given a standard drug (omeprazole or cimetidine). The negative control received the vehicle, which has no protective effect and the damage would be more severe. The positive control groups were used for comparing the effectiveness of the extract with, as their effects and mechanism of action were already known. The extracts and standard drugs used were freshly prepared in distilled water. The animals were then sacrificed by head blow followed with cervical dislocation for histological examinations. From a total of 16 groups of mice (5 each), 10 were used for the evaluation of EEP against ethanol and indomethacin-induced ulcers, 4 for the influence of indomethacin (i.p) pretreatment on the effects of EEP and the remaining 2 groups were used for histological experiments.

3.6. Phytochemical screening for the propolis extract

The major constituents in the propolis extract used in the present study were determined qualitatively and quantitatively with TLC and GC/MS, respectively. The extract was screened for the presence of polyphenols, phenolic acids, phytosteroids and withanoids, phenolic glycosides, flavonoids, terpenes, alcohols, and sugars. The preliminary phytochemical screening was conducted with TLC at Drug Research laboratory of EHNRI according to the method developed by Debella (2002). GC/MS analysis for the quantitative determination was carried out at the Institute of Organic Chemistry with Centre of Phytochemistry (IOCCP), Bulgarian Academy of Sciences, by Drs. Vassya Bankova and Milena Popova.

The TLC plate used was Silica gel 60 F254 coated in aluminum. The EEP sample was dissolved in 96% Ethyl alcohol and the aliquots were applied to the plates with a micro pipette. Two mobile phases were used, containing different concentrations of toluene, ethyl-acetate and formic acid: (5:4:1, V/V/V) and (3.6: 1.2: 1.5, V/V/V) (Kosalec et al., 2003). The mobile phase used for the TLC in the present sample was: Toluene: Ethyl-acetate: Formic acid (5:4:1V/V/V) because of its best resolution. The TLC chamber was saturated with the mobile phase at least 1 hour before analysis. The developed plates were air dried and heated for 10 minute at 110°C to facilitate the development of spots. The polyphenols and phenolic acids were visualized under long (366 nm) and short (254 nm) UV lights before and after spraying with reagents (3%AlCl₃, 1%FeCl₃+K₃Fe (CN)₆, 1% Fast Blue B + 0.1NNaOH, 1%vanillin.
and 9% \( \text{H}_2\text{SO}_4 \) for the presence of different plant metabolites. The position of the spots on the TLC plate was expressed as the retention factor (\( R_f \)), the distance the components traveled divided by the distance the solvent traveled from the base (Debella, 2002).

For GC/MS analysis, about 10g of the same sample of EEP was sent to Institute of Organic Chemistry with Centre of Phytochemistry, Bulgarian Academy of Sciences where Drs. Vassya Bankova and Milena Popova carried out the analysis as follows. The EEP sample was concentrated in vacuum and extracted three times successively with \( n \)-hexane. The hexane extract was evaporated to dryness and then subjected to column chromatography on silica gel with \( n \)-hexane-chloroform solvent gradient to produce several fractions. Different mixtures of the sample were isolated with the preparative TLC using \( n \)-hexane-diethyl ether as a mobile phase and then the chemical constituent of the mixtures were determined by GC/MS.

GC/MS was carried out on a Fisons GC 8000 gas chromatograph coupled to a Fisons MD 800 mass detector under electron impact ionization (70eV). The interface temperature 230°C and the MS scan range 35-450 atomic mass units (AMU) were used. The chromatographic column for the analysis was fused silica OV1 capillary column (25 m X 0.25 mm i. d.). The carrier gas used was helium at a flow rate of 10 ml/min (Karetal et al., 2002).

### 3.7. Gastroprotective effects of ethanol extracts of propolis

#### 3.7.1 Evaluation of EEP effects on ethanol induced gastric lesions

##### 3.7.1.1 Histological observation of EEP effects

Twenty four hour fasted mice were treated with either EEP (50mg/kg) or the vehicle (1% Tween 80 aqueous solution) in a volume of 0.3ml/30g. After fifty minute, absolute ethanol (99%) was administrated in a volume of 0.2ml intra-gastrically to induce gastric lesion (Mequanente et al., 2006). One hour later, the animals were sacrificed by blow on head followed by cervical dislocation. The stomach was excised and gross histological changes were assessed with the help of a hand lens (5 times magnification) and images were taken. Tissue samples from similar areas were taken for both treated and untreated groups, and then processed and embedded in blocks of paraffin wax. The tissue samples were sectioned at 5\( \mu \)m thickness with a microtome (Leica RM2125 Microsystems Nussloch GmbH, Germany) and stained with hematoxylin and eosin (H&E) and then examined under a light microscope (Leitz...
Dialux20 Wetzlar, Germany) for cellular damage, and comparison was made between treated and untreated groups.

### 3.7.1.2. Determinations of lesion index and total number of lesions

After 24 hours of fasting, the experimental animals were given various doses of EEP (25, 50 and 100mg/kg) orally by an intra-gastric tube (Zayachkivska et al., 2005). Equal volume of vehicle (0.3ml/30g 1% Tween 80 aqueous solution) was given for the negative control (placebo) group while omeprazole (50mg/kg), a standard drug, was given to the positive control group by the same route. After fifty minutes, absolute ethanol (99%) was administrated in a volume of 0.2ml intra-gastrically to induce gastric lesion (Mequanente et al., 2006). One hour after, the animals were sacrificed by blow on the head followed by cervical dislocation. The stomach was excised and injected with 3ml of 5% formalin solution. After 15 minutes, the stomach was opened along the greater curvature, rinsed with tap water to clear debris and remains of any wastes. Visual inspections for destructive mucosal lesions were done with the aid of hand lens (5 times magnification) and the measurement of lesion length was done with 6" (150mm) electronic digital caliper (Am-Tech, UK). The extent of damage was expressed as the sum length of all lesions (mm), which is a lesion index, and the mean total number of lesions in the glandular area of the stomach. The results were recorded for both experimental and control groups. Mean values were calculated for gastric lesions and number of lesions according to the method developed by Mequanente et al. (2006). The following formula was used to calculate the percentage inhibition of lesion index by EEP.

\[
\text{% Inhibition} = \frac{[\text{Lesion index in control} - \text{Lesion index in test}]}{\text{Lesion index in Control}} \times 100
\]

The percent inhibition of lesion number was also calculated similarly using the same formula.

### 3.7.2. Assessment of EEP effects on indomethacin induced gastric ulcers

After 24 hours of fasting, experimental mice were given varying doses of EEP (25, 50 and 100mg/kg). Equal volume of vehicle (0.3ml/30g 1% Tween 80 aqueous solution) and cimetidine (100mg/kg) were given for two other groups serving as negative (placebo) and positive (reference) controls, respectively. Fifty minutes later, indomethacin prepared in 2% NaHCO₃ solution was administered at an oral dose of 30mg/kg according to the method
described by Sartori et al. (1999). After six hours, each animal was sacrificed and the stomach was removed and injected with 5% formalin solution. After 15 minutes, the stomach was opened along the greater curvature, rinsed with tap water, and examined for ulcers. The ulcers were counted with the aid of a hand lens (5 times magnification power) and each was given a severity rating as follows: less than 1mm = 1; 1 - 2mm = 2; and greater than 2 mm = 3. The summation of the scores was divided by a factor of 10, to derive ulcer index for each animal as described by Makonnen (1996). The percent inhibition of ulcer was determined in the same way as that for ethanol-induced lesions.

3.7.3. Influence of indomethacin pretreatment on the gastroprotective effects of EEP

To determine whether mucosal protection by the extract was dependent on prostaglandins synthesis, indomethacin (20 mg/kg) prepared in 2% NaHCO₃ or equal volume of the vehicle (2% NaHCO₃ aqueous solution) was given intraperitoneally to 24hours fasted mice an hour before application of the extract (50mg/kg) or vehicle (1% Tween 80 aqueous solution). All the animals received 0.2ml absolute ethanol 50 minutes after extract administration and were sacrificed 1 hour later. Then the ethanol induced lesions were measured and the percentage inhibitions of lesion index and lesion number were calculated as described above.

3.8. Statistical analysis

The data were analyzed using one-way ANOVA. Post hoc comparisons between the experimental and control groups were made with Dunnett’s test using SPSS 10 statistical software package. When appropriate, independent student t-test was used. P value less than 0.05 was considered statistically significant. All data were expressed as mean ± standard error of the mean (M ±SEM).
4. RESULTS

4.1. Phytochemical screening

The phytochemical screening of the ethanol extract of propolis (EEP) with TLC showed the presence of polyphenols, steroids and withanoids, phenolic glycosides, sugars, and terpenoids. These were indicated by spots of different colors at different distances on the TLC plate upon spray by various chemical reagents as shown in Table 4.1 and Figure 4.1.

Table 4.1. Summary of chemical tests of propolis collected from Gedo area with TLC.

<table>
<thead>
<tr>
<th>No.</th>
<th>Spraying agents</th>
<th>Color observed on TLC after spray</th>
<th>No. of Spots</th>
<th>Chemical compounds detected</th>
<th>R&lt;sub&gt;f&lt;/sub&gt; values</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1%F. blue B + 0.1N NaOH</td>
<td>Blue &amp; brown spots</td>
<td>5</td>
<td>Phenolic acids</td>
<td>0.46, 0.58, 0.62, 0.88, 0.95</td>
</tr>
<tr>
<td>2</td>
<td>1%FeCl&lt;sub&gt;3&lt;/sub&gt; + K&lt;sub&gt;3&lt;/sub&gt;Fe(CN)&lt;sub&gt;6&lt;/sub&gt;</td>
<td>Green &amp; blue colored spots</td>
<td>3</td>
<td>Polyphenols (Phenolic comp. &amp; steroids)</td>
<td>0.2, 0.51, 0.6</td>
</tr>
<tr>
<td>3</td>
<td>1% Vanillin</td>
<td>Reddish pink colored spots</td>
<td>6</td>
<td>Terpenoids, Steroids &amp; withanoids</td>
<td>0.50, 0.54, 0.57, 0.60, 0.64, 0.84</td>
</tr>
<tr>
<td>4</td>
<td>9% H&lt;sub&gt;2&lt;/sub&gt;SO&lt;sub&gt;4&lt;/sub&gt;</td>
<td>Deep-pink &amp; black spots</td>
<td>4</td>
<td>phenolic glycosides, sugars, terpenoids</td>
<td>0.48, 0.58</td>
</tr>
<tr>
<td>5</td>
<td>3%AlCl&lt;sub&gt;3&lt;/sub&gt;</td>
<td>------</td>
<td>No</td>
<td>Not detected</td>
<td>------</td>
</tr>
</tbody>
</table>

Solvent system used for TLC was 96% Ethyl alcohol,

R<sub>f</sub>- Retention factor = distance of spot from the base
Distance the solvent moved from the base

TLC plate used was Silica gel- 60 F<sub>254</sub> coated in aluminum.
Figure 4.1. TLC Pattern of EEP using Toluene: Ethyl acetate: Formic acid (5:4:1V/V/V) as a mobile phase and 96% ethyl alcohol as a solvent system. Arrows indicate the spots.

Table 4.2. Depicts summarized results of EEP analysis by GC/MS. The result indicated that the sample contains aromatic acids (1.2%), alcohols (0.6%), esters (1.3%), sugars (24.9%), fatty acids (7.5%), diterpenoic acids (0.6), triterpenic alcohols (26.2%), glycerol (3.8) and shickimic acid (1.1%).
Table 4.2. Summary of major Composition of 70% EEP determined by GC/MS after Silylation

<table>
<thead>
<tr>
<th>Main constituents</th>
<th>% Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aromatic acids(p-hydroxybenzenacetic acid, cis &amp; trans- Caffeic acids)</td>
<td>1.2</td>
</tr>
<tr>
<td>Fatty acids &amp; hydroxyfatty acids (octadecanoic, oleic, hexadecanoic, mallic acid, butandioic acid, tetracozone acid, 2-hydroxypropeic acid &amp; dixhydroxybuteic acid et.c.)</td>
<td>7.5</td>
</tr>
<tr>
<td>Alcohols(trihydroxybutane, dixhydroxybenzene &amp; tetrahydroxybutane)</td>
<td>0.6</td>
</tr>
<tr>
<td>Esters (ethylolate, &amp; ethylhexadecanoate)</td>
<td>1.3</td>
</tr>
<tr>
<td>Diterpenic acids (isopimaric acid)</td>
<td>0.6</td>
</tr>
<tr>
<td>Triterpenic alcohols( α-Amyrin, β-Amyrin, cycloartenol &amp; 20,29-Lupen-3-one)</td>
<td>26.2</td>
</tr>
<tr>
<td>Sugars</td>
<td>24.9</td>
</tr>
<tr>
<td>Others(glycerol, shickimic acid, ethylamine phosphoric acid &amp; ethylphosphate)</td>
<td>5.5</td>
</tr>
</tbody>
</table>

* The figures correspond to the percent of total ion current (GC-MS). The ion current generated depends on the characteristics of the compound concerned and is not a true quantification.

4.2. Gastroprotective effects of EEP on ethanol induced gastric lesions

4.2.1. Histological changes as the result of EEP treatment

The gross histology in Figure 4.2 depicts the effect of EEP on acute gastric mucosal injury induced by 0.2ml absolute alcohol (99%) in mice. Multiple hemorrhagic erosions with acute edema were observed in the glandular area of mouse stomach 1hour after administration of alcohol (Figure 4.2A). The administration of EEP at a dose of 50 mg/kg markedly reduced the
hemorrhagic erosions and edematous lesions as can be observed in the antral portion of the stomach (Figure 4.2B).

The histological differences between vehicle treated and EEP treated groups were observed at the cellular level. Figure 4.3 presents the histological manifestations observed in the tissue that has been taken from the same areas of stomach (antral areas) processed and stained with hematoxylin and eosin. Hemorrhagic mucosal erosions and inflammatory cell infiltrations developed in the glandular stomach of mice 1 hour after the administration of ethanol (Figure 4.3A). The administration of EEP at dose of 50 mg/kg markedly reduced these changes (Figure 4.3B).

Figure 4.2. Gross histology of the glandular area of mouse stomach showing the hemorrhagic lesions. A: Vehicle treated (control) and B: Extract treated (50 mg/kg) Arrows indicate the ulcerated area.

Figure 4.3. Histological manifestation of hemorrhagic erosions and inflammatory cell infiltrations in the glandular stomach of mice 1 hour after the administration of ethanol. A: Vehicle treated (control) and B: Extract treated (50 mg/kg) The administration of EEP markedly reduced these changes.
4.2.2. Effects of EEP on lesion index and lesion number

The gastroprotective effects of EEP on absolute ethanol-induced gastric lesions are shown in Table 4.3. Vehicle treated control mice showed extensive number of gastric mucosal lesions and high ulcer index in the glandular segments (Figures 4.2A and 4.3A). Dose dependent gastroprotective effects of the extract against ethanol-induced lesion were observed with the varying doses of the extract. EEP at doses 25, 50 and 100mg/kg and omeprazole (50mg/kg) all significantly reduced lesion index (p<0.05) compared to the control. Low dose (25mg/kg) of the extract was less potent, while the high doses (50 and 100mg/kg) showed results similar to the ones produced by the standard drug, omeprazole (50mg/kg).

Table 4.3. Gastroprotective effects of EEP against alcohol-induced gastric lesion in mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dosage(mg/kg)</th>
<th>N</th>
<th>Lesion Index</th>
<th>% ILI</th>
<th>Lesion No.</th>
<th>% ILN</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>------</td>
<td>5</td>
<td>31.8±2.23</td>
<td>------</td>
<td>16.4±1.03</td>
<td>------</td>
</tr>
<tr>
<td>EEP</td>
<td>25</td>
<td>5</td>
<td>15.2±2.03*</td>
<td>52.17</td>
<td>6.2±1.46*</td>
<td>62.19</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>5</td>
<td>11.38±1.12*</td>
<td>64.24</td>
<td>4.4±0.51*</td>
<td>73.17</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>5</td>
<td>8.10±1.52*</td>
<td>74.54</td>
<td>3.8±0.97*</td>
<td>76.83</td>
</tr>
<tr>
<td>Omeprazole</td>
<td>50</td>
<td>5</td>
<td>8.98±1.52*</td>
<td>71.78</td>
<td>4.2±1.11*</td>
<td>74.39</td>
</tr>
</tbody>
</table>

*P<0.05= statistically significant relative to control (Dunnet’s test)
% ILI= percent inhibition of lesion index; %ILN= percent inhibition of lesion number
N= number of animals; EEP= Ethanol extract of propolis
4.3. Gastroprotective effects of EEP on indomethacin induced ulcer

Intragastric administration of indomethacin (30 mg/kg) resulted in production of gastric lesions on glandular segment of the stomach. The EEP showed significant gastroprotective effect against indomethacin-induced ulcers at all dose levels (25, 50 and 100mg/kg) compared to the control groups (P<0.05). The protective effect of the extract was increased with increasing dose. As shown in Table 4.4, higher doses of EEP showed similar effect to that of cimetidine at the same dose, i.e. 100mg/kg.

Table.4.4. Gastroprotective effects of EEP against indomethacin-induced gastric ulcer in mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose(mg/kg)</th>
<th>N</th>
<th>Ulcer Index</th>
<th>%IUI</th>
<th>Ulcer number</th>
<th>%IUN</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>-----</td>
<td>5</td>
<td>2.74 ± 0.19</td>
<td>----</td>
<td>13.2 ± 0.58</td>
<td>-----</td>
</tr>
<tr>
<td>EEP</td>
<td>25</td>
<td>5</td>
<td>1.86±0.16*</td>
<td>32.12</td>
<td>9.4 ± 0.51*</td>
<td>28.79</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>5</td>
<td>1.24±0.11*</td>
<td>54.74</td>
<td>5.4 ± 0.75*</td>
<td>59.10</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>5</td>
<td>0.64 ± 0.15*</td>
<td>76.64</td>
<td>3.8 ± 0.74*</td>
<td>71.21</td>
</tr>
<tr>
<td>Cimetidine</td>
<td>100</td>
<td>5</td>
<td>0.62 ± 0.11*</td>
<td>77.37</td>
<td>3.6 ± 0.51*</td>
<td>72.73</td>
</tr>
</tbody>
</table>

*P < 0.05 = statistically significant relative to control (Dunnet’s test)

%IUI= percent inhibition of ulcer index; N= number of the animal
%IUN= percent inhibition of ulcer number; EEP= Ethanol extract of propolis

4.4. Influence of indomethacin pretreatment on the gastroprotective effects of EEP

Indomethacin (20 mg/kg, i.p.) pretreatment significantly aggravated the ethanol-induced lesions (P < 0.05) compared to the vehicle (2% NaHCO₃ aqueous solution) treated mice as indicated on Figure 4.14. In the indomethacin-pretreated groups the lesion index due to ethanol was 44.4 ± 1.72 and total lesion number 20 ±1.05 for control group while the lesion index and total lesion number in EEP treated group was found to be 25.4±1.36 and 8.8±0.86, respectively (P<0.05) reducing the lesion index and total lesion number by 42.79% and 56%, respectively. For the vehicle (2% NaHCO₃) (i.p) pretreated group the lesion index and total lesion number were 33.2±2.06 and 13.6 ±1.03 for the untreated (distilled water group). Upon treatment with 50mg/kg EEP, the lesion index and total lesion number were reduced to 16.7±1.68 and 7.6 ±0.93 (P<0.05), indicating 49.7% and 44.12% inhibition, respectively.
Figure 4.4. The Influence of indomethacin pretreatment on gastroprotective effects of EEP

EEP = Ethanol extract of propolis; DW = distilled water; Veh = vehicle;

Dw = distilled water; Indo = indomethacin

* = P < 0.05 statistically significant compared to the control (Veh-Dw) (student t-test)
+ = p < 0.05 statistically significant compared to the control (Veh-Dw) (student t-test)
5. DISCUSSION

The gastric hyperacidity and ulceration of the stomach mucosa due to various agents are serious health problems of global concern. Moreover, there is growing evidence that oxygen-derived free radicals such as OH, O2⁻, RO⁻, and ROO⁻ play a role in the pathogenesis of various disorders of the digestive system including gastric ulcer (Dockmeci et al., 2005). A number of excellent drugs developed over the years, have proven useful in controlling hyperacidity and ulceration though their long-term use is reported to be associated with various side effects. The search for novel non-toxic, anti-ulcer preparations from medicinal plants is currently in vogue in order to obtain alternative sources of medicine for the management of gastric hypersecretion and gastroduodenal ulcers. In the developing nations, this turn of events has been prompted in part by the high cost of modern anti-ulcer medication, as well as the multiple side effects that result from their prolonged use (Tan et al., 2005). In the present study the gastro-protective effects of propolis from Ethiopian central high land were tested using alcohol and indomethacin as ulcerogenic agents. The major components of ethanol extract of propolis (EEP) were identified with TLC and GC/MS.

Thin layer chromatography (TLC) analysis showed the presence of polyphenols and phenolic acids, phenolic glycosides, sugars, terpenoids, steroids and withanoids in the present propolis sample. But flavonoids were not detected in the present EEP sample as expected. Further phytochemical analysis remains to be done to establish the fact.

The analysis of the same sample by GC/MS indicated that the major components in the sample were amyrin type triterpenic alcohol (α-amyrin, β-amyrin, cycloartenol and 20, 29-lupen-3-one), sugars, and fatty acids. Also, significant amount of esters, aromatic acids including caffeic acid, diterpenic acids, alcohols and others compounds like glycerol, shickimic acid, ethylamine phosphoric acid and ethylphosphate were found in the EEP in the present study. The GC/MS also confirmed the absence of flavonoids in the sample. Investigations on tropical and European propolis revealed that in many cases flavonoids are their important components although their plant origins are different (Bankova et al., 2000). Our present finding, however, did not indicate the presence of flavonoids in the sample.
Instead, new diterpenic acids and triterpenic compounds with valuable biological activities have been identified from tropical regions, which holds true for the present findings (Bankova et al., 2000). These include anti-tumor clerodane derivative, the cytotoxic substances like artepillin C and compounds with antibacterial activities (Banskota et al., 2001). Bankova et al. (2000) reported the presence of triterpenic alcohols of amyrin type (β-amyrin and cycloartenol) in propolis samples from Brazil and Egypt, both of which are from tropical regions that include Ethiopia. The major components in propolis of Brazilian origin were found to be terpenoids and prenylated derivatives of p-coumaric acids (Kumazawa et al., 2004). These compounds have recently been found to possess antibiotic activity against bacteria and fungi, and antioxidant activity similar to that of tocopherol (Banskota et al., 2001; Trushava et al., 2006). In the sample used in the present study, the total amount of phenolic compounds was found to be very low, though significant amount of cis-caffeic acids and trans-caffeic acids were detected. In agreement with the findings by Bankova et al. (2000), GC/MS analysis of our sample showed the presence of triterpenic and diterpenic alcohols. Triterpenic alcohols are typical for Brazilian propolis, the most abundant among them being β-amyrin (Trushava et al., 2006). Nevertheless, the high proportion of triterpenic alcohols in the present extract is thought to be unique to the Ethiopian propolis with reference to most samples for which extensive analysis have been done so far (Bankova, 2006; personal communication).

The ability of the gastric mucosa to resist injury by endogenous secretions and by ingested irritants (e.g. alcohol, NSAIDs) can be attributed to a number of factors that have been generally referred to as mucosal defense (Wallace, 2001). Ethanol-induced gastric ulcers have been widely used for the experimental evaluation of anti-ulcer activity. Disturbances in gastric secretion, damage to gastric mucosa, alterations in permeability, gastric mucus depletion and free-radical production like hydroxyl radicals are well known pathogenic effects of ethanol (Pandian et al., 2002). These effects of alcohol bring about depression in gastric defensive mechanisms leading to the formation of gastric mucosal lesions. On one hand, ethanol reduces mucus production, gastric mucosal blood flow, bicarbonate secretion, endogenous glutathione and prostaglandin (PG) levels. On the other hand, it increases the release of histamine, the influx of calcium ions and the generation of free radicals (de Barros et al., 2007). Recently, much attention has been focused on the role of ROS, including \( \mathbf{O}_2^- \), \( \mathbf{OH}^- \) and \( \mathbf{H}_2\mathbf{O}_2 \) in
mediating alcoholic tissue damage. Preventive endogenous antioxidants, such as SOD and catalase enzymes are the first line of defense against ROS. Reduced glutathione is a major scavenger of free radicals in the cytoplasm and an important inhibitor of free radical mediated lipid peroxidation. Various exogenous antioxidants such as melatonin and garlic have a protective effect against gastro-duodenal injury and reduce levels of ROS and thus decreasing ulcer formation in digestive tract (Dockmeci et al., 2005). The same could be true for the gastroprotective effects of propolis due to its antioxidant activity. Propolis might either potentiate the endogenous antioxidants or act as free radical scavengers (Banskota et al., 2001) and thus protect the gastric mucosa against injurious activity of alcohol.

In the alcohol-induced gastric lesions in the present study, EEP and omeprazole treated mice showed significant reduction in both the lesion index and total lesion numbers in the glandular area of their stomach. There was a dose dependent increase in the potency of the extract as the inhibition in ulcer index increased with increasing dose. It has long been known that intragastric administration of ethanol induces congestive hyperemia of the gastric mucosa and sub-mucosa, and that edema, necrosis and hemorrhage may arise in glandular areas of the stomach. That was why the control group that received alcohol alone showed extensive hemorrhagic lesions, and edematous epithelial cell infiltrations that cover large area of the glandular segment, while the EEP pretreated ones showed only few numbers of ulcers and reduced lesion index indicating the gastroprotective effects of the extract. The gastroprotective effect of EEP against mucosal damage induced by alcohol could be due to its antioxidant and/or free radical scavenging effects (Hegazi and Abd El Hady, 2002; Russo et al., 2002). The film forming nature of EEP may also contribute to its protective effects.

Many components of mucosal defense are regulated by PGs and NO. Endogenous PGs regulate mucosal blood flow, epithelial cell proliferation, epithelial restitution, mucosal immunocyte function, mucus and bicarbonate secretion, and basal acid secretion (Wallace, 2001). Prostaglandins induced protection of gastroduodenal mucosa involves increasing mucosal resistance on one hand and decreasing aggressive factors on the other hand, mainly acid and pepsin. Inhibition of cytoprotective prostaglandin synthesis probably weakens the gastric mucosal defense to resist luminal irritants, leading to disruption of gastric mucosal barrier and gastric lesions. The ulcerogenic effect of NSAIDs correlates well with its ability to suppress prostaglandin synthesis through their action on COX-pathway. Deleterious effects of
nonselective NSAIDs on gastroprotection results from their inhibition of COX-1 isoform, and indomethacin being one of such drugs, blocks both COX-1 and COX-2 and thus brings about gastric ulceration (Peura, 2002; Stillman and Stillman, 2007). Like PGs, NO has been shown to increase mucosal blood flow, stimulate mucus secretion, and inhibit neutrophil adherence (Wallace, 2001). In animals, NO-releasing NSAIDs, e.g. NCX-530 and NCX-4016, a NO-releasing aspirin, produce less gastric damage than their parent drugs, and they even promote ulcer healing (Wallace, 2001; Chan and Leung, 2002). The propolis extract exhibits anti-inflammatory effects against acute and chronic models of inflammations (Borrelli et al., 2002). Propolis might decrease the expression of inducible isoform of COX-2 and inducible NO synthase (iNOS) enzymes, though its exact mechanism of action remains to be established (Castaldo and Capasso, 2002; Tan-No et al., 2006). Though propolis has been observed to have anti-inflammatory activity, it may not inhibit COX-1 as the present study showed its anti-peptic ulcer effect.

Reactive oxygen species especially hydroxyl radicals play a major role in causing oxidative damage of the gastric mucosa in all types of ulcers including stress related gastric mucosal damage, NSAIDs-induced gastric lesions, and *H. pylori* mediated gastroduodenal ulcers (Demir et al., 2003; Jain et al., 2007). In the present study EEP significantly reduced the mucosal damage (i.e., ulcer index) induced by indomethacin. Its effect was comparable to that of cimetidine indicating that the extract is equipotent with cimetidine.

The inhibition of prostaglandin biosynthesis by indomethacin (i.p) pretreatment did not affect the gastroprotective effect of the extract against alcohol induced mucosal ulceration in mice in our study. This suggests that the presence of endogenous prostaglandins might not be essential to the expression of mucosal protective activity of the extract or the extract may increase mucus and/or prostaglandin secretion that counteract the reduction by the drug. This effect could be explained as the probable cytoprotective mechanism of the extract, which is in agreement with recent study on Brazilian green propolis on experimental gastric ulcers in rats (de Barros et al., 2007). The fact that i.p pretreatment by indomethacin did not abolish the gastroprotective effects of the EEP gives a clue that propolis, having an anti-inflammatory property, might act through inhibition of inducible prostaglandin synthesis (COX-2) preserving the housekeeping pathway (COX-1) (Tan-No et al., 2006). Agents which have cytoprotective and/or anti-acid secretory effect prevent gastric lesions induced by
indomethacin. Mequanente et al. (2006) suggested that the film forming property of *L. usitatissimum* gum extract could play important role for its gastroprotective effects. The gummy resin nature of the propolis extract which gives it film forming property could also be important in the gastroprotective effects of the material.

The gastroprotective effect of EEP shown in this study is attributed to the chemical make up of the extract. The phytochemical analysis confirmed that the extract contained high proportion of terpenoid and their derivatives, and also some phenolic compounds and their esters, and aromatic acids like caffeic acid. Anti-ulcerogenic terpenoids include triterpenes, diterpenes and terpenic derivatives have been isolated from plants. The triterpenic derivative carbenoxolone, for instance, has been extensively investigated for such mode of action. Carbenoxolone is an excellent stimulant of mucus synthesis, maintains the prostaglandin content of gastric mucosa at high levels and has been reported to inhibit pepsin secretion (Rodriguez et al., 2006).

Several terpenes or their derivatives have been shown to possess gastroprotective activity in different models of gastric lesions in animal and promoting healing of subacute gastric lesions in rats (Rodriguez et al., 2002). Hiruma-Lima et al. (1999) reported that the diterpene lactone dehydrocrotonin exhibited gastroprotective properties that could be due to an increase in prostaglandin E$_2$ release and non-competitive antagonism of H$_2$-receptors and/or of muscarinic receptors. As EEP contains diterpenes, triterpenes and aromatic compounds like caffeic acid, its anti-peptic ulcer effect could be attributed to the synergistic gastroprotective effects of these compounds. The antioxidant property of propolis is also attributed to its free radical scavenging activity against alkoxyl radicals (Padmavathi et al., 2006).

The wide diversity of propolis composition revealed in the last 10–15 years foretells much further research work and a distant horizon for the completion of the evaluation of the full potentiality of propolis chemistry and pharmacology (Salatino et al., 2005). The plant origin of propolis determines its chemical composition and this depends on the species of local flora present at the site of collection, and the geographic and climatic characteristics of the site.
6. CONCLUSION AND RECOMMENDATIONS

6.1. Conclusion

The present study showed that the propolis extract sample from Ethiopian central high land area consists of uniquely high proportion of amyrin type triterpenic alcohols. The absence of flavonoids, in contrast to most propolis samples from both temperate and tropical geographical regions, makes the Ethiopian propolis unique.
From the present findings, it can be concluded that EEP has anti-ulcerogenic property and the cumulative effect of its chemical constituents is responsible for this. The Ethiopian propolis has, therefore, the potential to be exploited as anti-peptic ulcer agent pending further investigation.

6.2. **Recommendations**

- Phytochemical screening with advanced instruments and fractionation of the propolis samples from different areas should be isolated, tested and analyzed to investigate its chemical make up, medicinal and nutritional values.

- The anti-ulcerogenic effects of Ethiopian propolis should be further evaluated with its biochemical interactions; its ulcer healing effects and toxicity tests in animal models to establish its uses in peptic ulcer managements.

- Samples from different agro-ecological zones covering larger area of the country should be assessed to come up with characteristics and potentiality of Ethiopian propolis.

7. **References**


Langen beck’s Arch Surg. 386:75–81.


Phytochemical compounds involved in the anti-inflammatory effect of propolis extract. 
Fitot. 73: S53–S63.


8. ANNEXES

Annex 1. Summary of the sources of plant cytoprotectors and their known physiological actions on GIT (Source: Zayachkivska *et al.*, 2005)

<table>
<thead>
<tr>
<th>Physiological actions</th>
<th>Origins</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gastroprotective and antiulcer</td>
<td>Grapefruit (<em>Citrus paradisi</em>) seeds</td>
</tr>
<tr>
<td>Induced changes in amount and</td>
<td><em>Panax ginseng</em></td>
</tr>
<tr>
<td>glycoprotein content of gastric mucus</td>
<td><em>Erica andrevalensis Cabezudo-Rivera</em></td>
</tr>
<tr>
<td>Preventive and curative effects</td>
<td>UL-409, herbal formulation</td>
</tr>
<tr>
<td>Inhibition the basal and histamineinduced</td>
<td><em>Azadirachta indica</em>, Chinese cinnamon</td>
</tr>
<tr>
<td>gastric acid secretion</td>
<td><em>Phellodendron amurense</em> Ruprecht</td>
</tr>
<tr>
<td>NO-induced rise in mucosal blood flow</td>
<td><em>Ginseng</em>, <em>Silybum marianum</em>, <em>Grapefruit seeds</em></td>
</tr>
<tr>
<td>Mucus and alkaline secretion</td>
<td><em>Bacopa monniera</em>, <em>Grape seeds</em></td>
</tr>
<tr>
<td>Prostaglandin release</td>
<td><em>Tasmannia lanceolata</em>, <em>Bacopa monniera</em></td>
</tr>
<tr>
<td></td>
<td><em>Azadirachta indica</em>, <em>Mikania cordata</em></td>
</tr>
<tr>
<td></td>
<td><em>Solon</em> <em>(Sophoradin)</em></td>
</tr>
<tr>
<td></td>
<td><em>Tasmannia lanceolata</em>, <em>Petasites hybridus</em></td>
</tr>
</tbody>
</table>
Ruta chalepensis L. (Rutaceae)

Hepatoprotective

Tinospora bakis (Menispermaceae)

Premna tomentosa (L. Verbanacae)

Anticancerogenic

Grapefruit seeds, Garinia kola

Grape seeds

Annex 2. Characteristic of propolis from different geographic origin
(Source: Bankova et al., 2000).

<table>
<thead>
<tr>
<th>Geographical origin</th>
<th>Plant source</th>
<th>Typical constituents (main components)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Europe, Asia, North America</td>
<td>Populus spp. (poplar)</td>
<td>pinocembrin, pinobanksin, pinobanksin-3-O-acetate, chrysin, galangin, caffeates (benzyl, phenylethyl, prenyl)</td>
</tr>
<tr>
<td>Northern Russia</td>
<td>Betula verrucosa (birch)</td>
<td>acacetin, apigenin, ermanin, rhamnocitrin, kaempferid, α-acetoxybetulenol</td>
</tr>
<tr>
<td>Brazil</td>
<td>Baccabris spp.</td>
<td>prenylated p-coumaric acids, prenylated acetophenones</td>
</tr>
<tr>
<td></td>
<td>Araucaria spp.</td>
<td>diterpenic acids</td>
</tr>
<tr>
<td>Canary Islands</td>
<td>unknown</td>
<td>furoruran lignans</td>
</tr>
</tbody>
</table>

Annex 3. Compounds responsible for the biological activity of different propolis types
(Source: Bankova, 2005).

<table>
<thead>
<tr>
<th>Propolis type</th>
<th>Antibacterial activity</th>
<th>Anti-inflammatory activity</th>
<th>Anti-tumor activity</th>
<th>Hepatoprotective activity</th>
<th>Antioxidant activity</th>
<th>Allergic action</th>
</tr>
</thead>
<tbody>
<tr>
<td>European (poplar type)</td>
<td>Flavanones, flavones, phenolic acids and their esters</td>
<td>Flavanones, flavones, phenolic acids and their esters</td>
<td>Caffeic acid phenethyl ester (CAPE)</td>
<td>Caffeic acid, ferulic acid, and caffeic acid phenethyl ester</td>
<td>Flavonoids, phenolic and their esters</td>
<td>3,3-Dimethoxy caffeate</td>
</tr>
<tr>
<td>Brazilian (Baccharis type)</td>
<td>Prenylated p-coumaric acids, labdane diterpenes</td>
<td>Unidentified</td>
<td>Prenylated p-coumaric acids, clerodane diterpenes, benzofuranes</td>
<td>Prenylated p-coumaric acids, flavonoids)</td>
<td>Prenylated p-coumaric acid, flavonoids</td>
<td>Not tested</td>
</tr>
<tr>
<td>Cuban</td>
<td>Prenylated benzophenones</td>
<td>Not tested</td>
<td>Not tested</td>
<td>Not tested</td>
<td>Preynlated benzophenones</td>
<td>Not tested</td>
</tr>
<tr>
<td>Taiwanese</td>
<td>Not tested</td>
<td>Not tested</td>
<td>Not tested</td>
<td>Not tested</td>
<td>Prenylated flavanones</td>
<td>Not tested</td>
</tr>
</tbody>
</table>