ADDIS ABABA UNIVERSITY SCHOOL OF GRADUATE STUDIES

EVALUATION OF THE ANTI PEPTIC ULCER ACTIVITY OF THE LEAF EXTRACT OF PLANTAGO LANCEOLATA IN RODENTS.

BY: ENDALE MELESE TSEGAYE

February, 2010

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BY

Endale Melese (B. PHARM)

A thesis submitted to the school of graduate studies of A.A.U. in partial fulfillment of the requirements for the degree of master of science in pharmacology in the department of pharmacology, school of pharmacy, A.A.U.

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3.2.3. Groupings of animals and dose determination 18

3.2.4. Acetic acid induced chronic gastric ulcer 19

3.2.5. Indomethacin induced gastric ulcer 20

3.2.6. Cysteamine HCl induced duodenal ulcer 20

3.2.7. Pylorus ligation induced gastric ulcer 21

3.2.8. Statistical analysis 23

4. RESULTS 24

4.1. Effect of the aqueous extract and mucilage of *P. lanceolata* in acetic acid induced chronic gastric ulcer. 24

4.2. Effect of the aqueous extract of *P. lanceolata* in indomethacin induced gastric ulcer. 25

4.3. Effect of the aqueous extract P. *lanceolata* on cysteamine HCl induced duodenal ulcer. . 26

4.4. Effect of the aqueous extract of *P.lanceolata* on ulcer score, total acidity, volume of gastric secretion, pH of gastric secretion and mucus secretion on pylorus ligation induced gastric ulcer. 28

4.5. Acute toxicity 32

5. DISCUSSION 33

5.1. Acetic acid induced chronic gastric ulcer 33

5.2. Indomethacin induced gastric ulcer 34

5.3. Cysteamine HCl induced duodenal ulcer. 35

5.4. Pylorus ligation induced gastric ulcer 36

5.5. Acute toxicity 37

6. CONCLUSION 39

SUGGESTIONS FOR FUTURE WORK 40

REFERENCES 40

LIST OF TABLES

Table 1: Effect of the aqueous extract and mucilage of *p. lanceolata* on ulcer index, % reduction in ulcer index and ulcer score in acetic acid induced chronic gastric ulce.....24

Table 2: Effect of the aqueous extract of *P. lanceolata* on ulcer score and % reduction in ulcer score in indomethacine induced gastric ulcer. 26

Table 3: Effect of the aqueous extract of *P. lanceolata* on ulcer score, ulcer area and % protection in cysteamine HCl induced duodenal ulcer. 26

Table 4: Effect of the aqueous extract of *P. lanceolata* on ulcer score, and mucus secretion in pylorus ligation induced gastric ulcer. 28

LIST OF FIGURES

Fig. 1. 1. Factors involved in the regulation of gastric acid secretion. 3
Fig. 1. 2. Gastric mucosal defense mechanism. 4
Fig. 1. 3. Pathogenesis of PUD by NSAIDs..... 10
Fig. 1.4. Picture of *Plantago lanceolata*..... 14
Fig. 3.1. Calibration curve for Alcian blue 8GX in aqueous solution..... 22
Fig. 4. 1. Pictures of acetic acid induced chronic gastric ulcers in mice 25
Fig. 4. 2. Effect of the aqueous extract of *P. lanceolata* on ulcer index in cysteamine HCl induced duodenal ulcer..... 27
Fig. 4. 3. Pictures of cysteamine induced duodenal ulcers in mice 27
Fig. 4. 4. Effect of the aqueous extract of *P. lanceolata* on the total acidity of the gastric secretion. 29
Fig. 4. 5. Effect of the aqueous extract of *P. lanceolata* on volume of gastric secretion in ml/100g of rat. 30
Fig. 4. 6. Effect of the aqueous extract of *P. lanceolata* on pH of gastric secretion..... 30
Fig. 4. 7. Pictures of pylorus ligation induced gastric ulcers in rats. 31

ACRONYMS

5-HT	5- Hydroxy tryptamine (Serotonin)
Ach	Acetylcholine
ANP	Antral natriuretic peptide
AP	Anterior pituitary gland
BT	Body temperature
CCK	Cholecystokinine
CCK_1	Cholecystokinine receptor sub type 1
CCK_2	Cholecystokinine receptor sub type 2
CD11/CD18	Cluster of differentiation
CGRP	Calcitonin gene-related peptide
COX-1	Cyclooxygenase-1
COX-2	Cyclooxygenase-2
CRH	Corticotropin-releasing factor
DMN	Dorsal motor nucleus of the vagus nerve
DU	Duodenal ulcer
DVC	Dorsal vagal complex
ECL	Enterochromafin-like
ICAM-1	Intercellular adhesion molecule-1
IL-12	Interleukin-12
M_3	Muscarinic receptor sub type 3

NA	Nucleus ambiguous
NSAIDs	Non steroidal anti inflammatory drugs
NTS	Ncleus tractus solitarius
PACAP	Pituitary adenylate cyclase-activating peptide
P-CABs	Potassium competitive acid blockers
PGs	Prostaglandins
PP	Pancreatic polypeptide
PPG	Posterior pituitary gland
PPIs	Proton pump inhibitors
PPY	Peptide Y
PUD	Peptic ulcer disease
PVN	Paraventricular nucleus of the hypothalamus
ROS	Reactive oxygen species
SRMD	Stress related mucosal damage
SSTR1-5	Somatostatine receptors sub type 1-5
TNFα	Tumour necrosis factor alpha
TRH	Thyrotropin-releasing hormone.
WHO	World health organization
Y1	Peptide Y receptor sub type 1
Y2	Peptide Y receptor sub type 2
αvp	Arginine vasopressin

ABSTRACT

The lesion of peptic ulcer disease (PUD) is a disruption in the mucosal layer of the stomach or duodenum. An ulcer is distinguished from erosion by its penetration through the muscularis mucosa or the muscular coating of the gastric or duodenal wall. PUD results from the imbalance between defensive factors that protect the mucosa and offensive factors that disrupt this important barrier. There are several different types of modern drugs for the treatment of PUD though they are associated with clinically significant drug interactions, side effects, and relapse. Thus, there is a need to develop new drugs, for which natural products are potential candidates. *Plantago lanceolata* is used traditionally for the treatment of upper respiratory inflammation, urinary conditions, uterotonic, vascular disorders, wound, cough, ulcer etc.

The present study was undertaken to evaluate the anti ulcer activity of the aqueous extract, and mucilage of *P. lanceolata* using different rodent models of PUD. Negative controls were given distilled water. Whilst positive controls received ranitidine in acetic acid induced chronic gastric ulcer, cysteamine HCl induced duodenal ulcer and pylorus ligation induced gastric ulcer models; misoprostol was used in indomethacin induced gastric ulcer. One group of animals received 200 mg/kg (for mice) and the other group received 400 mg/kg (for mice) of the aqueous extract in each model. In addition, one group of animals received 172 mg/kg of the mucilage in acetic acid induced chronic gastric ulcer model.

The aqueous extract showed a better healing of ulcer than the mucilage in the acetic acid induced chronic gastric ulcer as evidenced by the higher percent reduction in ulcer index (77.9% vs 51.9%) and ulcer score (Table 1). The aqueous extract was therefore, used for further evaluation. The effect of the aqueous extract at 400 mg/kg was more effective in healing and/ or protecting ulcer in virtually all models than 200 mg/kg.

The extract at both 200 mg/kg and 400 mg/kg produced reduction in ulcer index ($P<0.05$ and $P<0.001$ respectively) as compared to the negative control in acetic acid induced chronic gastric ulcer. However, it was only 400 mg/kg of the extract that produce a significant reduction ($P<0.01$) in the ulcer score as compared to the negative control. In addition, the percent reduction in the ulcer index by 400 mg/kg of the extract was higher than ranitidine (77.9 % vs 76.2%). Similar results were also found on the ulcer score in pylorus ligation induced gastric ulcer. The aqueous extract at 400 mg/kg was found to offer significant

protection in indomethacin induced stomach ulcer as compared to the negative control ($P<0.001$) as well as to the 200 mg/kg of the extract ($P<0.01$). Moreover, it appeared to reduce the ulcer score by 86.4% while misoprostole reduced it by only 68.2%. Ranitidine protected the ulcerogenic effect of cysteamine HCl by 73.3% while 400 mg/kg offered protection by 60% indicating ranitidine is more effective in inhibition of acid secretion than the extract.

In conclusion, the aqueous extract at 400 mg/kg showed a better activity against gastric as well as duodenal ulcer than the standard drugs. Moreover, some of the possible mechanisms by which the extract showed the activity were corroborated to be anti secretory and cytoprotection. Thus, the present work up holds the traditional use of the plant for PUD.

Keywords: Peptic ulcer disease, Acetic acid induced chronic gastric ulcer, Pylorus ligation induced gastric ulcer, Cysteamine HCl induced duodenal ulcer, Indomethacine induced gastric ulcer, and *P. lanceolata.*

1. INTRODUCTION

1.1. Regulation of gastric acid secretion

The acid secreting parietal cells are the principal cells in gastric glands. Three major pathways activating parietal acid secretion include neuronal stimulation via the vagus nerve, paracrine stimulation by local release of histamine from enterochromafin-like (ECL) cells, and endocrine stimulation via gastrin released from antral G cells (Weiner *et al.*, 1996).

In neuronal pathway, acetylcholine (ACh) released by vagal nerve; emanating from two vagal complex nuclei, the dorsal motor nucleus and ambiguous nucleus; upon stimulation by thyrotropin-releasing hormone (TRH) or other stimulants, directly stimulates muscarinic M_3 receptors on the basolateral membrane of parietal cells (Konturek *et al.*, 2004). Likewise, injection of TRH or its stable analog RX77368 in to the cisternae magna or dorsal vagal complex (DVC) increased efferent vagal discharge activity leading to vagal stimulation of gastric acid, pepsin, histamine, etc. secretions through vagal dependent muscarinic mediated mechanisms as shown in Fig. 1.1. Sites of actions of TRH have been located in the DVC and Central amygdala. Several data indicate that medullary TRH may play role in cold restraint stress-induced gastric lesions. Furthermore, ACh indirectly stimulates release of histamine from ECL cells in the fundus and gastrin from the G cells in the gastric antrum (Weiner *et al.*, 1996; Kiraly *et al.*, *2000).*

ECL cells, the sole source of gastric histamine, are present in close proximity to parietal cells. Histamine released from ECL cells activates parietal cells in paracrine fashion by binding to H_2 receptors. Gastrin is primarily present in antral G cells. Release of gastrin is under regulation of central neural activation, local distension, and chemical composition of gastric content. In addition, gastrin stimulates parietal cells by binding with gastrin receptors. Gastrin also exerts its action in an indirect manner by causing the release of histamine from ECL cells as shown in Fig.1.1 (Weiner *et al.*, 1996).

Furthermore, gastrin stimulates the secretion of hydrochloric acid through activation of chole cystokinine 2 (CCK_2) receptors located on parietal cells or histamine-releasing ECL cells. Furthermore, CCK can either mimic the excitatory action of gastrin on acid secretion, or inhibit

the stimulated acid output: this latter effect appears to be related to the release of somatostatin from gastric D cells, mediated by CCK_1 receptors (Blandizzi *et al.*, 1999).

Somatostatin released from gastric D cells acts locally as a paracrine hormone to inhibit gastric acid secretion. Antisecretory effects of somatostatin result mainly from a direct inhibition of histamine release from ECL cells and, to a lower extent, from a direct inhibitory effect on parietal cells. Although the various biological effects of somatostatin are mediated through five different receptor sub types ($SSTR_{1-5}$), convincing evidence supports that $SSTR_2$ receptors, located mainly on ECL cells, are primarily involved in the gastric antisecretory action of the peptide. A lot of studies showed that various regulatory peptides inhibiting gastric acid secretion exert their action through, partially or totally, somatostatin dependent pathways. These peptides stimulate the release of gastric somatostatin that in turn inhibits acid secretion through the stimulation of $SSTR_2$ receptors on ECL cells and parietal cells (Komasaka *et al.*, 2002; Piqueras *et al.*, 2004).

In addition, there are a lot of endogenous peptides that are involved in the regulation of acid secretion by different mechanisms: for example, PPY, galanin etc. Circulating PYY may enter the brain through the area postrema and portions of the nucleus tractus solitarius (NTS) to inhibit through Y_2 receptors and to stimulate through PYY preferring, Y_1 like receptors, the activity of cholinergic vagal efferent neurons in the dorsal motor neuron (DMN). At the peripheral level, one of the mechanisms for PYY to inhibit gastric acid secretion is to reduce histamine release, which is mediated by Y_1 receptors located on the ECL cells (Yang, 2002). Galanin mediated inhibition of gastric acid secretion is exerted through a direct effect on ECL cells via galanin receptors resulting in inhibiting histamine release (Jr *et al.*, 2000; Piqueras *et al.*, 2004).

There are also numerous peptides that have a negative influence in the regulation of gastric acid secretion. These include calcitonin gene-related peptide (CGRP), adrenomodulin, amylin, pituitary adenylate cyclase-activating peptide (PACAP), antral natriuretic peptide (ANP) and pancreatic polypeptide (PP). Finally, the E series of PGs are especially important, in the regulation of secretion of acid, and pepsinogen. It is generally believed that PGE_2 negatively regulates gastric acid secretion. There are many reports of PGE suppressing gastric acid secretion in experimental animals and humans (Konturek *et al.*, 2004; Kato *et al.*, 2005).

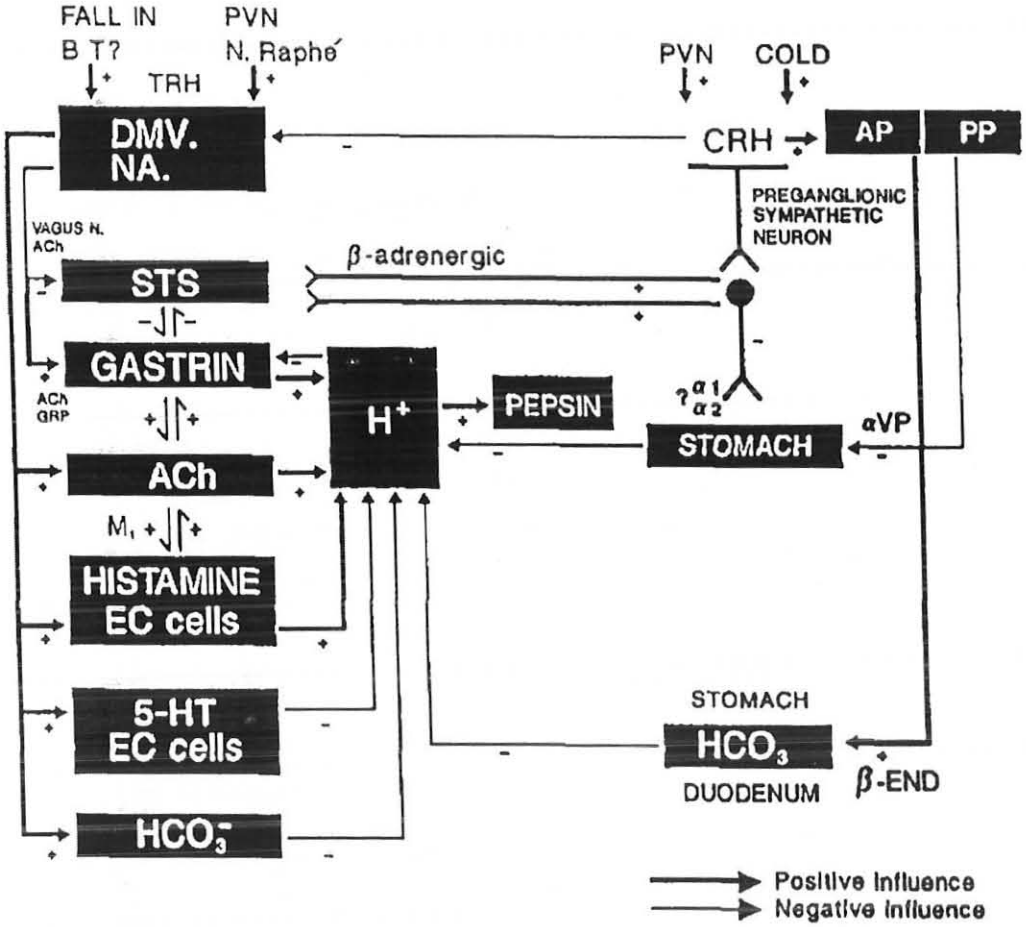


Fig.1. 1. Factors involved in the regulation of gastric acid secretion. Note in particular the regulatory influences at the level of the DMV and NA, and of the stomach. Cold and a fall in body temperature (BT) release TRH and CRH. TRH mainly increases gastric acid (H) secretion by acetylcholine (Ach) and histamine acting respectively through muscarinic (M) and histamine receptors in the stomach. Gastrin plays a major role in regulating H secretion but is counter-regulated by antral acidification, and its own release is inhibited by somatostatin (STS) which is under vagal and CRH control. Gastrin, ACh, and histamine potentiate each other's activities in promoting H secretion. This figure highlights some of the known factors influencing acid secretion. Adopted from Weiner, 1996 .

1.2. Cytoprotection

Defense mechanisms permit the gastric mucosa to withstand frequent exposure to damaging factors like gastric acid, pepsin, NSAIDs, *H. pylori*, alcohol, stress etc. As it is clearly depicted

The local gastric mucosal defense mechanisms, among others, include mucus-bicarbonate-phospholipid barrier. surface epithelial cells. continuous generation of PGE and PGI, and mucosal blood flow. The mucus-bicarbonate-phospholipid barrier constitutes the first line of mucosal defense (Fig. 1. 2). This barrier is formed by mucus gel, bicarbonate, and surfactant phospholipids, which cover the mucosal surface. This unstirred layer retains bicarbonate secreted by surface epithelial cells to maintain a neutral microenvironment (pH 7.0) at the surface of epithelial cells and prevents penetration of pepsin and thus proteolytic digestion of the surface epithelium (Laine *etal*., 2008).

Moreover, local gastric mucosal defense includes mechanisms which resist oxygen-induced injury since peptic ulcer disease (PUD) has been reported to involve ROS (reactive oxygen species) activity. Antioxidants act via different mechanisms such as blocking free radical formation, removal of oxidants from biological targets, scavenging of reactive species or transforming them into inert compounds, stabilizing biological membranes, and removing substances that catalyze free radical induced damage (Ligumsky *et al*., 2005).

The next line of mucosal defense is formed by a continuous layer of surface epithelial cells, which secrete mucus and bicarbonate and generate PGs. Because of the presence of phospholipids on their surfaces. these cells are hydrophobic, repelling acid and water soluble damaging agents. Interconnected by tight junctions, the surface epithelial cells form a barrier preventing back diffusion of acid and pepsin (Laine *et al*., 2008).

In addition to local mucosal factors, gastric mucosal defense is regulated, at least in part, by the central nervous system. Central vagal activation increases mucus gel, serotonin and bicarbonate. For example, studies using isolated parietal cells demonstrated that cholinergic agonists such as carbachol induce expression of cyclooxygenase-(COX)-2 in these cells via several signaling pathways leading to an abundant production of PGs protecting these cells and the entire surface epithelium of gastric mucosa against damage caused by secreted H^+. Constitutively expressed COX-1, normally present in gastric mucosa, generates PG, providing day-to-day gastric mucosal protection against gastric acid and any other irritant as PGs inhibit acid secretion, stimulate mucus, bicarbonate, and phospholipids secretion, increase mucosal blood flow and accelerate epithelial restitution and mucosal healing (Weiner *et al*., 1996; Konturek *et al*., 2004).

1.3. Peptic ulcer disease

"Peptic" is derived from the Greek peptikos, which means "conducive to digestion." Histologically,"peptic ulcer" means a mucosal defect deeply eroding the muscle layer. Moreover, "Peptic ulcer" may be narrowly defined to mean ulcers in the stomach or duodenum, although the definition could also include ulceration of mucosa in the esophagus and jejunum (Mou, 1999). It can also be considered as a breach in the gastric or duodenal mucosa with obvious depth. Small or shallow breaches are termed "erosions". Peptic ulcer has been a major threat to the world's population over the past two centuries, with a high morbidity and substantial mortality (Malfertheiner *et al.*, 2009).

The two most common causes of peptic ulceration are *H. pylori* infection and NSAIDs. Rare causes include gastric adenocarcinoma or lymphoma, Zollinger–Ellison syndrome, Crohn's disease etc. In addition to these factors, psychological and/or physiological stresses are also implicated in PUD (Jones, 2005). Moreover, a few studies have suggested that several factors, such as inflammation, or muscle spasm may cause PUD (Lu *et al.*, 2004). Indeed, alcohol use and cigarette smoking are also worth mentioning as causative factors (Barros *et al.*, 2008).

PUD continues to have a significant impact on society, with a cumulative life time prevalence of 8% to14%. Epidemiologic studies have reported that 50% to 90% of patients with duodenal ulcers (DU) are infected with *H. pylor*i. The use of NSAID can be identified in up to 60% of ulcer cases. Though the majority of patients with PUD are infected with *H. pylori* or have been taking NSAIDs, it would be an over simplification to suggest that the story ends there owing to the fact that epidemiologic studies in patients with PUD have suggested that as many as 50 % have no evidence for *H. pylori* infection or NSAID use. The explanation for PUD in this subgroup of patients has been elusive though there are a number of disease states that have been associated with an increased risk of PUD (Saad and Chey, 1999). For example, patients with hepatic cirrhosis have an increased risk of developing PUD with a prevalence ranging from 10% to 49% and an annual incidence of up to 4.3% (Wu *et al.*, 1995; Saad and Chey, 1999).

Patients who have developed PUD often present with dyspepsia, recurrent abdominal pain, or complications of bleeding, perforation, and obstruction (Wong *et al.*, 2006).The predominant symptom of uncomplicated peptic ulcer is epigastric pain, which can be accompanied by other

cells and promotes a Th1-type cytokine profile to enhance the inflammatory response to infection *(González et al.. 2005). H. pylori* are common in asymptomatic persons and its prevalence exceeds 70% among adults in the developing world. However, only a small proportion of carriers develop PUD. Factors determining whether the infection will produce disease are the pattern of histological gastritis induced, changes in homeostasis of gastric hormones and acid secretion, gastric metaplasia in the duodenum, interaction of *H. pylori* with the mucosal barrier and immune pathogenesis, and ulcerogenic strains. The two best studied bacterial determinants of disease are the presence of cytotoxin-associated gene A (cagA) and the cag pathogenicity island and the vacuolating cytotoxin (vacA) genotype (Thfm *et al.*, 2001). VacA enhances urea diffusion from capillaries across epithelial cells as well as induces apoptosis in collaboration with Cag protein (Joseph and Krischner, 2004).

Nonselective NSAIDs induce predictable gastric mucosal injury. In general, the properties of NSAIDs that contribute to ulcerogenesis can be divided into two categories: (i) topical irritancy, and (ii) the suppression of prostaglandin synthase activity (Wallace, 2000; Malfertheiner *et al.*, 2009).

Initial early injury with agents such as aspirin or other NSAIDs may occur because of a topical effect. Most nonselective NSAIDs are weakly acidic. Thus, in gastric juice, they are relatively nonionized and lipophilic, allowing them to move across cell membranes into the interior of cells. Once in the neutral intra-cellular environment, the drugs are converted to an ionized state and cannot diffuse out. This has been referred to as ion trapping. As the drug accumulates within the epithelial cell, the osmotic movement of water into the cell results in swelling of the epithelial cell eventually leads to the point of lysis. Furthermore, breaking of the barrier permits the back diffusion of acid, which leads to the rupture of mucosal blood vessels and then gastric injury (Laine *et al.*, 2008).

Another mechanism that could explicate the topical irritant property of NSAIDs is their ability to decrease the hydrophobicity of the mucus gel layer, which is the primary barrier to acid induced damage, in the stomach. NSAIDs have been shown to associate with the surface active phospholipids within the mucus jell layer thereby reducing hydrophobic properties (Wallace, 2000).

Although direct topical injury may occur, the major mechanism via which NSAIDs cause ulcers and GI complications is thought to be systemic: inhibition of COX mediated PG synthesis. COX-1 and COX-2 are key enzymes in the biosynthesis of PGs. COX-1 is constitutively expressed in many tissues, whereas COX2 has little or no expression in most tissues but is rapidly induced in response to growth factors and cytokines. Endogenous PGs are involved in the regulation of most of the defensive factors, for example, mucus and bicarbonate secretion by the gastric and duodenal epithelium, mucosal blood flow, epithelial cell proliferation, and epithelial restitution (Tomisato *et al*., 2004; Jones *et al*., 2005; Jansson *et al*., 2007).

The component of mucosal defense that appears to be most profoundly altered by NSAIDs is the gastric microcirculatory response to injury. When the mucosa is exposed to an irritant, or when superficial epithelial injury occurs, mucosal blood flow substantially increases. This is probably a response aimed at removing any toxins or bacterial products that enter the lamina propria, neutralizing back diffusing acid and contributing to the formation of a microenvironment at the surface of the mucosa that is conducive to repair. NSAIDs can reduce gastric mucosal blood flow and profoundly alter the behavior of neutrophils flowing through the gastric microcirculation (Wallace, 2000). PGs of the E and I series are potent vasodilators that are continuously produced by the vascular endothelium, so the inhibition of their synthesis by NSAID leads to a reduction in vascular tone. Several lines of evidence have suggested that damage to the vascular endothelium is an early event following the administration of NSAIDs to experimental animals. Endothelial injury is also an early event in the pathogenesis of gastrointestinal damage associated with ischemia and reperfusion, in which neutrophils have been demonstrated to play a critical role as mediators of endothelial injury.

Studies showed that administration of NSAIDs results in rapid and significant increase in the number of neutrophils adhering to the vascular endothelium in both gastric and mesenteric venules. This effect typically seen within15-60 min after the administration of NSAID; consistent with the period of time required for the suppression of PG synthesis by these drugs. Subsequent studies demonstrated that this adherence was dependent on the expression of the β-integrins (CD11/CD18) on the neutrophil and intracellular adhesion molecule-1(ICAM-1) on the vascular endothelium (Fig. 1. 3). Furthermore, the up regulation of ICAM-1on the vascular endothelium in the gastric micro circulation was shown to occur within 15-30 min of

administration of a NSAID to rats, and this could be prevented by the administration of exogenous PGs (Soll, 1998).

Of course, the fact that neutrophils adhere to the vascular endothelium following NSAID administration does not necessarily mean that these cells contribute to the endothelial injury of the mucosa. Rather, it was suggested that tumor necrosis factor alpha (TNFα) might be the key signal for NSAID-induced neutrophil adherence within the gastric microcirculation. This is because of the fact that release of TNFα from macrophages and mast cells has been shown to be suppressed by PGs, and TNFα is a well-characterized stimulus for adhesion molecule expression. Another group of mediators that might contribute to the increase in neutrophil adherence following NSAID administration is the leukotrienes as they have been shown to be capable of altering the susceptibility of the gastric mucosa to injury, at least in part through stimulatory effects on neutrophil adherence to the vascular endothelium (Wallace, 2000).

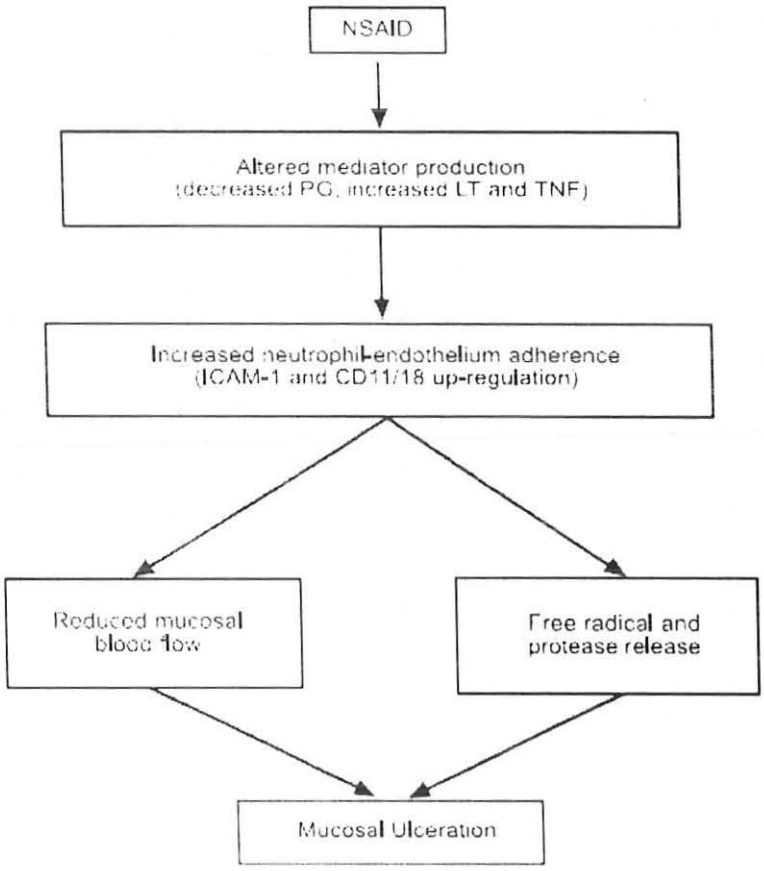


Fig.1. 3. Pathogenesis of PUD by NSAIDs. Adopted from Wallace *et al., 2000.*

combined with acid suppressing medications to allow for an environment that is less acidic as the body heals itself. Many different combinations of these medications have been used successfully. A small role exists for drugs enhancing mucosal resistance (Graham, 1997; Godshall, 2003; Malfertheiner *et al.*, 2009; Miehlke and Gustafson, 2009).

However, clinical evaluation of these drugs has shown incidence of relapse, side effects, for example, hypersensitivity, arrhythmia, impotence, gynecomastia and hematopoietic changes and drug interactions (Lima *et al.*, 2006). Apart from this, accessibility and affordability is one of the major problems to the public at large especially in the developing and underdeveloped countries. Thus, there is a need to develop new drugs, for which natural products are potential candidates.

1.4. Natural products and PUD

Plants have been utilized as a medicine for thousands of years. More recently, a WHO study has shown that about 80% of the world's population still relies on traditional medicine. A growing numbers of plants have been reported for antiulcer activity. These include *Ocimum sanctum, Allophylus serratus, Desmodium gagenticum, Azadirachta indica, Hemidesmus racemosus, Panax japonicas, Kochia scoparia, Asparagus racemosus, and Musa sapientum* (Dharmani *et al.*, 2006).

1.4.1. *Plantago lanceolata*

Plantago is the most important genus of Plantaginaceae family and is used in traditional medicine around the world for different purposes. *Plantago coronopus* L., *Plantago media* L., *Plantago major* L., and *Plantago lanceolata* L., are the most commonly used species of *Plantago* in traditional medicine in Turkey (Celik *et al.*, 2006).

The plant is known by various common names, among others, Ribwort, Plantain, Snake Plantain, Black Plantain, Long Plantain, Ribble Grass, Black Jack, Jackstraw, Lamb's Tongue, Hen Plant, Wendles, Kemps, Cocks, Quinquenervia, and Costa Canina. The plant is a rosette forming perennial herb, with leafless, silky, hairy flower stems (10-40 cm). The basal leaves are lanceolate spreading or erect, scarcely toothed with 3-5 strong parallel veins narrowed to short petiole. Several grooved flower stalks may grow tipped by a short spike of tune white flowers

2. OBJECTIVES

2.1. General objective

To evaluate the antiulcer activity of the extracts of *P. lanceolata* leaves and its acute toxicity on rodent models of gastric and duodenal ulcers.

2.2. Specific objectives

-To study the effect of aqueous extract of the fresh leaves of *P.lanceolata* and the mucilage isolated from it on gastric ulcer healing in acetic acid induced chronic gastric ulcer.

- To study the acute toxicity.

-To further evaluate the extract for its gastric cytoprotective, antisecretory and anti duodenal ulcer effect using the following models of ulcer:

- indomethacin induced gastric ulcer.,
- cysteamine HCl induced duodenal ulcer., and
- pylorus ligation induced gastric ulcer.

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Chemicals and reagents

Glacial Acetic acid, diethyl ether, and acetone (Research – Lab fine Chem Industries, Mumbai (India)), Indomethacin (Merck Shsrp and Dohm, Haarlem-Netherlands), Misoprostol (Cipla Ltd, India), Ranitidine injection (Cadila Pharmaceuticals, India), Cysteamine HCl and Alcian Blue 8GX (Himedia Laboratories PVT. Ltd, Mumbai, India), Magnisium chloride, Phenolphethalin, NaOH, Sodium acetate (May and Baker Ltd Dagenham, England), Sucrose, Carboxy methyl cellulose (Bdh Chemicals Ltd Poole, England), Normal saline (Euro- Med® Laboratories Phil.,Inc) were purchased from the stated sources.

3.1.2. Plant material

The plant was collected on November 2008 from sidist kilo, Addis Ababa and identified as *P. lanceolata* by a taxonomist and a voucher specimen (EM 022653) was deposited at the National Herbarium, Science Faculty, Addis Ababa University.

3.1.3. Experimental animals

Female Swiss albino mice (25–35 g) and male Sprague-Dawley rats (150–250 g) bred at the animal houses of School of pharmacy, Addis Ababa University, and Ethiopian Health and Nutrition Research Institute were used. The animals were fed standard pellets with free access to tap water under standard conditions of 12 h dark/12 h light cycle, humidity (60±1.0%) and temperature (21±1%). The care and handling of animals were in accordance with the internationally accepted standard guidelines for use of animals (Hiruma-Lima *etal.*, 2006).

3.2. Methods

3.2.1. Extraction

Aqueous extraction

The fresh leaves were extracted with decoction to simulate the traditional uses. The extract was then dried in vacuum drier. The percentage yield of the leave was found to be 14.2%.

Preparation of mucilage

Mucilage was prepared from the dried aqueous extract using acetone and methanol. Initially, 15g of the dried extract was dissolved in about 60 ml of distilled water and warmed on water bath for a few min to increase solubility. The solution was then filtered using filter paper and 300 ml of acetone was added to the filtrate and the mucilage was precipitated. After 24 h, the supernatant was decanted and then the mucilage was put in a vacuum drier. The dried mucilage was again dissolved in distilled water and to this solution methanol was added and a similar procedure as above was followed in order to get more purified mucilage. The percentage yield of the mucilage was found to be 43% of the dried aqueous extract.

3.2.2. Acute toxicity study

The acute oral toxicity study was performed according to OECD guideline for testing of chemicals (OECD, 2001). A limit test was employed so as to determine the LD50. A test dose of 2000 mg/kg was administered sequentially to five rats. The animals were followed for any sign of acute toxicity for two weeks.

3.2.3. Groupings of animals and dose determination

In all animal models, except for the acetic acid induced chronic gastric ulcer, there were four groups six animals each. In acetic acid chronic gastric ulcer, there were five groups, each comprising six animals. Negative controls were treated with the vehicle used for reconstitution, whereas positive controls were treated with ranitidine 50 mg/kg (for rats) and misoprostol 140 µg/kg (for mice) as a standard antisecretory and cytoprotective agents, respectively (Rahul Pathaka *et al.*, 2007). The other groups were treated with two different doses (200 mg/kg and

400 mg/kg for mice) of the extract. However, one group of mice in the acetic acid induced chronic gastric ulcer was treated with the mucilage (172 mg/kg). The aqueous extract, the mucilage and the standard drugs were always administered orally (by gavage) or by intraduodenal route. The doses for the extract were selected to be 200 mg/kg and 400 mg/kg for mice after having considered the safety of the plant and the daily dose of human being used in folk medicine; about 9 g of the plant is taken per day (EPO, 2009). Finally, dose conversions were done from human to mice and from mice to rats whenever necessary (Paget and barnes, 1964). In the case of cysteamine HCl induced duodenal ulcer, the dose of cysteamine HCl for mice was determined based on the pilot study that was done before the commencement of the experiment. Doses below 650 mg/kg didn't produce any duodenal ulcer whereas 800 mg/kg caused death of animals. Consequently, 650 mg/kg was selected as an optimum dose to induce duodenal ulcer. The dose for the mucilage group of mice was calculated based on the percentage yield of the extract out of which the mucilage was prepared i.e., 43 % of 400 mg is 172 mg.

3.2.4. Acetic acid induced chronic gastric ulcer

The method described by Asad *et al.* (2001) was followed. Briefly, under ether anesthesia the abdomen was opened by midline incision below the xyphoid process and the stomach was exposed. Glacial acetic acid (0.025 ml) was added to the cylindrical mould of 4 mm diameter placed tightly over the anterior serosal surface of the stomach and this was allowed to remain there for 30 sec. The acid solution was removed after 30 sec and the mould was rinsed with normal saline to avoid damage to surrounding tissues. The abdomen was then closed and the animals were treated once daily with ranitidine 70 mg/kg, the aqueous extract 200 mg/kg and 400 mg/kg for 10 days. The negative controls were given distilled water 1 ml/100 g. On the 11^{th} day, the animals were sacrificed and the stomach was removed and cut along the greater curvature. The total mucosal area and total ulcerated area were measured. Finally, the ulcer index was calculated using the following relation Ganguly, 1969 :

$$\text{Ulcer index} = 10/x$$

Where x=total mucosal area/ ulcerated area.

Furthermore, the score for intensity of gastric lesions were measured. The scores were assigned based on the following index; 0 no ulcer, 1 superficial mucosal erosion, 2 deep ulcer or rans-mural necrosis, and 3 perforated or penetrated ulcer.

3.2.7. Pylorus ligation induced gastric ulcer

Rats were fasted for 36 h before pylorus ligation with water *ad libitum* and were placed individually in cages to avoid coprophagy and cannibalism. Normal saline (1 ml/rat *p.o.*) was administered twice daily to all animals. Under ether anesthesia, the abdomen was opened by midline incision below the xiphoid process. The pyloric portion of the stomach was slightly lifted out and ligated, avoiding damage to its blood supply. Animals received either distilled water, ranitidine (50 mg/kg, p.o), or aqueous extract (140 mg/kg or 280 mg/kg) intraduodenaly immediately after ligation. The stomach was placed back carefully and the abdominal wall was closed with sutures. The animals were deprived of food and water during the postoperative period and were sacrificed 6 h after pylorus ligation by over dose of ether anesthesia. The stomachs were isolated and opened and the contents of the stomach were collected and centrifuged. The volume of gastric secretion was measured. Whilst the gastric juice was used for estimation of pH using pH meter (Metteler Toledo in lab expert PLC, England) and total acidity (Pathak *et al.*, 2007; Shine *et al.*, 2009), the glandular portion of the stomach was used for estimation of the mucin content. Ulcer scores were given based on the method described by Hemmati *et al.* (1973) and Gupta *et al.* (1974).

Determination of total acidity

Gastric juice (1 ml) was taken into a 100 ml conical flask, to which 2-3 drops of phenolphthalein solution was added and titrated with 0.01N sodium hydroxide until a definite pink color appeared. The volume of alkali added was noted. This volume corresponds to total acidity. Acidity was calculated by using the following formula (Hawk *et al.*, 1947):

$$\text{Acidity} = \frac{\text{volume of NaOH} \times \text{Normality of NaOH} \times 100 \text{ mEq/litr}}{0.1}$$

Estimation of mucin content

After collection of the gastric juice, the glandular portions of the stomach was excised and opened down the lesser curvature. The weight of the tissue was noted. The everted stomach was soaked for 2 h in 1% Alcian blue 8GX dissolved in 0.16 M sucrose solution buffered with 0.05 M sodium acetate. The uncomplexed dye was then removed by two successive washes for 15 and 45 min in 0.25 M sucrose solution. The dye complexed with mucin was obtained by immersion

in 10 ml aliquots of 0.5 M magnesium chloride for 2 h. The resulting blue solutions were then shaken briefly with equal volume of diethyl ether and the absorbance of the aqueous phase was measured at 605 nm using UV-Visible spectrophotometer (CECIL, model UV 1601, England). Standard calibration curve for Alcian blue was generated with 95% confidence interval (Fig.3.1) by taking different concentrations (2.5, 5, 10, 15, 20, 25, 30 and 35µg) of Alcian blue so as to determine the content of Alcian blue attached to mucin of glandular tissues of the stomach in all groups of mice.

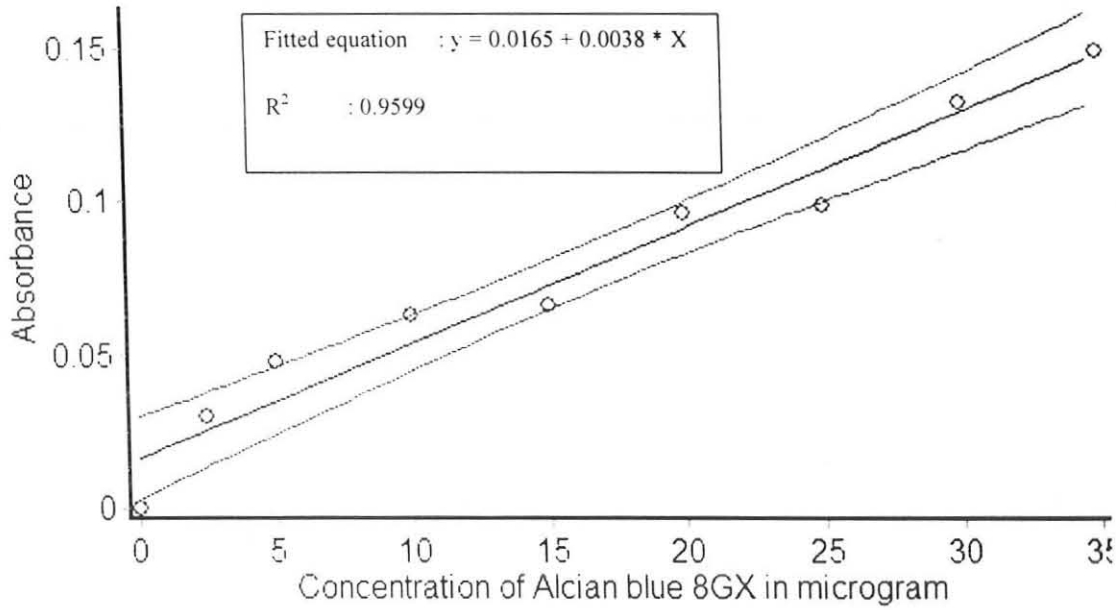


Fig.3.1. Calibration curve for Alcian blue 8GX in aqueous solution

The equation for the linear calibration graph was Y = 0.0165 + 0.0038 * X, where "Y" represents the dependent variable (Absorbance) and "X" stands for the independent variable (concentration of Alcian blue in micro gram). Thus, the value of the absorbance measured in the different treatment groups was used to get the corresponding concentration of Alcian blue which was complexed with the mucin on the wall of the glandular portion of the stomach. Finally, the amount of mucin per gram of glandular tissue was calculated using the formula shown below (Corne *et al.*, 1974; Shine *et al.*, 2009).

$$\text{Mucin content} = \frac{\mu g \text{ Alcian blue}}{\text{gm wt of glandular tissue}}$$

3.2.8. Statistical analysis

The values were expressed as mean ± SEM and $p<0.05$ was considered significant. The statistical significance was assessed by graph pad instat software (USA) using one-way analysis of variance (ANOVA) followed by Tukey's comparison test. For comparing nonparametric ulcer scores, one way ANOVA followed by non-parametric Dunn post test was used.

4. RESULTS

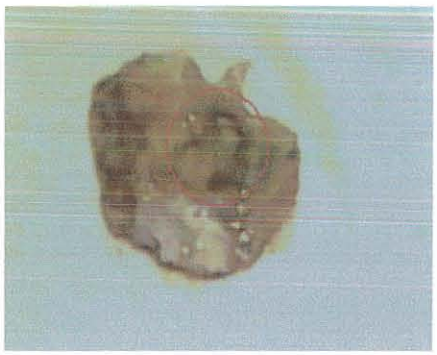
4.1. Effect of the aqueous extract and mucilage of *P. lanceolata* in acetic acid induced chronic gastric ulcer.

The aqueous extract of *P. lanceolata* showed a significant reduction in ulcer index in both 200 mg/kg ($P<0.05$) and 400 mg/kg ($P<0.001$) treated mice compared to the negative control. However, the extract at a dose of 200 mg/kg did not produce a statistically significant reduction in the ulcer score. Ranitidine 70 mg/kg did also reduce the ulcer index ($P<0.001$) and ulcer score ($P< 0.01$) as compared to the negative control (Table 1). However, the percent reduction in ulcer index differed in value in all the treatment groups of mice. More specifically, the percent reduction in the ulcer index increased as the dose of the plant extract increased from 200 mg/kg to 400 mg/kg (50.85% vs. 77.92%) and 400 mg/kg of the extract exhibited a slightly higher activity than ranitidine in reducing the ulcer index. The mucilage also reduced the ulcer index as well as the ulcer score ($P<0.05$) as compared to the negative control. Nevertheless, the aqueous extract at 400 mg/kg and ranitidine 70 mg/kg produced a much higher reduction than the mucilage in both the ulcer index and ulcer score (Table 1). Fig. 4. 1 shows the effect of the extracts on ulcer index and ulcer score as compared to the control and the standard drug. The ulcerated area and the intensity of ulceration are clearly visible in the control (Fig. 4. 1. A), and in 200 mg/kg treatment groups (Fig. 4. 2. B), but not in 400 mg/kg (Fig. 4. 1. C) and ranitidine (Fig.4. 1. D) treatment groups indicating that the higher dose of the extract and ranitidine produced a significant healing as compared to the negative control.

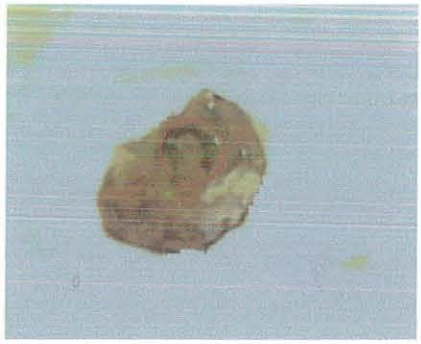
Table 1: Effect of aqueous extract and mucilage of *P. lanceolata* on ulcer index, % reduction in ulcer index and ulcer score in acetic acid induced chronic gastric ulcer.

Treatment	Ulcer index	% reduction in ulcer index	Ulcer score	% reduction in ulcer score
Vehicle (control)	0.643±0.132	-	2.667±0.211	-
Extract (200 mg/kg)	0.316±0.067[1]	50.85	1.500±0.224	43.75
Mucilage (172 mg/kg)	0.309±0.068[1]	51.94	1.167±0.167[1]	56.24
Extract (400 mg/kg)	0.142±0.037[3]	77.92	0.833±0.167[2]	68.77
Ranitidine (70 mg/kg)	0.153±0.035[3]	76.21	0.833±0.167[2]	68.77

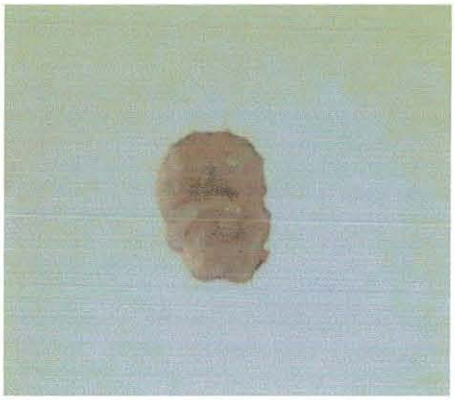
Values are mean ± SEM, n=6, [1]:$p<0.05$, [2]: $p< 0.01$, [3]: $p< 0.001$.



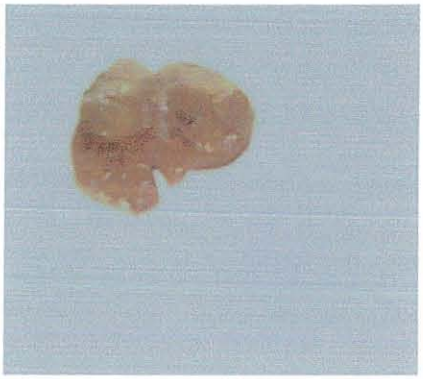
A. Control



B. Extract 200 mg/kg



C. Extract 400 mg/kg



D. Ranitidine 70 mg/kg

Fig. 4. 1. Pictures of acetic acid induced chronic gastric ulcer of mice. The encircled areas represent ulcer.

4.2. Effect of the aqueous extract of *P. lanceolata* in indomethacin induced gastric ulcer.

Among the treatment groups it was only 200 mg/kg of the extract that failed to reduce ulcer score (Table 2). Both 400 mg/kg of the plant extract and the standard drug misoprostol reduced the ulcer score ($P<0.001$ in both cases) as compared to the negative control. Moreover, the higher dose of the extract ($P<0.01$) as well as the standard ($P<0.05$) brought about significant reduction in ulcer score compared to 200 mg/kg of the extract, with 400 mg/kg being more effectively reducing ulcer score (86.4%) than misopristol (68.2%).

Table 2: Effect of the aqueous extract of *P. lanceolata* on ulcer score and % reduction in ulcer score in indomethacin induced gastric ulcer.

Treatment	Ulcer score	% reduction in ulcer score
Control	1.833±0.167	
Extract (200 mg/kg)	1.333 ±0.211	27.28
Extract (400 mg/kg)	0.25±0.112 [a3.b2]	86.36
Misoprostol (286 µg/kg)	0.583±0.201[a3b1]	68.19

Values are mean ±SEM, n=6, [a]: against control, [b]: against 200 mg/kg; [1]:p <0.05, [2]: p< 0.01, [3]:p<0.001.

4. 3. Effect of the aqueous extract P. *lanceolata* on cysteamine HCl induced duodenal ulcer.

As can be seen in Table 3, the aqueous extract at 200 mg/kg did not show significant reduction in the ulcer score as compared to the negative control, whereas the same extract at 400 mg/kg reduced (P< 0.05) the ulcer score compared to the negative control. In addition, ranitidine at 70 mg/kg caused a significant reduction in the ulcer score (P< 0.01) as compared to the negative control.

Table 3: Effect of the aqueous extract of *P. lanceolata* on ulcer score, ulcer area and % protection in cysteamine HCl induced duodenal ulcer.

Treatment	Ulcer score	Ulcer area	% protection
Control	2.500±0.224	52.667±8.337	-
Extract (200 mg/kg)	1.500±0.224	5.000±1.155[3]	40.00
Extract (400 mg/kg)	1.000±0.258[1]	3.000±0.817[3]	60.00
Ranitidine (70 mg/kg)	0.667±0.211[2]	0.833±0.307[3]	73.32

Values are mean ±SEM, n=6, [1]:p <0.05, [2]: p< 0.01, [3]:p<0.001.

It is also shown in Table 3 that the plant extracts at doses 200 mg/kg and 400 mg/kg and ranitidine at 70 mg/kg resulted in a significant reduction (P< 0.001) in the ulcerated area as compared to the negative control, with ranitidine conferring maximum protection. Moreover, the standard antisecretory drug, ranitidine showed a better reduction in the ulcer index and protected from the ulcerogenic effect of cysteamine better than the plant extracts (Fig. 4. 2.). Fig. 4.3 also clearly shows the difference in the ulcer area among the treatment groups.

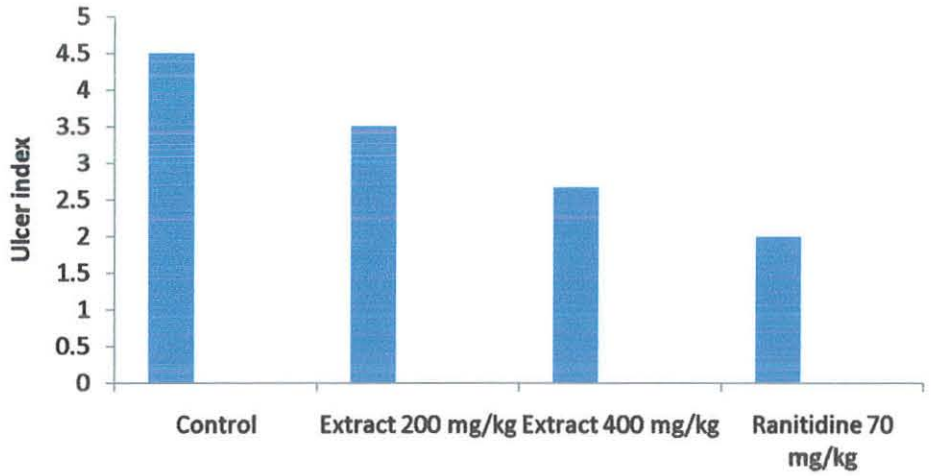
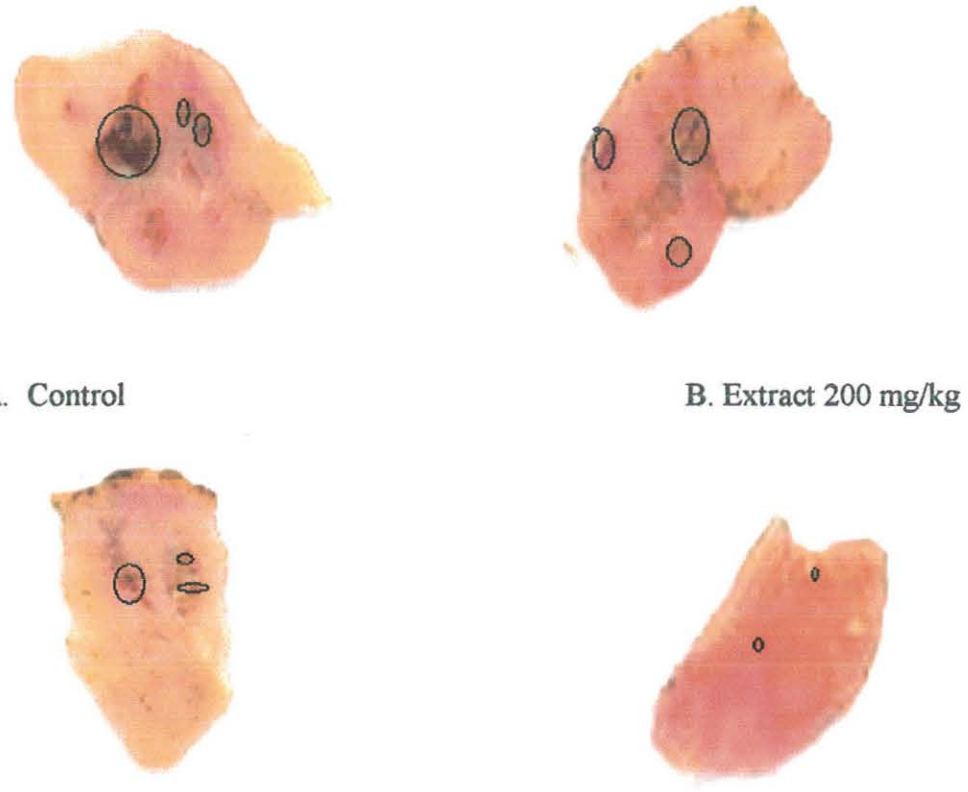


Fig. 4.2. Effect of the aqueous extract of the fresh leaves of P. lanceolata on ulcer index in cysteamine HCl induced duodenal ulcer. The ulcer index decreased as the dose increased from 200 mg/kg to 400 mg/kg. Ranitidine produced the highest reduction in the ulcer index.



A. Control

B. Extract 200 mg/kg

C.Extract400 mg/kg

D. Ranitidine 70 mg/kg

Fig. 4.3. Pictures of cysteamine HCl induced duodenal ulcer of mice. The encircled areas are ulcers.

4.4. Effect of the aqueous extract of *P.lanceolata* on ulcer score, total acidity, volume of gastric secretion, pH of gastric secretion and mucus secretion on pylorus ligation induced gastric ulcer.

The plant extract at a dose of 140 mg/kg did not produce significant reduction in ulcer score as compared to the negative control. In contrast, 280 mg/kg of the extract ($P<0.01$) and 50 mg/kg ranitidine ($P<0.05$) did show a significant reduction in ulcer score as compared to the negative control, with 280 mg/kg providing better protective than ranitidine (Table 4). The effect of the extracts and ranitidine on the ulcer was also shown in Fig. 4. 7.

Table 4: Effect of the aqueous extract of *P. lanceolata* on ulcer score, and mucin secretion in pylorus ligation induced gastric ulcer.

Treatment	Ulcer score	Mucin content µg/gm
Control	2.667±0.211	8.347±0.324
Extract (140 mg/kg)	1.250±0.250	15.490 ± 1.763^{a3c3}
Extract (280 mg/kg)	0.667 ± 0.105^{a2}	18.387 ± 1.147^{a3c3}
Ranitidine 50 mg/kg	0.833 ± 0.105^{a1}	8.323±0.328

Values are mean ±SEM, n=6, [a]: against control, [c]: against ranitidine 50mg/kg; [1]:$p<0.05$, [2]: $p<0.01$, [3]:$p<0.001$

Table 4 also shows that the aqueous extract of *P. lanceolata* in both 140 mg/kg and 280 mg/kg treated rats increased mucin secretion significantly ($P<0.001$) against the negative control. However, the mean mucin content was 15.8% higher in value in those rats that were treated with 280 mg/kg of the plant extract than those that received 140 mg/kg of the same extract. In addition, ranitidine did not produce a significant increase in mucin secretion as expected.

One can also clearly see from Fig. 4. 4 that the aqueous extracts at both 140 mg/kg and 280 mg/kg ($P<0.05$ in both cases) and ranitidine ($P<0.001$) reduced the total acidity as compared to the negative control. Apart from this, the reduction in the total acidity by ranitidine and 280 mg/kg of the extract was statistically significant ($P< 0.01$) as compared to 140 mg/kg of the extract.

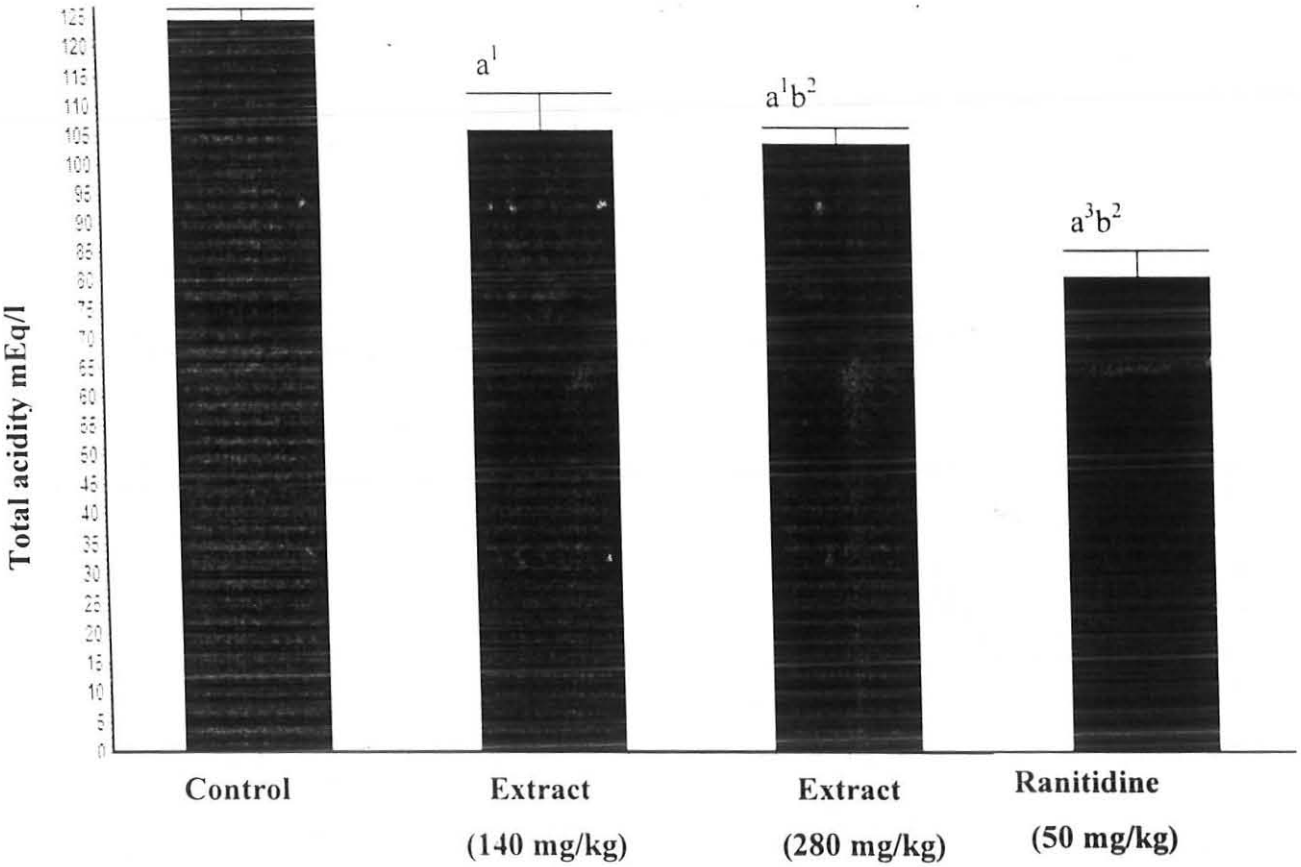


Fig. 4. 4. Effect of the aqueous extract of the leaves of P. lanceolata on the total acidity of the gastric secretion. [a]*: against control,* [b]*: against 140 mg/kg,* [1]*:p <0.05,* [2]*: p< 0.01,* [3]*:p<0.001.*

As regard to gastric secretion, 140 mg/kg of the extract did not produce a statistically significant reduction in the volume of gastric secretion as compared to the negative controls (Fig. 4. 5). However, 280 mg/kg of the extract and ranitidine decreased volume of secretion ($P<0.01$, and $P<0.001$, respectively) as compared to the negative controls. Besides, ranitidine 50 mg/kg reduced the volume of gastric secretion significantly ($P< 0.001$) as compared to 140 mg/kg while the reduction in the volume of gastric secretion by 280 mg/kg was not statistically significant when compared to 140 mg/kg. Moreover, it is evident from Fig. 4. 6 that the extract at a dose of 280 mg/kg and the standard drug raised the pH of the gastric juice significantly ($P<0.05$) as compared to the negative control. However, the aqueous extract of the plant at 140 mg/kg produced no statistically significant increase in the pH of the gastric juice as compared to the negative controls.

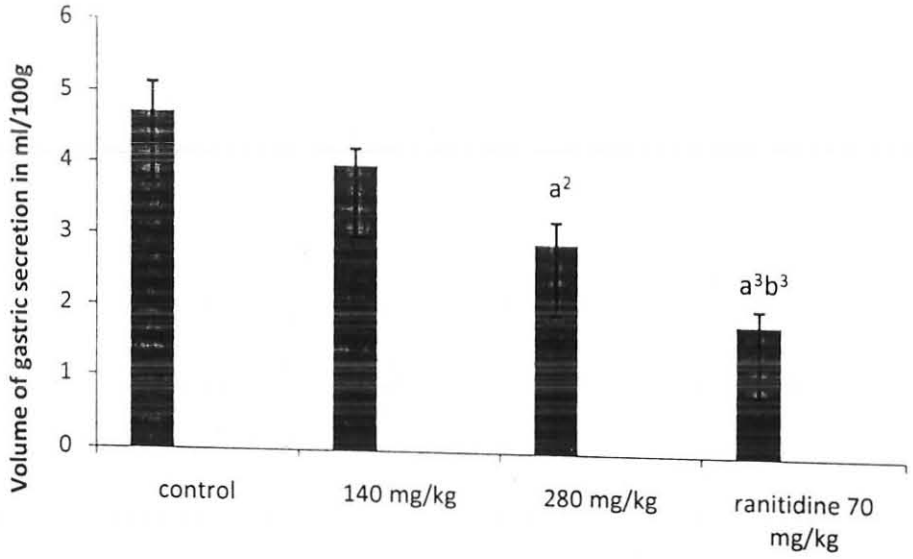


Fig.4.5. Effect of aqueous extract of the fresh leaves of P.lanceolata and ranitidine on volume of gastric secretion in ml/100g of rat. 400 mg/ kg of the extract showed a significant reduction in the volume of gastric secretion. Ranitidine produced the highest reduction. [a]*: against control,* [b]*: against 140 mg/kg;* [1]*:p <0.05,* [2]*: p< 0.01,* [3]*:p<0.001.*

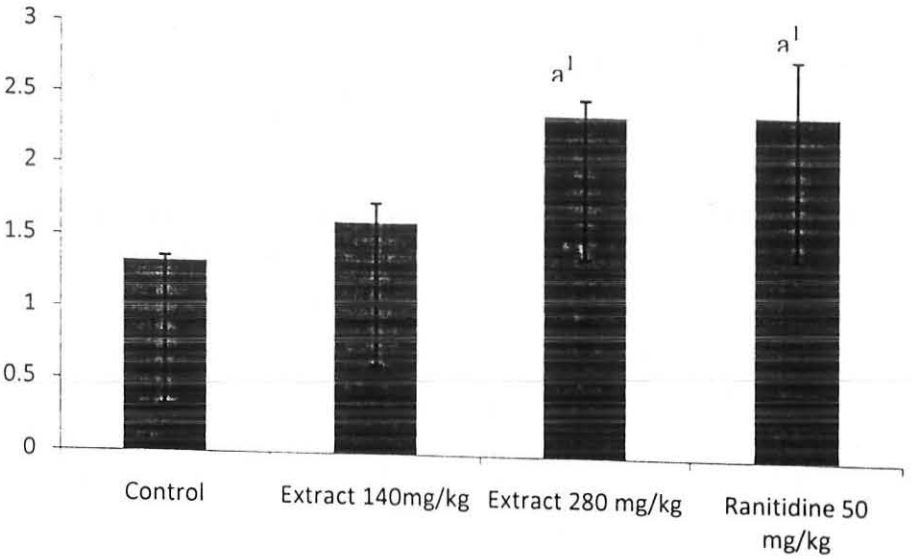
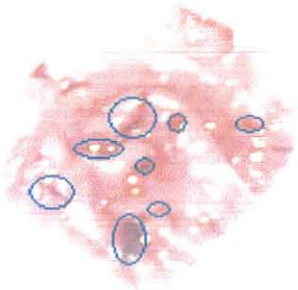
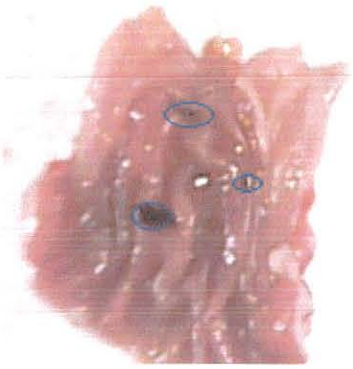


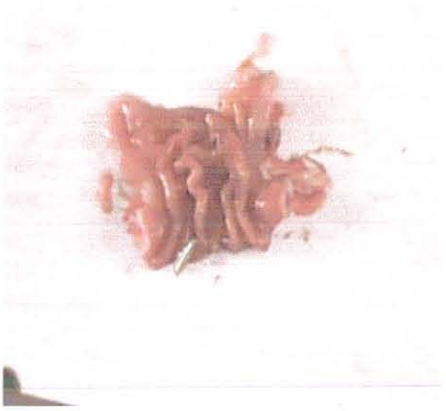
Fig. 4. 6. Effect of the aqueous extract of the fresh leaves of P.lanceolata on pH of gastric secretion. 280 mg/kg of the extract raised the pH significantly so did Ranitidine 50 mg/kg.



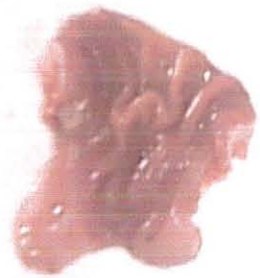
A. Control



B. Extract 140 mg/kg



C. Ranitidine 50 mg/kg



D. Extract 280 mg/kg

Fig.4.7. Pictures of pylorus ligation induced stomach ulcers of rats. The black spots in the circles (A and B) indicate ulcer. The extract at 400 mg/kg (D) and ranitidine (C) prevented ulcer formation.

As it was anticipated misoprostol 286 µg/kg produced statistically significant reduction in the ulcer score as compared to the negative control. However, it did not reduce the intensity of ulcer as much as 400 mg/kg of the extract as indicated by the % reduction in ulcer score or the mean ulcer score. This result indicates that 400 mg/kg of the extract produced a better protection than misoprostol. This may be because misoprostol produces cytoprotection only via enhancing synthesis of prostaglandins whereas the extract may have exerted its action by other mechanisms other than production of prostaglandin synthesis as indicated above. In addition, this result supports the clinical use of cytoprotective agents in patients who are on treatment with NSAIDs so as to prevent erosion of the stomach.

The statistically significant increase in the mucin secretion by 200 mg/kg that was observed in pylorus ligation induced gastric ulcer model will enable one to infer that it will also produce a statistically significant reduction in the intensity of ulcer in indomethacin induced gastric ulcer. However, it failed to produce a statistically significant reduction in the ulcer score unlike 400 mg/kg of the extract, which means the mucin secretion by 200 mg/kg was not adequate to offer sufficient protection in the presence of NSAIDs. Thus, higher doses of the extract has to be used in order to produce full protection against the ulcerogenic effect of NSAIDs during treatment with these class of drug.

5.3. Cysteamine HCl induced duodenal ulcer.

Cysteamine (SH- CH_2-CH_2-NH_2, β-mercaptoethylamine) is a reducing amino thiol. *Invitro* studies demonstrated that the cytotoxic effect of cysteamine in a variety of cells depends on the generation of H_2O_2 (Khomenko *et al.*, 2003; Khomenko *et al.*, 2004; Khomenko *et al.*, 2009).It is also known that cysteamine's ulcerogenic effect depends, among others, on depletion of somatostatin which is an inhibitor of acid secretion (Drago and Montoneri 1997; Piqueras *et al.*, 2004). Therefore, in the present study, cysteamine induced duodenal ulcer model was used to evaluate the free radical scavenging activity and antisecretory effect of the aqueous extract.

The aqueous extract at a dose of 400 mg/kg was found to be effective in this model as evidenced by the reduction in the ulcer score and ulcer area. Ranitidine showed a better healing than all the treatments for it has reduced the ulcer index much higher than the extracts. Moreover, the mean ulcer area as well as the mean ulcer score was also reduced to a greater extent by ranitidine than

the extracts. These results may therefore point to two important notions: (i) inhibition of acid secretion might have played the major role in preventing the ulcerogenic effects of cysteamine and (ii) the extract perhaps might have worked mainly via antisecretory mechanism.

Flavonoids constitute one of the most characteristic classes of compounds in *Plantago* and they have been reported to possess antiulcer activity by decreasing histamine secretion (Borrelli *et al.*, 2000; Gálvez *et al.*, 2003). Therefore, the antisecretory activity of the extract could have been due to its flavonoids content. In addition, flavonoids have been found to be free radical scavengers (Borrelli *et al.*, 2000). Thus, the protective effect of the extract may be partly due to its free radical scavenging activity.

5.4. Pylorus ligation induced gastric ulcer

Pylorus ligation-induced ulcer is used to study the effect of drug on gastric acid and mucus secretion. The ligation of the pyloric end of the stomach causes accumulation of gastric acid in the stomach. This increase in the gastric acid causes ulcers in the stomach. Agents that decrease gastric acid secretion and increase mucus secretion are effective in protecting the ulcers induced by this method (Pathak *et al.*, 2007).

This model surely indicated that 400 mg/kg of the extract had got antisecretory activity as evidenced by the statistically significant reduction in the total acidity as well as volume of secretion. Additionally, the statistically significant rise in pH of the gastric secretion is also another important evidence of the anti secretory activity of the extract. 200 mg/kg of the extract reduced the total acidity of gastric secretion significantly which may give clue that, it could also reduce volume of secretion and raise the pH of gastric secretion significantly. However, it did not reduce volume of gastric secretion and raised the pH of the secretion significantly. This should not be considered as if it doesn't have effect on volume of gastric secretion and pH of gastric secretion for it tended to decrease the volume of secretion and raised the pH. Moreover, it would probably have a statistically significant effect if the sample size had increased.

The cytoprotective activity of the extract, that have been shown by indomethacin induced gastric ulcer model, might have been mainly due to its ability to increase mucin secretion because it has produced a statistically significant increase in mucus secretion as compared to the negative control.

Even though ranitidine was found to be more active antisecretory agent than the aqueous extracts a dose of the extract at 400 mg/kg showed a better reduction in the ulcer score than ranitidine suggesting that the extract was more effective in healing the ulcer in this model.

5.5. Acute toxicity

The acute toxicity study confirmed that the aqueous extract did not produce acute toxicity in mice at 2000 mg/kg. Therefore, the ingestion of the plant for any of its therapeutic effect will be safe as the dose which is much higher than that used in folk medicine has not produced acute toxicity in mice. Moreover, this result suggests that the LD_{50} of the extract is above 2000 mg/kg.

6. CONCLUSION

The aqueous extract of the fresh leaves of *P. lanceolata* at 400 mg/kg showed a better activity against gastric as well as duodenal ulcer than the standard drugs. Moreover, some of the possible mechanisms by which the extract showed the activity were corroborated to be anti secretory and cytoprotection. The extract was also safe when taken orally as it did not produce any toxicity in rodents. Thus, the present work holds up the traditional use of the plant for PUD.

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Declaration

I, the under signed, declare that this thesis is my original work and had not been presented for a degree in any other university.

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