KWAME NKRUMAH UNIVERSITY OF SCIENCE & TECHNOLOGY, KUMASI, GHANA

PHARMACOLOGICAL AND TOXICOLOGICAL EVALUATION IN RATS OF *DISSOTIS ROTUNDIFOLIA*, A MEDICINAL PLANT TRADITIONALLY USED IN GHANA FOR THE MANAGEMENT OF PEPTIC ULCER

by

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DECLARATION

The experimental work described in this thesis was carried out at the Department of Pharmacology, KNUST, the Department of Biochemistry, University of Cape Coast and the Centre for Plant Research Akuapem-Mampong. This work has not been submitted for any other degree.

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ABSTRACT

The ethnopharmacological importance of *Dissotis rotundifolia* (Melastomacea) is evidenced by its traditional use by various tribes in Ghana for several illness including gastrointestinal disorders. Despite its widespread use in the treatment of peptic ulcer, the plant extract has not been subjected to any systematic pharmacological investigations to ascertain its efficacy and safety. This study aimed at evaluating the gastroprotective activity, gastrohealing effects and establishing the possible mechanism of action of the extract of whole plant of *Dissotis rotundifolia* (DRE) using chemical and physiological stress-induced ulcer models in rats. Peptic ulcer was induced using, acetylsalicylic acid, ethanol and cold restraint stress and either a pre-treatment with DRE (100, 300 and 500 mg/kgbwt) and positive drugs (30 mg/kg bwt omeprazole and 50 mg/kgbwt ranitidine) before ulcer induction or post-treatment with DRE and positive drugs after ulcer induction. The antisecretory effect of DRE was evaluated in a bid to examining the possible antiulcer mechanism. The *in vitro* anti-*Helicobacter pylori* activity of the extract was also assessed in addition to antioxidant effects *in vitro* and *in vivo*. The phytochemical screening of the major chemical constituents of DRE was also done. The potential toxicological effect of the extract in Sprague-Dawley rats was evaluated in acute and sub-acute toxicity studies. The results show a marked percentage protection and curative indices of pre-treated and treated groups in the cold stress, acetylsalicylic acid and ethanol induced ulcers in rats. This was coupled to a significant reduction in ulcer indices. The 300 mg/kg bwt extract pre-treated / post-treated group recorded a significant reduction in ulcer index compared to DRE doses of 100 and 500 mg/kgbwt group and ranitidine group. Meanwhile statistical analysis showed no significant difference between the 300 mg/kgbwt DRE and 30 mg/kgbwt omeprazole treated / pretreated group. The observation was seen in almost all the ulcer models employed. Also in the antisecretory studies it was observed that the pretreatment with 300 mg/kg DRE significantly increased gastric secretion (pH, mucus and titrable acidity) and showed a marked reduction in gastric juice volume ($p < 0.01$), when compared with the groups treated with ulcerogen (acetylsalicylic acid) only without any form of pretreatment. In contributing to elucidating the possible mechanism of action, results from anti-*Helicobacter pylori* activity indicates that plant extracts (200-800 mg/ml) and standard antibiotic drugs (amoxicillin, clarithromycin) showed antimicrobial activity against clinical isolates of *H. pylori*. The zones of inhibition ranged from 13-30 mm. DRE showed statistically lower zones of inhibition compared to standard drugs (clarithromycin, amoxicillin) ($p<0.05$). Metronidazole revealed no zone of inhibition indicating resistance by *Helicobacter pylori*. Meanwhile maximal effect was found at 400 mg/mL concentration of DRE. *In vivo* activity of H^+ / K^+ - ATPase in negative control CRS, ASA, and EtOH ulcerated rats was found to be elevated compared to normal, omeprazole and DRE

treated groups ($p< 0.05$). It was also observed that H^+ / K^+ - ATPase enzyme activity was low in DRE and omeprazole treated rats in all models compared to negative control groups ($p<0.05$). A marked increase in glycoproteins (sialic acid, hexose, hexosamine and fucose) was observed in all the pretreated groups compared to negative control ulcer groups. There was also an increase in the total carbohydrate / protein ratio of the gastric mucosa in DRE treated rats. Histopathological examination of stomach of ulcerogens (cold, ASA, EtOH) without drug pretreatment animals indicated that there was disruption of the surface epithelium and glandular structure. Drugs (DRE, omeprazole and ranitidine) pretreated group showed mild damage to the gastric mucosa indicative by a mild disruption of the surface epithelium with mild edema and leucocyte infiltration into the submucosal layer. This is indicative of a marked inhibition of gastric ulcer by DRE, omeprazole and ranitidine. This result demonstrates that the plant extracts possesses antiulcer effect against cold, acetylsalicylic acid and ethanol induced ulcers in rats. The anticholinergic experiment showed that the extract decreased the propulsive movement of charcoal meal through the gastrointestinal tract (GIT). This observation was significantly ($P < 0.01$) different from what was seen in the negative control group. The delayed gastric emptying time increases the absorption of orally administered drugs. *In vitro* free radical scavenging activity suggest that aside the other mechanisms, DRE possesses antioxidant activity evidenced by an excellent DPPH, nitric oxide, superoxide, hydroxyl radical-scavenging activities and profound Fe^{2+} - ascorbate lipid peroxidation inhibitory activity. Results from the effects of DRE on the gastric mucosa antioxidant *in vivo* in ethanol induced ulcer model shows that there was a significant increase in catalase (CAT) and superoxide dismutase (SOD) activities, a rise in reduced glutathione (GSH) content and a marked decrease in malondialdehyde (MDA) levels in DRE and omeprazole pretreated group compared to negative control ($p< 0.05$). The preliminary phytochemical screening, showed the presence of tannins, saponins, terpenoids, flavonoids, alkaloids and reducing sugars. In the toxicological study, there was no significant difference in almost all the parameters measured. This was confirmed by the absence of any damage observed in micrographs obtained from histopathological examination. The results showed that DRE had no overt organ specific toxicity in rats within the 14-day administration of the extract thus have a high safety profile. In conclusion the study demonstrates that the extract is able to perform its antiulcer effect through inhibition of H^+ / K^+ - ATPase, increasing mucus content and mucin activity, acts as an antagonist at muscarinic cholinergic receptor and inhibition of *Helicobacter pylori*. The study also shows that DRE performs its antiulcer effect by acting as a scavenger of Hydroxyl, nitric oxide, DPPH radical and also as anti-lipid peroxidative. The *in vitro* scavenging property was confirmed by results obtained from the *in vivo* antioxidant experiment indicating that DRE significantly inhibited the effects of ethanol on lipid

peroxidation, gastric GSH levels depletion and increased CAT and SOD activities. Implicitly it can be stated that the antiulcer effects of DRE is multifaceted. DRE is acting as an antiulcer agent by serving as a proton pump inhibitor, increasing mucin activity, gastric mucus content, pH and titrible acidity, inhibiting growth of *H. pylori*, serving as a scavenger of OH, DPPH, NO_2 radicals and anti-lipid peroxidative. Overall these findings provide substantial evidence-based data to support the traditional medicinal use of the whole plant extract of *Dissotis rotundifolia* in the management of peptic ulcer.

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ABBREVIATIONS

MCHC- Mean corpuscular haemoglobin concentration

NSAIDs - Non steroidal anti-inflammatory drugs

PLT- Platelet

T Pro - Total protein

Ach - Acetylcholine

ALB- Albumin

ALP- Alkaline phosphatase

ALT- Alanine aminotransferase

ASA-acetylsalicylic acid

AST- Aspartate aminotransferase BUN-

Blood urea nitrogen cAMP - Cylic

adenosine monophosphate

CAT - catalase

CCK - Cholecystokinin

CO - Carbon monoxide

COX - cyclooxygenase

CRS-cold restraint stress

CYP- Cytochrome P450

D-BIL- Direct bilirubin

DPPH- 2, 2-diphenyl-1-picrylhydrazyl

DRE-*Dissotis rotundifolia* extract

ECF- Extracellular fluid

ECL - enterochromaffin–like cells

EDTA- Ethylene diamine tetra acetic acid EtOH-ethanol

GFR- Glomerular filtration rate

GGT- Gamma glutamyl transpeptidase

GIT-gastrointestinal tract

GLP- Good Laboratory Practice

GLP-1-glucagon-like peptide-1

GPx- Glutathione peroxidase

GR- Glutathione reductase

GSH- Reduced glutathione

GSSG- Oxidized glutathione

HCT- Haematocrit

HP-NAP - *Helicobacter pylori* neutrophil-activating protein

I-BIL- Indirect bilirubin

ICF- Intracellular fluid

LPO- Lipid peroxidation

MCH- Mean corpuscular haemoglobin

MCV- Mean corpuscular volume

MCHC- Mean corpuscular haemoglobin concentration

NSAIDS- Non steroidal anti-inflammatory drugs

MDA- Malondialdehyde

MIC- Minimum inhibitory concentration

NADPH- reduced Nicotinamide Adenine Dinucleotide Phosphate

NADP- oxidized Nicotinamide Adenine Dinucleotide Phosphate

NBT- Nitroblue tetrazolium chloride

OECD- Organization of Economic Cooperation and Development PG- Prostaglandin

PGD_2 - prostaglandins D_2

PGE_2 - Prostaglandin E_2

$PGF\alpha_2$ - prostaglandins $F_2\alpha$

PGI_2 - prostaglandins I_2 (prostacyclin)

PUDs-peptic ulcer diseases

ROIs-reactive oxygen intermediates

ROS- Reactive oxygen species

SD- Spraque-Dawley

SEM- Standard error of mean

SOD- Superoxide dismutase

T-BIL- Total bilirubin

TBA- Thiobarbituric acid

TBARS- Thiobarbituric acid reactive substance

TCA- Trichloroacetic acid

TTC-Triphenyl Tetrazolium Chloride

TXA_2 - thromboxanes A_2

WBC- White blood cell

Chapter 1

GENERAL INTRODUCTION

1.1 BACKGROUND

The use of natural products for the management of various ailments is as old as human civilization. Plants and plant-based medications, which are natural products, form the basis of many of the modern pharmaceuticals. The use of plant medicine is now considered as a keystone of health care services in most developing countries.

Presently there is indication that about 80 % of the world's population depend on herbal medicine for healthcare and about 70-95 % of developing countries rely on plant medicine for primary healthcare (WHO, 2011). In Ghana about 80% of the populace rely on plants for primary healthcare. This is an indication that plant medicine continues to make an important role in the maintenance of health, in spite of the great advances made in modern or orthodox medicine. WHO also endorses the use of herbal medicine, especially in countries where access to conventional treatment is inadequate and inaccessible (WHO, 2011).

It is known that a large number of plant species and their derivatives are used in different parts of the world either as food, or for the treatment of different diseases. Various people around the world have used 35,000 - 70,000 species of plants as medicines and at least about 7,000 plant-derived medicinal compounds have been introduced into Western Pharmacopoeia (Lewington, 1990). For sometime now, interest in drugs obtained from plants has increased marginally. It is projected that about 25 % of medicines are directly or indirectly derived from plants (Calixto, 2000). According to Dokosi (1998), more than 3000 different medicinal plant species can be found in Ghana and almost all are used in traditional and herbal medicine.

In Ghana, medicinal plants are assuming greater importance in health care systems. A large number of plant species are used in different parts of the country as food

and also as medicine for the management of several diseases including rheumatism, arthritis, diarrhoea, inflammation, coughs, hypertension and placenta retention. Others are menstrual disorders, erectile dysfunction, infertility, lactation failure and peptic ulcers (Mshana *et al*., 2000).

Dissotis rotundifolia is a common plant that has been reported to have various ethno-medicinal uses in tropical Africa (Dokosi, 1998; Mshana *et al*., 2000). It is among the most widely used medicinal plants in Africa. Its folkloric use has been reported in traditional herbal medicine systems in Nigeria (Odugbemi, 2008), Tanzania (Hamisy *et. al.*, 2000), Cameroun (Jiofack *et al*., 2009; Noumi, 2010), Liberia (Watt and Breyer-Brandwijk, 1962), and Ghana (Mshana *et al*., 2000; Darko, 2009). Implicitly, there are indications that the plant is used traditionally in the management of several illnesses including gastrointestinal disorders.

Peptic ulcer affects many people worldwide and is considered a global health problem (Chan and Leung, 2002). In Ghana, about 300, 000 cases of peptic ulcer are reported annually (WHO, 2004). Despite the fact that several drugs are in use for managing the disease, research shows that most of them have serious undesirable effects such as erectile dysfunction, diarrhoea, and hypergatrinemia (Waldum *et al*., 2005; Kovacs *et al*., 2009; Sheen and Triadafilopoulos, 2011). This has necessitated the need for research into natural products from plant sources for the management of peptic ulcer.

Traditional knowledge of the existence and use of *Dissotis rotundifolia* (Sm.) Triana in the management of peptic ulcer in Ghana has been published by Mshana *et al*. (2000), Addo-Fordjour *et al.* (2008) and Darko, (2009). Though this plant is being used medicinally, its pharmacological and safety profiles have not been investigated scientifically.

1.2 *DISSOTIS ROTUNDIFOLIA* PLANT

Dissotis rotundifolia, also known as Spanish shawl, trailing Tibouchina or pink lady, has synonymic names such as *Osbeckia rotundifolia* sm, *D. plumosa* (G. Don) Hook.f, *D. prostrate* (Thonn.) Hook.f and *D. deistelli* (Gilgex) Engl. *Dissotis rotundifolia* has common vernacular names in Africa such as; Adongo gbe in Ewe (Ghana) (Darko, 2009), Adolea in Nzema (Ghana) (Darko, 2009), Awede in Yoruba (Nigeria) (Abere *et al.*, (2010), Kinzasu in Kiliguru (Tanzania) (Hamisy *et al.*, 2000) and Boreadaso in Twi (Ghana) (Mshana *et al*., 2000). *Dissotis rotundifolia* belongs to the kingdom, *Plantae* and family, Melastomataceae.

Dissotis rotundifolia is a perennial decumbent herb, which is found mostly in the tropics. The stem of *Dissotis rotundifolia* is ascending, rooting at the nodes and can grow up to 40 cm long (Abere *et al.*, 2009). In cross section, the stem is rectangular and is lightly covered with soft hairs hence it is described as pilose. The leaves are arranged opposite each other and are commonly ovate or ovatelanceolate measuring about 1.5 – 6 cm in length and 1 – 2.5 cm wide (Agyakwa and Akobundu, 1998), with petioles as long as 1.5 cm (Liogier, 1995). A picture of the plant is shown in Figure 1.1. *Dissotis rotundifolia* produces flowers throughout the year (Wicken, 1975), bearing one to three flowers, at the tip of the stem. The calyx is covered with bristles bearing terminal star shaped projections and corolla of five free parts, obovate and deep pink in colour. The plant can be propagated either by seeds or vegetatively (Mshana *et al*., 2000). *Dissotis rotundifolia* consists of a primary taproot, arising from the nodes, which bear numerous, filamentous secondary and tertiary roots. The young roots are covered in a clear, thick jelly that protects them from mechanical damage until they are fully matured (Whistler, 2000; Loigier, 1995; Wicken, 1975). In Ghana, the plant can be found in the Ashanti, Western, Brong-Ahafo, Central and Eastern regions (Darko, 2009).

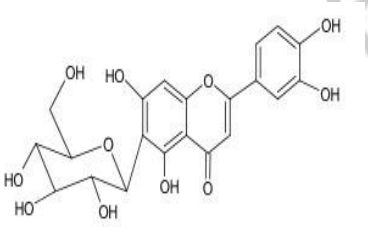
x1/4

Figure 1.1: A picture of *Dissotis rotundifolia* whole plant (Fieldwork, 2011)

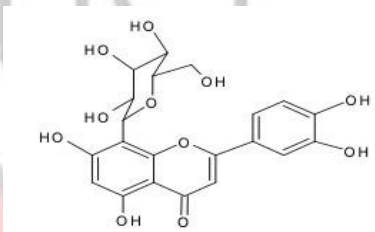
Phytochemical analysis of leaves of *Dissotis rotundifolia* revealed the presence of saponins and tannins (Gills, 1992). Abere *et al.* (2010) also reported that the active components of the leaf of *Dissotis rotundifolia* contain saponins, tannins, and cardiac glycosides. Also, some constituents of the whole plant extract of *Dissotis rotundifolia* have been characterized and *C*-glycosylflavones from the methanolic extract of the plant were isoorientin, orientin, vitexin and isovitexin (Rath *et al.*, 1995). The structures of representative compounds of Cglycosylflavones are shown in Figure 1.2.

Researchers have reported pharmacological effects of *Dissotis rotundifolia* in various test models. These include anti-diarrhoea effects, antimicrobial and antitrypanosomiasis. In castor oil-induced diarrhoea model, the ethanolic extracts of the leaves of *Dissotis rotundifolia* was able to stop the release of wet faeces in rats (Abere *et al*., 2010). Ethanolic extracts of *Dissotis rotundifolia* leaves have also been shown to possess antimicrobial effects against *Pseudomonas aeruginosa, Escherichia coli, Salmonella typhi and Staphylococcus aureus* (Abere *et al*., 2010). A pharmacological study on Trypanosome brucei showed that ethanolic extract of

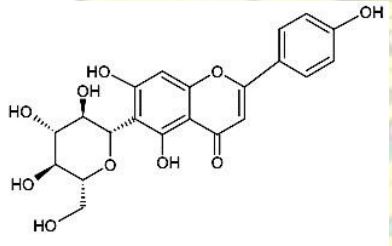
Dissotis rotundifolia leaves was able to reduce parasitemia in rats (Mann *et al*., 2009).



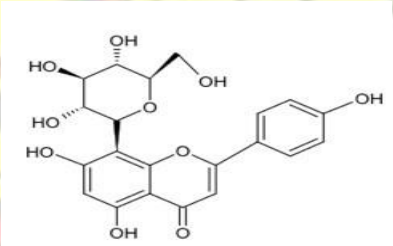
Isoorientin (luteolin-6-*C*-β-glucopyranoside)



Orientin (luteolin-8-*C*-β-glucopyranoside)



Isovitexin (apigenin-6-*C*- β-glucopyranoside)



vitexin (apigenin-8-*C*- β-glucopyranoside)

Figure 1.2: Structures of representative compounds of glycosylflavones isolated from methanolic extract of whole plant of *Dissotis rotundifolia* (adopted from Mohammed et al., 2014)

1.3 PEPTIC ULCER

Peptic ulcer, which is also known as *ulcus pepticum* in Latin (Cohen, 2006) is a breach, break, sore, or lesion in the inner layer of the GIT. Peptic ulcer is an ulcer in or adjacent to an acid producing part of the GIT. This can occur in the mucosa, submucosa and muscularis mucosa as shown in Figure 1.3 and in certain instances, the inner layers of the wall of the muscle (Pounder, 1994). When the breach in the gastrointestinal mucosa is more than 5 mm or 0.5 cm diameter and penetrating the muscularis mucosa, it is sometimes considered as an ulcer. On the other hand, a breach that is less than 5 mm or 0.5 cm in diameter and not penetrating the muscularis mucosa, or partial thickness mucosal defect, is referred to as an erosion. Meanwhile, the term peptic ulcer is generally used to describe a sore on the stomach or duodenum lining (NIH, 2010) and thus, ulcers and lesions are all collectively referred to as peptic ulcers.

Peptic ulcers usually occur in the stomach, proximal duodenum and less commonly, in the lower oesophagus, the distal duodenum or in the jejunum. When the ulceration is located in the stomach, it is called gastric ulcer, if it surfaces in the duodenum, it is referred to as duodenal ulcer and oesophageal ulcer if seen in the oesophagus (Schafer, 2011). These three types of peptic ulcers are shown in figure 1.4. Though peptic ulcer can be gastric, duodenal or oesophageal, the two most common types are the gastric and duodenal ulcers (Schafer, 2011).

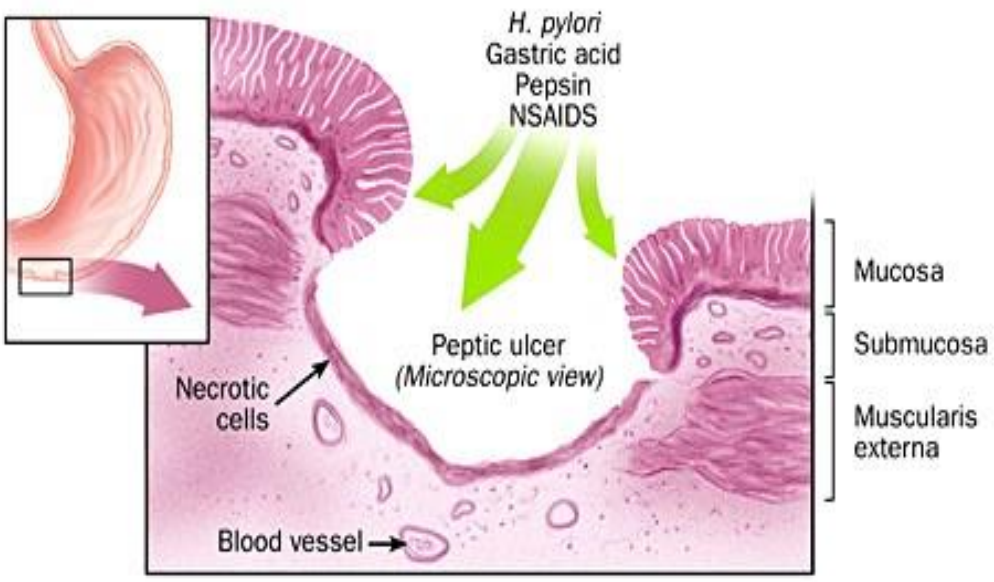


Figure 1.3: pathogenesis of peptic ulcer disease in the gastrointestinal tract (Adopted;http://www.hopkinsmedicine.org/gastroenterology_hepatology/_pdfs/esophagus_stomach/peptic_ulcer_disease.pdf. accessed 02/10/2014)

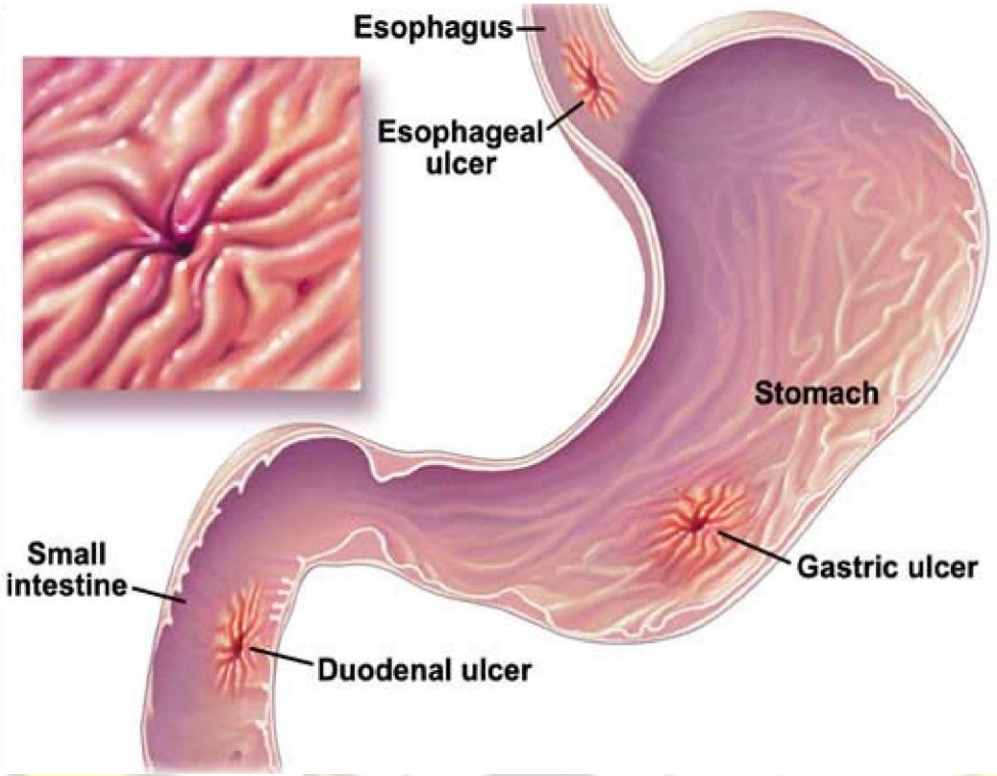


Figure 1.4: Schematic diagram indicating the different types of peptic ulcer in the gastrointestinal tract (Adopted from http://www.medicinenet.com/imagecollection/peptic_ulcer_picture/picture.htm. retrieved 13/06/2014)

1.3.1 Physiology of gastric acid secretions

The stomach, the distended part of the gastrointestinal tract (GIT), is the main organ of attack during peptic ulceration. The stomach is described as a J–shaped organ that is divided into four regions namely, the narrow upper cardiac zone, the dome shaped fundus, the large central body and the terminal pylorus (Figure 1.5). Anatomically, the stomach is located in the upper left of the abdominal quadrant just inferior to the diaphragm and it is continuous with the oesophagus superiorly and empties into the first part of the small intestines; that is the duodenum (Van de Graff, 2001).

There are several physiological functions of the stomach namely, motility of bowel, excretion of bile salts, gastric acid secretions and others, but gastric acid secretions is more relevant to the present study and thus will be discussed.

The human stomach (parietal cells of the fundus and body) normally secretes about 2.5 L of gastric juice a day. Gastric juice is composed of pepsin, hydrochloric acid and electrolytes such as sodium ions, potassium ions, hydrogen carbonate ions and chloride ions (D' Alessio *et al*., 2001; Altman, 2001). According to Wallace (2008), gastric juice also contains certain substances such as immunoglobulins and lactoferrin that possess the ability to reduce bacterial colonization though there are few microbes such as *Helicobacter pylori* that are able to survive the low pH in the stomach (Clyne *et al*., 1995).

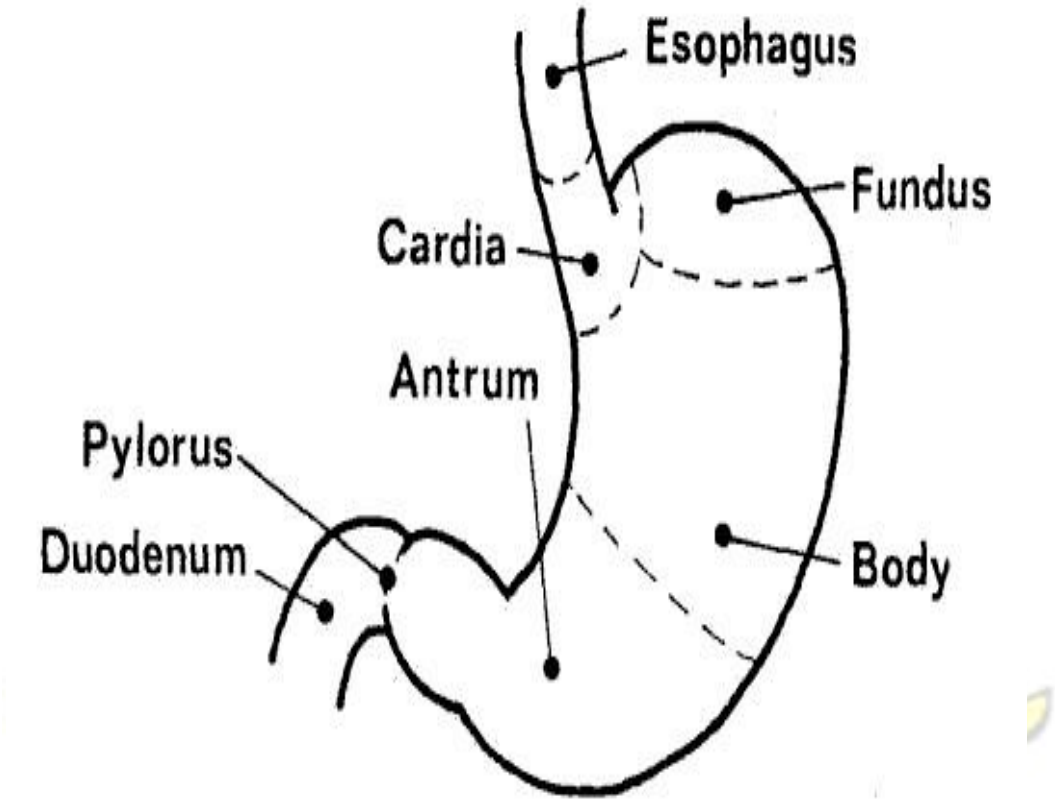


Figure 1.5: The regional parts of the stomach (adopted from Mansy, 2009) The critical role of gastric acid in mucosal defensive factor is seen in the observations made by Giannella *et al*., (1973), that low or no stomach acid in gastric juice increases the chance of bacterial colonization and certain parasitic infections. There is an inverse relationship between bacterial load in the stomach and duodenum, and the level of gastric acid secretion (Gray and Shiner, 1967). Gastric acid plays its role by aiding in food digestion and also killing bacteria. Gastric acid also ensures that the optimal pH of 1.8-3.5 is created for the correct functioning of pepsin.

An important enzyme for gastric acid secretion is the Hydrogen / Potassium ATPase. This enzyme is produced by the parietal cells. It makes use of the energy

derived from ATP to pump intracellular hydrogen ions into the lumen, in exchange for extracellular potassium ions thus it is also refered to as proton pump. The hydrochloric acid is produced through the interaction between chloride ions of arterial blood and hydrogen ions from parietal cells. When the cells are at rest or unstimulated, H^+ / K^+-ATPase are found in vesicles inside the parietal cells. When the cells are stimulated, particularly during a meal, these vesicles fuse with the plasma membrane, thus increasing the plasma membrane surface area. This ultimately leads to increase in the number of proton pumps in the membrane (D' Alessio *et al*., 2001).

The secretion of gastric juice and contraction of smooth muscle in the stomach wall is controlled by both nervous and hormonal mechanisms. The control process occur in three overlapping phases namely cephalic, gastric and intestinal phase.

The cephalic phase comprise of reflexes initiated by sensory receptors in the head. Even before food enters the stomach, the cephalic phase stimuli namely smell, sight, and taste or thought of food intiates this reflex. During the cephalic phase, parasympathetic impulses from nuclei in the medulla oblongata are transmitted through the vagus nerves. These nerve impulses stimulate peristalsis movement in the stomach smooth muscle and stimulate the secretion of pepsinogen, HCl and mucus into the stomach chyme whilst gastrin enters the blood stream.

The key factor in the gastric phase is the presence of food in the stomach. Once food gets into the stomach, sensory receptors in there initiate both neural and hormonal mechanisms to ensure that gastric secretions and motility continue. During gastric phase, reflexes within the stomach continue to stimulate whipping contractions and secretory events. The presence of food in the stomach raises the pH in the stomach. The increase in pH serves as a stimulus to inhibit somatostatin secretion (Page and Blackshaw, 1998).

The intestinal phase takes place as a result of receptor activation in the small intestines. While reflexes initiated during the cephalic and gastric phase stimulate secretory activity and motility in the stomach. On the contrary those happening in intestinal phase have inhibitory effects. During intestinal phase, neural and hormonal reflexes initiated in the small intestine exert an inhibitory effects on secretion by the stomach and motility of the stomach. This slows the exit of chyme from the stomach and prevents overloading the duodenum. Examples of stomach inhibitory hormones include, cholecystokinin (CCK), and secretin (Altman, 2001).

1.3.2 Chemical mediators of peptic ulceration

Generally three biomolecules are known to stimulate gastric acid release, these are gastrin, acetylcholine and histamine. There is one other regulatory biomolecule called somatostatin that is inhibitory. Figures 1.6 and 1.7 show how these regulatory biomolecules interact to stimulate or inhibit gastric acid secretions.

Parietal cells are directly stimulated by acetylcholine and histamine to increase gastric acid secretion. Acetylcholine (Ach), is released by enteric neurons of parasympathetic innervations into stomach (vagus). The release of acetylcholine causes not only an increase in acid secretion but also motor activities of the gastrointestinal tract (GIT). Acetylcholine (Ach) binds to its receptors that are present on the parietal cells of the stomach and thus increases the levels of calcium intracellularly. Calcium ion (Ca^{2+}) binds to calmodulin and activates calcium calmodulin-dependent protein kinase C, which phosphorylates and activates proton pump in the stomach to increase hydrogen ion secretion (D' Alessio *et al*., 2001).

Histamine another chemical mediator is synthesized from the amino acid histidine and stored as granules. In rats, it is derived from enterochromaffin cells while in dogs and in humans; it is released from mast cells. Gastric parietal cells are equipped with specific types of receptors for histamine, which are known as

histamine (H_2) receptors. These receptors aside from the stomach are also found in the uterus where they cause contractions and in the heart where they increase heart rate. In the stomach, histamine activates its receptors and subsequently stimulates the cAMP signal transduction system which after a series of reactions, leads to phosphorylation of proton pump.

Phosphorylation of proton pump leads to the release of gastric HCl from parietal cell into the lumen (Guyton, 1991). HCl secretions increase markedly due to release of histamine while histamine secretions increase under the influence of gastrin and acetylcholine (Figure 1.6). Implicitly it can be said that histamine, acetylcholine and gastrin all work through the proton pump H^+ / K^+ -ATPase to allow the parietal cells to produce HCl as shown in Figure 1.6. On the other hand, somatostatin release decreases histamine secretions as shown in Figure 1.7.

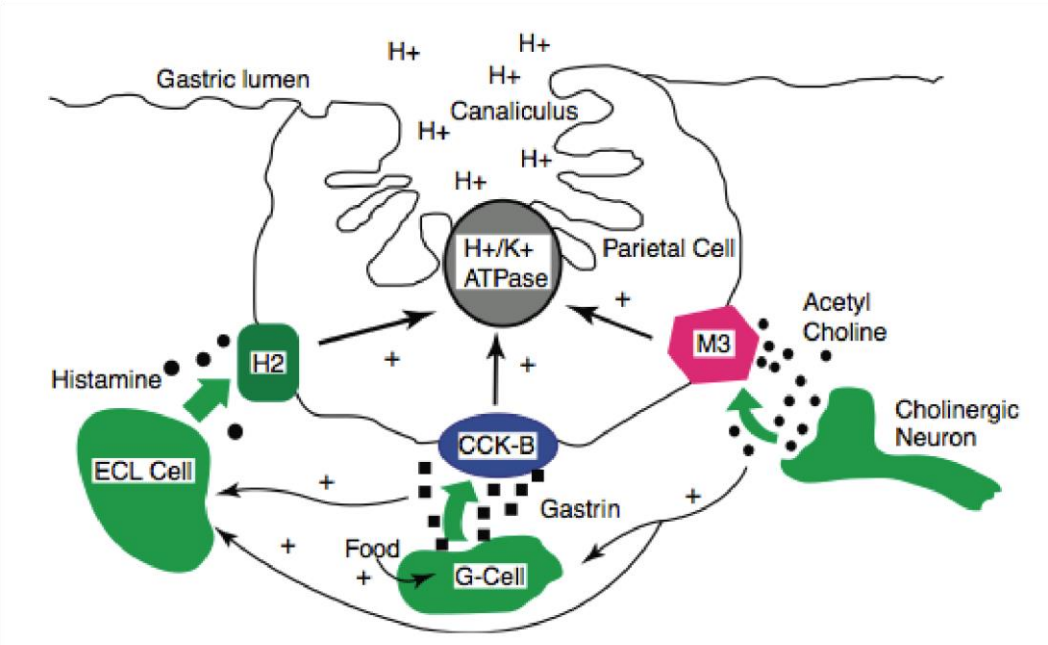


Figure 1.6: Gastric acid secretion by the H^+ / K^+ - ATPase in the parietal cell is driven by acetylcholine, gastrin and histamine (Adopted from Bamford, 2009).

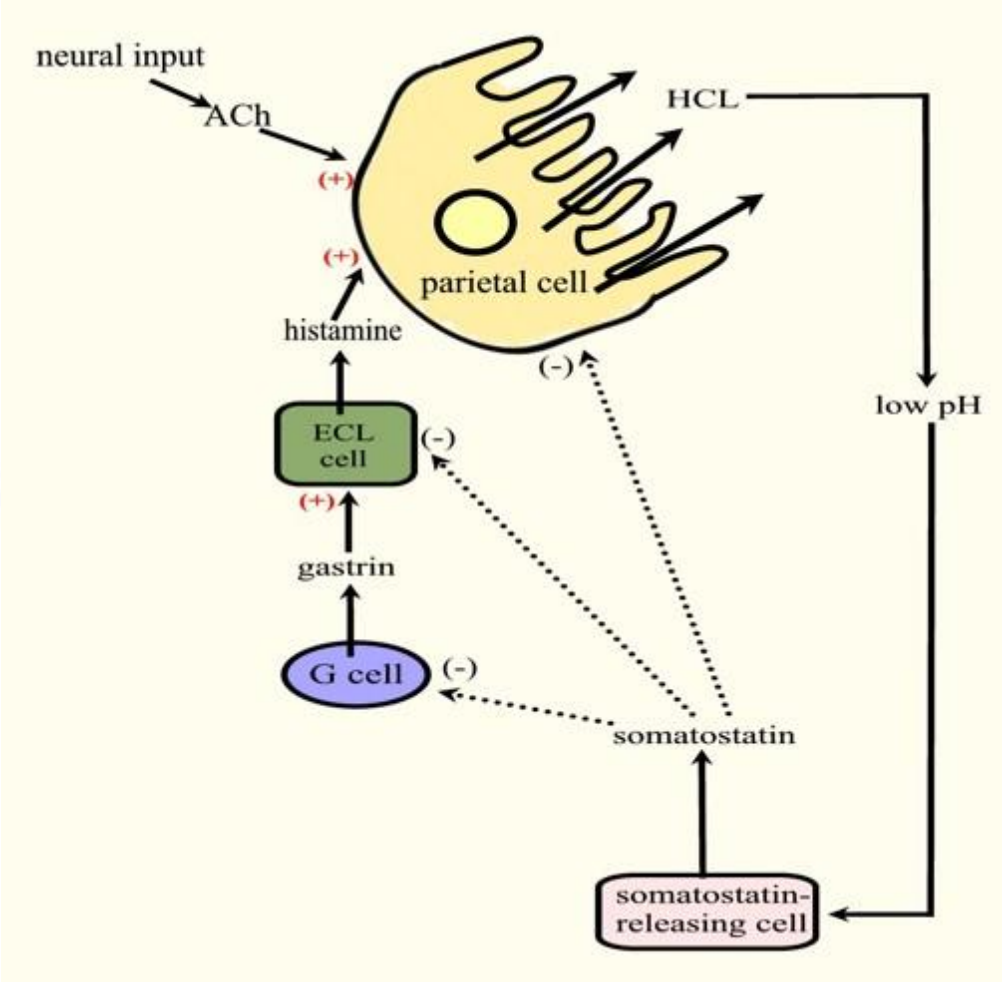


Figure 1.7: Stimulation of parietal cells in the stomach to produce HCl (Adopted from D' Alessio et al., 2001).

Gastrin is a peptide hormone secreted by G-cells of the gastric antrum in epithelium of GIT. There are three main forms of gastrin; G_{34}, G_{17} and G_{14} which contain 34, 17 and 14 amino acids residues, respectively. According to D' Alessio *et al*., 2001, in humans, G_{17} remains the most potent stimulant of gastric acid secretion. Gastrin

is secreted into the blood, and transported to the gastric glands where it stimulates histamine release from ECL cells and ultimately gastric acid secretions as shown in figure 1.7. Gastrin also increases the secretions of pepsinogen and also stimulates cellular proliferation in parietal cells. When the pH in the stomach drops below 2, there is inhibition of gastrin secretions by a negative feedback mechanisms. This mechanism ensures the maintenance of an optimum pH in the stomach.

Somatostatin is an inhibitory hormone secreted by cells of the gastric epithelium. When there is a drastic reduction in pH of the stomach, the release of somatostatin is stimulated. Once somatostatin is released, it inhibits gastric acid secretions by its direct effects on parietal cells, ECL and G cells to release HCl, histamine and gastrin respectively, as shown in figure 1.7. There is a change in the levels of these biomolecules when food is ingested.

1.3.3 Protective factors of gastric mucosa

Mucosal defence is a terminology that is used to depict the elements and components that make the gastric mucosa (Wallace and Granger, 1996). Gastric mucosa is not impervious to injury. Though gastric mucosal injury often occurs, it does not necessarily lead to serious disruption, of the role of gastric mucosal tissue. The explanations for this include the presence of several mucosal defences, with secondary factors, which become extra important when several components are breached. Additionally, a swift repair process occurs when injury to the gastric lining happens (Wallace and Granger, 1996). Besides, the numerous segments of gastric mucosal defence can be regulated by several elements, within the gastric mucosa such as mucus, bicarbonate, restitution, prostaglandins and gastric mucosal blood flow (Wallace, 2008).

Mucus secreted by the stomach epithelial cells functions to lubricate and protect the stomach mucosa from physical injury by ingested substances. When the epithelium

is destroyed, amazingly, re-establishment happens within mins after damage. This fast process of reparation is known as restitution. Restitution encompasses the movement of beneficial epithelium cells, to satiate defects created by the shedding off injured epithelial cells (Wallace *et al*., 1982; Morris and Wallace, 1981). The mucus released from injured epithelium and plasma discharged slowly from the mucosal vascular system, combines to form a protective layer over the shed area which is referred to as the *mucoid cap* (Wallace and McKnight, 1990; Wallace and Whittle, 1985). When the gastric acid levels in the stomach is raised, the mucoid cap pH is kept at almost neutral. This phenomenon is key to permit restitution to occur. This is another component of mucosal defence for which there is indication of alteration by prostaglandins.

The mucus layer is comprised of glycoprotein that is thick and possesses gelforming characteristics, which enables the formation of a continuous layer of water at the luminal epithelial surface. The mucus and water layers together lessen the damage by the acidic environment. It also acts as a trap for bacteria (Belley *et al*., 1999) except *Helicobacter pylori*. The mucus plays an essential role structurally in creating an undisturbed stable layer, which supports the continuance of a close to neutrality pH at that mucosal surface (Ho *et al*., 2004; Allen *et al*., 2005).

Secretion of bicarbonate by the epithelial cells at the duodenal mucosa (Altman, 2001) is within the mucus, thus generating an environmental pH near neutral (Bahari *et al*., 1982; Garner *et al*., 1984; Chu *et al*., 1999). The secreted bicarbonate also shields the gastric epithelium by neutralizing gastric acid.
Bicarbonate secretion helps to neutralize gastric acid that enters the intestine from the stomach.

Prostaglandins (PG) are synthesized *de novo* in response to different stimuli and perform various physiological functions. They belong to the class of substances

called eicosanoids. Prostaglandins are derived from arachidonic acid and prostaglandins E_2, I_2, D_2, F_2 and thromboxane A_2 (TXA_2) are the most important products. Different types of prostaglandins are synthesized in various tissues due to differences in subsequent steps of arachidonate metabolism in every cell. Prostaglandin E_2 is synthesized in the GIT (Guyton, 1991; Walter and Israel, 1996). Different receptors for prostaglandins are found on different cells of the body. These include receptors for PGD_2, PGF_2, PGI_2, TXA_2, and PGE_2 are DP, FP, IP, TP and EP. The types present in the GIT are the EP that is further subclassified into EP_1, EP_2 and EP_3. (Gregersen *et al*., 2000). Two cyclooxygenase (COX) enzymes are involved in catalysing the formation of prostaglandins from arachidonic acid as shown in Figure 1.8. Prostaglandin E_2 (PGE_2) improves gastric mucosal defence to tissue damage by

I. Decreasing basal and stimulated gastric acid secretion
II. Enhancing epithelium, bicarbonate secretion, mucus production, cell turnover, and flow of blood locally.

The final protective factor is blood flow (Valle, 2005). The preservation of a fairly elevated pH in the stomach environment is reliant on uninterrupted gastric blood flow. When there is interruption of flow of blood to the stomach, the pH within the mucoid cap falls and haemorrhagic injuries occur (Wallace and McKnight, 1990).

Prostaglandins play an important function in maintaining the flow of blood throughout the essential phase of repair, since studies have shown that these occurrences can be averted by the use of a prostaglandin (Wallace and McKnight, 1990).

On the contrary, there are some situations where gastric mucosal defence is weakened. These include excessive intake of non-steroidal anti-inflammatory

drugs (NSAIDs), stress and *Helicobacter pylori* colonization. All these make the mucosal barrier more prone to injury.

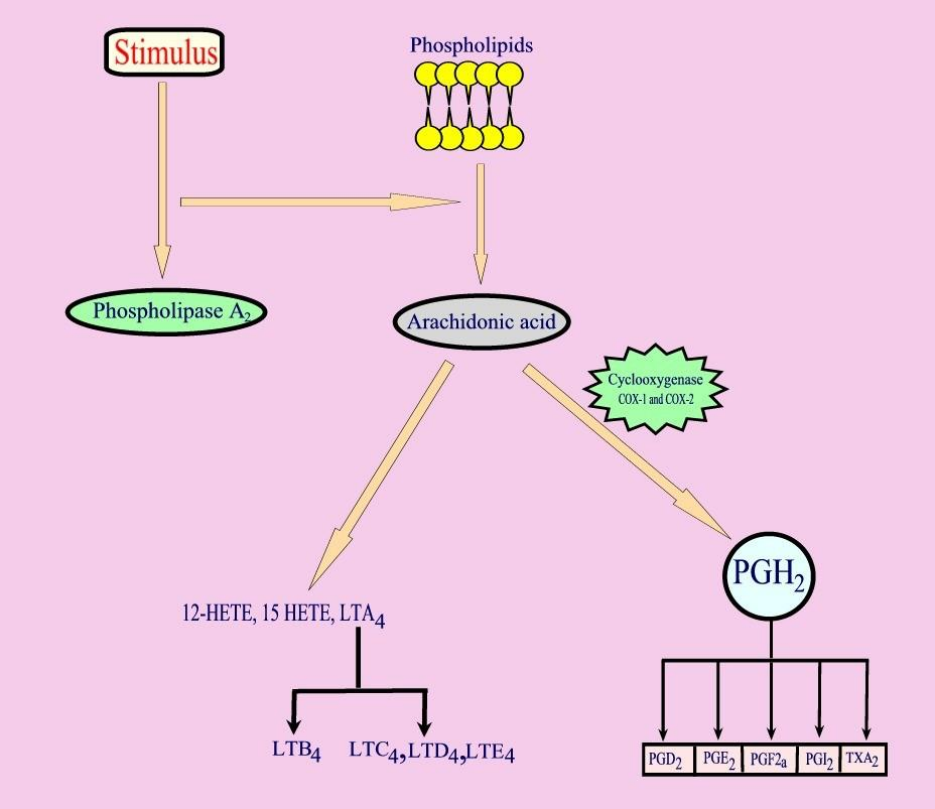


Figure 1.8: Biosynthesis of Eicosanoids

(adopted from **http://www.arthritis.co.za/nsaids2.htm. retrieved 05/04/2014)**

1.3.4 Epidemiology of Peptic ulcer

Peptic ulcer diseases (PUDs) affect many people all over the world, and is currently considered a health problem. The prevalence of the disease is higher in males than females. The prevalence of peptic ulcer in the general population is 912 %; 12 % for men and 9 % for females (Sung *et al*., 2009). Peptic ulcer is considered a worldwide health problem with the United States of America recording the highest

mortality rate (Cook and Zumla, 2009). Approximately 4500 people in the United Kingdom (UK) and 15,000 in United States (US) die each year from complications of PUDs (Valle, 2005). Although, the prevalence of peptic ulcer in Africa is not well documented, some sources based on the known statistics in the United States of America, i.e. US Census Bureau, International Database, have extrapolated the prevalence rate of other countries using their populations as at 2004 (Cook and Zumla, 2009). The highest incidence in Africa is in Egypt, recording 1,035,420 incidences annually (Cook and Zumla, 2009). Swaziland is said to record the lowest incidence of 15,905 cases per year Cook and Zumla, 2009). According to Cook and Zumla (2009), parts of West Africa, Rwanda, Burundi, eastern Zaire, western Tanzania, southwestern Uganda and the Ethiopian highlands are the areas of high prevalence in Africa. Ghana is estimated to have about 300, 000 cases of peptic ulcer per year (Cook and Zumla, 2009). In a study carried out in Kumasi, Ghana, it was reported that perforated gastric ulcer was more common than perforated duodenal ulcer and this was attributed to the increased usage of NSAIDs (Cook and Zumla, 2009). Another study conducted at the Korle-Bu Teaching hospital in Ghana, from January, 1995 to December, 2002 revealed that out of 6977 patients, 3777 males and 3200 females, within the age range of 1 year 8 months to 93 years, 19.6 % had duodenal ulcer, 12.7 % had acute gastritis, 10.2% had duodenitis and 7.5 % had oesophagitis (Aduful *et al*., 2007).

1.3.5 Pathophysiology of gastric ulceration

Peptic ulcer is one of the major GIT disorders characterized by mucosal damage secondary to excessive pepsin and gastric acid secretion. The pathophysiology of peptic ulcer has been centralized on an imbalance between offensive and defensive factors for gastric mucosal integrity (Cohen, 2006). The offensive or damaging factors include gastric acid-pepsin secretion and bile salts, while defensive factors includes bicarbonate, mucosal layer, secretion of mucus, gastric mucosal blood flow, cell regeneration of the epithelial layer and protective endogenous substances such as prostaglandins (Valle, 2005).

Peptic ulcer arises when there is a disturbance in the normal equilibrium caused by either enhanced aggression or weakened mucosal defence. These disturbances or imbalance involve increased gastric acid–pepsin secretion, impaired neutralization by bicarbonate, and impaired secretion of mucus (Tytgat, 2011).

Several aetiological agents have been implicated in this imbalance between aggressive and defensive factors but the two main ones are *Helicobacter pylori* – a bacterium and the continuous use of Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) (Soll and Graham, 2009). Other factors that put one at risk of peptic ulcer are stress, smoking, alcohol, nutritional deficiencies, genetic predisposition, bile acids, and steroids (Kavanagh, 1988).

Peptic ulcers caused by *Helicobacter pylori*, account for the highest percentage (70-75%) of peptic ulcer cases worldwide (Shrester and Lau, 2009). *Helicobacter pylori* infection prevalence has reduced in developed and developing countries over the past decade, due to widespread use of antibiotics (Malfertheiner *et al*., 2009). Gastric acid secretion increases in *Helicobacter pylori*-associated duodenal ulcer patients. This happens when the levels of gastrin goes up, leading to parietal cell proliferation and the consequence is high gastric acid secretion.

Gastrin hormone release goes up by two ways

I. The NH_3 produced by *Helicobacter pylori* creates an alkaline environment thus stimulating gastrin release
II. Studies have shown that the number of antral D cells is decreased in *Helicobacter pylori* infected individuals, leading to reduced somatostatin levels and increased gastrin levels. *Helicobacter pylori* reduces duodenal hydrogen carbonate ion secretion thus wearing the protective mechanism of the duodenal mucosa.

1.3.5.1 *Helicobacter pylori*

Helicobacter pylori is a spiral-shaped gram-negative bacterium and the most common cause of non-NSAID-associated peptic ulcer disease. Studies have shown the role of *Helicobacter pylori* as an aetiological agent of gastritis and peptic ulcers (Sipponen and Hyvärinen, 1993). Other species that have been classified in the genus Helicobacter include *Helicobacter cinaedi*, *Helicobacter fennelliae* and *Helicobacter mustelae*. The *Helicobacter cinaedi*, *Helicobacter fennelliae* were initially isolated from homosexual men with proctitis, proctocolitis and / or enteritis while *Helicobacter mustelae* was isolated from ferrets. *Helicobacter cinaedi* and *Helicobacter fennelliae* may colonize the GIT without causing diseases. *Helicobacter cinaedi* has been found to be the primary cause of bacteraemia in AIDS patients.

Helicobacter pylori can be distinguished from other species due to its multiple sheathed flagella, hydrolysis of urea and unique fatty acid profile. It is the only organism that shows golden colonies when cultured on brain heart infusion agar supplemented with sheep or human blood and 40 mg/L of Triphenyl Tetrazolium Chloride (TTC).

Helicobacter pylori are mostly found in the mucus–secreting epithelial cells of gastric mucosa. It can grow in air with increased CO_2 content (10 %), reduced oxygen concentration (5 %) and increased nitrogen content (85 %). Various tests are used to distinguish the different species of Helicobacter, and this is shown in Table 1.1.

Table 1.1: Biochemical characteristics of some common Helicobacter species

Test reaction	***H. pylori***	***H. cinaedi***	***H. fenneliae***

Growth at;			
25 ^{O}C	-	-	-
37 ^{O}C	+	+	+
42 ^{O}C	-	-	-
Reaction to;			
Oxidase test	+	+	+
Catalase test	+	+	+
Urease test	+	-	-
Nitrate reduction test	-	+	-
Hipurate hydrolysis test	-	-	-
Susceptibility to;			
Nalidixic acid	R	S	S
Cephalothin	S	S	S

+ = Active, - = Inactive, S = Sensitive, R = Resistant (Adopted from Collier *et al*., 1998 *with modification)*

Mechanism of peptic ulcer induction by Helicobacter pylori

Helicobacter pylori are able to survive in the low pH of the stomach environment. They are acid-resistant because of their capability to produce the enzyme urease, which converts urea in the gastric mucosa to ammonia (Olivera-Severo *et al*., 2006). In the course of infection, the bacterium finds its way into the gastric lumen where the urease supports its survival by producing ammonia that buffers it from the acidic environment. Ammonia also combines with water to form ammonium hydroxide, forming an alkaline "cloud" around *Helicobacter pylori*, whereas hemin stimulates growth of *Helicobacter pylori* (Yoshiyama and Nakazawa, 2000). This serves as a

competitive advantage not exhibited by most bacteria and thus, protecting *Helicobacter pylori* from the acidic environment of the stomach as shown in Figure 1.9.

Meanwhile the ammonium hydroxide formed by the enzyme urease is cytotoxic and triggers gastric epithelial cell damage. The ammonia also inhibits cell proliferation and causes mucosal injury by stimulating neutrophils and lipopolysaccharides (endotoxins). Bacterial lipopolysaccharide attracts inflammmatory cells in response to proliferating *Helicobacter pylori* organism, leading to gastritis in the mucosa whereas neutrophils release myeloperoxide.

Other virulence factors such as lipase and proteases and other enzymes secreted by the bacteria also contribute to damage of the gastric mucosa. Phospholipase, neuraminidase, fucosidase, and alcohol dehydrogenase damage the gastric mucosa by degrading phospholipids and glycoprotein and generating reactive oxygen metabolites that are precursors of ulcerogenic components.

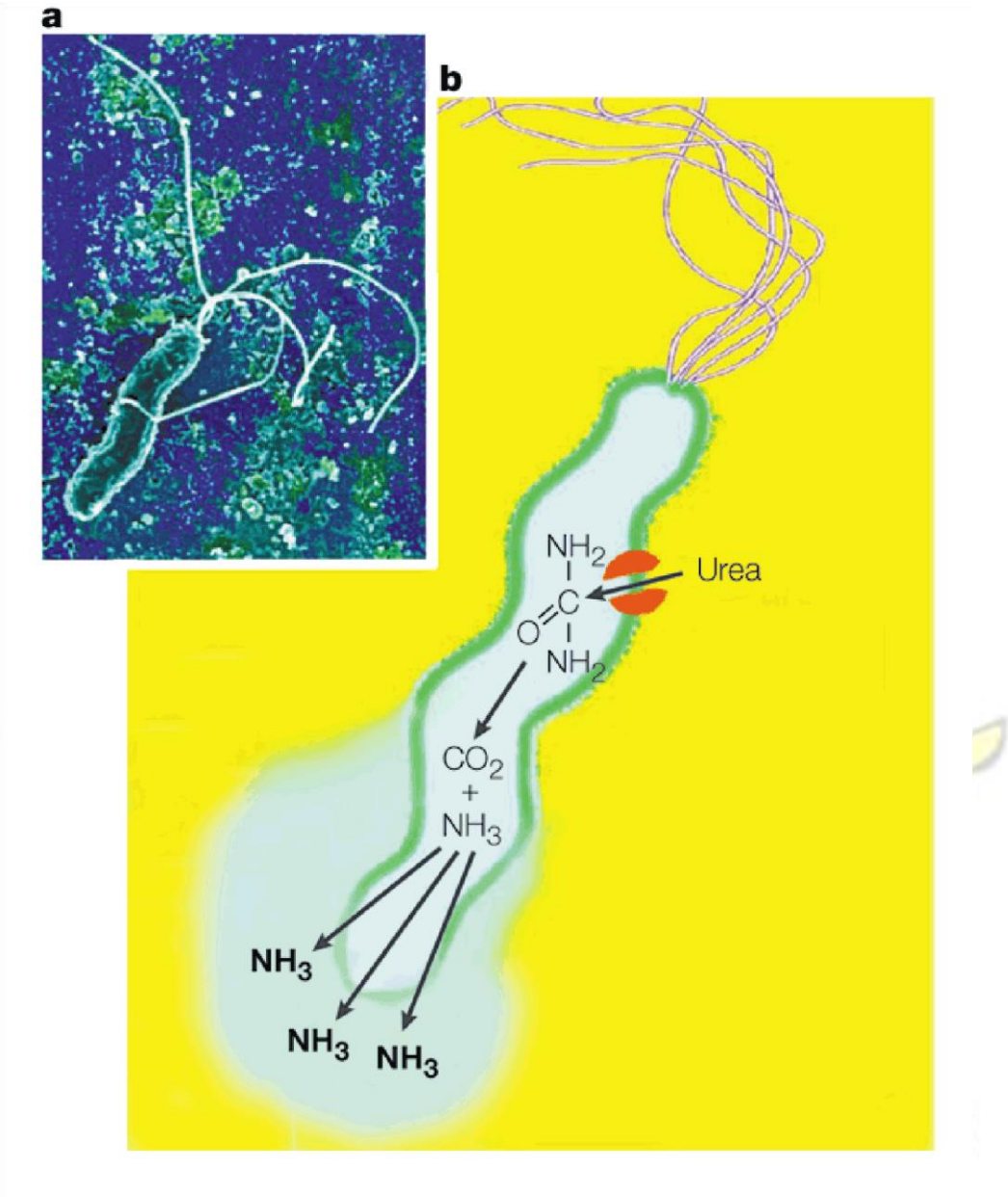


Figure 1.9: (a) Electron micrograph and (b) schematic representation showing shape, polar flagella, urease, H^+-gated urea channel and the production of ammonia, which neutralizes the **yellow** acidic environment and the cytosol and the immediate environment around the bacterium (light green). (Adopted from Montecucco and Rappuoli, 2001).

The presence of sheathed flagella with terminal bulbs together with the spiral shape of the helicoidal bacterium, allow for extraordinary propulsion capabilities of the bacterium. The bacterium is propelled by the flagella into the mucus layer and let it get into the epithelium cells, where it attaches itself. *Helicobacter pylori* then introduces a special protein called cagA protein into the host cells and discharges other toxins such as *Helicobacter pylori* neutrophil-activating protein (HP-NAP) and VacA (Montecucco and Rappuoli, 2001). VacA provokes modifications of junctions and the formation of large vacuoles. The HP-NAP moves across the epithelium and recruits neutrophils and monocytes, which force out liquid and cause tissue injury by liberating reactive oxygen intermediates (ROIs). The combined toxicity of VacA and ROIs brings about gastric tissue injury as shown in figure 1.10 (Montecucco and Rappuoli, 2001).

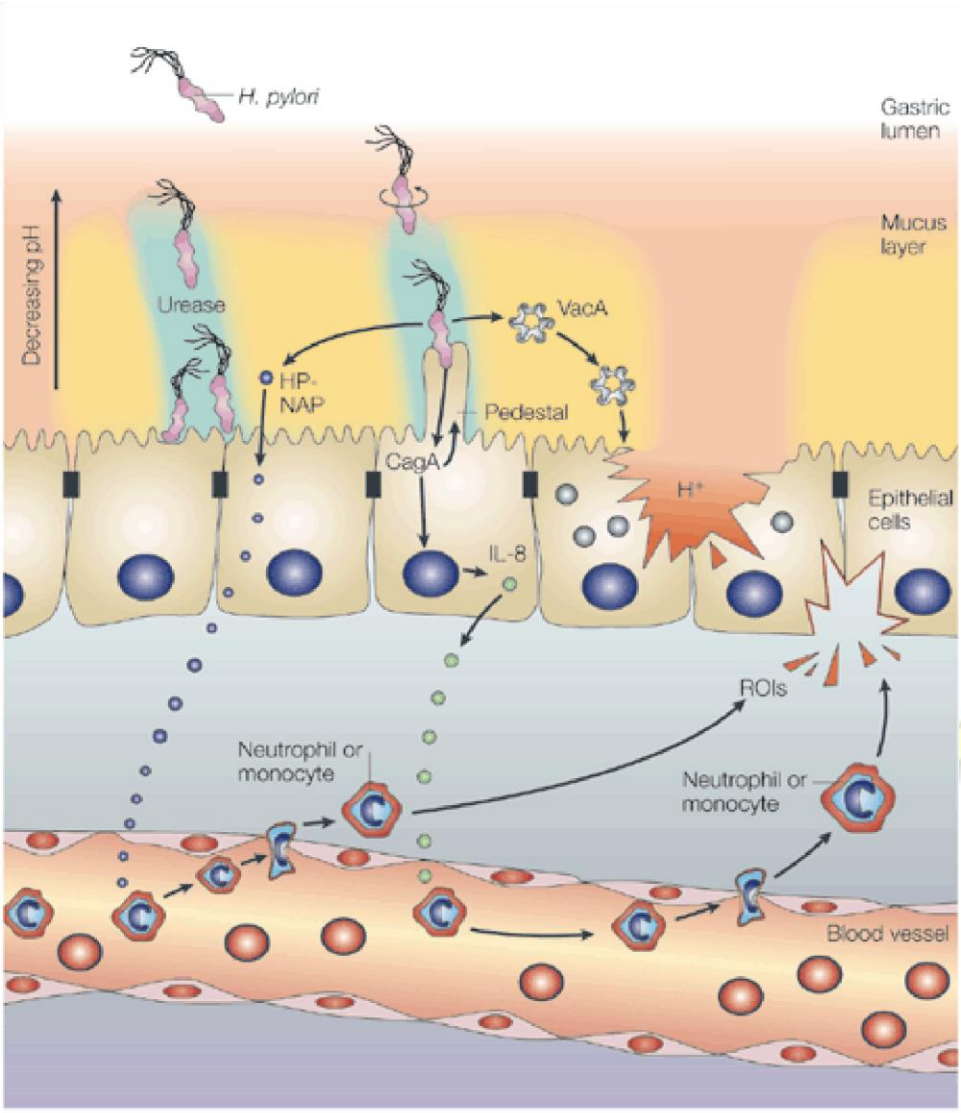


Figure 1.10: Diagramatic representation of the stomach mucosa showing colonization by *Helicobacter pylori* (Adopted from Montecucco and Rappuoli, 2001).

1.3.5.2 NSAIDs and gastric ulceration

NSAIDs are amongst the most frequently prescribed drugs for arthritis and pain associated diseases though they are known to be responsible for triggering several

adverse effects in the GIT. Most of these side effects have frequently been attributed to NSAID's inhibitory effect on COX enzyme and the consequence being prostaglandin (PG) deficiency. NSAID's-related GIT destruction is likely to yield topical as well as systemic injury, in both the stomach and intestines. The pathophysiological processes of NSAID-induced mucosal injury differ in stomach from that of the small intestines.

The vital role that prostaglandins (PGs) play in the stomach, makes it possible to explain, NSAID-induced mucosal injury when PGs are deficient. On the contrary, this explanation cannot be used in the case of the small intestine.

The main processess by which NSAIDs cause injury is categorized into local and systemic effects The local effects are prostaglandin (PG)-independent while the systemic ones, are PG- dependent.

Mechanism of PG independent NSAID induced gastric damage

The PG independent effects of NSAIDs on the gastric epithelium encompass several mechanisms that are independent of PG synthesis. There is disruption in the layer of phospholipids on the surface of the mucosa by NSAIDs. (Lichtenberger *et al*., 2006). Such action renders the gastric mucosa weak. Moreover most NSAIDs, are also weak acids thus can directly damage epithelial cells (Allen *et al*., 1993). Several processes have been proposed to be responsible for these effects, but the two generally well understood and accepted ones include (i) the initiation of lysis subsequent to the trapping of charged NSAIDs (Schoen and Vender, 1989) referred to as, the "trapping theory" and (ii) loss of the epithelial cell due to the uncoupling of oxidative phosphorylation.

NSAID's move across gastric epithelium into the cytoplasm which has a neutral pH. NSAIDs re-ionizes and become lipophilic. This makes it possible for them to be trapped and consequently accumulate within the cell causing injury. The

mechanism is what is referred to as, “trapping theory” mechanism of epithelial cell death. This trapping theory is not applicable to small intestines mucosa, where luminal pH is closer to neutrality. According to Matsui *et al*. (2011) though “trapping theory” cannot be used to explain small intestine injury, it may accelerate the degree of injury.

The epithelium cell death regulation has also been suggested as a second role of mitochondria, in addition to cell energy metabolism (Ott *et al.*, 2007). The respiratory chain that takes place in the mitochondria is the main source of ROS that is generated at Complex I and III. The NSAIDs impede oxidative phosphorylation process to disintegrate the mitochondrial transmembrane potential. This leads to release of ROS thus triggering the activation of caspases 3 and 9 and cellular lipid peroxidation (Brand *et al*., 2004). The outflow of Ca^{2+} ions from mitochondria, and loss of control over intracellular junctions, ends in increase in permeability and ultimately damage to mucosa (Brzozowski *et al*., 2000).

Mechanism of PG dependent NSAID induced gastric damage

NSAIDs also trigger systemic injury to the GIT lining, mostly as a result of decline in prostaglandin synthesis. This type of stomach injury can also be referred to as prostaglandin-dependent NSAID induced gastric damage. Prostaglandins play a crucial function in supporting gastric mucosal defence.

This is critical to the development of NSAIDs-induced gastric injury (Cryer *et al*., 2000; Cryer and Spechler, 2006). The two COX isoforms namely COX-1 &2 have been identified to be present in mammals. COX-1 is found in tissues including the stomach tissue and has been reported to be responsible for sustaining gastric integrity. COX-2 on the other hand is involved in inflammation. Implicitly gastric mucosal injury could be attributed to PGs deficiency caused by inhibition of COX-1 enzyme.

Wallace and his colleagues demonstrated that gastric ulcer happens only when both enzymes are inhibited (Wallace *et al*., 2000). This result contradicts the earlier concept on the house keeping role of COX - 1. The opinion that both enzymes make vital contributions to mucosal defence is additionally supported by studies carried out by Tibble *et al*., (2001) in mice. Another research by Laine *et al*., (2008), hinted that both enzymes may be performing a function in the synthesis of PG and also in preserving mucosal integrity. According to the conclusion made by these researchers, COX-2 enzyme plays a supportive role by alleviating PG deficiency which is provoked by the inhibition of COX-1 enzyme.

Aside these two main mechanisms of NSAIDs-induced gastric injury, NSAIDs also produce gastric injury by reducing the secretion of mucus and bicarbonate thus reducing the efficiency of the juxtamucosal pH gradient in shielding the epithelial cells (Figure 1.11) (Baumgartner *et al*., 2004). NSAIDs also reduces the ability of epidermal growth factor (EGF) to promote epithelial repair (Kajanne *et al*., 2007).

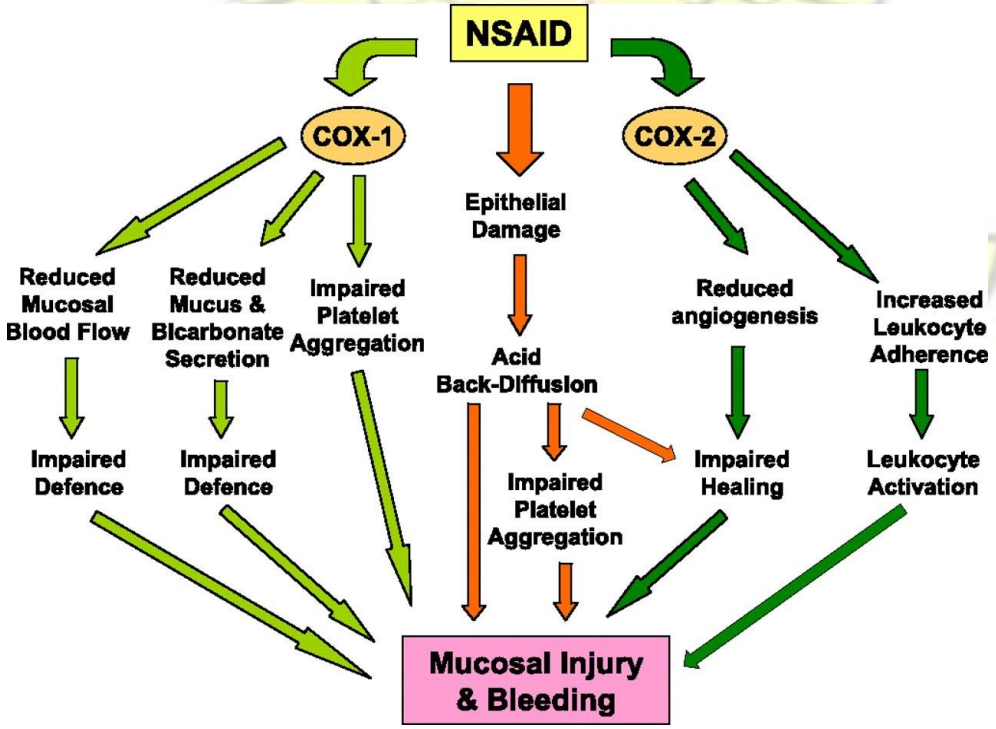


Figure 1.11: Pathophysiology of NSAID-induced gastric injury and bleeding (Adopted from Wallace, 2008)

Another proposed mechanism is that, NSAID excreted into the bile may flow back into the stomach and this can lead to injury in the epithelium tissue (Figure 1.11) (Whittle *et al*., 1985; Brune *et al*., 1993; Hawthorne *et al*., 1999).

Mechanisms of NSAIDs-induced small intestine damage

The mechanism by which NSAIDs induce small intestinal injuries is not satisfactorily understood compared to gastric injuries. A three-step hypotheses has been suggested (Bjarnason *et al*., 1993). This is illustrated in Figure 1.12. In the first place, in the mitochondria, NSAIDs absorbed in the cell of intestinal lining inhibit oxidative phosphorylation. The second step involves, inhibition of oxidative phosphorylation. This leads to impairment in the function of the junctions intracellularly and ultimate increase in the permeability of the intestine (Higuchi *et al*., 2009). The third step is that through mucosal barrier which becomes more permeable, when exposed to luminal contents leading to mucosal injury (Feldman, 2006).

The effect of deficiency in PGs in NSAIDs-induced injury in small intestine is not well understood. This has created a controversy with respect to PGs deficiency being the main agent of small intestine damage. Many studies have shown the role of PGs in protecting injury on small intestine caused by NSAID's (Laine *et al*., 1996; Kunikata *et al*., 2001; Takeuchi *et al*., 2002; Takeuchi *et al*., 2003). However, other experimental studies have revealed that PGs deficiency is not key to small intestine injuries (Wallace, 1997; Reuter *et al*., 1997). Somasundram *et al*. (2000), demonstrated in rats and concluded that, deficiency in the synthesis of PGs is not adequate to alter permeability in the intestine

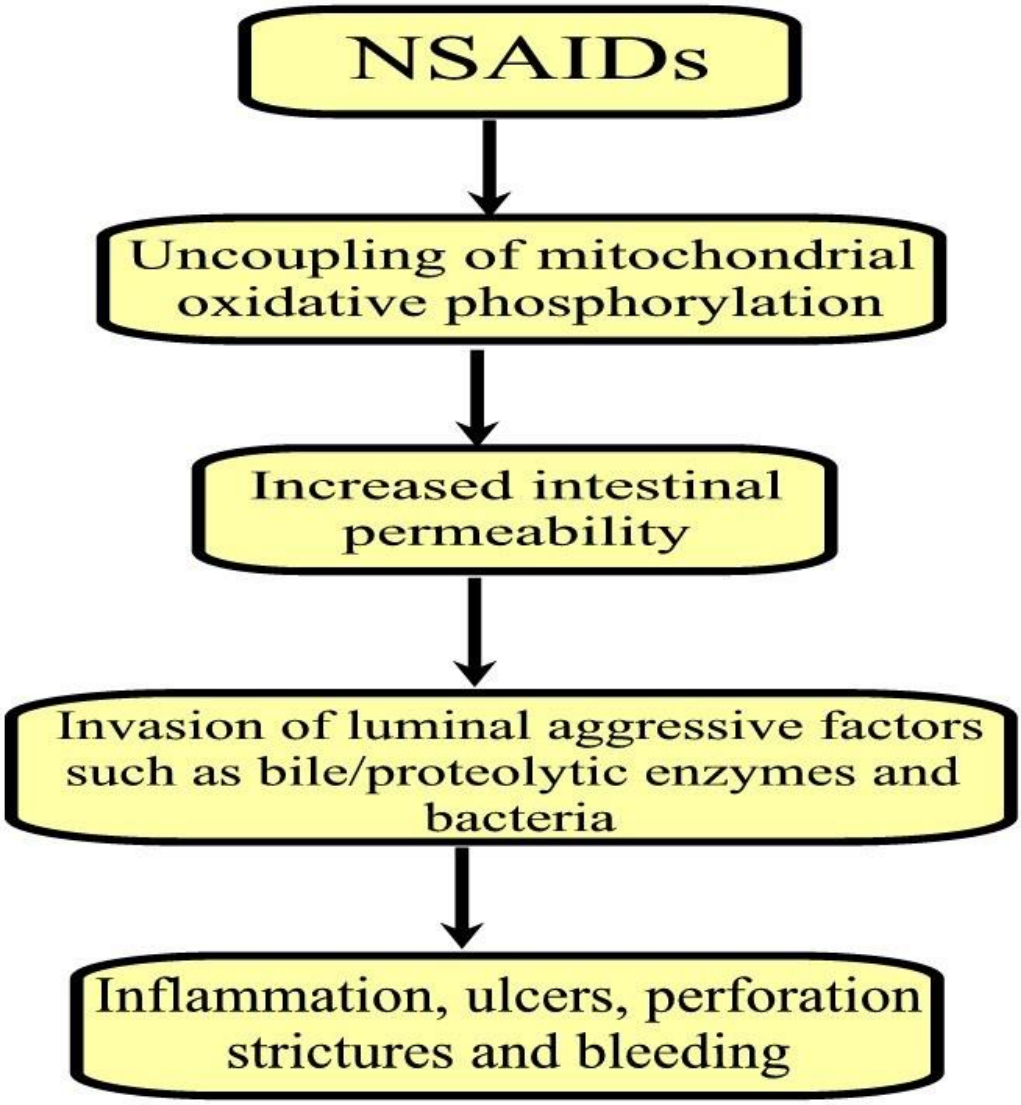


Figure 1.12: The pathophysiology of NSAID-induced small intestinal mucosal injury (Adopted from Wallace, 2008)

1.3.5.3 Acid hypersecretion

Acid hypersecretion is regarded as another causative factor of peptic ulcer (Clark *et al*., 1992). Zollinger-Ellison syndrome, a gastrin-secreting tumour of the nonβ-cells of the pancreas leads to amplified gastric acid secretions.

1.3.5.4 Other aetiological factors

The inactive precursor of pepsin known as pepsinogen, has a role in peptic ulcer development due to its ability to impair gastric blood flow, healing and also inhibit pancreatic bicarbonate production. Cigarette smoking, caffeine ingestion, excessive alcohol intake, and hereditary predisposition are also linked to the disease (Malfertheiner *et al*., 2009).

Cigarette smoking is a known risk factor in peptic ulcer. It increases the risk of ulcer complications such as ulcer bleeding, stomach obstructions and perforations. It also results in ulcer medication treatment failure. Studies have shown that people who smoke have slow rate of peptic ulcer healing and also smoking contributes to peptic ulcer recurrence (Malfertheiner *et al*., 2009).

Other factors such as alcohol, spicy foods and chronic psychological stress are also contributors of peptic ulcer disease development. Spicy foods are known to aggravate peptic ulcer and not necessarily cause ulcer on their own. Stress precipitates increases in gastric motility, vagal acidity, reduced gastric blood flow and reduces synthesis of prostaglandins. Chronic stress which may be hunger, emotional, environmental as in cold conditions also triggers peptic ulcer by the release of histamine that is known to enhance gastric acid secretions and reduce mucus production (Valle, 2005).

Hereditary predisposition is another factor involved in peptic ulcer disease

(Barros *et al*., 2008). Peptic ulcer may be affected by diseases such as malignant tumours, cirrhosis and kidney disease (Valle, 2005). Most of these factors and how they contribute to peptic ulcer disease (PUDs) is illustrated in Figure 1.13

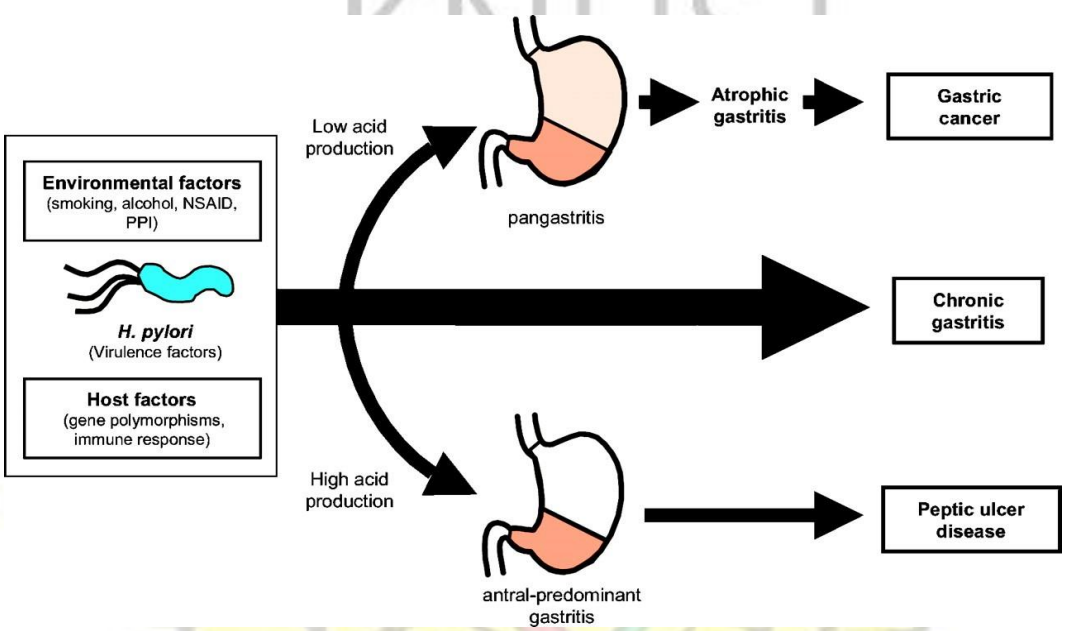


Figure 1.13: Diagramatic representation of the factors that contribute to gastric pathology and disease outcome in PUDs (Adopted from Kusters et al., 2006)

1.3.6 Role of reactive oxygen species in gastric ulceration

Reactive oxygen species (ROS) have been implicated in the manner of development of many diseases including peptic ulcer (Bafna and Balaraman, 2005). Upsurge in ROS and/or a decline in the levels of endogenous antioxidant trigger oxidative stress, which plays an important role in the devlopment of peptic ulcer (Tian *et al*., 2007).

The mechanism of gastric injury by free radicals also encompasses peroxidation of lipid, which damages cell membranes. There is also release of other components such as lysosomal enzymes, which are responsible for further damage of the gastric tissue. The free radicals also facilitate destruction of the epithelial membrane and comprehensive alteration of metabolic processes in the cell leading to mucosal injury (Demir *et al*., 2003; Repetto and Llesuy, 2002; Davies and Rampton *et al*., 1994; Harris, 1992).

Free radicals are responsible for the oxidation of bases in DNA, making them mutagenic, cytotoxic and cross-linking agents, which in turn cause uncontrolled expression of certain genes causing increased cellular proliferation and gastric cancer.

Various endogenous antioxidant enzymes like superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) and reduced glutathione (GSH) control the accumulation of reactive oxygen species. Any imbalance in the activity of these enzymes usually leads to a faulty disposal of these free radicals. Antioxidants have a protective role in gastric ulcers and carcinomas. Drugs with antioxidant properties have been shown to have beneficial effects in alleviating gastric lesions (Demir *et al*., 2003).

Humans and rodents have several antioxidant systems, which scavenge ROS and prevent their destructive action. The major antioxidative enzyme is SOD (Brzozowski *et al*., 2001). Three types of SOD; cytoplasmic, mitochondrial and extracellular. Superoxide dismutase catalyses the dismutation of superoxide radical anion (O_2•-) into less harmful substance hydrogen peroxide, which is further broken down by the aid of the enzymes; catalase and glutathione peroxidase. Catalase catalyses the break down of H_2O_2 into water and oxygen (Halliwell, 1996). The second pathway for metabolism of H_2O_2 depends on glutathione peroxidase (GPx)

and glutathione reductase activities. The reduction of H_2O_2 into water by GPx is accompanied by the conversion of glutathione from the reduced form (GSH) into oxidized form (GSSG) (Togashi *et al*., 1990). This is shown in figure 1.14

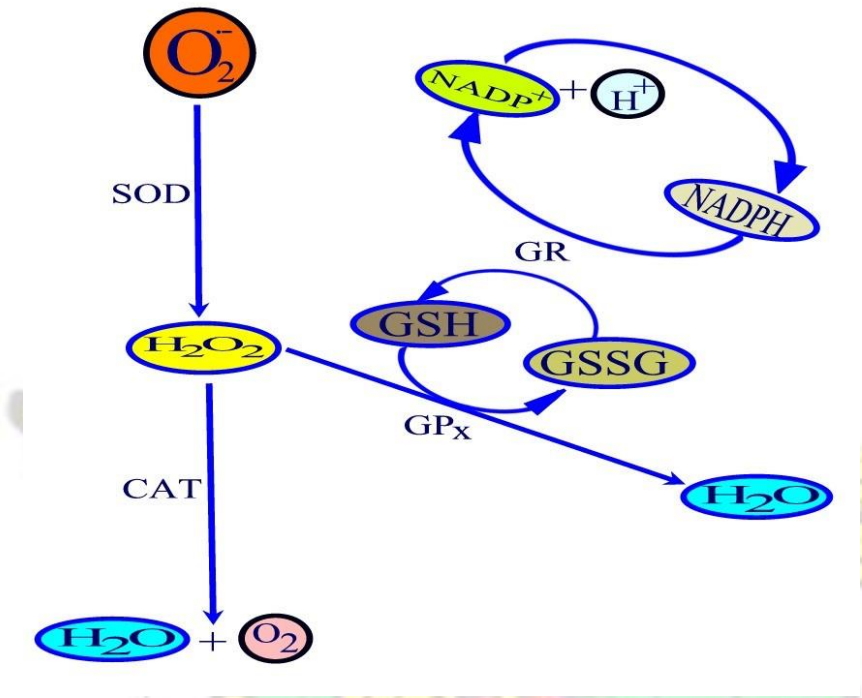


Figure 1.14: Metabolism of reactive oxygen species in an organism (Adopted from Brzozowski et al., 2001)

1.4 SIGNS, SYMPTOMS AND COMPLICATIONS OF PEPTIC ULCER

Peptic ulcer triggers many symptoms, and these vary from person to person. Some persons with the disease have minimal, unusual, or even no symptoms at all. Others may have all the known symptoms. The most common symptoms of peptic ulcer is dyspepsia. However, peptic ulcers can occur without dyspepsia or any other GIT symptom (asymptomatic), especially when they are caused by NSAIDs or at the

initial stages of *Helicobacter pylori* infection. The type of signs and symptoms depends on the type of ulcer (NDDIC, 2004).

The occurrence of gastric ulcer symptoms, are worsened by eating. Pain is experienced soon after meals, and food would not improve symptoms. There is a burning sensational pain, gnawing, boring, and aching, which occur towards the left side of epigastrium. Duration of pain is less marked compared to duodenal ulcers. Moreover the pain in this case never occurs at night. Vomiting is common and it relieves the pain. Patients have good appetite but afraid to eat because food aggravates the pain, this makes patients suffering from this condition usually lean (NDDIC, 2004).

Duodenal ulcers mostly trigger symptoms, when there is no food in the stomach. A person suffering from this can be relieved by eating foods such as alkaline milk, egg and fish. The symptom of pain, which resembles a sensation of hunger, include, burning, gnawing, boring, and aching and occurs at the right side of epigastrium. Pain may also occur during the night and thus may awake the patient. There are also periodic attacks of pain, which is episodic and recurrent and may last for several days, weeks or months with an interval of 2-3 months or more. There is also severe general abdominal pain that indicates perforation in the peritoneal cavity. Appetite is good so individuals suffering from this condition usually do not lose weight which is contrary to gastric ulcer patients.
Vomiting is unusual until pyloric stenosis has developed. Malaena is more common than hematemesis.

It is also noteworthy that the symptoms enumerated may happen as a result of other conditions such as gastro-oesophageal reflux disease (GERD), chronic dyspepsia, gall bladder disease. Thus, it is not advisable to depend solely on these symptoms for medication (Ehrlich, 2009).

The development of peptic ulcer can lead to two main complications namely; acute and chronic. Acute peptic ulcers are those that do not penetrate the muscularis mucosae. The common site is the stomach but may occur in the duodenum too. Acute peptic ulcers are thought to be due to the disruption of the gastric, mucosal barrier and regarded as extension of acute erosive gastritis.

Chronic peptic ulcers are those that penetrate the muscularis mucosa. It is the chronic peptic ulcer for which the term peptic ulcer is usually used. The common sites are the duodenum, stomach, oesophagus, margins of stoma of gastroenterostomy, Meckl's diverticulum and Gastro-jejunum.

1.5 DIAGNOSIS OF PEPTIC ULCER

Diagnosis is based on characteristic signs and symptoms, family history of the disease, biochemical laboratory test results, endoscopy, imaging and microbial laboratory culturing test for *Helicobacter pylori*. It could also be based on the history of risk factors including medications, smoking and drinking habits (Ehrlich, 2009). The use of medical history, signs and symptoms, exposure of risk factors such as smoking, drinking, and abuse of NSAIDs medications assist in diagnosis and prognosis. Physical examination may also be done and this includes check up of the abdomen, chest and rectum to look for signs of bleeding (NDDIC, 2004).

Various methods are available for the detection of *Helicobacter pylori*. These methods are divided into (i) invasive and (ii) non-invasive tests. A combination of two or more methods is often employed. For example, a stool test that detects *Helicobacter pylori* antigens is combined with other test for accurate diagnosis, to avoid false positives since the detection of *Helicobacter pylori*-specific antibodies does not ultimately reflect current infection. In routine clinic, the use of a single

test is generally acceptable. For daily diagnostic purposes, histology is often used, whereas the use of serology is most appropriate for research purposes. In hospitals, most people undergo endoscopy. In children, faecal antigen tests offer the opportunity to assess *Helicobacter pylori* presence without employing endoscopy (Greenberger *et al.*, 2009).

1.5.1 Urea breath test

Urea breath test is also done, where patients are given a capsule, liquid or pudding containing urea labelled with a special carbon atom, and after a few mins patients are allowed to breath into a container. The presence of the carbon atom in the exhaled air indicates that *Helicobacter pylori* are present (NDDIC, 2004).

1.5.2 Endoscopy

Endoscopic test is usually done to expose the ulcer if present. Gastric and duodenal mucosa is directly visualized and positive results are obtained in more than 95% of cases.

1.5.3 Gastroscopy

This is a diagnostic technique whereby a flexible tube shaped device with special light conducting properties is put down the throat of patients for a clear visibility of lesions or ulcerations and also to obtain tissue samples from the stomach for the determination of cancer status (NDDIC, 2004).

1.5.4 Barium meal test

Another diagnostic test is the Imaging test. This is where an X–ray of the stomach, duodenum or oesophagus is performed after the patient has been administered with Barium meal which makes the ulcer visible on the X–ray (NDDIC, 2004).

1.6 MANAGEMENT OF PEPTIC ULCER

1.6.1 Conventional management

Management of PUDs has the objective of obtaining a relief of common symptoms such as pain, reducing gastric acid secretion, promoting healing of ulcer, supressing or killing *Helicobacter pylori* if present, preventing the recurrence of the disease and avoiding complications. These conventional treatment options involve non-pharmacological and pharmacological therapy (NDDIC, 2004).

1.6.1.1 Non-pharmacological treatments

Earlier, surgical procedures such as vagotomy, truncal vagotomy, pyloroplasty and omental implantation were involved in the management of peptic ulcers (Kavanagh, 1988), but have subsequently been replaced by pharmacotherapy, which is currently considered as a better treatment option for PUD management. With the achievement of pharmacotherapy, surgery has a limited role in managing peptic ulcer diseases. Potential indications for surgery includes the following;

I. Refractory, symptomatic peptic ulcers II. Perforation.
III. Obstruction
IV. Bleeding (NDDIC, 2004).

A suitable surgical procedure is dependent on the location and nature of the disease. Some of these procedures include, vagotomy and pyloroplasty, vagotomy and antrectomy with gastroduodenal reconstruction, vagotomy and antrectomy with gastrojejunal reconstruction and lastly selective vagotomy.

Vagotomy involves cutting the vagus nerve that transmits impulses from the brain to the stomach. However this surgery may interfere with the stomach emptying, by interrupting impulses sent through the vagus nerve. A variation to this is the

selective or highly selective vagotomy. This involves cutting only parts of the nerve that control the acid-secreting cells of the stomach, thereby avoiding the parts that influence stomach emptying i.e. reduce acid secretion (NDDIC, 2004).

1.6.1.2 Pharmacological treatments

Formerly, before the advancements in medicine, peptic ulcer was considered a chronic lifetime disease. Today, it can be totally cured through pharmacotherapy. Treatment takes different forms depending on the extent of damage and the classification of the ulcer. The classes of drugs presently used in managing peptic ulcers include antacids, anticholinergics, mucosal protective agents, prostaglandin analogues, proton pump inhibitors (H^+ / K^+ ATPase inhibitors), histamine (H_2)receptor antagonists and antibiotics (NDDIC, 2004).

Most patients at the initial stages of the disease may require antacids, which are given to increase the pH in the stomach (Kavanagh, 1988). Antacids are classified as systemic and non-systemic. Non-systemic antacids that come either in liquid or tablet form are calcium, magnesium or aluminium-containing agents. These antacids are administered orally to neutralize gastric acidity in the stomach thus, increasing the stomach and duodenal pH. Most antacid products contain one or more of four active alkaline ingredients namely (i) Aluminium salts eg. ($AlOH_3$), (ii) Magnesium salts e.g. $MgCO_3$, $Mg(OH)_2$ (milk of magnesia), (iii) Calcium Carbonate e.g. $CaCO_3$ / chalk / limestone (iv) Sodium bicarbonate e.g. Baking soda, $NaHCO_3$. Several salts are formulated with combinations of magnesium and aluminium salts e.g. Magaldrate ($AlMg(OH)_5$ to neutralize stomach acid, to decrease the action of pepsin and to prevent deleterious side effects including constipation and diarrhoea. In addition, many types of bismuth mineral salts e.g. Bismuth subsalicylate which are multipurpose intestinal medicinal agents are used as antacids because they increase alkaline secretions to counteract any acid production in the stomach. As an antiulcer agent, bismuth subsalicylate also coats

and protects irritated and inflamed gastrointestinal lumen tissue. Effervescent antacids are those products that contain sodium bicarbonate and citric acid. These products give effervescent action when dissolved in water because of the evolution of carbon dioxide gas when sodium bicarbonate and citric acid react in a hydrated environment. It has been reported that the release of CO_2 by sodium bicarbonate during this reaction may provide relief from the discomfort of over eating by inducing belching, which aids in the expulsion of swallowed air. Sodium citrate, which is also produced in the reaction of Sodium bicarbonate and citric acid, may also accept Hydrogen ions and revert back to citric acid. Thus the Sodium citrate that is produced when effervescent antacids are dissolved in water also acts as stomach acid-neutralizing antacid. Some antacids contain additional active ingredients, including analgesics to treat headaches and also antigas / antibloating products such as Simethicone, Magnesium trisilicate to treat uncomfortable feelings which are often associated with heartburn as a result of overeating.

Calcium and aluminium antacids often cause constipation as side effects whereas magnesium products often produce diarrhoea. Antacids containing both aluminium and magnesium attempt to balance these constipating and diarrhoeal effects. Calcium antacids are a factor in gastric acid rebound syndrome. These non-systemic antacids also may interfere with the absorption of other drugs from the bowel. The increase in pH may convert some drugs to a predominantly hydrophilic form that is less able to penetrate the intestinal mucosa. Drugs may be chelated with antacids e.g. tetracycline with Calcium antacids (Shayne and Miller, 2010). In contrast to the local neutralizing effects of non-systemic antacids, systemic antacids decrease acid production in the stomach.

Anticholinergic drugs are another class of antiulcer drugs that act as antagonist of acetylcholine secreted at postganglionic fibres. They act by antagonizing muscarinic cholinergic receptors. They depress gastric motility and reduce

secretions such as pepsin in the stomach. By using these drugs, basal acid secretion is decreased by one half and acid secretion subsequent to food ingestion is decreased by 30 %. The main problem with this drug is that the dosages required to achieve therapy is very large and thus, ends up causing numerous adverse effects such as blurred vision and dryness of mouth. Examples of anticholinergic drugs include propantheline, pirenzepine and telenzepine (Hoogerwerf and Pasricha, 2001).

Mucosal protective or defence agents are another class of antiulcer drugs. An example is sucralfate (carafate) (sucrose aluminium sulphate) which is known to boost the healing of peptic ulcer without affecting the secretion of gastric acid. These are drugs that strengthen the gut lining against attacks by acidic digestive juice. They bind to positively charged proteins in exudates and form a viscous adhesive substance that protects the GI lining against peptic acid and bile salts (Shayne and Miller, 2010). Unlike H-2 blockers and proton pump inhibitors, protective agents do not inhibit the release of gastric acid but rather shield the stomach's mucous lining from the damaging effects of gastric acid.

Cytoprotectants that are mainly prostaglandin analogues such as Misoprostol (cytotec), can prevent peptic ulcer in patients taking NSAID. Misoprostol replaces depleted prostaglandin E1 in prostaglandin inhibiting therapies (Shayne and Miller, 2010), thereby protecting the stomach lining from the attack of gastric secretion. These agents have the ability to induce prostaglandin synthesis in GIT.

A class of drugs called Histamine antagonists (H_2 blockers) are designed to block competitively the action of histamine on H_2 receptors of gastric parietal cells, resulting in reduced gastric secretions; reduced gastric acid volume and reduced hydrogen ion concentration. Examples of such drugs include Cimetidine

(Tagamet), Ranitidine (Zantac), Nizatidine (Axid), and Famotidine (Pepcid) (Altman, 2001).

Another class of drugs called Proton-pump inhibitors (PPIs) are used in ulcer therapy. This class of drugs bind to H^+ / K^+ - ATPase pump and inhibit the secretion of hydrogen ions (acid) into gastric lumen. Due to this action, they are also referred to as inhibitors of H^+ / K^+ - ATPase. They relieve pain and promote peptic ulcer healing more rapidly than the H-2 antagonists. It is of a special value in patients who do not respond to H_2 receptor antagonists. A standard dosage will inhibit about 90 % of the 24-hr acid secretion compared to the 50-80 % with H-2 receptor antagonists (Shayne and Miller, 2010). Such drugs include Omeprazole (Prilosec®), Esomeprazole (Nexium®), Lansoprazole (Prexacid®), Rabeprazole (Aciphex®), and Pantoprazole (Protonix®) (Cohen, 2006). These drugs are generally taken 30 mins before meals.

The use of antibiotics since the discovery of the link between peptic ulcers and *Helicobacter pylori* is also another pharmacological treatment option (Walsh and Peterson, 1995). Antibiotics used include amoxicillin, clarithromycin, metronidazole, tetracycline etc. Without antibiotic therapy there is 75 % recrudescence but with antibiotics there is only 1 % chance of ulcer reoccurrence (Waldum, *et al*., 2005). According to the American College of Gastroenterology practice guidelines (1996), the most effective proven treatment comprises of a two-week course called "Triple therapy". This involves a combination of two antibiotics – clarithromycin and amoxicillin, with a proton pump inhibitor (PPI). A bismuth quadruple therapy that involves the use of PPI, bismuth, metronidazole and tetracycline, for treating peptic ulcer caused by *Helicobacter pylori* is also recommended. There are also reports of two weeks of therapy with two drugs; one antibiotic and one acid suppressor. This therapy is known as 'dual therapy'

(Waldum *et al*., 2005). The dual therapy has not been proven to be as effective as the triple therapy.

Meanwhile, two weeks of therapy involving the use of two antibiotics; an acid suppressor and a gastric epithelial lining shield which is called 'bismuth triple therapy' or 'quadruple therapy' looks promising in treatment though there may be many adverse effects (NDDIC, 2004).

Aside all these conventional treatments, peptic ulcer management has continued to be a health challenge to medical practitioners. There are reports of relapse, recrudescence and several adverse effects such as erectile dysfunction (Zathas *et al*., 2008; Kovacs *et al*., 2009). There is an urgent need to consider other options from botanical sources for the management of PUDs.

1.6.2 Conservative management and the use of herbal remedies

Dietary life style and use of herbal medicine are other alternatives for the management of peptic ulcer. Dietary life style involves preventive measures such as avoidance of spicy foods that aggravate the pain, xanthine containing beverages, alcohol, NSAIDs, heavy meals, corticosteroids and smoking. Relief of anxiety and stress are options to consider if anxiety and stress are suspected to contribute to the aetiology of peptic ulcer.

Plant medicines have been used for centuries in the management of peptic ulcer all over the world. Carbenoxolone, the first systemically effective antiulcer agent, was isolated from the plant, *Glycyrrhiza glabra* (Brown *et al*., 1959). It is the first plant drug that proved effective in treatment of gastric ulcers (Mac Donald and Stoller, 1973; Robson and Sullivan, 1968). Gefarnate, another effective compound isolated from the juice of wild cabbage, was found to be effective against gastric ulcer (Adami *et al*., 1964). It was shown to improve the gastric defensive mechanism,

by increasing mucus synthesis in the mucosa through enhanced synthesis of prostaglandins (Barbara *et al*., 1974).

In Ghana, many plants are used for the management of gastrointestinal diseases including peptic ulcer but only a few have been experimentally proven to be effective. Some of these plants that are used to manage peptic ulcer, include *Ocimum gratissimum* leaves, *Psidum guajava* Linn, *Azadirachta indica, Spathodea campanulata, Musa sapientum, Bryophyllum, pinnatum, Aframomum melegueta, Piper guineense, Paullinia pinnata, Newbouldia laevis, Tetrapleura tetrapleura, Dissotis rotundifolia and Launaea taraxacifolia* (Noumi, 2010; Mann *et al*., 2009; Mshana *et al*., 2000; Noamesi *et al*., 1994).

1.7 EXPERIMENTAL MODELS FOR EVALUATION OF ANTI PEPTIC ULCER DRUGS

Peptic ulcers can be induced by physiological, pharmacological or surgical manipulations in different animals. Nevertheless, many of the experiments are done in rodents. Numerous models are used for evaluating antipeptic ulcer effects of agents, and these include the following:

i. Cold-restraint stress
ii. NSAIDs
iii. Ethanol
iv. Acetic acid
v. Histamine

Though there are several models that are used to evaluate potential antiulcer agents, the choice of an appropriate model has shown to be difficult since each has important advantages and disadvantages. The selection of a model is determined by availability of local resources, the objectives of the present study and the

question being answered (Adinortey *et al*., 2013). The choice of model may also depend on the applicability to the type of peptic ulcer disease under study. These models might also serve as tools that could help to better understand the antisecretory, gastrocytoprotective and healing mechanisms of agents that possess antiulcer activities (Adinortey *et al*., 2013).

1.8 EVALUATION OF DRUG TOXICITY

Establishing drug safety requires information about the toxic effects of such a drug. Toxicity studies are employed in predicting the safety associated with the use of drugs for short and long-term duration. The results of acute and subacute toxicity studies are used for the planning of chronic toxicity studies. Chronic toxicity testing becomes important in situations where the dose level at which the animal can live free of side effects over a period of time is to be determined. The results of chronic toxicity experimentation collected with other results from acute and subacute toxicity tests and other pharmacological experiments help to evaluate the possible hazardous properties of a new drug.

The duration of studies on a drug under investigation is a subject of controversy. The World Health Organization (WHO) defines a short-term study of less than three months as sub-acute and from three to six months as long-term i.e subchronic or chronic studies (WHO, 1992).

The human population is exposed to an ever-increasing number of chemical substances used to improve the quality of life. With the introduction of new drug agents, an equally great probability exists for the incidence of adverse effects upon the populace. Subsequently, various legislative bodies are becoming increasingly aware of the need for establishing not only the effectiveness of such drugs but their safety as well. The use of animal experimentation for providing prospective and

retrospective information profiling for chemical safety is of great importance. It should be noted that animal investigations cannot conclusively establish human safety but are conducted to detect the deleterious effects of compounds to establish whether they should be tested further in humans.

1.9 JUSTIFICATION FOR THE STUDY

Peptic ulcer is a global health problem. Several conventional drugs presently used in peptic ulcer treatment include antacids, anticholinergics, proton pump inhibitors and H_2-receptor blockers (Lakshimi *et al*., 2009). Although these drugs have brought about notable improvement in peptic ulcer management, the efficacy of these drugs is still debatable.

There are problems of poor patient compliance, high cost of combination therapy, occurrence of resistance in *Helicobacter pylori* strains and non availability of drugs in some rural settings. Moreover reports on clinical evaluation of these drugs show that there are incidences of relapses, notwithstanding a healing rate of 80 - 100 % after 4 - 8 weeks of treatment (Miller and Faragher, 1986). The rate of PUDs recrudescence within 1 year after stopping the treatment is between 40 and 80 % (Hoogerwerf and Pasricha, 2001). There are also reported cases of undesirable side effects such as gynecomastia and erectile dysfunction during ulcer therapy. Hence, the search for other anti-peptic ulcer drugs continues. The search is now extended to plants for new and novel molecules, which may afford better protection and decrease the incidence of relapse in peptic ulcer patients. Research effort in this direction, is necessary. Many plants have been used as traditional remedies for prevention and treatment of gastrointestinal disorders for years and their anti-gastro-duodenal and anti-*Helicobacter pylori* activity have been widely demonstrated *in vivo* and *in vitro* (Ndip *et al*., 2007; Twardowschy *et al*., 2008; Lemos *et al*., 2011).

Reports based on community knowledge of existence and application indicate that the plant *Dissotis rotundifolia* is used as anti-peptic ulcer, analgesic, antipyretic and anti-diarrhoea in different parts of Africa including Ghana (Mann *et al*., 2000; Mshana *et al*., 2000; Noumi and Yomi, 2001; Addo-Fordjour *et al* ., 2008). This plant preparation is peddled and patronized by some Ghanaians who have little access to orthodox medicine. Though this plant is in use traditionally, it has not been investigated scientifically for its medicinal values. Based on the above, the purpose of this research was to explore the potential of *Dissotis rotundifolia* plant extract as a dual-target phytotherapy to prevent and treat peptic ulcer diseases.

1.10 HYPOTHESIS

Based on the ethnomedicinal use of *Dissotis rotundifolia* in the treatment of peptic ulcers, it is hypothesized that *Dissotis rotundifolia* has anti-peptic ulcer activity.

1.11 AIM OF THE STUDY

The aim of this research work was to evaluate the traditionally acclaimed antiulcer activity and potential toxicological effects of whole plant extract of *Dissotis rotundifolia* in rats

1.12 SPECIFIC OBJECTIVES

The specific objectives of the present study were to:

1. Determine the phytochemicals present in the whole plant extract of *Dissotis rotundifolia*.

2. Investigate the cytoprotective effects and gastric ulcer healing activity of *Dissotis rotundifolia* whole plant extract using different *in vivo* peptic ulcer models in rats.

3. Examine the possible mechanisms of antiulcer effects of *Dissotis rotundifolia* whole plant extract *in vitro* and *in vivo* by:
    - Assessing the anticholinergic effects
    - Estimating H^+ / K^+-ATPase enzyme activity *in vivo*
    - Determining mucus content in gastric wall
    - Measuring the index of mucin activity
    - Determining the antimicrobial activity of *Dissotis rotundifolia* whole plant extract against *Helicobacter pylori in vitro*
    - Determining the free radical scavenging activity *in vitro* using hydroxyl ion, superoxide anion, nitric oxide ion and 2, 2-diphenyl-1-picrylhydrazyl radical scavenging models
    - Evaluating the scavenging ability of the extract on reactive oxygen species *in vivo* by measuring GSH, SOD, CAT and MDA

4. Assessing *Dissotis rotundifolia* whole plant extract for safety in rats using acute and subacute toxicity models.

Chapter 2

COLLECTION, EXTRACTION AND PHYTOCHEMICAL ANALYSIS OF *DISSOTIS ROTUNDIFOLIA* WHOLE PLANT

2.1 INTRODUCTION

Dissotis rotundifolia is used in traditional herbal medicine practice in managing wounds, dysentery, rheumatoid arthritis and peptic ulcer in countries such as; Nigeria (Odugbemi, 2008), Tanzania (Hamisy *et. al.*, 2000), Cameroun (Jiofack *et al*., 2009; Noumi, 2010), Liberia (Watt and Breyer-Brandwijk, 1962) and in Ghana (Mshana *et al*., 2000; Darko, 2009). It is evident from ethnomedicinal reports that, the plant has potential in treating a number of disorders including gastric hyperacidity and gastroduodenal ulcers. Even though the whole plant is used as medicinal herb, there is paucity of information about it's phytoconstituents that contribute to the medicinal properties. The objective of this chapter was to screen

for the major phytochemical constituents of whole plant extract of *Dissotis rotundifolia*.

2.2 CHEMICALS, DRUGS AND REAGENTS

The following chemicals, drugs and reagents were used for the experiments:, Potassium ferricyanide, ascorbic acid and tannic acid were purchased from BDH, Poole, England. Methanol (98%), vanillin, iron chloride ($FeCl_3$), sulphuric acid, sodium hydroxide, Fehling's solution (A and B), hydrochloric acid (HCl), Mayer's reagent, Dragendorff's reagent, ferric chloride, ammonia solution, chloroform ethanol (99.8%), sodium carbonate and dichloromethane were purchased from Merck Chemical Supplies (Darmstadt, Germany). All other chemicals used were of analytical grade.

2.3 COLLECTION AND EXTRACTION OF PLANT MATERIAL

The whole plant was collected from around the Kakum National Park, Cape Coast, Ghana in January 2012 and authenticated by the Curator at the Herbarium of the Department of Environmental Science, School of Biological Sciences, University of Cape Coast. A voucher specimen # (107346) was prepared and deposited at the herbarium. The whole plant was washed thoroughly with clean tap water, shade-dried for three weeks, oven-dried at 40 ^{O}C for 3 hrs and then pulverized into powder.

A two-step sequential method of extraction involving the use of dichloromethane and 70 % methanol was employed in the preparation of the crude whole plant extract, according to the method described by Rath *et al*. (1995). One hundred and fifty (150 g) of *Dissotis rotundifolia* powder was transferred into a 2 L flask and 1.5 L of dichloromethane was added to the powdered sample in a fume chamber. The flask was tightly corked with a cotton wool plug which was covered with an

aluminium foil and placed on an IKA® KS260 basic orbital shaker at a speed of 200 rpm for 48 hrs.

The resulting mixture was then filtered into a 2 L flat bottom flask using a Whatman No.1 filter paper. The residue was dried at 40 ^{0}C on a water bath and the filtrate was discarded appropriately. Methanol (70 %, 1.5 L) was added to the dried residue corked with a cotton wool plug and placed on an orbital shaker at 200 rpm for 48 hrs. The resulting mixture was later filtered using a Whatman No.1 paper. The filtrate was subsequently concentrated using a rotary evaporator and later dried at 40 ^{0}C in an oven to obtain a brown coffee-coloured extract of *Dissotis rotundifolia* and this was labelled as *Dissotis rotundifolia* extract (DRE). The crude DRE of 8.3 % yield was kept in a crucible and covered with aluminium foil and stored in a freezer at -20 ^{0}C until ready for use.

2.4 PHYTOCHEMICAL ANALYSIS

Phytoconstituents are chemical compounds that occur naturally in plants, affect health, but are not established as essential nutrients. Phytochemical screening of DRE for tannins, saponins, alkaloids, flavonoids, glycosides, anthraquinone glycosides, cardiac glycosides, cyanogenic glycosides, terpenoids, carbohydrates, sugars, steroids, resins and coumarins was carried out using standard procedures as described by Harbourne (1998) and Trease and Evans (2002).

2.4.1 Methodology for qualitative analysis of phytoconstituents

2.4.1.1 Test for tannins (Iron complex test)

The extract (0.05 g) was dissolved in 5 mL distlled water followed by addition of 5 drops of 10 % ferric chloride ($FeCl_3$) solution (Trease and Evans, 2002;

Harborne, 1998).

Condensed tannins (Lead acetate test)

The extract (0.05 g) was dissolved in 5 mL distlled water followed by addition of few drops of 1% lead acetate (Trease and Evans, 2002).

Hydrolysable tannins

The extract (0.05 g) was dissolved in 5 mL distilled water followed by addition of 5ml of 10 % ammonia solution and shaken for some 10 sec (Trease and Evans, 2002).

2.4.1.2 Test for saponins

The extract (0.05 g) was dissolved in an aliquot of 5 mL of distilled water in a test tube, which was shaken vigorously for 30 sec and was subsequently allowed to stand for 45 min (Harborne, 1998; Sofowora, 1993).

Confirmatory test for saponins

The foamy suspension was mixed with 3 drops of olive oil and shaken vigorously for about 30 secs. The mixture was observed for the formation of emulsion (Trease and Evans, 2002).

2.4.1.3 Test for alkaloids

The extract (0.02 g) was dissolved in 5 mL of methanol and 3 drops of 1 % hydrochloric acid was added to it. Then the mixture was heated in a water bath for about 2 mins and allowed to cool. The extract was tested carefully with alkaloidal reagents as follows:

Mayer's test

Mayer's reagent [Mercuric chloride (1.4 g) dissolved in 60 mL of distilled water and potassium iodide (5.0 g) was dissolved in 10 mL of distilled water, the two solutions were mixed and volume topped up to 100 mL with distilled water]. To about 1 mL of extract, 2-3 drops of Mayer's reagent was added by the side of the tube (Harborne, 1998).

Dragendorff's test

Few drops of Dragendorff's reagent (two solutions in 1:1 ratio: 'solution A' with 0.85 g of basic bismuth nitrate, 10 mL of glacial acetic acid and 40 mL of distilled water and 'solution B' with 8 g of KI in 30 mL of distilled water) were added to aliquot of 1 mL of extract (Harborne, 1998).

Wagner's test

Wagner's reagent (2 g of Iodine crystals and 6 g of KI in 100 mL of distilled water). Few drops of Wagner's reagent was added to 1 mL of extract and observed for turbidity or reddish brown precipitate (Trease and Evans, 2002).

2.4.1.4 Test for flavonoids

About 1 mL of distilled water was added to 20 mg of the semi-solid extract and shaken in a test tube. Then few drops of concentrated H_2SO_4 were added. Yellow colouration in the sample which disappeared on standing after adding 0.5 mL of dilute ammonia solution indicates the presence of flavonoids (Harborne, 1998).

2.4.1.5 Test for glycosides

Fifty milligrams (50 mg) of the extract was hydrolysed in 10 mL concentrated hydrochloric acid for 2 hrs on a water bath and filtered. The filtrate was subjected to following tests:

Bornträgor's test for general glycosides

To 2 mL of filtrate, 3 mL of chloroform was added and shaken, chloroform layer was separated and 10 % of ammonia solution was added to it and observed for colour change.

Test for cyanogenic glycoside

About 2 mL of filtrate was poured into a conical flask. A piece of picric acid paper was moistened with 5 % Na_2CO_3 solution. The piece of sodium picric paper was then suspended in the flask containing the filtrate with the aid of a cork placed in the neck of the flask. It was warmed gently for about 2 mins and allowed to stand for observation (Trease and Evans, 2002).

2.4.1.6 Test for terpenoids

Five mililitres (5 mL) of extract was mixed in 2 mL of chloroform, and 3 mL concentrated H_2SO_4 was carefully added to the mixture by the side of the test tube and observed (Sofowora, 1993).

2.4.1.7 Test for reducing sugars

Fehling's solution prepared by mixing equal volumes of Fehling's solution A (7 % $CuSO_4 . 5H_2O$) and B (12 g of NaOH and 35 g of Sodium Potassium tartrate in 100 mL of distilled water). About 2 mL of 5 mg/ml of water extract was added to 2 mL of Fehling's solution in a test tube and boiled for 5 mins and colour observed (Harborne, 1998).

2.4.1.8 Test for steroids

About 500 mg of extract was dissolved in an aliquot of 2 mL of acetic anhydride and the mixture was cooled to 0 - 4 ^0C, when a few drops of 12 N sulphuric acid were carefully added and observed for colour change (Sofowora, 1993).

2.4.1.9 Test for resins

An aliquot of 2 mL of 1 % copper acetate solution was added to 2 mL of diluted extract (0.05 mg/mL) and the mixture was shaken vigorously. A green colour indicates the presence of resin (Sofowora, 1993).

2.4.1.10 Test for Coumarins

About 2 mL of extract was added to 0.5 mL of 10 % ammonia solution in tubes and viewed under UV light. The appearance of a blue-green fluorescence indicates the presence of coumarin (Harborne, 1998).

2.5 RESULTS

The phytochemical screening of the *Dissotis rotundifolia* whole plant extract revealed the presence of flavonoids, alkaloids, terpenoids, condensed tannins and reducing sugars but not hydrolysable tannins, cyanogenic glycosides, steroids, coumarins and resins (Table 2.1).

Table 2.1: Phytochemical screening test results of *Dissotis rotundifolia* extract

Test	Observation	Inference
Saponins	appearance of froth	Present
Tannins		
Condensed	bluish black precipitate	Present
Hydrolysable	no emulsion formed	Absent
Flavonoids	yellow colouration	Present
Alkaloids		
Mayer's	appearance of creamy precipate	Present
Wagner's	turbid solution formed	Present
Dragendoff's	development of an orange red precipitate	Present
Reducing sugars	brick red precipitate	Present
Glycosides		
Bornträgor's test	formation of pink colour	Present
Cyanogenic glycosides	no colour change in filter paper	Absent
Terpenoids		
Salkowski test	appearance of a reddish brown colour at the interface	Present
Steroids	no change in colur	Absent
Resins	no colour change	Absent
Coumarins	no blue green fluorescence seen	Absent

Tests were carried out in triplicate

2.6 DISCUSSION

Phytochemical screening of plant extracts is necessary in identifying the types of compounds present. In this study the presence of some phytochemicals were screened using standard methods.

The presence of saponins, tannins, flavonoids, alkaloids, glycosides, reducing sugars and terpenoids in the whole plant extract of *Dissotis rotundifolia* observed in this study, is consistent with previous reports by Abere *et al*., (2010) on the leaves of the same plant. Gills (1992) also reported the presence of saponins and tannins in the leaves of *Dissotis rotundifolia* plant.

Flavonoids occur as aglycones, glycosides and methylated derivatives. In plants, flavonoid aglycones (i.e., flavonoids without attached sugar) occur in a variety of structural forms (Middleton, 1984). Flavonoids comprise a vast array of biologically active compounds that are ubiquitous in plants, many of which have been used in phytomedicine for thousands of years. It is well known that many flavonoids display several pharmacological activities including antisecretory and cytoprotective properties (Zayachkivska *et al*., 2005).

Alkaloids are naturally occurring chemical compounds containing basic nitrogen atoms. The name derives from the word alkaline and is used to describe any nitrogen-containing based compound and organic compound. Alkaloidal compounds isolated from different parts of many plants have been reported to posses several pharmacological activities (Falcão *et al*., 2008; Patel *et al*., 2012). Atropine, the optically inactive form of hyoscyamine, is used widely as an antidote to cholinesterase inhibitors such as physostigmine and organophosphate insecticides. Morphine and codeine are narcotic analgesics. Codeine is an antitussive agent, which is less toxic and has less tendency to cause dependency than morphine. Caffeine, which occurs in coffee, tea and cola, is a central nervous

system stimulant; it is used as a cardiac and respiratory stimulant and as an antidote to barbiturate and morphine poisoning. Emetine, the key alkaloid of ipecac root (*Cephaelis ipecacuanha),* is used in the treatment of amoebic dysentery and other protozoal infections (Patel *et al*., 2012).

Saponins occur widely in plant species and exhibit a range of biological properties, both beneficial and deleterious (Price *et al*., 1987). Saponins are a class of chemical compounds found in abundance in various plant species. Saponins are a group of naturally occurring plant glycosides (amphipathic glycosides), characterized by their strong foam-forming properties when shaken in aqueous solution. Structurally, they have one or more hydrophilic glycoside moieties combined with a lipophilic triterpene derivative. The presence of saponins have been reported in more than 100 families of plants out of which at least 150 kinds of naturally occuring saponins have been found to possess significant pharmacological activities. There are several distinct classes of saponins including dammaranes, tirucallanes, lupanes, hopanes, oleananes, taraxasteranes, ursanes, cycloartanes, lanostanes, cucurbitanes and steroids. Due to the great variability in their structures, most saponins always display biological effects (Man *et al*., 2010).

Terpenoids, also referred to as terpenes, are the largest class of natural products, with more than 23,000 known compounds. Among this group, many interesting compounds are extensively used in the food, pharmaceutical and cosmetic industries as flavours, fragrances, spices, perfume and cosmetics products. Many terpenes also have pharmacological activities and are used for medicinal purposes. For example, the antimalarial drug artemisinin and the anticancer drug paclitaxel (Taxol®) are two of a few terpenes with established medicinal use. Natural products from plants continue to be one of the most important sources of lead compounds for the pharmaceutical industry. Terpenoids indisputably continue to be important compounds for drug discovery.

Several plants that produce tannins are used in plant medicine for treating different diseases. The usefulness of tannins as drugs is mainly related to their astringent properties. These properties are due to the fact that tannins react with tissue proteins with which they come into contact. In gastric ulcers, this tanninprotein complex layer protects the stomach by promoting greater resistance to physiological, chemical and mechanical injury. Some tannins have been shown to possess antioxidant activity, promote tissue repair, exhibit anti-*Helicobacter pylori* effects in many gastric ulcer models (Neyres *et al*., 2012). The presence of tannins in most plants may contribute to their anti-ulcer effects.

The presence of these phytoconstituents (flavonoids, tannins, saponins, alkaloids, glycosides, terpenoids and reducing sugars) in many plant extracts have been reported to be responsible for the medicinal properties of most plants (Narendhirakannan *et al*., 2007; Nayak *et al*., 2011). It is plausible that these constituents identified in DRE may contribute to its pharmacological effects.

2.7 CONCLUSION

Phytochemical screening of crude *Dissotis rotundifolia* whole plant extract indicate the presence of flavonoids, condensed tannins, saponins, alkaloids, glycosides, reducing sugars and terpenoids.

Chapter 3

ANTIULCER ACTIVITY OF METHANOLIC EXTRACT OF *DISSOTIS ROTUNDIFOLIA* WHOLE PLANT IN RAT MODELS

3.1 INTRODUCTION

Peptic ulcer is one of the major gastrointestinal disorders, which occur due to an imbalance between gastric acid secretion and gastric mucosal integrity factors in the stomach (Hoogerwerf and Pasricha, 2001). Implicitly, reduction of gastric acid production as well as re-inforcement of gastric mucosal protection can be a major approach to the management of peptic ulcer diseases.

More and more orthodox drugs, with gastroprotective effects, antisecretory activities and healing potential are being offered as newer and better options for treatment of peptic ulcer. These drugs include proton-pump inhibitors, H_2 receptor blockers, cytoprotective agents, anticholinergics and antacids. Most of these drugs exhibit adverse effects like arrhythmias, impotence, gynaecomastia, hyperplasia and haemopoeitic changes in humans. Also, there is 40-80 % recurrence of the diseases in future after treatment (Waldum *et al*., 2005). Pharmacological studies to assess possible antiulcer effects of some plant extracts used in peptic ulcer diseases (PUDs) in Ghana is scanty. Scientific information on antiulcer properties of *Dissotis rotundifolia* is limited. It is therefore worthwhile to undertake a study to assess its efficacy. This chapter reports on the evaluation of the gastroprotective and gastrohealing effects of *Dissotis rotundifolia* extract (DRE) against physiological and chemical induced gastric ulcers in rats.

3.2 MATERIALS AND METHODS

3.2.1. Drugs, reagents and solvents

The following chemicals and drugs were used for the experiments: water soluble aspirin, omeprazole, and ranitidine were purchased from Biomed Pharmacy, Cape Coast. Ethanol (99 %) and sodium carbonate were obtained from Merck Chemical Supplies (Darmstadt, Germany).

3.2.2 Plant collection and preparation of plant extract

The plant was collected and prepared as described in chapter 2.

3.2.3 Animals

Sprague Dawley (SD) rats of either sex (180-200g) obtained from the Centre for Plant Research, Akuapem-Mampong, Ghana were used for the study. The study was approved by the institutional Review Board of the University of Cape Coast

and experiments were conducted according to the guidelines on the use and care of experimental animals. The experimental protocol followed the Principles of Laboratory Animal Care (US NH publication no. 83-85, revised 1985).

Animals were fed with commercial rat food supplied by AGRIFEEDS (AGRICARE Ltd Kumasi, Ghana) and water *ad libitum*. They were maintained in a 12 hr light/dark cycle at 25 ± 2 ^{0}C. All animals were acclimatized for at least two weeks, before the start of the experiment. The present study was performed in compliance with the Good Laboratory Practice (GLP) of the Organization for Economic Cooperation and Development guidelines (OECD, 1997).

3.2.4 Determination of median lethal dose (LD50)

The LD_{50} values of the extract in rats was determined according to the method of Finney (1964). *Dissotis rotundifolia* extract at a maximum dose level 5000 mg/kg *p.o.* was used for acute oral toxicity study. A total of 60 rats; 10 rats (5 males and 5 females) per group was used. Group 1 served as control (received distilled water) and the other five groups were treated with extracts at different doses (10, 100, 500, 2500 and 5000 mg/kg body weight). A single dose of the extract was administrated orally to each animal. Animals were observed for changes in skin, fur, eyes, mucous membrane, salivation, defecation, drowsiness, tremors etc.

Mortality, if any was determined over a period of 14 days.

3.2.5 Antiulcer activity

The antiulcer experiments were done using the following animal models: cold restraint-stress (CRS) - induced ulcer, acetylsalicylic acid (ASA) - induced ulcer and ethanol (EtOH) - induced ulcer. Since traditional uses of this plant is based on oral administration of a decoction prepared from 40 g of dry powdered herb in 1 L of water, taken at (5 ml) four times daily (GHP, 2007), for a person of body weight 60-80 kg, this administration represents approximately 300 mg/kg/day.

The dosages of the extract were chosen from results obtained from the acute toxicity study as well as translation of traditional dosage using the equation:

$$\text{Dosage (mg/kg)} = \frac{\text{volume of extract (mL)} \times \text{Conc of extract (mg/mL)}}{\text{Weight of animal (kg)}}$$

The extract was administered orally at 100, 300 and 500 mg/kg. The effect of DRE was studied in gastric induced ulcers in Sprague-Dawley rats using gastroprotective and gastrohealing models.

3.2.5.1 Gastroprotective studies

In this study, three animal models were employed namely; cold restraint-stress (CRS) - induced ulcer, acetylsalicylic acid (ASA) - induced ulcer and ethanol (EtOH) - induced ulcer. In all models, animals were kept each separately in wire mesh cage for at least 2 weeks to allow for acclimatization followed by random assignment into groups.

Acetylsalicylic acid (ASA) - induced gastric ulcer

The experiment was carried out according to the method described by Williamson *et al*., 1986. The Sprague-Dawley rats were divided into seven treatment groups with five animals in each group. All groups were pretreated with extract or standard drugs for 14 days as follows:

Group I, rats were pretreated with 100 mg/kg of DRE

Group II received pretreatment with 300 mg/kg of DRE

Group III were dosed with 500 mg/kg of DRE as pretreatment

Group IV were given 30 mg/kg of omeprazole as pretreatment

Group V rats received pretreatment with 50 mg/kg of ranitidine as pretreatment
Groups VI and VII served as negative and normal controls respectivley

On the 13th day after treatment with DRE and standard drugs, animals in groups IVI were fasted for 24 hrs prior to receiving the last oral dose of the extract. On the 14 day, after 60 mins of extract / drug administration, animals in groups I-VI were orally treated with 150 mg/kg of acetylsalicylic acid (ASA) for gastric ulcer induction. Four hours after ASA administration, all animals were sacrificed by cervical dislocation. Their stomachs were excised and analyzed as described under the section of stomach analysis (section 3.2.5.3).

Ethanol (EtOH) - induced acute gastric ulcer

The experiment study was carried out according to the method described by Hollander *et al*., 1985. The Sprague-Dawley rats were divided into seven treatment groups with five animals in each group. Groups were pretreated for 14 days.

Group I rats were pretreated with 100 mg/kg of DRE
Group II received pretreatment with 300 mg/kg of DRE
Group III were dosed with 500 mg/kg of DRE as pretreatment
Group IV were given 30 mg/kg of omeprazole as pretreatment
Group V rats received pretreatment with 50 mg/kg of ranitidine
Groups VI and VII served as negative and normal controls respectively

On the 13th day after treatment with DRE and standard drugs, animals in groups IVII were fasted for 24 hrs prior to receiving the last oral dose of the extract and standard drugs. On the 14th day, after 60 mins of extract / drug treatment, all animals in groups I-VI were orally treated with 1 mL of absolute ethanol / 200 mg/kg for the gastric ulcer induction. An hr later after ulcerogen administration, animals were sacrificed by cervical dislocation, and their stomachs excised and analyzed as described under section of stomach analysis (section 3.2.5.3)

Cold restraint stress (CRS) - induced gastric ulcer

The experiment was done using the method described by Takagi and Okabe (1968). The Spague-Dawley rats were put into seven treatment groups (n=5) in each group. Groups I-V were pretreated with extract or standard drugs for 14 days as follows:

Group I, rats were pretreated with 100 mg/kg of DRE
Group II received pretreatment with 300mg/kg of DRE
Group III were dosed with 500 mg/kg of DRE as pretreatment
Group IV were given 30 mg/kg of omeprazole as pretreatment
Group V rats received pretreatment with 50 mg/kg of ranitidine
Groups VI and VII served as negative and normal controls respectively

On the 13th day after treatment with DRE and standard drugs, animals in groups IVI were fasted for 24 hrs prior to receiving the last extract oral dose of the extract, standard drug and vehicle (water 2 ml). On the 14th day, after 60 mins of extract / drug administration, animals in groups I-VI were exposed to cold stress for gastric ulcer induction. After 17 hrs of exposure to cold between 15-17 ^{0}C in a restraint position, all animals in the various groups were sacrificed by cervical dislocation and their stomach excised and analyzed as described under the section of stomach analysis (section 3.2.5.3).

3.2.5.2 Gastrohealing studies

Acetylsalicylic acid (ASA) - induced acute gastric ulcer

The testing was done out using the method described by Williamson *et al*., 1986. The Sprague-Dawley rats were put into seven treatment groups. Animals in groups I-VI were fasted for 24 hrs prior to ulcer induction and were orally administered with 150 mg/kg bwt of acetylsalicylic acid for gastric ulcer induction. After 4 hrs of ASA administration, animals in groups I-VI were orally treated with extract / drugs.

Group I rats were exposed to 100 mg/kg of DRE
Group II received treatment with 300 mg/kg of DRE
Group III were dosed with 500 mg/kg of DRE
Group IV were given 30 mg/kg of omeprazole
Group V rats received treatment with 50 mg/kg of ranitidine
Groups VI and VII which served as negative and normal controls respectively received 2 mL distilled water each.

The extract / drug post-treatment continued for 14 days. On the 15th day, all animals in the study were sacrificed by cervical dislocation and their stomachs excised and analyzed as described under the section of stomach analysis (section 3.2.5.3).

Ethanol - induced acute gastric ulcer

The experiment study was carried out according to the method described by Hollander *et al*., 1985. The Sprague-Dawley rats were divided into seven treatment groups of five animals each. Animals in groups I-VI were fasted for 24 hrs prior to ulcer induction and were orally treated with 1 mL of absolute ethanol / 200g/kgbwt for gastric ulcer induction. After 1 hr of ethanol administration to induce ulcer, animals in groups I-VII were orally treated with extract / drugs.

Group I, rats were given 100 mg/kg of DRE
Group II received treatment with 300 mg/kg of DRE
Group III were dosed with 500 mg/kg of DRE
Group IV were given 30 mg/kg of omeprazole
Group V rats received treatment with 50 mg/kg of ranitidine
Groups VI and VII which served as negative and normal controls were administered 2 mL distilled water each.

This post-treatment continued for 14 days. On the 15th day, all animals were sacrificed by cervical dislocation, and their stomachs excised and analyzed as described under the section of stomach analysis (section 3.2.5.3).

Cold restraint stress (CRS) - induced acute gastric ulcer

The experiment was carried out according to the method described by Takagi and Okabe (1968). The Sprague-Dawley rats were put into seven treatment groups. Rats in groups I-VI were fasted for 24 hrs prior to ulcer induction and were exposed to cold for gastric ulcer induction. After 17 hrs of exposure to cold between 15-17 ^{0}C under restraint condition for ulcer induction, animals in group I-V were orally treated with extract / drugs as follows.

Group I, rats were exposed to 100 mg/kg of DRE
Group II received treatment with 300 mg/kg of DRE
Group III were dosed with 500 mg/kg of DRE
Group IV were given 30 mg/kg of omeprazole
Group V rats received treatment with 50 mg/kg of ranitidine
Groups VI and VII which served respectively as negative and normal controls were administered 2 ml distilled water each.

This post-treatment continued for additional 13 more days. On the 15th day, all animals in the study were sacrificed by cervical dislocation and their stomachs excised and analyzed as described under the section of stomach analysis (section 3.2.5.3)

3.2.5.3 Stomach analysis

Macroscopic examination

The excised stomachs were washed thoroughly with normal saline to remove traces of gastric content and blood clots. They were spread on a card board with the mucus

surface upwards, avoiding corrugation and observed for ulcerations and photographs were taken.

The total area of stomach and ulceration were traced by placing a transparency on it. Stomach area and lesions / ulcers in the glandular part of the stomach were measured under an illuminated magnifying microscope (x10). The traced total area and the area of ulceration were each superimposed on a graph paper having a mm^2 scale and measured. The ulcer index was then calculated according to the method described by Ganguly, 1969. The total ulcerative area in relation to the total area of each stomach was employed in the calculation of the relative area.

The ulcer index of the relative area was determined from Table 3.1 as reported by Ganguly, 1969.

$$\text{Relative area} = \frac{\text{Total mucosal area}}{\text{Total ulcerated area}}$$

$$\text{Percentage Protective ratio} = \frac{([\text{UI untreated}] - [\text{UI pretreated}])}{\text{UI untreated}} \times 100$$

$$\text{Percentage Curative ratio} = \frac{([\text{UI untreated}] - [\text{UI posttreated}])}{[\text{UI untreated}]} \times 100$$

where UI = Ulcer index

3.2.5.4 Histopathological evaluation

Stomach of all rats in the experiment design were excised, rinsed with ice cold saline solution to remove debris and blood clots. Sections of washed stomachs were cut and stored in 10 % phosphate buffered formalin. Excised tissue were cleaned in saline, fixed in Bouin's fluid, dehydrated in increasing concentrations of ethanol and embedded in paraffin wax. Thereafter, sections of tissues were cut at 5 µm

with a rotary microtome, mounted on clean glass slides and stained with haematoxylin. The stained tissues were observed through an Olympus microscope and photographed by charecouple device camera at magnifications X 100.

Table 3.1: The relative area and their corresponding ulcer index.

Relative area/mm^2	Ulcer index	Relative area/mm^2	Ulcer index
No ulcer	0	221–230	0.006
451–460	0.00001	211–220	0.007
441–450	0.00002	201–210	0.008
431–440	0.00003	191–200	0.009
421–430	0.00004	181–190	0.01
411–420	0.00005	171–180	0.02
401–410	0.00006	161–170	0.03
391–400	0.00007	151–160	0.04
381–390	0.00008	141–150	0.05
371–380	0.00009	131–140	0.06
361–370	0.0001	121–130	0.07
351–360	0.0002	111–120	0.08
341–350	0.0003	101–110	0.09
331–340	0.0004	91–100	0.1
321–330	0.0005	81–90	0.2
311–320	0.0006	71–80	0.3
301–310	0.0007	61–70	0.4
291–300	0.0008	51–60	0.5
281–290	0.0009	41–50	0.6
271–280	0.001	31–40	0.7
261–270	0.002	21–30	0.8
251–260	0.003	11–20	0.9
241–250	0.004	1–10	1.0
231–240	0.005	Perforation	-

Adopted from Ganguly, 1969

3.2.5.5 Data Analysis

Data were analysed using one-way analysis of variance (ANOVA), followed by Bonferroni's pairwise posthoc test where P-value was less than 5 %. Values were presented as mean ± standard error of the mean (S.E.M.).

3.3 RESULTS

3.3.1 Acute toxicity

Acute toxicity study did not show any signs of abnormality in rats even at 5000 mg/kgbwt. Rats treated with a single dose of DRE did not show any signs of abnormal alteration in the stomach lining after macroscopic examination following 14 days observation.

3.3.2 Gastroprotective and gastrohealing activity of DRE in ASA model

Pre-treatment and post-treatment of ASA-induced ulcer in rats with DRE (100, 300 and 500 mg/kg), Omeprazole (30 mg/kg) and ranitidine (50 mg/kg) reduced ulcer index significantly in comparison with negative control ($p<0.01$), (Table 3.2 and Table 3.3 respectively). The group pretreated with 300 mg/kg recorded a significant lower ulcer index compared with 500 mg/kg group ($p<0.05$). No marked difference in ulcer index was observed amongst groups pretreated with 300 and 100 mg/kg of DRE, omeprazole and ranitidine ($p>0.05$). However, there was a marked increase in percentage ulcer protective and curative ratios in groups pretreated or post-treated with 300 mg/kg compared to 500 mg/kg dose group ($p<0.05$) (Table 3.2 and Table 3.3). In the ASA gastroprotective model, protection from ulcers were 80.00, 96.15, 64.10, 96.74 and 90.77 % for groups pretreated with 100, 300 and 500 mg/kg bwt of DRE, omeprazole and ranitidine respectively (Table 3.2). In the ASA gastrohealing model, rats treated with DRE, omeprazole and ranitidine at different doses significantly led to a reduction in ulcer indices compared to negative control group ($p<0.05$). The percentage curative ratios in ASA model were 93.12, 98.35, 91.47, 97.15 and 92.94% for 100, 300 and 500 mg/kg of DRE, omeprazole and ranitidine groups respectively (Table 3.3).

Table 3.2: Effect of whole plant extract of *Dissotis rotundifolia* (DRE), omeprazole and ranitidine on acetylsalicylic acid (ASA), ethanol (EtOH) and cold restraint stress (CRS)-induced gastric ulcers in rats during gastroprotective study

Dosage (mg/kgbwt)	ASA		EtOH		CRS	
	Ulcer index	%Protection	Ulcer index	%Protection	Ulcer index	%Protection
Negative control	0.78± 0.021	-	0.88±0.020	-	0.72±0.049	-
Normal control	-	100±0.00	-	100±0.00	-	100±0.00
DRE 100	0.15±0.052[a]	80.00±6.70	0.26±0.080[a]	70.23±9.05	0.08±0.019[a]	89.44±2.66
300	0.03±0.017[a]	96.15±2.15	0.04±0.011[a]	95.96± 1.25	0.03±0.017[a]	95.75±2.41[b]
500	0.28±0.102[a]	64.10± 13.07	0.40±0.071[a]	54.55± 8.03	0.26±0.051[a]	63.89±7.08
Omeprazole	0.03±0.012[a]	96.74± 1.58	0.20±0.090[a]	77.21± 10.07	0.11±0.039[a]	84.78±5.38
Ranitidine	0.07±0.037[a]	90.77±4.77	0.32±0.071[a]	63.19± 16.38	0.031±0.019[a]	95.64±2.57

Values are expressed as mean ± Standard error of mean (SEM) for 5 animals in each group (a) Significant when group was compared to negative control, (b) Significant when group was compared to omeprazole treated group (c) Significant when group was compared to ranitidine treated group (d) Significant when group was compared to 300mg/kgbwt treated group

Table 3.3: Effect of whole plant extract of *Dissotis rotundifolia* (DRE), omeprazole and ranitidine on acetylsalicylic acid (ASA), ethanol (EtOH) and cold restraint stress (CRS) induced-gastric ulcers in rats during gastrohealing study

Dosage (mg/kgbwt)	ASA		EtOH		CRS	
	Ulcer index	% Curative	Ulcer index	% Curative	Ulcer index	% Curative
Negative control	0.68± 0.058	-	0.78±0.02	-	0.60±0.032	-
Normal Control	-	100±0.00	-	100±0.00	-	100±0.00
DRE 100	0.05±0.018[ab]	93.12±2.70	0.08±0.005[a]	89.23±0.65	0.05±0.022[a]	91.00±3.71[d]
300	0.01±0.009[a]	98.35±1.44	0.04±0.015[a]	94.77± 1.88	0.02±0.011[a]	96.70±1.76
500	0.06±0.018[a]	91.47± 2.69	0.30±0.105[a]	61.54± 13.44	0.14±0.059[a]	77.33±4.36[bcd]
Omeprazole	0.02±0.013[a]	97.15± 1.94	0.06±0.037[a]	92.38± 4.77	0.07±0.018[a]	88.67±2.95[c]
Ranitidine	0.05±0.020[a]	92.94± 2.99	0.17±0.054[a]	78.46± 6.94	0.04±0.012[a]	94.13±2.02[b]

Values are expressed as mean ± Standard error of mean (SEM) for 5 animals in each group (a) Significant when group was compared to negative control, (b) Significant when group was compared to omeprazole treated group (c) Significant when group was compared to ranitidine treated group (d) Significant when group was compared to 300 mg/kgbwt treated group.

3.3.3 Gastroprotective and gastrohealing activity of DRE in ethanol model

In the ethanol-induced ulcer model, it was seen that pretreatment with DRE (100, 300 and 500 mg/kg), omeprazole (30 mg/kg) and ranitidine (50 mg/kg) significantly reduced ulcer index in comparison with negative control group ($p<0.05$) in both gastroprotective and gastrohealing models. No gross difference in ulcer indices was observed amongst groups pretreated with 300 and 100mg/kg of DRE, omeprazole and ranitidine ($p>0.05$). The percentage protection from ulcers were 70.23, 95.96, 54.55, 77.21 and 63.18 for groups pretreated with 100, 300 and 500 mg/kg of *Dissotis rotundifolia* and positive controls (omeprazole and ranitidine), respectively Table 3.2. In the ethanol-induced ulcer model, it was seen that post-treatment with DRE (100, 300 and 500 mg/kg) and omeprazole (30 mg/kg) showed significant therapeutic effect in ethanol induced ulcer rats compared with negative control group ($p < 0.05$). The percentage curative ratios were 89.23, 94.77, 61.54, 92.38, and 78.46 for groups treated with 100, 300 and 500 mg/kg of *Dissotis rotundifolia*, omeprazole and ranitidine respectively. Table
3.3.

3.3.4 Gastroprotective and gastrohealing activity of DRE in cold stress model

Regarding stress ulcer, no marked difference in ulcer index was observed amongst groups pretreated with 300 and 100 mg/kg of DRE, omeprazole and ranitidine ($p>0.05$) in gastroprotective model. On the contrary, in the gastrohealing model, treatment of rats with 300 mg/kg DRE recorded a significantly lower ulcer index compared to treatment with 500 mg/kg ($p<0.006$). Moreover animals treated with ranitidine recorded a lower ulcer index compared to those treated with 500 mg/kg bwt of DRE. In the gastrohealing model, post-treatment with *Dissotis rotundifolia* extract (300 mg/kg) and ranitidine significantly recorded a higher percentage curative ratio than omeprazole. The percentage curative ratios were 96.70, 94.13 and 88.67 for groups exposed to 300 mg/kg of DRE, ranitidine and omeprazole respectively (Table 3.3).

3.3.5 Appearance of mucosae tissues

3.3.5.1 Appearance of gastric mucosae in ASA-induced ulcer model

Rats pretreated with omeprazole, ranitidine and three doses of DRE showed considerably reduced areas of gastric ulcer formation compared to negative controls (ASA only without drug treatment) (Plate 3.1). Mucosal damage seen as dark spots was also notably decreased in size and severity in DRE and positive control pretreated animals. On the other hand, negative control group exhibited severe ulcer formation (Plate 3.1). Results shown are for gastroprotective experiment, similar results were obtained for gastrohealing studies.

3.3.5.2 Appearance of mucosae tissues in ethanol induced-ulcer model Rats pretreated with omeprazole, ranitidine and three doses of DRE exhibited significant reduced areas of gastric ulcer formation compared to negative controls (Plate 3.2). Severe haemorhage was seen as red patches on gastric mucosae. Gastric ulcer seen as haemorhage was significantly reduced in size and severity in DRE and positive control pretreated animals. Meanwhile, there was severe ulcer formation in negative control animals (ethanol only without any form of pretreatment) (Plate 3.2). Results shown are for gastroprotective experiment, similar results were obtained for gastrohealing.

3.3.5.3 Appearance of mucosae tissues in cold stress-induced ulcer model Regarding the appearance of gastric mucosa, rats pretreated with omeprazole and ranitidine and three doses of DRE showed marked decrease in areas of gastric ulcer formation compared to negative controls (CRS only without drug treatment) Plate 3.3. Ulcer was seen mostly with several mucosal fold as dark spots. Flattening of the folds of gastric mucosal was also seen in controls (omeprazole and ranitidine) and DRE treated rats. Additionally, area of ulceration was drastically reduced in size in DRE treated animals similar to positive controls. Negative control animals

showed severe ulcer formation (Plate 3.3). Results shown are for gastroprotective experiment, similar results were obtained for gastrohealing.

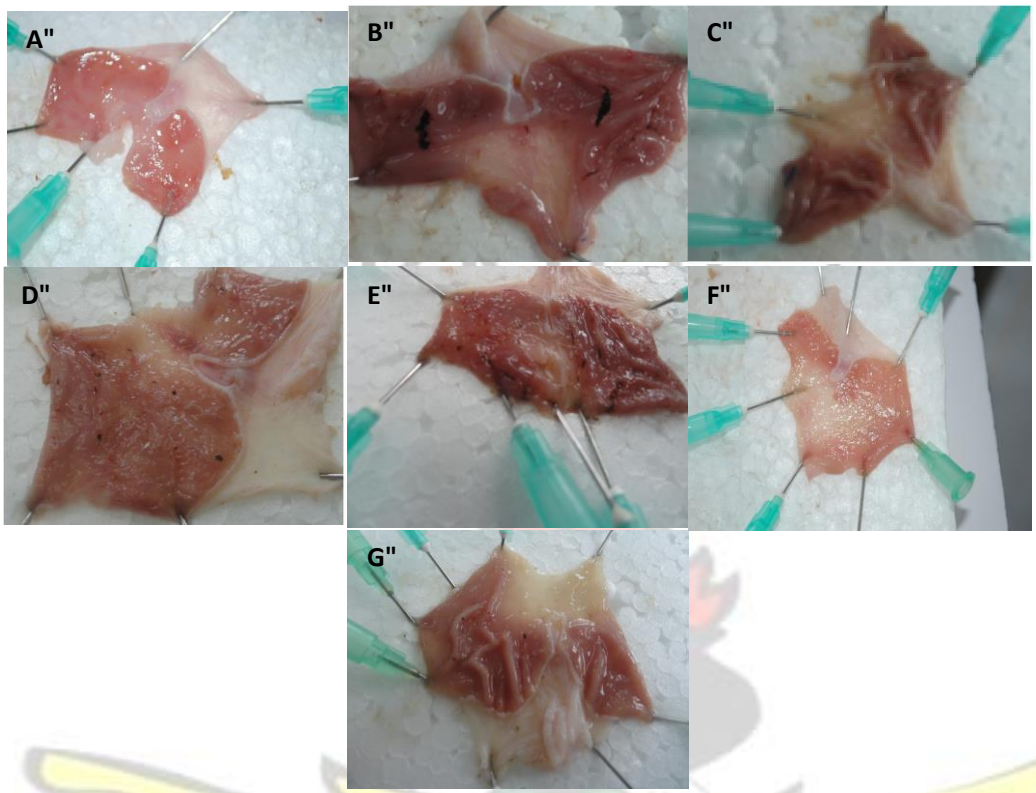


Plate 3.1: Representive appearance of gastric mucosa of rats pretreated with DRE, omeprazole and ranitidine before ulcer induction with acetylsalicylic acid. (A) Rats pretreated with vehicle without ulcerogen (normal control). Normal mucosal lining. (B) Rats treated with only ulcerogen (cold) without any form of DRE or positive control treatment. Severe injuries are observed in the gastric mucosa. ulcer in mucosa appears as dark spots on the surface (C) Rats pretreated with 100 mg/kg DRE. Moderate injuries to gastric mucosa are seen. (D) Rats pretreated with 300 mg/kg DRE. Mild injuries to gastric mucosa are seen and flattening of gastric mucosa is observed. (E) Rats pretreated with 500 mg/kg DRE. Moderate injuries to gastric mucosa are observed though not like 100 mg/kg DRE (F) Rats pretreated with omeprazole. Injuries to gastric mucosa are much milder compared to injuries observed in ulcerogen treated only and ranitidine group. (G) Rats pretreated with ranitidine. Injuries to gastric mucosa are much milder compared to injuries observed in only ulcerogen treated.

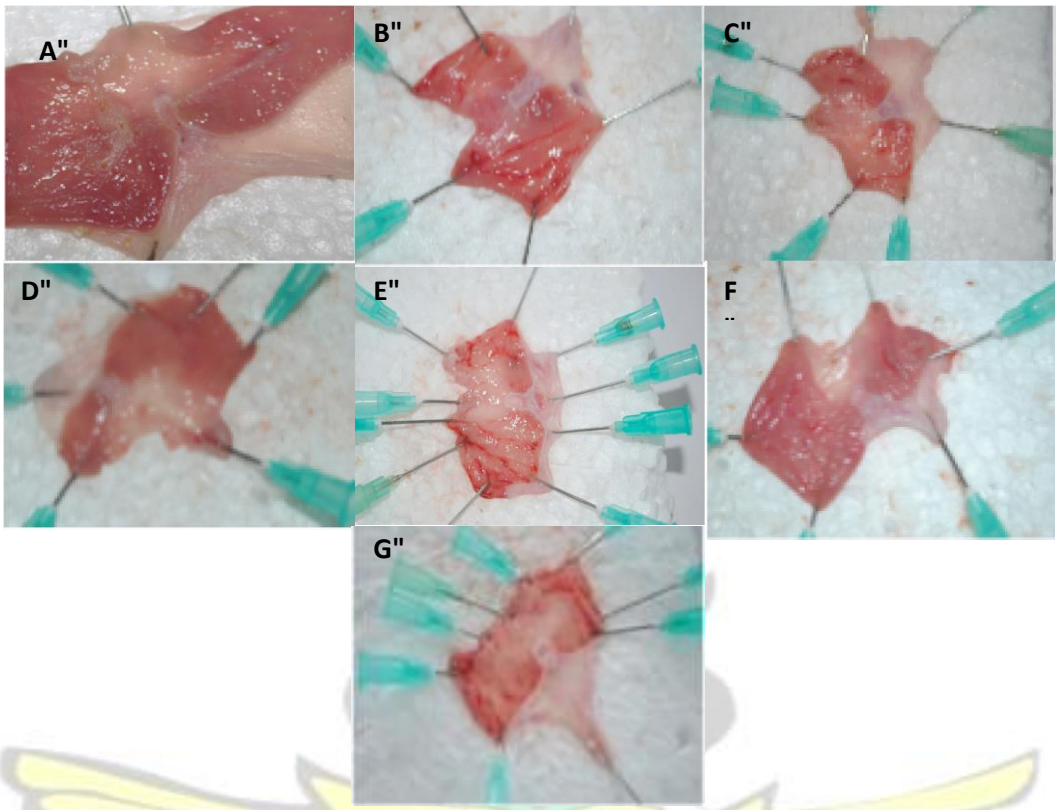


Plate 3.2: Representative appearance of gastric mucosa of rats pretreated with DRE, omeprazole and ranitidine before ulcer induction with ethanol. (A) Rats pretreated with vehicle without ulcerogen (normal control). Normal gastric mucosa appearance. (B) Rats treated with only ulcerogen (ethanol) without any form of drug treatment. Severe injuries are observed in the gastric mucosa. Extensive visible haemorrhage necrosis observed (C) Rats pretreated with 100 mg/kg DRE. Moderate injuries to gastric mucosa are observed. (D) Rats pretreated with 300 mg/kg DRE. Mild injuries to gastric mucosa are observed (E) Rats pretreated with 500 mg/kg DRE. Moderate injuries to gastric mucosa are observed though not as 100 mg/kg DRE (F) Rats pretreated with omeprazole. Injuries to gastric mucosa are much milder compared to injuries observed in ranitidine group. (G) Rats pretreated with ranitidine. Injuries to gastric mucosa are much milder compared to injuries observed in ulcerogen treated only.

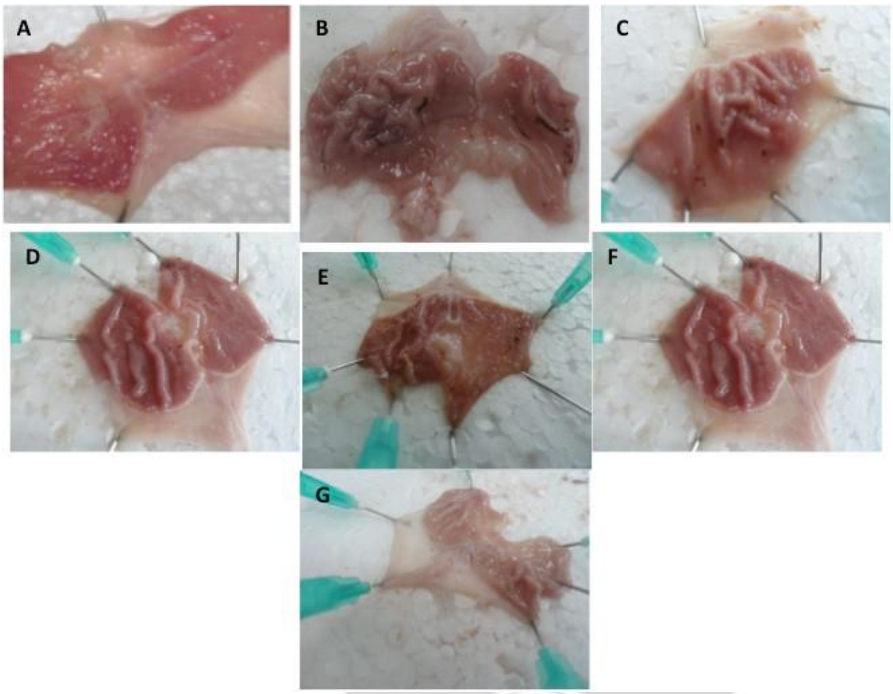


Plate 3.3: Representative appearance of gastric mucosa of rats pretreated with DRE, omeprazole and ranitidine before ulcer induction with cold stress. (A) Rats pretreated with vehicle without ulcerogen (normal control). normal macroscopic appearance of gastric mucosa with intact stomach. (B) Rats treated with only ulcerogen (cold stress) without DRE or positive control treatment. Severe injuries are seen in the gastric mucosa. (C) Rats pretreated with ranitidine. Injuries to gastric mucosa are milder in comparison to injuries seen in ulcerogen treated only. (D) Rats pretreated with omeprazole. Injuries to gastric mucosa are much milder in comparison to injuries seen in ulcerogen treated only and ranitidine group. (E) Rats pretreated with 100 mg/kg DRE. Moderate injuries to gastric mucosa are seen. (F) Rats pretreated with 300 mg/kg DRE. Mild injuries to gastric mucosa are observed and flattening of gastric mucosa is seen. (G) Rats pretreated with 500 mg/kg DRE. Moderate injuries to gastric mucosa are observed though not like 100 mg/kg DRE.

3.3.6 Histopathological appearance of mucosal tissues

3.3.6.1 Histopathological appearance of mucosal tissues in ASA-induced ulcer model

In the acetylsalicylic acid model, histopathological examination of stomach mucosa shows that pretreatment with DRE (300 mg/kg) and omeprazole protected the mucosal epithelium from the damage caused by the ulcerogen-aspirin. The aspirin group without extract pretreatment shows ulcerated mucosa with haemorrhage and

discontinuity of the lining epithelium while extract and positive control pretreated group shows the normal mucosa with mild hyperplasia and mild oedematous submucosa (Plate 3.4). Results shown are for gastroprotective experiment, similar observations were made on micrographs taken from gastrohealing studies.

3.3.6.2 Histopathological appearance of mucosal tissues in ethanol-induced ulcer model

Histological examination of mucosae in ethanol-induced ulcer model indicated that there was disruption of the surface epithelium and glandular structure, while the histological examination of the groups pretreated with 300 mg of DRE and omeprazole showed mild damage to the gastric mucosa. There was a mild disruption of the surface epithelium with mild oedema and leukocyte infiltration into the submucosal layer (Plate 3.5). Results shown are for gastroprotective experiment, similar observations were made on micrographs taken from gastrohealing studies.

3.3.6.3 Histopathological appearance of mucosal tissues in cold stress-induced ulcer model

Micrographs obtained from histopathological examination of the normal control group in cold stress model indicated that there was no disruption of the surface epithelium, while in the negative control group, the histopathological examination showed extensive damage to the gastric mucosa, with necrotic lesions penetrating deeply into the mucosa accompanied by extensive oedema and leukocyte infiltration of the submucosal layer. The 30 mg/kg bwt omeprazole and 300 mg/kg bwt extract pretreated groups exhibited mild to moderate disruption of the surface epithelium (Plate 3.6). Similar observation pattern was made on micrographs taken from gastrohealing studies.

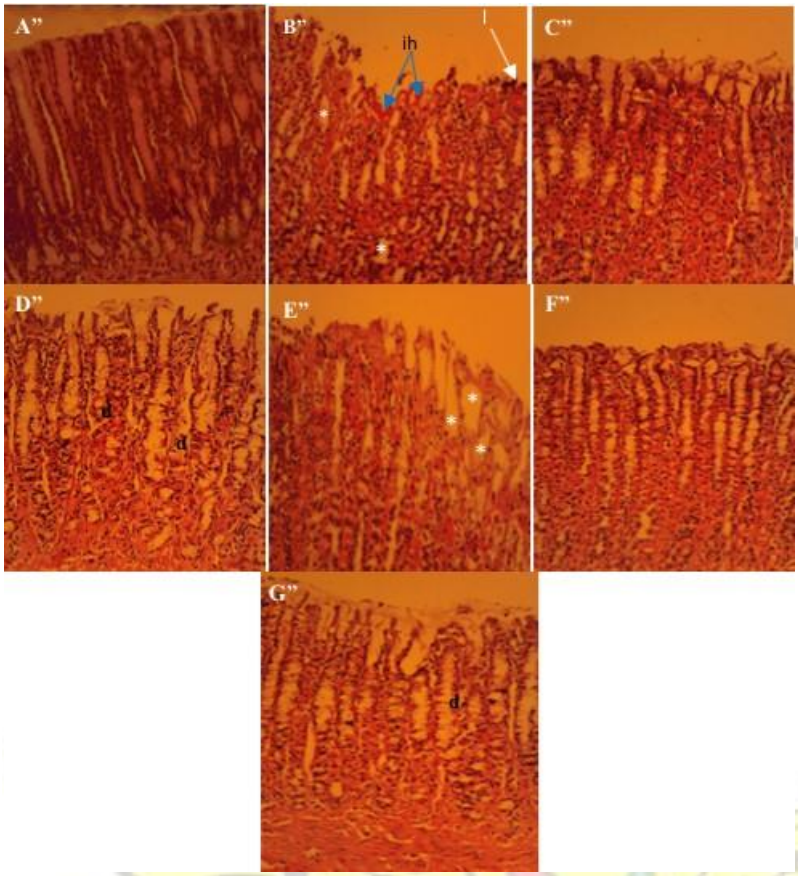


Plate 3.4: Representative histopathological transverse section microscopic appearance of gastric mucosa of rats pretreated with DRE, ranitidine and omeprazole before ulcer induction with acetylsalicylic acid using H&E stain. **(A)** Rats pretreated with vehicle without ulcerogen (normal control) Normal gastric Mucosa. **(B)** Rats treated with only ulcerogen (ethanol) without any form of drug treatment. Section shows extensive ulceration to the exterior of epithelium and lesions penetrate deeply into mucosa accompanied by intra-and intermucosal haemorrhage (*ih*) with leucocyte infiltration. Also mild mucosal oedema are seen within mucosal layer. **(C)** Rats pretreated with 100 mg/kg DRE. Section reveals normal gastric mucosa with mild distortion in the arrangement of mucosal layer. **(D)** Rats pretreated with 300 mg/kg DRE. Section reveals normal gastric mucosa with mild oedema and distorted mucosal layer (*d*). **(E)** Rats pretreated with 500 mg/kg DRE. Section shows extensive superficial erosion of gastric mucosa, with reduced haemorrage, accompanied by extensive oedema (white asterisk) at the superficial layer. **(F**) Rats pretreated with omeprazole. Section reveals normal gastric mucosa in comparison to the normal control group **(G)** Rats pretreated with ranitidine. Section shows normal gastric mucosa with mild distortion (*d*) in the mucosal layer as compared to the normal.

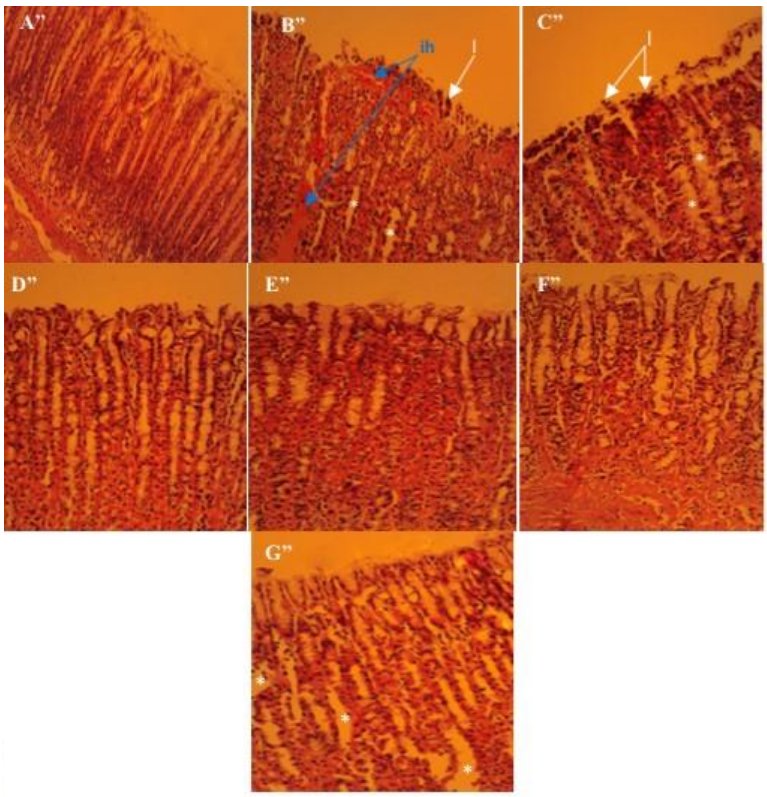


Plate 3.5: Representative histopathological transverse section microscopic appearance of gastric mucosa of rats pretreated with DRE, omeprazole and ranitidine before ulcer induction with ethanol using H&E stain. **(A)** Rats pretreated with vehicle without ulcerogen (normal control). Normal gastric Mucosa. **(B)** Rats treated with only ulcerogen (ethanol) without any form of drug treatment. Section displays extensive ulceration to the surface epithelium and lesions penetrate deeply into mucosa accompanied by intra- and intramucosal haemorrhage (*hi*) with leukocyte (*l*) infiltration. Also mucosal oedema (white asterisk) are seen within mucosal layer. **(C)** Rats pretreated with 100 mg/kg DRE. Section shows extensive oedema with reduced haemorrage, and leukocyte (*l*) infiltration at the superficial layer. **(D)** Rats pretreated with 300 mg/kg DRE. Section reveals normal gastric mucosa. **(E)**. Rats pretreated with 500 mg/kg DRE. Section reveals normal gastric mucosa with mild distortion in the arrangement of mucosal layer. **(F**) Rats pretreated with omeprazole. Section reveals normal gastric mucosa. **(G)** Rats pretreated with ranitidine. Section shows normal gastric mucosa but conspicous intra-and intermucosal oedema (*white asterisks*).

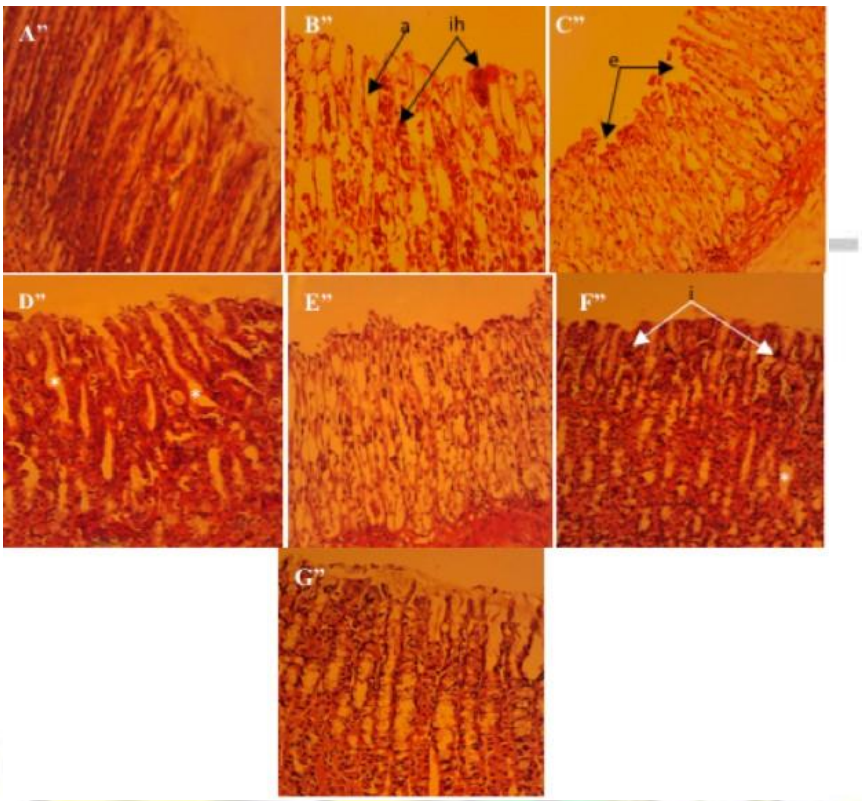


Plate 3.6: Representative histopathological transverse section microscopic appearance of gastric mucosa of rats pretreated with DRE, omeprazole and ranitidine before ulcer induction with cold stress using H&E stain. **(A)** Rats pretreated with vehicle without ulcerogen (normal control). Normal gastric Mucosa. **(B)** Rats pretreated with only ulcerogen (cold) without any form of drug treatment. Section shows extensive intra-and intermucosal haemorrhage (*ih*), accompanied by "thinning" of the mucosal layer as a result of atrophied (*a*) epithelial cells of the mucosa. **(C)** Rats pretreated with 100 mg/kg DRE. Section shows extensive superficial erosion (*e*) of gastric mucosa, with reduced haemorrage, accompanied by atrophy of mucosal layer. **(D)** Rats pretreated with 300 mg/kg DRE. Reduced ulceration with intra-and intermucosal oedema (white asterisk) evident, and absence of atrophied mucosal cells, with distorted arrangement of mucosal layer. **(E)** Rats pretreated with 500 mg/kg DRE. Mild apical erosion with absence of haemorrage and mild form of atrophied mucosal layer. **(F)** Rats pretreated with omeprazole. Section shows normal gastric mucosa with little oedema (white asterisks) and leukocyte infiltration (*l*). **(G)** Rats pretreated with ranitidine. Section shows normal gastric mucosa as compared to the normal control.

3.4 DISCUSSION

Acute toxicity studies did not show signs of abnormality even at 5000 mg/kg, an indication of a wide safety margin of the DRE extract. Normal rats observed after 14 days did not show abnormal changes in the stomach lining suggesting that DRE has no damaging effect on the integrity of the stomach.

Peptic ulcer can mainly be accounted for by hyperacidity in the stomach. Abuse of alcohol, NSAIDs and stress are among other factors reported to be contributing to development of peptic ulcer diseases (PUDs) (Barocelli *et al*., 1997). Currently, there are many orthodox drugs for peptic ulcer management ranging from antiacids to anticholinergics. However, majority of these drugs have adverse effects and are expensive, limiting their use. Thus, current research for potential antiulcer agents is focused on a cheaper, more effective and less toxic agent. A candidate for an effective drug against peptic ulcer should basically act either by reducing the aggressive factors on gastroduodenal mucosa or by increasing mucosal defense against them and ultimately contribute to ulcer healing.

Medicinal plants play a vital therapeutic role in the management of many human illnesses including peptic ulcer and have been employed in the health care system in Ghana. Some of the plant extracts are attractive sources of new drugs and have been shown to yield promising results in the treatment of gastric ulcers. The ethnomedicinal use of *Dissotis rotundifolia* in Ghana for the management of peptic ulcer diseases with very little scientific evidence of efficacy and safety necessitated this study.

For this purpose, the antiulcer effects of DRE were evaluated using rat ulcer models (cold, acetylsalicylic acid and ethanol) that simulate human ulcers. In this study, different ulcer models caused by the most common aetiological agents namely stress, acetylsalicylic acid and ethanol were employed to evaluate the gastroprotective activity and gastrohealing properties of DRE in rats. Though all

animals were put in groups, they were maintained separately in cages with mesh beneath to allow faeces to drop. This arrangement was put in place to prevent coprophagy and cannibalism during the fasting period.

The cold stress model was the first to be used to assess the possible antiulcer properties of the extract. This model was employed because it has been stated by Nyarko *et al*. (2005) to be useful for assessing the general antiulcer activity of any potential drug. The cold stress model employs the restraint technique developed by Brodie and Hanson, 1960 coupled with the cold-water or ordinary-water immersion method by Levine, 1971. The combination of these methods is reported to be synergistic in inducing acute stress lesion in rats (Senay and Levine, 1967) arising primarily from physiological distress. Gastric ulcers induced by cold-water-restraint stress (CWRS) or cold-restraint stress (CRS) or water-immersion stress (WIS) in rats are known to resemble human peptic ulcers, both grossly and histopathologically (Konturek *et al*., 2003). The pathophysiology of stress-induced ulcers is complex. The ulcers are produced due to the release of histamine, leading to an upsurge in gastric acid secretion, a reduction in mucus production (Kitagawa *et al*., 1979), pancreatic juice reflux, and poor blood flow to gastric mucosa (Guth, 1972).

In the current study regarding cold stress ulcer, DRE significantly reduced the ulcer index in rats pretreated and post-treated with it compared to negative control group. This also reflected in the percentages for the protective and curative ratios. This is an indication of the possible therapeutic effect of the extract in managing stress related ulcers in man. Several folds observed in the stomach of negative control animal may be explained by the fact that stress triggers an increase in gastrointestinal motility resulting in folds in the stomach (Peters and Richardson, 1983). The insignificant difference recorded between the effects of 300 mg/kg animal treated group and positive controls (omeprazole and ranitidine) shows that

the extract at that dose is comparable to the standard drugs. The results in this study suggest that DRE could be acting as an histamine receptor antagonist or anticholinergic agent.

Nonsteroidal anti-inflammatory drugs (NSAIDS), such as acetylsalicylic acid are known to contribute to peptic ulcers during usage by inhibiting prostaglandin synthetase in the cycloxygenase pathway (Rainsford, 1987). This has been exploited in developing the model. In the stomach, prostaglandins play a crucial role in protecting the stomach mucosa lining, stimulating the secretion of bicarbonate and mucus, maintaining mucosal blood flow, and regulating mucosal cell turnover and repair (Hayllar and Bjarnason, 1995). Therefore the suppression of prostaglandin synthesis by NSAIDS results in increased susceptibility to mucosal injury and ultimately gastric ulcers. In the ASA model, DRE displayed significant reduction in gastric ulcer formation as depicted by the reduced ulcer index and increased percentage protective and curative ratios. The significant inhibition of gastric ulcer in DRE-treated groups compared to untreated group in this study suggest the possible improvement of prostaglandin synthesis or mucus production in the antiulcer effect of the plant extract.

Ethanol (EtOH) is metabolized in the body and releases superoxide anion and other free radicals. According to a study by Glavin and Szabo, (1992), ethanol induces ulcer by reducing the gastric mucosal blood flow and mucus production in the gastric lumen, endogenous glutathione and prostaglandin levels and by increasing ischaemia, gastric vascular permeability, acid 'back diffusion', release of histamine, efflux of sodium and potassium, influx of calcium, generation of free radicals and leukotrienes.

Furthermore, disturbances in gastric secretion, damage to the gastric mucosa, alterations in permeability, gastric mucus depletion and free-radical production are

observed after the administration of ethanol (Salim, 1991). In the present study, the negative control group treated orally with ethanol noticeably produced the expected characteristic zone of necrotizing mucosal lesions (Plate 3.4). Pretreatment and post-treatment with DRE drastically decreased ulcer index, and also increased percentage curative and protective ratios. These results indicate that DRE extract shows an antiulcerogenic effect related to cytoprotective activity and gastrohealing potential.

Histological examination was performed to ascertain whether ulcer induced affected mucosa layers and also assess the protective effects of DRE. Histopathological observation after staining further confirmed the ability of DRE to inhibit CRS, ASA and EtOH-induced gastric damage in the superficial layers of the gastric mucosa when compared to the normal control group. In the micrographs obtained from pretreated animals' stomachs showed normal arrangement of gastric cells in control rats. Ulcerogens (cold, ASA and EtOH) induced rats showed damage to mucosal epithelium in the stomach. Meanwhile the marked reduction of size of ulcers in 100 and 300 mg/kg of DRE, 30 mg/kg omeprazole and 50 mg/kg of ranitidine pretreated rats was confirmed by a mild damage to the epithelium in micrographs. This is indicative of a marked inhibition of gastric ulcer by DRE, omeprazole and ranitidine. The results from histopathological studies confirm that the plant extract possesses antiulcer effect against cold, acetylsalicylic acid and ethanol in rats.

3.5 CONCLUSION

The data from the present study as demonstrated in CRS, ASA, and EtOH- induced ulcer models show that DRE displays gastroprotective properties and gastrohealing activities in rats. These findings corroborate the traditional use of DRE in stomach ulcer treatment.

Chapter 4

IN VIVO INHIBITION OF GASTRIC ACID SECRETION BY THE METHANOLIC EXTRACT OF *DISSOTIS ROTUNDIFOLIA* IN RATS

4.1 INTRODUCTION

One of the modern approaches to control gastric ulceration is to inhibit gastric acid secretion (Al Mofleh, 2010). Most of the antisecretory drugs used to control increased acid secretion and acid related disorders caused by stress, NSAID's, and alcohol abuse, present adverse effects and relapses (Martelli *et al*., 1998; Wolfe and Sachs, 2000). More research is required to be done in identifying and evaluating new drugs of plant origin that possess the capacity to reduce acid secretion.

It has been demonstrated in chapter 3 that *Dissotis rotundifolia* extract possesses antiulcer activity. This chapter seeks to examine the possible antisecretory activity of *Dissotis rotundifolia* extract as part of understanding the mechanism of the antiulcer property of the plant extract.

4.2 MATERIALS AND METHODS

4.2.1. Drugs, reagents and solvents

The following chemicals and drugs were used for the experiments: water soluble aspirin, omeprazole, ranitidine. Ethanol (99%), Sodium carbonate and other chemicals were obtained from Merck Chemical Supplies (Darmstadt, Germany).

4.2.2 Plant collection and preparation of plant extract

The plant was collected and prepared as described in chapter 2.

4.2.3 Animals

Sprague Dawley (SD) rats of either sex (180-200g) obtained from the Centre for Plant Research, Akuapem-Mampong, Ghana were used for the study as reported in chapter 3.

4.2.4 Studies on the effect of DRE on normal intestinal transit

The method, described by Rao *et al.* (1997), was employed in this study. The animals were fasted for 12 hrs prior to the experiment but were allowed free access to water. The animals were divided into groups namely; control (saline, 2 mL p.o.), positive control (pirenzepine 2 mg/kgbwt p.o.) and test groups (extract 100, 300 and 500 mg/kg p.o.) containing five rats in each group. After 60 mins, standard charcoal meal (1 mL / rat of a 5% deactivated charcoal suspension in 5% tragacanth) were given to the rats orally. Animals were sacrificed 30 mins after administration of charcoal meal and the small intestine immediately isolated. Peristaltic index for each rat was expressed as percentage of the distance travelled by the charcoal meal

relative to the total length of the small intestine from pyloric sphincter to ileo-caecal junction of each animal.

Persitalic index and percentage inhibition were calculated.

$$\text{Peristaltic index} = \frac{\text{Distance moved by charcoal in ST}}{\text{Total length of the ST}}$$

$$\text{Peristaltic inhibition} = \frac{\text{Total length of ST} - \text{Distance moved by charcoal in ST}}{\text{Total length of the ST}} \text{X } 100$$

Where ST is small intestine

4.2.5 Determination of the activity of H^+ / K^+-ATPase enzyme

In this experiment, all three gastroprotective antiulcer models were used; cold restraint stress, acetylsalicylic acid and ethanol induced models. The method described in chapter 3 was followed but only the 300 mg dose of extract was used.

4.2.5.1 Preparation of gastric mucosal tissue homogenate

In this experiment, after sacrificing the animals and excising their stomachs, the stomach tissues were cut into two. The first portion was used for H^+ / K^+-ATPase enzyme assay, and the second portion was used for determination of mucosal glycoproteins. For the first portion, gastric mucosal lining was scraped using a glass slide and weighed. About 1 g of this tissue was homogenized in 100 mL Tris-HCl pH 7.0 at 3000 rpm for 5 mins at 4 ^{0}C. The homogenate was divided into two and stored at -20 ^{0}C untill use.

4.2.5.2 Preparation of gastric parietal cells

Gastric microsomes were prepared by three successive centrifugations. One portion of homogenate was centrifuged at 3000 rpm for 10 mins at 4 ^{0}C and the supernatant was subsequently centrifuged at 20,000 rpm for 30 mins twice at 4 ^{0}C. The resultant supernatant was discarded and the microsome pellet obtained was dissolved and homogenized in mannitol buffer solution (250 mM mannitol, 2 mM magnesium chloride and 2 mM Tris buffer maintained at pH 7.4) and stored at -20 ^{0}C. The gastric parietal cells that were obtained were used as enzyme source.

4.2.5.3 Measurement of protein concentration in the parietal cell

The amount of protein in the parietal cell was measured using the Bradford, Method (1976). The method used for this assay is based on a colour reaction of protein molecules with cupric ions. This colour reaction is known as the Biuret colour reaction. This procedure was first described by Riegler, (1914) and later modified by Weichselbaum, (1946) and Gornall *et al*., (1948) where sodium potassium tatrate was introduced to stabilise the cupric ions in the alkaline reagent.

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Protein forms a violet coloured complex when it reacts with cupric ions in the alkaline solution. The intensity of the violet colour is directly proportional to the amount of protein in the tissue and this is measured at 540 nm.

The parietal cell homogenate (1 mL) was measured into a test tube. 2 mL of the The biuret reagent [2 mL] (prepared as follows; 0.3 mg copper sulphate and 0.9 mg sodium potassium-tartarate in 50 mL of 0.2 M NaOH solution, 0.5 KI was added and the volume was topped up to 100 mL using the NaOH solution) was added to the content in the test tube and gently swirled to mix. The content was then incubated at 37 ^{0}C for 10 mins and the absorbance was read using the spectrophotometer at 540 nm. This was done in triplicate. A standard curve using

bovine serum albumin (BSA) was prepared and the protein concentration in parietal homogenate was estimated from the standard curve.

4.2.5.4 Estimation of H^+ / K^+ ATPase anzyme activity in rats pretreated with DRE

The H^+ / K^+ -ATPase activity of the stomach of treated rats was determined by the method of Reyes-Chilpa *et al*. (2006) on gastric microsomes. The enzyme activity was assayed by measuring the amount of inorganic phosphate released from ATP.

The homogenate (0.1mL) containing enzyme from rats stomach was mixed with 0.1 mL of distilled water and pre-incubated at 37 ^{0}C for 60 min. After incubation, 0.2 mL of Tris–HCl (20 mM, pH 7.4); 0.2 mL of $MgCl_2$ (2 mM); 0.2 mL of KCl (2 mM); was added to the reaction mixture. The reaction was initiated by adding 0.2 mL of ATP (2 mM) and incubated at 37 ^{0}C for 30 mins. The reaction was terminated by addition of 1 mL of ice cold 10 % TCA and after centrifugation at 2000 x g for 10 mins. The supernatant obtained was used for determination of inorganic phosphorus generated.

The amount of inorganic phosphorus (Pi) liberated from ATP in the supernatant was determined using the method by Shyla *et al*., (2011). The supernatant (1mL) was added to 0.5 mL of 5.54 mM Ammonium Molybdate, 0.5 mL of 0.25 N H_2SO_4, 1 mL of 2 % thiourea and incubated at room temperature for 30 mins. Finally 7 mL distilled water was added and absorbance measured at 840 nm using water as blank. A calibration curve was also prepared using $Na_2H_2PO_4$. $2H_2O$ as standard phosphate. The specific activity was calculated and expressed in nmoles of inorganic phosphorus liberated/min/mg of protein using the formula.

Specific activity (nmol/min/mg protein) =

[generated inorganic phosphate (nmol) / reaction time (min) / protein (mg)]

4.2.6 Determination of levels of mucosal glycoproteins

4.2.6.1 Preparation of defatted gastric mucosal tissues

The second portion of gastric tissue was first defatted before analysis was done on it (Folch *et al*., 1957). A known weight about 1 g of the tissue was homogenized at 5000 rpm for 5 mins in 14 mL of 99.6 % methanol. The contents were filtered and the residue was homogenized in 28 mL of chloroform at 5000 rpm for 3 mins. This was filtered again and the residue was successively homogenized in chloroform-methanol (2:1 v/v) twice and each time the homogenate was filtered. The residue obtained was the defatted tissues which was used for determination of glycoproteins. It was collected and stored at -20 ^{0}C till use.

4.2.6.2 Estimation of levels of glycoproteins

The defatted tissue was hydrolyzed with 0.1N HCl at 80 ^{0}C for 1 hr. This hydrolysis step releases bound sialic acids without degradation. Aliquots were used for sialic acid estimation. To the remaining solution, 0.1 N NaOH was added to neutralize it and aliquots were used for the estimation of protein, hexose, hexosamine and fucose.

The estimation of proteins was according to the method described by Bradford, 1976, total hexoses was determined according to the method by Niebes, 1972, hexosamine content was carried out as described by Wagner, 1979, fucose was determined as described by Dische and Shettles, 1948 and sialic acid was estimated by the method of Warren, 1959. The results were expressed in mg/g tissue. The ratio of total carbohydrates (TC) [sum of hexoses, hexosamine, fucose and sialic acid] to protein (P) represents the index of mucin activity.

Determination of protein concentration in the parietal cell homogenate The amount of protein in the gastric tissue was estimated by the method described by Bradford, (1976) as described earlier.

Estimation of Sialic acid

Hydrolysed gastric tissues (0.3 mL) was added to 0.1 mL. of the 0.2 M periodate solution prepared in 9.0 M phosphoric acid. The tubes were vortexed and allowed to stand at room temperature for 20 mins. Sodium arsenite solution (1 mL, 10%) prepared in 0.5 M sodium sulphate, was added and the tubes were corked and vortexed until the yellow-brown colour disappeared. An aliquot of 3 mL of 0.6% TBA solution prepared in 0.5 M sodium sulphate solution, was added, capped tightly and vortexed and heated on a boiling water bath for about 15 mins. The tubes were then removed and placed in cold water for about 5-10 mins. About 2 mL of solution was transferred to another tube which contains 2 mL of cyclohexanone. The tubes were vortexed twice and then centrifuged for 5 mins at 2000 x g. The red organic cyclohexanone phase was transferred into another tube and absorbance was measured at 549 nm using a spectrophotometer. The test was carried out on 0.1 N HCl which was used as blank (Warren, 1959). Sialic acid was used to prepare a standard curve which was used to extrapolate the unknown sample concentration.

Determination of Hexosamine content

The level of hexosamine was measured using the method described by Wagner, (1979). To 0.5 mL of neutralized hydrolysate gastric tissue sample, 0.6 mL of freshly prepared acetyl-acetone reagent (1mL of acetyl acetone in 50 mL of 0.5 N sodium carbonate solution) was added, mixed and boiled at 100 ^{0}C on a water bath for 30 - 40 mins. After cooling, 5 mL of 95 % ethanol was added to the mixture, followed by 2 mL of Ehrlich reagent (0.8 g para-dimethylaminobenzaldehyde in 30 mL methanol and 30 mL conc HCl). The content was vortexed and the mixture diluted to 10 mL with 95 % ethanol, and allowed to stand for 30 mins. The absorbance of a pink coloured chromogen solution was measured at 540 nm against a blank. The concentration of hexosamine was extrapolated from a calibration curve using glucosamine as standard. The hexosamine content was presented in mg/g of defatted tissues.

Determination of fucose content

For fucose estimation, 0.5 mL of aliquot was treated with 4.5 mL of H_2SO_4 and boiled for 3 mins; 0.1 mL of cysteine hydrochloride reagent was then added. After 75 mins in the dark, the absorbance was read at 430 nm. A standard curve of fucose was prepared and used for extrapolating fucose content. The fucose level was presented as mg/g of defatted tissue (Dische and Shettles, 1948).

Determination of Hexose content

The method of Niebes, 1972 was employed. To 0.5 mL of neutralized sample, 7 mL Orcinol reagent was added slowly to the tubes kept on ice water bath. The contents were thoroughly mixed and then heated at temperature of 80°C for 15 mins. The absorbance of the dark coloured solution developed after cooling was read at 540 nm using a spectrophotometer. A standard curve was prepared using glucose and distilled water was used as blank. The Hexose level was expressed as mg /g of defatted stomach tissue.

4.2.7 Determination of biochemical parameters of gastric juice and mucus content in gastric wall

In this experiment only the acetylsalicylic acid model was employed and the method described in chapter 3 was adopted. Four hrs after ASA administration, all animals in the study were sacrificed by cervical dislocation and gastric juice was collected and gastric wall mucus was scraped for analysis.

4.2.7.1 Determination of biochemical parameters for gastric juice

The stomachs of animals pretreated with extract and standard drugs were removed after dissection, put on a watch glass and cut along the greater curvature. With the help of a syringe, gastric juice secretions was collected and used for subsequent analysis. The volume of gastric juice was measured and the pH also measured. The

total acidity in gastric secretions was determined by titration to pH 7.0 using NaOH solution and phenolphthalein as indicator.

Measurement of volume of gastric juice

The gastric juice was transferred into a measuring cylinder and the volume measured.

Determination of pH

A volume of juice was diluted with an equal volume of distilled water and pH was measured with the aid of a portable pH meter.

Determination of total acidity

A known volume of gastric juice which was diluted with distilled water was transfered into a 50 mL flask and 2-3 drops of freshly prepared 1% phenolphthalein was added to it and titrated with 0.01N NaOH until a pink colour was seen. The volume of 0.01N NaOH used was noted. The total acidity / total acid output was calculated by using the formula below and was expressed as mEq/L per 100 g of body weight.

$$\text{Acidity (mEq/L)} = \frac{\text{Vol. of NaOH} \times \text{Normality} \times 100}{0.1}$$

normality = 0.01N

4.2.7.2 Determination of mucus in gastric wall

Gastric wall mucus was determined in ASA-induced ulcer model according to the method of Corne *et al*., (1974). The glandular segments from stomachs were collected by gently scraping using a glass slide and weighed. Each tissue segment was transferred into 10 ml 1% Alcian blue solution (0.16 M sucrose buffered in 0.05 M sodium acetate, pH 5.8). Tissue was stained for 2 hrs in Alcian blue and

excess dye was removed by two successive washing with 10 ml of 0.25 mM sucrose solution, first for 15 mins and then for 45 mins. Mucus-dye complexed was extracted by placing the tissue segments in 10 ml of 0.5 mM $MgCl_2$ that was intermittently shaken for 1 min at 30-minute intervals over a 2 hr period. An equal volume of diethyl ether solution was added to the blue dye extract and shaken vigorously. The resulting emulsion was centrifuged at 4000 x g for 10 mins and the absorbance of a supernatant clear upper aqueous layer was measured at 600 nm. The quantity of Alcian blue extracted (ug/ml/g of tissue) was then calculated using a standard curve of Alcian blue.

4.2.8 Data analysis

All tests were performed in triplicate, expressed as mean ± S.E.M. Statistical analysis was performed on data using the Graph Pad prism version 4.0. ANOVA was used to determine differences in mean values and also if there was any significant difference in mean values Bonferroni's pairwise posthoc test was done. Values were considered significant when $P<0.05$

4.3 RESULTS

4.3.1 Intestinal transit in rats

The oral pretreatment of rats with DRE (100, 300 and 500 mg/kgbwt) did alter the charcoal transit (peristaltic index) to 53.30, 47.29 and 62.85 % respectively compared to negative control (100 %) (Table 4.1). Pirenzepine (2 mg/kg, p.o) used as positive control reduced this parameter by (48.31%) (Table 4.1). This observation was significantly ($P < 0.01$) different from the negative control group (Table 4.1).

Table 4.1: Effects of Pirenzepine and DRE on small intestinal transit in rats

Treatment	Dose (mg/kg bwt)	Peristalic index	% Inhibition
Water	-	100±0.00	-
Pirenzepine	2	48.31±3.35[a]	51.69±3.35 [bc]
DRE	100	53.30±1.16[a]	46.70±1.16 [cd]
	300	47.29±1.80[a]	52.71±1.80 [b]
	500	62.85±5.39[a]	37.15±5.39[d]

Values are expressed as mean ± Standard error of mean (SEM) for 5 animals in each group P<5% was considered significant. ([a]) Significant when group was compared to negative control. ([b]) Significant when group was compared to 100 mg group. ([c]) Significant when group was compared to 500 mg group. ([d]) Significant when group was compared to 300 mg group.

Table 4.2: Effect of whole plant extract of *Dissotis rotundifolia* (DRE) and omeprazole on the levels of H^+/K^+-ATPase enzyme on gastric mucosa of rats

Treatment (mg/kgbwt)	H^+/K^+-ATPase (nmol Pi/min/mg protein)
1ml distilled water	1.19±0.04 [abc]
DRE only	1.13±0.03 [abc]
Omeprazole only	1.15±0.04 [abc]
ASA only	2.04±0.10
DRE + ASA	1.08±0.15[a]
Omeprazole + ASA	1.11±0.05 [a]
CRS only	1.92±0.07
DRE + CRS	0.94±0.10[b]
Omeprazole + CRS	1.07±0.04 [b]
EtOH only	2.29±0.07
DRE + EtOH	1.21±0.14[c]

Omeprazole + EtOH	1.17±0.08 [c]

Values are expressed as mean ± Standard error of mean (SEM) for 5 animals in each group. P<5% was considered significant ([a]) Significant when group was compared to negative control (ASA), ([b]) Significant when group was compared to negative control (cold), ([c]) Significant when group was compared to negative control (ethanol)

4.3.2 Activity of H^+ / K^+ - ATPase

The activity of H^+ / K^+ - ATPase in negative control CRS, ASA, and EtOH ulcerated rats was observed to be higher compared to normal, omeprazole and DRE treated group ($p< 0.05$). However enzyme activity was reduced in DRE treated rats in all models compared to negative control group ($p<0.05$) (Table 4.2).

4.3.3 Mucin activity

A marked increase in glycoproteins (sialic acid, hexose, hexosamine and fucose) was recorded in all the pretreated groups compared to negative control group. There was also an increase in the total carbohydrate / protein ratio (TC: P) of the gastric mucosa in DRE treated rats (Tables 4.3 and 4.4).

4.3.4 Volume, pH and total acidity of gastric juice

In the antisecretion studies, using acetylsalicylic acid as an ulcerogen, pretreatment with *Dissotis rotundifolia* extract (300mg/kgbwt), omeprazole (30 mg/kgbwt) and ranitidne (50 mg/kgbwt) decreased the volume of gastric juice, total acidity and elevated gastric pH significantly ($p<0.05$) in comparison with the negative control group (Table 4.5).

4.3.5 Gastric wall mucus

The amount of gastric wall mucus in normal control rats was reduced from (326.02±0.79) µg/ml of Alcian Blue / g of tissue to 247.31±0.27 µg/ml of Alcian

Blue / g of tissue in the ulcerated untreated rats. Oral administration of DRE 300 mg/kg enhanced the amount of mucus (357.75±5.61) µg/ml of Alcian Blue / g of tissue when compared to ulcerated rats (247.31±0.27) µg/ml of Alcian Blue / g of tissue ($p<0.05$) (Table 4.5).

Table 4.3: Effect of whole plant extract of *Dissotis rotundifolia* (DRE) and omeprazole on the sialic acid, fucose, hexose and hexosamine levels in gastric mucosa of rats

Treatment (mg/kgbwt)	**Sialic acid (mg/g tissue)**	**fucose (mg/g tissue)**	**hexose (mg/g tissue)**	**hexosamine (mg/g tissue)**
1ml distilled water	1.90±0.05 [abc]	2.38±0.04 [abc]	15.44±0.06 [abc]	8.41±0.18 [abc]
DRE only	2.11±0.05 [abc]	2.65±0.05 [abc]	15.91±0.18 [abc]	8.94±0.09 [abc]
Omeprazole only	1.92±0.03 [abc]	2.50±0.05 [abc]	15.57±0.07 [abc]	8.81±0.20 [abc]
ASA only	0.58±0.04	1.78±0.07	7.77±0.14	2.56±0.27
DRE + ASA	1.87±0.02 [a]	2.27±0.06 [a]	14.16±0.08 [a]	8.03±0.08 [a]
Omeprazole + ASA	1.80±0.03 [a]	2.25±0.07 [a]	13.97±0.05 [a]	7.80±0.23 [a]
CRS only	0.69±0.04	1.89±0.05	9.99±0.11	2.85±0.03
DRE + CRS	1.85±0.02 [b]	2.44±0.02 [b]	14.40±0.31 [b]	8.02±0.04 [b]
Omeprazole + CRS	1.86±0.03 [b]	2.34±0.08 [b]	14.00±0.04 [b]	8.00±0.04 [b]
EtOH only	1.51±0.02	1.43±0.04	6.43±0.21	1.94±0.07
DRE + EtOH	3.06±0.16 [c]	2.35±0.03 [c]	13.77±0.22 [c]	7.94±0.03 [c]
Omeprazole + EtOH	3.04±0.16 [b]	2.30±0.04 [c]	13.72±0.26 [c]	7.84±0.07 [c]

Values are expressed as mean ± Standard error of mean (SEM) for 5 animals in each group. P<5% was considered significant ([a]) Significant when group was compared to negative control (ASA), ([b]) Significant when group was compared to negative control (cold), ([c]) Significant when group was compared to negative control (ethanol)

Table 4.4: Effect of whole plant extract of *Dissotis rotundifolia* (DRE) and omeprazole on the levels of protein, total protein bound carbohydrate and mucin activity in gastric mucosa of rats

Treatment (mg/kgbwt)	Protein (mg/g tissue)	TPBC (mg/g tissue)	mucin activity (TPBC : P)
1ml distilled water	10.31±0.08 [abc]	28.14±0.27 [abc]	2.73±0.03 [abc]
DRE only	10.35±0.06 [abc]	29.61±0.20 [abc]	2.86±0.03 [abc]
Omeprazole only	10.27±0.07 [abc]	28.84±0.14 [abc]	2.81±0.01 [abc]
ASA only	6.36±0.51	12.69±0.31	2.03±0.18
DRE + ASA	9.60±0.39 [a]	26.33±0.17 [a]	2.80±0.15 [a]
Omeprazole + ASA	9.07±0.39 [a]	25.82±0.25 [a]	2.86±0.12 [a]
CRS only	6.53±0.09	15.42±0.12	2.36±0.04
DRE + CRS	9.87±0.39 [b]	26.70±0.33 [b]	2.72±0.15 [b]
Omeprazole + CRS	9.29±0.56 [b]	26.17±0.09 [b]	2.72±0.15 [b]
EtOH only	6.14±0.15	10.31±0.32	1.68±0.08
DRE + EtOH	8.52±0.52 [c]	25.84±0.23 [c]	2.75±0.11 [c]
Omeprazole + EtOH	8.54±0.44 [c]	25.60±0.29 [c]	3.01±0.13 [c]

Values are expressed as mean ± Standard error of mean (SEM) for 5 animals in each group. P<5% was considered significant ([a]) Significant when group was compared to negative control (ASA), ([b]) Significant when group was compared to negative control (cold), ([c]) Significant when group was compared to negative control (ethanol)

Table 4.5: Effect of whole plant extract of *Dissotis rotundifolia* (DRE), omeprazole and ranitidine on biochemical parameters of gastric secretions in ASA - induced ulcer in rats

Treatment (mg/kgbwt)	pH	Gastric juice (mL)	Total acidity (mEq/L/4h)	Mucus (ug Alcian blue /ml / g tissue)
1ml water	3.30±0.04[c]	0.51±0.02[c]	44.23±1.48[c]	326.02±0.79[c]
150 ASA only	1.54±0.02	3.22±0.03	120.42±4.49	247.31±0.27
300 DRE+150 ASA only	3.14±0.04[ac]	0.64±0.03[a]	75.53± 2.00[a]	357.75±5.61[a]
30 omeprazole+ASA	4.65±0.04[ac]	0.54±0.02[a]	59.94±2.15[a]	339.18±1.53[a]
50 ranitidine+ASA	4.97±0.05[a]	0.59±0.02[a]	61.70±3.27[a]	334.78±1.45[c]
300 DRE only	7.31±0.09[abc]	0.56±0.02[a]	48.37±2.05[a]	327.61±2.56[a]
30 omeprazole only	7.20±0.02[acd]	0.62±0.02[ad]	45.79±2.01[a]	322.81±0.54[ad]
50 Ranitidine only	7.46±0.03[ad]	0.54±0.03[a]	46.76±1.54[a]	310.33±1.02[ad]

Values are expressed as mean ± Standard error of mean (SEM) for 5 animals in each group. (a) Significant when group was compared to negative control (b) Significant when group was compared to omeprazole treated group (c) Significant when group was compared to ranitidine treated group (d) Significant when group was compared to 300mg/kgbwt treated group

4.4 DISCUSSION

The beneficial effects of several plant extracts in the prevention and healing of gastric ulcers have been evaluated in several experimental studies. Several mechanisms including H^+/K^+ - ATPase inhibition, antisecretory, anticholinergic, cytoprotective, stimulation of epithelial cell proliferation, improved antioxidant defense system and anti-*Helicobacter pylori* effects have been mentioned as responsible for the pharmacological effects (Al Mofleh, 2010; Megala and Geetha, 2012). This chapter reports on the anticholinergic, H^+/K^+ - ATPase inhibition, cytoprotective and the antisecretory effects of the *Dissotis rotundifolia* extract.

Anticholinergics are another class of antiulcer drugs that act as antagonist of acetylcholine secreted at postganglionic fibres. They act by antagonizing muscarinic cholinergic receptors. They depress gastric motility and secretions Examples of anticholinergic drugs, include pirenzepine, propantheline, and telenzepine (Hoogerwerf and Pasricha, 2001). In this study, the extract decreased the propulsive movement of charcoal meal through the gastrointestinal tract (GIT). The observation that gastric emptying time of DRE treated group was significantly reduced compared to negative control group depicts the involvement of the cholinergic system in the antiulcer activity of the DRE. According to Bertaccini and Scapignato (1981), the delayed gastric emptying time increases the absorption of orally administered drugs. The significant delay in gastrointestinal transit caused by DRE would therefore be of benefit in peptic ulcer management.

A modest method to control ulceration is by inhibiting H^+ / K^+ - ATPase enzyme. Although several ATPase inhibitors are being used to control acid secretion, they produce adverse effects (Waldum *et al*., 2005). This experiment was conducted to further explore the mechanism through which the extract works as an antiulcer

agent as reported in chapter 3. In this experiment, the levels of H^+ / K^+ -ATPase enzyme in ulcerated rats in CRS, ASA and EtOH models was studied. A significant reduction in enzyme activity found in DRE treated rats ($p<0.05$) suggest that it may be one of the mechanisms by which the extract exhibits its antiulcer effects.

Glycoproteins are widely distributed proteins that contain one or more covalently linked carbohydrate chains. They are constituents of extracellular membranes, blood group antigens, enzymes etc. The main sugars present in glycoproteins are hexose, hexosamine, fucose, and sialic acid, which are essential for their diverse biological functions. For instance, protein-bound hexose imparts hydrophilic nature to the cell membrane, and hexosamine through its cationic charges makes the cell membrane more polar. L-fucose, a deoxy-hexose is a component of many N- and O-linked glycoproteins and participates in biological recognition events. Sialic acid, a terminal monosaccharide of glycoconjugates plays a pivotal role in cell-to-cell and cell to matrix interactions (Goel *et al*., 1994). The appreciable increase in glycoproteins observed in all the pretreated groups compared to untreated ulcer group comfirms the significant percentage inhibition / protection provided by DRE treatment. The carbohydrate / protein ratio (TC: P) is taken as reliable marker for mucin activity (Goel *et al*., 1985). The increase in (TC: P) confirms the increase in the glycoprotein content of the gastric mucosal cells of rats treated with DRE. Implicitly, the antiulcer effects of DRE on gastric mucosa may be partly accounted for by its mucin activity.

In assessing the effect of DRE on secretion, only the acetylsalicylic acid model was used and this is because according to Nyarko *et al*., 2005, the model is dependent on gastric acid secretions and thus reveals possible changes in parameters such as gastric secretion volume, pH and titrable acidity compared to EtOH which is independent of gastric acid secretions and the CRS model which produces less gastric juice due to the cold conditions (Nyarko *et al*., 2005). The significant reduction in gastric juice volume and total acidity, and increase in pH in animals

pretreated with *Dissotis rotundifolia* extract (300 mg/kg) and positive control (ranitidine and omeprazole), in comparison with negative control group ($p<0.05$) suggest that the extract interfered with gastric acid secretion. According to Altman, (2001), bicarbonate secreted by the gastric epithelial cells can be concentrated within the mucus, creating an environment with a pH nearer to neutrality than what is observed in the luminal gastric juice. The secreted bicarbonate also shields the gastric epithelium by neutralizing gastric acid (Chu *et al*., 1999). This may be responsible for the increased pH values recorded in extract and positive control groups in this study.

It has been stated that certain antiulcer drugs cause an increase in gastric mucus secretion in the mucosa (Ibrahim *et al*., 2012). Gastric mucus operates as first line of defense against ulcerogens. It is known to play an important role as a defensive barrier against gastrointestinal damage (Davenport, 1968). Mucus is secreted by the mucus neck cells and serves as a cover for gastric mucosa thus preventing physical damage and back diffusion of hydrogen ions (Williams and Turnberg, 1980). Gastric wall mucus depletion provoked by chemical irritants such as acetylsalicylic acid (Thirunavukkarasu *et al*., 2010) is among the mechanisms responsible for gastric injury.

Pretreatment of rats with DRE, ranitidine and omeprazole in this study significantly increased bound Alcian blue which is directly proportional to the gastric mucus content in ASA model. This increase may be due to stimulation of epithelia cells that enhanced mucus secretions which serves as a barrier against necrotizing agents such as ASA .

The increase in mucus content could also be accounted for by the individual mucopolysaccharides. Mucopolysaccharide is the major constituent of mucus and also responsible for the viscous nature and gel-forming properties of the mucus. The gel is reported to be resistant to a number of ulcerogens including acid, ethanol

and NSAIDs, eg. indomethacin (Bell *et al*., 1985). Hence increase in synthesis of mucus may be one of the important contributing factors for the protective role of DRE in peptic ulceration.

Overall, these results show that the antiulcer activity of DRE observed in this study is not only related to a local neutralization of gastric juice content, increase in mucin activity and increased gastric mucus secretion, but participation of H^+ / K^+ -ATPase enzyme inhibition and antagonism of cholinergic receptors.

4.5 CONCLUSION

Taking in account previous results in chapter 3, it is concluded here that the antiulcer effects of DRE is multifaceted; DRE is acting as a proton pump inhibitor, mucin preserver and an antagonist at muscarinic receptors.

Chapter 5

EFFECT OF *DISSOTIS ROTUNDIFOLIA* WHOLE PLANT EXTRACT ON GROWTH OF *HELICOBACTER PYLORI*

5.1 INTRODUCTION

Peptic ulcer, a gastrointestinal disorder, is a pathological condition in which the biological balance between aggressive and defense factors is disturbed. *Helicobacter pylori* is reported to be the main etiological agent of peptic ulcer, which has a prevalence rate of about 40 % in developed countries and over 80 % in developing countries among peptic ulcer patients (Ndip *et al*., 2008; Tanih *et al*., 2009). *H. pylori* is a major cause of at least 90 % of duodenal ulcers and 70 % of gastric ulcers (NIH consensus Development Panel, 1994).

Eradication of *H. pylori* has been shown to result in peptic ulcer healing, prevention of peptic ulcer recurrence and reduction in the prevalence of gastric cancer in high-risk populations (Sepulveda and Coelho, 2002). However, resistance of the organism to drugs (Sherif *et al*., 2004), poor patient compliance, undesirable adverse effects and high cost of combination therapy (O'Gara *et al*., 2000; Smith *et al*., 2001), supports the importance of seeking alternative therapies of managing *H.*

pylori infections. *H. pylori* is an organism reported to weaken the protective mucosal lining of the stomach and duodenum thus allowing gastric hydrochloric acid to easily penetrate through to the sensitive submucosal lining of the mucosa.

Over the years, medicinal plants have been used to treat gastrointestinal diseases and other ailments particularly in the developing world where these diseases are endemic and modern health facilities and services are inadequate (Samie *et al*., 2005; Ishikawa *et al*., 2008). The use of phytotherapy has shown great promise in managing several diseases. One such plant currently used is *Dissotis rotundifolia*. The whole plant of *Dissotis rotundifolia* plant, also known as pink lady is popularly used for treatment of gastric disorders. This plant has been reported to be active against *Staphylococcus aureus*, *Salmonella typhi*, *Escherichia coli*, *Pseudomonas aeruginosa* and other organisms (Abere *et al*., 2010). The reported antimicrobial activity of the extract and the present findings in chapter 3 and 4 that the plant has antiulcer activity prompted investigation into the effect of the extract against *H. pylori*. This is believed to be the leading report on the activity of DRE against clinical isolates of *H. pylori*.

5.2 MATERIALS AND METHODS

5.2.1 Chemicals and drugs

The following chemicals and drugs were used for the experiments: Brain Heart Infusion (BHI) agar, Brain Heart Infusion (BHI) broth, Skirrow's supplement (SR69) Oxoid England, glycerol, cephalotin (30 µg) and nalidixic acid (30 µg) antiobiotic disc obtained from Mast Group Ltd., Merseyside, UK. GasPak EZ Campy Container System obtained from Becton Dickinson, USA. Sodium Hydroxide, magnesium chloride, sucrose, glucose, ethanol (99.8%), were obtained from British Drug House, (BDH) (London, UK) England, Nitroblue tetrazolium

salt (NBT) was obtained from Sigma Aldrich. Expired blood was obtained from the Blood Bank of the Cape Coast Regional hospital, Cape Caost.

5.2.1.1 Preparation of media

Sterile saline solution

Normal saline was prepared by weighing 0.064 g of NaCl and dissolving it in 7.5 mL of distilled water. It was then autoclaved at 121 $^{\circ C}$ for 15 mins.

Amended Brain heart infusion blood agar

Dehydrated powdered Brain Heart Infusion agar (5.20 g) was transferred into a 250 mL Erlenmeyer flask. Distilled water (100 mL) was added. The mixture was heated briefly in a microwave until the agar was completely dissolved. The flask was corked with cotton wool plug and it was autoclaved at 121 ^{0}C for 15 mins and allowed to cool to approximately 50 ^{0}C . Expired human blood (7 mL) was then added to the Brain Heart Infusion agar and 0.1 g of tetrazolium salt dissolved in 10 mL sterile distilled water was added. Skirrow's supplement (SR69) (Oxoid, England); vancomycin (5 mg), polymyxin B (2.5 mg) and trimethoprim (2.5 mg) were also added to the medium to suppress the growth of other bacteria and fungi. The mixture was swirled and was poured aseptically into sterile Petri dishes. The agar plates were then allowed to solidify.

Brain Heart Infusion broth as transport medium

Brain Heart Infusion (BHI) broth was used as the transport medium for gastric biopsy samples obtained from the Endoscopy Laboratory of Life Sciences Laboratory, Cape Coast. The broth was prepared by transferring 4.2624 g of the Brain Heart Infusion broth powder into a clean conical flask and 114.48 mL of sterile distilled water was added. Glycerol (28.8 mL) representing about 20 % of total solution, was added to the solution and swirled until it was uniformly mixed. Cysteine solution (0.72 mL) of 0.2 g/L prepared by dissolving 0.5 g of cysteine

powder in 2.5 mL of 1N HCl was also added to the mixture. The pH of the mixture was adjusted to 7.40 by adding diluted sodium hydroxide (NaOH) solution in drops until the desired pH was attained. The BHI broth was then transferred into eight McCartney's bottles. Each of the bottles containing 18 mL of the broth was autoclaved at 121 °C for 15 mins and stored in the refrigerator.

Brain Heart Infusion Broth for antimicrobial activity

The broth was prepared by transferring 2.96 g of the Brain Heart Infusion (BHI) into a clean conical flask and adding 76 mL of distilled water. It was then autoclaved at 121 $^{\circ}C$ for 15 mins and allowed to cool. Sterile inactivated human serum (4 mL) representing 5 % of medium, was added and the mixture swirled until it was uniformly mixed. Skirrow's reagents was also added as a supplement. Then 5 mL of the mixture were transferred into clean test tubes which were corked with cotton wool and stored at -20 $^{\circ}C$ until it was needed. Sterility control was performed by incubating an aliquot of the broth mixture at 37°C for 48 hrs.

Christensen's urea

Peptone (0.08 g), 0.4 g NaCl, 0.16 g H2PO4, 0.08 g D-glucose, and 1.6 g of agar powder were transferred into a conical flask. Distilled water (72 mL) was added and the mixture was microwaved to ensure complete dissolution. It was then allowed to cool sufficiently to 50 ^{0}C before phenol red (480 µL) was added. The pH of the mixture was adjusted to 6.30 and the medium was autoclaved at 121^{0}C for 15 mins. Urea (1.6 g) was dissolved in 8 mL of sterile distilled water and added to the medium. The medium was poured aseptically into test tubes, allowed to solidify in a slanted position and stored until the were needed.

5.2.2 Isolation of organism

5.2.2.1 Collection of gastric biopsy

Antral and corpus gastric mucosal biopsy specimens were taken from patients referred to Life Science Laboratory, Cape Coast for endoscopy after informed

consent was obtained. Biopsies confirmed to be positive for *H. pylori* using urease test kit were immediately placed in sterile McCartney bottles containing 0.2 g/l of cysteine and 20 % glycerol in brain heart infusion (BHI) broth and transported on ice to the laboratory within 1 hr of collection for isolation and subsequent analysis.

5.2.2.2 Inoculation of gastric biopsy

The BHI broth containing the gastric biopsy was homogenized using the WiseTis homogenizer HG–15D, Germany at 5000 rpm for 30 secs to produce an evenly distributed solution of biopsy tissue in the broth. The homogenized biopsy was then inoculated onto the brain heart infusion blood agar. The inoculation was done by dipping a flamed inoculation loop that has cooled for some time, into the McCartney's bottle containing the homogenized biopsy tissue. The loop was used to streak the surface of an amended Brain Heart Infusin blood agar plate. The inoculation loop was flamed until they were red hot after usage. The inoculated plates were then packed in an inverted manner into gaspak air tight container and a gaspak kit that generates microaerophilic conditions (80 % N_2, 10 % CO_2, 5 % O_2) for the growth of *H. pylori* was added to the plates. The entire process was carried out in the Laminar Air Flow Hood which provides a sterile environment. The inoculated plates together with the gas generating kit (Plate 5.1) were incubated at 37 ^{0}C for 3-6 days.

Plate 5.1: Plates in gaspak container with gas generating kits used in this study

5.2.2.3 Sub culturing of bacterial isolates

After 3-6 days of incubation, sparkling colonies were observed on the amended BHI blood agar. A flamed inoculation loop was used to pick a single sparkling colony and streaked on the surface of a fresh amended BHI blood agar plate. The inoculated agar plates were then packed into gaspak kits (80 % N_2, 10 % CO_2, 5 % O_2), and incubated at 37 ^{0}C for another 3-6 days. After the incubation period, pure cultures of *H. pylori* were obtained with sparkling colonies (Plate 5.2).

5.2.3 Identification of *Helicobacter pylori* organism

H. pylori isolates were identified based on colony morphology, Gram staining reaction, oxidase, urease and catalase tests and antibiotic (cephalotin and nalidixic acid) susceptibility test (Jerris, 1995).

5.2.3.1 Gram Staining reaction test

A heat fix smear was prepared by dropping a loopful of sterile distilled water on a clean glass slide. The inoculation loop was then flamed, allowed to cool and used to pick a single colony of the bacteria. The colony picked was smeared in a drop

of water in a concentric manner. The glass slide was then briefly passed through the flame to allow it to dry. The slide carrying the heat fixed smear was placed on a staining tray. It was gently flooded with crystal violet and allowed to stand for a min. It was then rinsed under running tap water. The smear was flooded with iodine and allowed to stand for a minute before washing under running tap water. It was then decolourized using a 1:1 solution of acetone- ethanol and immediately washed under running tap water. The decoloured smear obtained was flooded with safranin solution and allowed to stand for 2 mins before washing under running tap water. The slide was blot- dried using blotting paper and kept for microscopic examination.

5.2.3.2 Urease Test

Urease activity is detected by a rise in pH, which follows the production of ammonium ions from urea. The rise in pH induces a colour change of phenol red indicator in the culture medium from yellow to pink or red. Colonies were picked from the agar plate with a sterile loop and then inoculated on urea agar slant containing phenol red as an indicator and incubated at 25 ^{0}C (Cheesbrough, 1998). A positive result is detected by a colour change from yellow to pink within 15-40 mins and after 24 hr, while a negative test result is obtained when there is no colour change and the medium remains yellow.

5.2.3.3 Catalase Test

Catalase test makes use of hydrogen peroxide (H_2O_2) and a positive test is indicated by effervescence on the slide when the organism is emulsified in H_2O_2, while for a negative test no effervescence is recorded (Cheesbrough, 1998). A sterile inoculation loop was used to pick a small amount of a single colony which was transferred onto a glass slide. A drop of H_2O_2 was then added. Production of gas bubbles within 5-15 secs indicated a positive reaction.

5.2.3.4 Oxidase Test

Few drops of freshly prepared oxidase reagent (1% N, N-dimethyl-p-phenylenediamine) was added on a strip of filter paper. Test organism was smeared on the strips by using a sterile woody stick. A positive reaction was indicated by an intense deep purple colour appearing within 5-10 seconds (Cheesbrough, 1998).

5.2.3.5 Susceptibility Test for H. pylori to nalidixic acid and cephalothin disc

Preparation of bacterial suspension

A bacterial suspension was prepared by using a sterile inoculation loop to pick 3 discrete colonies of pure culture of *H. pylori* growing on BHI blood agar amended with skirrow's supplement and TTC dye. These colonies were transferred into a test tube containing 5 mL of sterile normal saline and the bacterial suspension was briefly vortexed to ensure even distribution of bacteria.

Susceptibility Test

About 0.1 mL of the *H. pylori* bacterial suspension was transferred unto the surface of an amended BHI blood agar plate and spread over the entire surface of the agar plate. With the aid of sterile forceps, cephalothin and nalidixic acid discs were placed on the surface of inoculated plate and incubated at 37 ^{0}C for 3-6 days under microaerophilic conditions.

5.2.4 Evaluation of plant crude extract for anti-*Helicobacter pylori* activity The agar-well diffusion method was used in accordance with the method previously described by Boyanova *et al*., 2005. Bacterial suspensions of *H. pylori* were adjusted to the McFarland turbidity standards corresponding 1.5x10^{8} CFU/mL. Agar wells were punched in sterile amended BHI blood agar plates using a sterile stainless 6 mm cork borer and allowed to dry for 3-5 mins. The wells were then filled with 65 µL each of different concentrations of the crude plant extract (200, 400, 600, 800 mg/mL), while water was used as negative control. and 0.05 µg/mL clarithromycin, 0.05 µg/mL amoxicillin and 500 µg/mL metronidazole as positive

controls. The inoculated plates were incubated at 37^0C for 3-6 days under microaerophilic condition. After the incubation period, the diameter of the clear zones around the agar wells were measured. The tests were done in triplicate. The mean zones of inhibition were calculated and recorded (CLSI, 2008).

5.2.5 Data analysis

Statistical analysis was performed on data using the Grap Pad prism version 4.0. ANOVA was used to determine differences in mean values and also if there was any statistically significant difference in the diameter of zones of inhibition of the DRE samples and antibiotics. Values were considered significant when $P<0.05$ followed by Bonferroni's pairwise posthoc test where there was a significant difference amongst means

5.3 RESULTS

5.3.1 Identification of *Helicobacter pylori*

In this study *H. pylori* was successfully isolated from the gastric biopsy of peptic ulcer patients as shown on Plate 5.2. After the incubation period, pure cultures of *H. pylori* were obtained with sparkling colonies (Plate 5.2). Five isolates, identified as *H. pylori*, were Gram-negative, urease, catalase and oxidase positive (Table 5.1 and Plate 5.3) and showed characteristic morphology as spiral-shaped bacteria under the microscope. The organism was also found to be susceptible to cephalothin and resistant to nalidixic acid (plate 5.4).

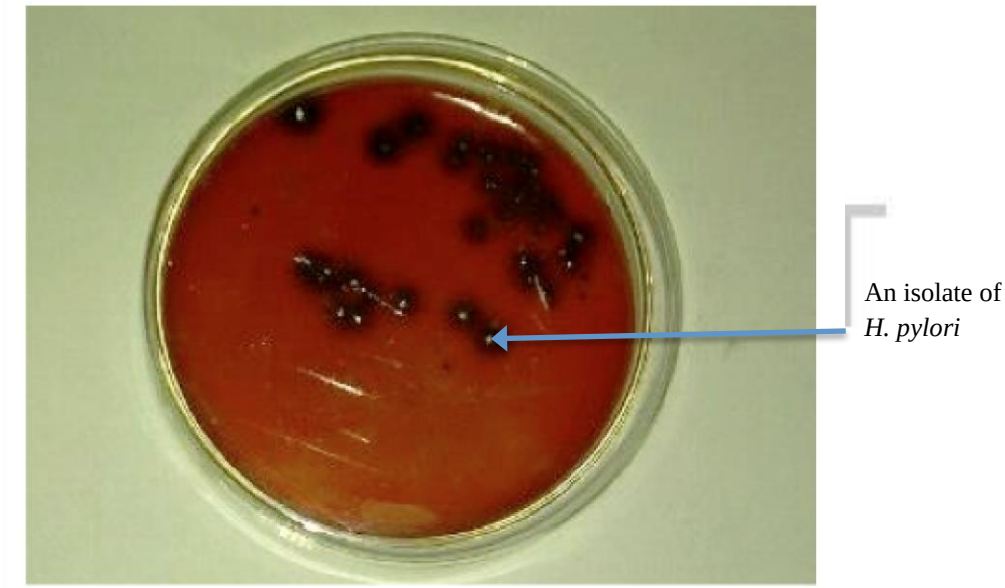


Plate 5.2: Pure cultures of *H. pylori* showing sparkling colonies growing on amended BHI blood agar after 3-6 days incubation at 37 ˚C under microaeorophilic conditions

5.3.2 Anti-*Helicobacter pylori* activity

The whole plant of *Dissotis rotundifolia* extract (DRE) was screened for its potential in inhibiting growth of *H. pylori*. The plant extracts and standard antibiotic drugs showed antimicrobial activity against 5 confirmed *H. pylori* isolates (Plate 5.3). The diameters of the zones of inhibition ranged from 13-30 mm (figure 5.1). Autoclaved distilled water used as negative control, showed no inhibitory effect or activity. DRE showed significant lower zones of inhibition compared to standard drugs (clarithromycin, amoxicillin) ($p<0.05$) with the exception of metronidazole which revealed no zone of inhibition. DRE has an inhibitory effect on growth of *H. pylori*. The inhibitory effect of DRE was observed from a concentration of 200 mg/ml to 800 mg/ml meanwhile maximal effect was observed at 400 mg/ml concentration (figure 5.1).

Table 5.1: Biochemical tests for bacterial isolates

Isolate #	Gram stain	Urease	Oxidase	Catalase
H1	-	+	+	+
H2	-	+	+	+
H3	-	+	+	+
H4	-	+	+	+
H5	-	+	+	+

+ positive reaction, - negative reaction

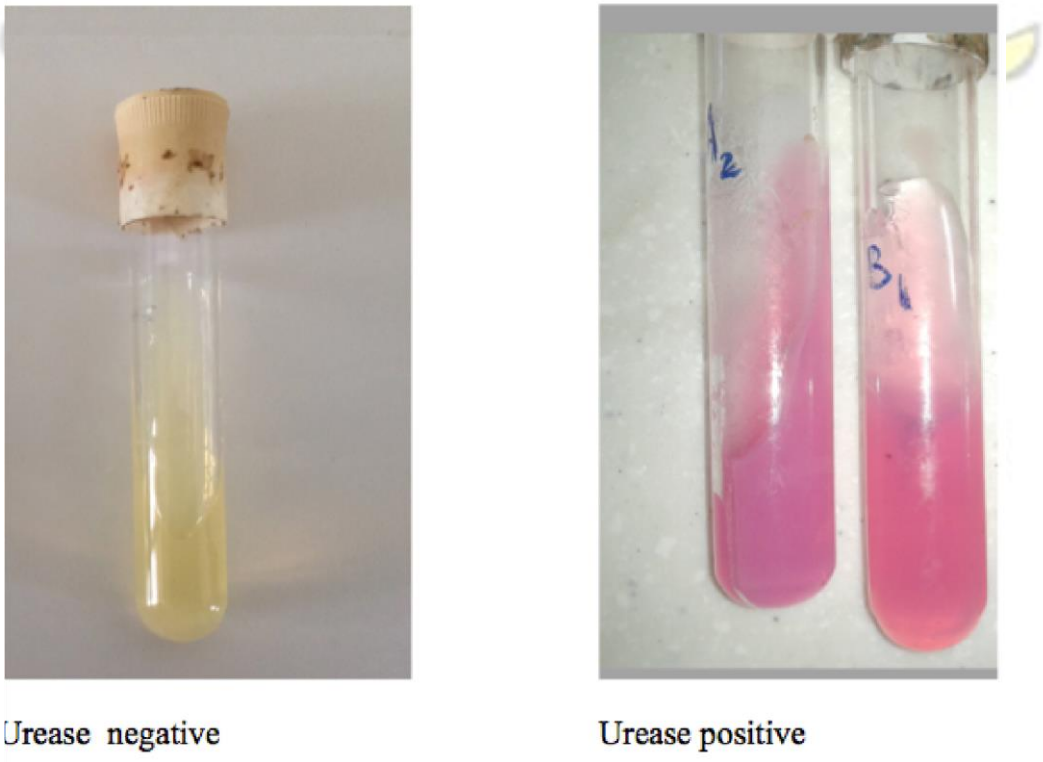


Plate 5.3: Urease test for *H. pylori* showing negative and positive results

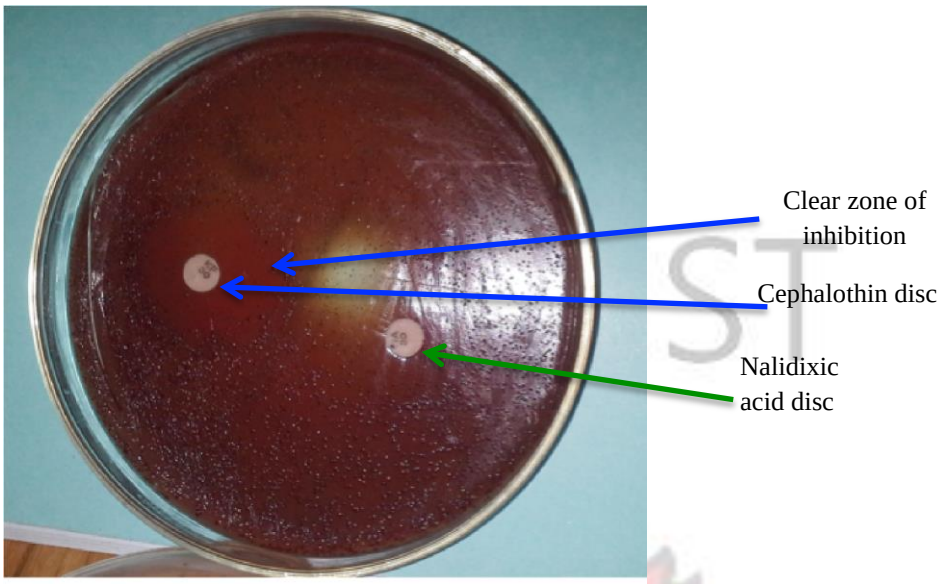


Plate 5.4: Results from susceptiblity test of *H. pylori* using nalidixic acid and cephalothin antibiotic disc showing clear zones of inhibition around the antibiotic discs.

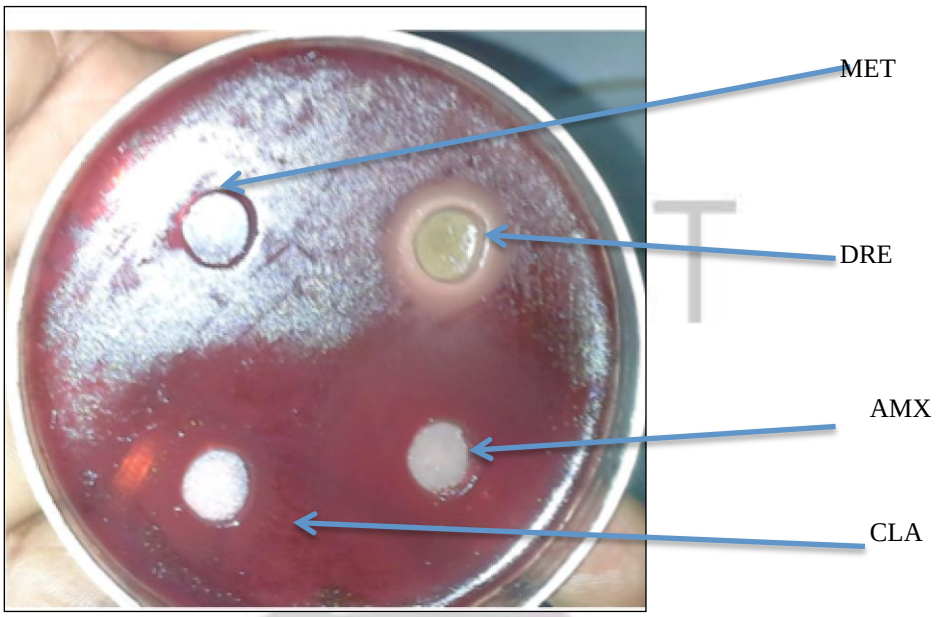


Plate 5.5: Inhibitory effects of *Dissotis rotundifolia* extract and different standard antibiotics on growth of *Helicobacter pylori* on BHI agar. *Dissotis rotundifolia* extract (DRE), Amoxicillin (AMX), Clarithromycin (CLA), Metronidazole (MET)

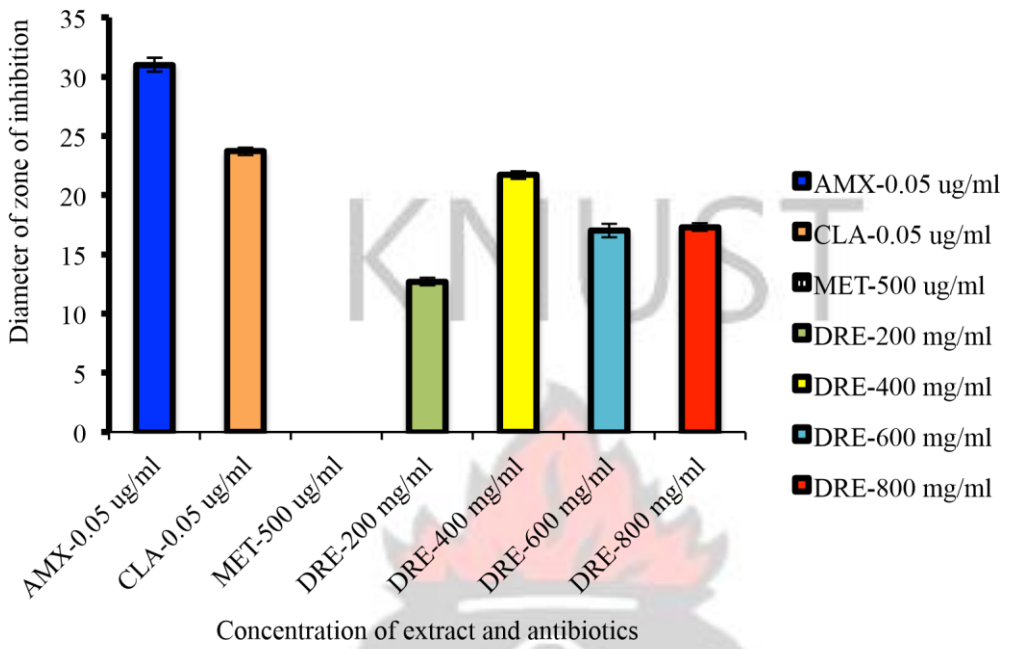


Fig. 5.1: Anti-*Helicobacter pylori* activity of *Dissotis rotundifolia* extract and antibiotics. DRE- *Dissotis rotundifolia* extract, CLA- Clarithromycin, AMX- Amoxicillin, MET- Metronidazole

5.4 DISCUSSION

H. pylori is a major aetiological agent of peptic ulcer diseases (NIH consensus Development Panel, 1994). The organism has been reported to have developed resistance against some antibiotics, especially metronidazole, limiting its use in the treatment (Stamatisa *et al*., 2003; Ndip *et al*., 2008; Dube *et al*., 2009; Tanih *et al*., 2010). Other factors including poor patient compliance, the high cost of combination therapy, and the non-availability of medications in rural areas, especially in remote areas of Africa, supports the need to develop alternative approaches to prevent or / and cure the infection.

The use of natural product could be an attractive alternative treatment regime for *H. pylori* infected individuals (Calvet *et al*., 2000). Garlic and honey have been found to be valuable in the treatment of *H. pylori* infections (Al Somal *et al*., 1994;

Mahady and Pendland, 2000). Medicinal plants such as *Thymus kotschyanus*, *Calophyllum brasiliense* (Camb), *Ageratum conyzoides* (Linn), *Scleria striatinux* (De Wild), *Mallotus phillipinese* (Lam) Muell, *Myrisctica fragrans* Houtt, *Phyllanthus urinaria* (Linn.), *Lycopodium cernua* (Linn) Pic. Serm and *Curcuma amada* Roxb, have shown potential against *Helicobacter pylori* infections (Mahady *et al*., 2005; Ndip *et al*., 2007).

Although in this study, DRE showed statistically lower zones of inhibition compared to standard drugs [amoxicillin and clarithromycin] ($p<0.05$), the results corroborate the reported ethnomedicinal use in managing gastric disturbances. In this study, the *H. pylori* showed resistance to metronidazole, confirming previous reports by Ndip *et al*., 2008; Dube *et al*., 2009 and Tanih *et al*., 2010. Some flavonoids and isoflavonoids isolated from licorice such as licochalcone A, licoisoflavone B, and gancaonols have been reported to inhibit growth of *H. pylori* (Fukai *et al*., 2002). C-glycosylflavones compounds namely vitexin and isovitexin have been isolated from the methanolic extract of the whole plant *Dissotis rotundifolia* (Rath *et al*., 1995). Vitexin and isovitexin have also been isolated from the leaves of *Piper carpunya* and have been reported to possess pharmacological activity aganist growth of *H. pylori* (Quílez *et al*., 2010). Implictily the presence of these compounds in *Dissotis rotundifolia* plant extract may be contributing to the inhibitory effects of the plant on *H. pylori* observed in this study.

The smaller zone of inhibition demonstrated by the crude extracts *in vitro*, compared to standard drugs does not necessarily imply that the extract demonstrates weak antimicrobial effects *in vivo*. Bever (1986) and Garcia *et al*. (2003) had demonstrated immuno-modulation of chemical compounds isolated from medicinal plants, many of which had been proven to be inactive or weakly active *in vitro* against pathogens. Also, as with some drugs, some of these plants may be more potent *in vivo* due to metabolic biotransformation of their components into highly active intermediates (Ngemenya *et al*., 2006).

5.5 CONCLUSION

Dissotis rotundifolia extract inhibits the growth of *Helicobacter pylori* clinical isolates. This finding support the antiulcer activity of the extract.

Chapter 6

ANTIOXIDANT EFFECTS OF METHANOLIC EXTRACT OF *DISSOTIS ROTUNDIFOLIA*

6.1 INTRODUCTION

Earlier in chapters 3 and 4, it was shown that DRE has an antiulcer property and acts by inhibiting H^+ / K^+ - ATPase pump and increasing mucin activity. In chapter 5, it was demonstrated that DRE inhibits the growth of *H. pylori*. Several reports suggest that peptic ulcer development due to abuse of NSAIDs and alcohol and infection caused by *H. pylori* is mediated through the generation of reactive oxygen species especially hydroxyl radical (Akyon, 2002; Bandyopadhyay *et al*., 2000). Therefore an antiulcer drug which possesses the ability to inhibit growth of *H. pylori* with an antioxidant activity coupled to its H^+/K^+-ATPase inhibitory potential and mucin activity could be an ideal antiulcer agent. Whether DRE has an antioxidant activity in addition to the observed actions is unknown. This chapter seeks to assess the antioxidant potential of DRE *in vitro* and *in vivo* as part of an effort in determining the possible mechanism of action of the plant extract.

6.2 MATERIALS AND METHODS

Nitro blue tetrazolium (NBT), phenazonium methosulphate (PMS), naphthylenediamine dichloride, phosphoric acid, reduced glutathione (GSH), 5, 5^1-Dithiobis (2-nitrobenzoic acid) (DTNB), nicotinamide adenine dinucleotide (NADH), phenazine methosulphate (PMS), were obtained from Sigma Chemical Company, USA. Iron, EDTA, DMSO, ammonium acetate, glacial acetic acid, and acetyl acetone, Trichloroacetic acid (TCA) and sulphanilamide were obtained from British Drug House, England.

6.2.1 Determination of *in vitro* antioxidant activity

6.2.1.1 Hydroxyl radical scavenging activity

Hydroxyl radical scavenging activity was estimated by generating the hydroxyl radical *in vitro* using ascorbic acid-iron-EDTA solution. The hydroxyl radicals formed by the oxidation process react with DMSO to yield formaldehyde. The formaldehyde formed produces intense yellow colour with Nash reagent (Mohan-Kumari *et al*., 2011).

$$HO^{\cdot} + (CH_3)_2SO \longrightarrow H_2CO + \text{Nash reagent}$$

The intensity of the yellow colour formed is reduced in the presence of an antioxidant and the absorbance is measured at 412 nm. Hydroxyl radical is neutralized by an antioxidant compound to form water.

$$HO^{\cdot} + \text{H-Antioxidant} \longrightarrow H_2O$$

The hydroxyl radical scavenging effects of the DRE and standards (citric acid and EDTA) were determined according to the method described by Klein *et al.,* (1991). Accurately, 0.5 mL of various concentrations (20 - 200 µg/mL) of DRE and standards were mixed individually, with 1.0 mL of iron-EDTA solution (0.13 % ferrous ammonium sulphate and 0.26 % EDTA all mixed in a ratio of 1:1).

Then, 0.5 mL of EDTA solution (0.018 %), and 1.0 mL of DMSO (0.85 % v/v in 0.1 M phosphate buffer, pH 7.4) were added. The reaction was initiated by adding 0.5 mL of ascorbic acid (0.22 %) and incubated at 80 - 90°C for 15 mins. After incubation, 1.0 mL of ice-cold TCA (17.5 % w/v) was added to terminate the reaction. Then, 3 mL of Nash reagent (75.0 g of ammonium acetate, 3.0 mL of glacial acetic acid, and 2.0 mL of acetyl acetone mixed and the volume raised to 1 L with distilled water) was added and left at room temperature for 15 mins. All experiments were conducted in triplicate. The reaction mixture without the test sample was used as a control. The intensity of the chromophore formed was

measured spectroscopically at 412 nm against an appropriate reagent blank which contains all constituents except ascorbic acid.

The percentage inhibition of hydroxyl radical was evaluated using the formula:

$$\% \text{ inhibition of hydroxyl radical} = X\ 100 \frac{[\text{Abs control} - \text{Abs sample}]}{[\text{Abs control}]}$$

Where Abs is absorbance

6.2.1.2 Superoxide (SO) anion-scavenging activity

Superoxide anion radical scavenging assay was based on the reduction of nitro blue tetrazolium (NBT) in the presence of NADH and phenazonium methosulphate (PMS) under aerobic condition (Nishikimi *et al*., 1972). The nonenzymatic phenazine methosulfate–nicotinamide adenine dinucleotide (PMS/NADH) coupling system generates superoxide anion radicals, which reduce nitro blue tetrazolium (NBT) to a purple formazan.

The superoxide anion scavenging activity was measured as described by Robak and Gryglewski (1988). The superoxide anion radicals were generated in 3.0 mL of Tris-HCl buffer (16 mM, pH 8.0), containing 0.5 mL of NBT (0.3 mM), 0.5 mL NADH (0.936 mM) solution, 1.0 mL extract or standards (20 - 200µg/mL) in Tris-HCl and 0.5 mL Tris-HCl buffer (16 mM, pH 8.0). The reaction was started by adding 0.5 mL PMS solution (0.12 mM) to the mixture, incubated at 25°C for 5 mins and then the absorbance change was measured at 560 nm against a blank sample containing Tris-HCl only. The tests were done in triplicate and the mean calculated. Ascorbic acid was used as standard antioxidant. The decrease in absorbance at 560 nm as concentration increased was measured against an appropriate blank to determine the quantity of formazan generated.

The percentage of inhibition of superoxide anion was computed using the following equation:

$$\% \text{ inhibition of superoxide radical} = \frac{[\text{Abs control} - \text{Abs sample}]}{[\text{Abs control}]} X\ 100$$

Where Abs is absorbance

6.2.1.3 Nitric oxide radical scavenging activity

Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide (NO) which interacts with dissolved oxygen to produce nitric and nitrous acids. This method is based on the inhibition of nitric oxide radical generation from sodium nitroprusside. The liberated nitric and nitrous acid reacts with Griess reagent to form a purple azo dye. In the presence of antioxidants, the amounts of nitric and nitrous acids decreases and the degree of this reduction in formation of purple azo dye reflect the extent of scavenging measured spectrophotometrically at 540 nm (Marcocci *et al.*, 1994). Thus, scavengers of nitric oxide compete with oxygen, leading to reduced production of nitrite ions and formation of a pink chromophore

$$Na_2[Fe(CN)_5NO]\cdot 2H_2O \longrightarrow NO + \text{oxygen} \longrightarrow HNO_3 + HNO_2$$

The nitric oxide radical scavenging activity was determined according to the method described by Green *et al.*, (1982) with slight modifications. A volume of 2 mL of 10 mM sodium nitroprusside which was prepared in 0.025 M of phosphate buffer (pH 7.4) was individually mixed with 0.5 mL of DRE and standards, (gallic acid and ascorbic acid) at concentrations of (20-200µg/mL). The mixture was incubated at 25 °C for 150 mins. A volume of 0.5 mL of the incubated solution was mixed with 0.5 mL of Griess reagent (prepared by mixing in a ratio 1:1, 1 % w/v

sulphanilamide reagent prepared in distilled water and heated on a water bath at 100 ^{0}C and 0.1 % w/v naphthylenediamine dichloride dissolved in 5 % phosphoric acid). The mixture was further incubated at room temperature for 30 mins after which the absorbance of the chromophore formed during diazotization of nitrite with sulphanilamide and its subsequent coupling with naphthylenediamine dichloride was measured at 540 nm against a phosphate buffer blank. Control experiment without the test sample but with equivalent amount of the solvent used to dissolve the sample was conducted in an identical manner. All experiments were conducted in triplicate.

$$\% \text{ inhibition of nitric oxide radical} = X\ 100 \frac{[\text{Abs control} - \text{Abs sample}]}{[\text{Abs control}]}$$

6.2.1.4 DPPH-radical scavenging activity (Hydrogen donating ability) DPPH radical-scavenging activity has been widely used to assess the *in vitro* antioxidant activity of crude plant extracts. DPPH is a stable free radical at room temperature which acts as hydrogen radicals acceptor to become a stable diamagnetic molecule (Soares *et al*., 1997). This assay involves hydrogen atom transfer processes in alcoholic solution in the presence of a hydrogen-donating antioxidant (Kaviarasan *et al.,* 2007). A stable, deep violet or purple 2, 2diphenyl-1-picrylhydrazyl radical is reduced to a yellow 1, 1-diphenyl-2picrylhydrazine after reacting with hydrogen donating antioxidants:

$$DPPH\bullet + AH \longrightarrow DPPH\text{-}H + A\bullet$$

As 2, 2-diphenyl-1-picrylhydrazyl radical picks up a hydrogen atom in the presence of a free radical scavenger, the absorption decreases resulting in a colour change from purple to yellow.

The method of Blois (1958) with modification was employed in determining the scavenging ability of extract to DPPH free radical. Approximately 1.0 mL of DPPH (0.135 mM) prepared in absolute methanol was mixed with 1.0 mL of dissolved extracts and standards (ascorbic acid and gallic acid) of concentration ranging from (20- 200 µg/mL). The reaction mixture was shaken vigorously and left in the dark at room temperature for 30mins. The absorbance was measured spectrophotometrically at 517 nm against a reagent blank containing only methanol. All experiments were conducted in triplicate. Control experiment contained only methanol and DPPH free radical.

The percentage scavenging activity of DPPH-radical was calculated using the formula:

$$\% \text{ inhibition of DPPH radical} = X\ 100 \frac{[\text{Abs control} - \text{Abs sample}]}{[\text{Abs control}]}$$

6.2.1.5 Determination of anti-lipid peroxidation

A thiobarbituric acid-reactive species (TBARS) method described by (Dasgupts and De, 2004) was used to measure the lipid peroxide formed using egg-yolk homogenates as a lipid-rich media. About 0.5 mL of egg yolk homogenate (10% v/v in distilled water) and 0.1 mL of the extract and standard (20-200 µg/mL) were mixed separately in a test tube and 1 mL of distilled water was added. Finally, 0.05 mL Fe_2SO_4 (0.07 M) was added to the mixture and incubated for 30 mins to provoke lipid peroxidation. Thereafter, 1.5 mL of 20 % acetic acid (pH adjusted to 3.5 with NaOH) and 1.5 mL of 0.8 % TBA (w/v) (prepared in 1.1 % sodium dodecyl sulfate) and 0.05 mL 20% TCA were added, vortexed and heated in a boiling water bath at 100 ^{0}C for 60 mins.

To eliminate non-malonaldehyde (MDA) interference, another set of samples was treated in the same way, but incubating without TBA, so as to subtract the absorbance of the non-MDA interference from the test and standard absorbance. After cooling, the coloured TBA–MDA complex was extracted with 5 mL butanol by vigorously shaking and centrifuging at 3000 x g for 10 min. The absorbance of the organic upper layer was determined at 532 nm using a spectrophotometer.

For control, 0.1 mL of distilled water was used instead of the extract or standard.

$$\% \text{ antilipid peroxidation} = \frac{[\text{Ao} - \text{A1}]}{[\text{Ao}]} X\ 100$$

where A_0 is the absorbance of the control reaction and A_1 is the absorbance in the presence of the sample or standard. Where Ao is the absorbance value of the fully oxidized control and A_1 is $\{(A_{532} + \text{TBA})–(A_{532}– \text{TBA})\}$.

6.2.2 Determination of *in vivo* antioxidant property

6.2.2.1 Preparation of gastric homogenate from the stomach of EtOH-induced experimental rats

Stomach tissue mucosal scrapings obtained from the rat stomach of absolute EtOH-induced gastric ulcer experiment (chapter 3) was used for the determination of antioxidant enzymes (superoxide dismutase, catalase), reduced glutathione (GSH) and malondialdehyde (MDA).

The mucosal scrapings was homogenized for 30 s in 0.9 % ice cold saline, in (1:10 w/v) and was centrifuged initially at 800 x g for 10 mins and later at 12,000 x g for 15 mins at 4 ^{0}C . The clear supernatant collected containing the mitochondrial fraction was used for the following estimations.

6.2.2.2 Determination of reduced glutathione (GSH)

Reduced glutathione (GSH) was determined according to the method described by

Ellman 1957. Aliquots 0.1 mL of 10 % tissue homogenate were mixed with 2.4 mL of 0.02 M EDTA solution and kept on ice bath for 10 mins. Then 2 mL of distilled water and 0.5 mL of trichloroacetic acid (TCA) 50 % (w/v) were added and centrifuged at 3000 × g for 20 mins at 4 ^{0}C to remove precipitate. The supernatants 1 mL were mixed with 2.0 mL of Tris buffer (0.4 M, pH 8.9) and 0.05 mL of 5' - dithiobisnitro benzoic acid (DTNB) solution – Ellman's reagent (10 mM) was added and vortexed thoroughly. The absorbance was measured at 412 nm against a reagent blank with no homogenate after addition of DTNB and incubation at room temperature for 5 mins. The glutathione values were extrapolated from a glutathione standard curve and expressed as μM/mg of protein.

6.2.2.3 Determination of superoxide dismutase (SOD) activity

Superoxide dismutase activity was measured as described by Kakkar *et al*. (1984).

It is based on the inhibition of the formation of nicotinamide adenine dinucleotide (NAD), phenazine methosulfate (PMS) and amino blue tetrazolium formazan. In the presence of SOD, NBT reduction is inhibited because the enzyme converts the superoxide radical to peroxide. SOD activity was determined at 560 nm using a spectrophotometer. Enzyme activity leading to 50 % inhibition was considered as 1 unit /mg protein.

Tissue homogenate (0.5 mL) was mixed with 0.4 mL of ethanol and chloroform mixture (1:1) and centrifuged. To the supernatant, assay mixture, sodium pyrophosphate buffer (0.052 M, pH 8.3), phenazine methosulphate (186μM), nitroblue tetrazolium (300 μM) and reduced nicotinamide adenine dinucleotide (NADH) (780 μM) was added and incubated at 30 ^{0}C for 3 mins. The reaction was arrested by the addition of 1 mL glacial acetic acid. The reaction mixture was shaken with n-butanol, allowed to stand for 15 mins and centrifuged. The intensity of the colour developed in butanol layer was measured at 560 nm using butanol as blank with a spectrophotometer. The results are expressed as the quantity of SOD

necessary to inhibit the rate of NBT reduction by 50 % in units of enzyme per milligram of protein (mmol/min/mg protein). The enzyme activity was calculated using the following equations.

$$\text{Rate } (R) = \frac{\text{Final Absorbance} - \text{initial absorbance}}{3 \text{ mins}}$$

$$\% \text{ Inhibition} = X\ 100 \frac{\text{Absorbance of blank} - R}{\text{Absorbance of blank}}$$

$$1 \text{ Enzyme unit } (U) = \frac{\% \text{ Inhibition}}{50} X \text{ common dilution factor}$$

50 % inhibition =1 enzyme unit

$$\text{Specific activity } (\text{units /mg protein}) = \frac{\text{activity in units of enzyme}}{\text{mg protein}}$$

6.2.2.4 Determination of catalase (CAT) activity

Catalase (CAT) was assayed colorimetrically at 620 nm as described by the method of Sinha 1972. It is based on the ability of the enzyme source to break down H_2O_2. The reaction mixture of 1.5 mL contained 1.0 mL of 0.01 M, pH 7.0 phosphate buffer, 0.1 mL of tissue homogenate (supernatant) and 0.4 mL of 2.0 M H_2O_2. The reaction was stopped by the addition of 2.0 mL of dichromate-acetic acid reagent (5 % potassium dichromate and glacial acetic acid mixture 1:3). One unit of CAT was defined as the amount of enzyme required to decompose 1mmol of H_2O_2 per minute, at 25 ^{0}C (pH 7.0). Results obtained were expressed as units (U) of CAT activity/mg protein or mmol/min/mg protein .

Moles of H_2O_2 consumed/min (units/mg protein)=

$2.3/\Delta t$ x ln (ABS initial /ABS final) x 1.63x 10^{-3}

ABS = Absorbance at 620 nm,

Δt = time required for a decrease in the absorbance.

6.2.2.5 Malondialdehyde (MDA) determination

The lipid peroxidation (LPO) was estimated in gastric mucosal supernatant in terms of malondialdehyde (MDA) content which was determined as per the method of Ohkawa et al. (1979). MDA levels were determined by the MDA- Thiobarbituric acid (TBA) test. In this method, TBA reacts with MDA, a secondary by-product from lipid peroxidation, and generates a red / pink colour, which is detected spectrophotometrically. This method is used to indicate the extent of lipid peroxidation in biological systems (Shlafer and Shepard, 1984).

To 0.2 mL of supernatant of tissue homogenate, 0.2 mL of 8.1% SDS, 1.5 mL of 20 % acetic acid and 1.5 mL of 0.8 % TBA were added. The mixture was diluted to 4 mL with distilled water and heated in a water bath at 95 $^{\circ}C$ for 60 mins. The mixture was left to cool and sample was extracted with butanol; 1mL of water and 5 mL of n-butanol were added, shaken vigorously and centrifuged at 4000 rpm for 10 mins. The supernatant (organic layer) was collected and its absorbance measured at 532 nm. The procedure was repeated using 1,1,3,3-tetramethoxypropane as standard and the level of MDA measured was expressed as nmol MDA/g of tissue.

6.2.3 Data analysis

Statistical analysis was performed on data using the Grap Pad prism version 4.0. ANOVA was used to determine differences in mean values in percentage scavenging activity and enzymatic and non enzymatic activities. Values were

considered significant when $P<0.05$ followed by Bonferroni's pairwise posthoc test where there was a significant difference amongst means.

6.3 RESULTS

6.3.1 *In vitro* antioxidant activity

6.3.1.1 Hydroxyl radical-scavenging activity

In this study the hydroxyl scavenging ability of the EDTA, citric acid and DRE at 200 µg/mL were 93±2.28, 90±0.32, and 63±1.28 % respectively as shown in Figure 6.1. The concentrations of EDTA, citric acid and DRE needed for 50 % inhibition of hydroxyl radical generated from reaction mixture was found to be 143.3±0.75, 143.3±0.42, 106.8±1.70 µg/mL respectively.

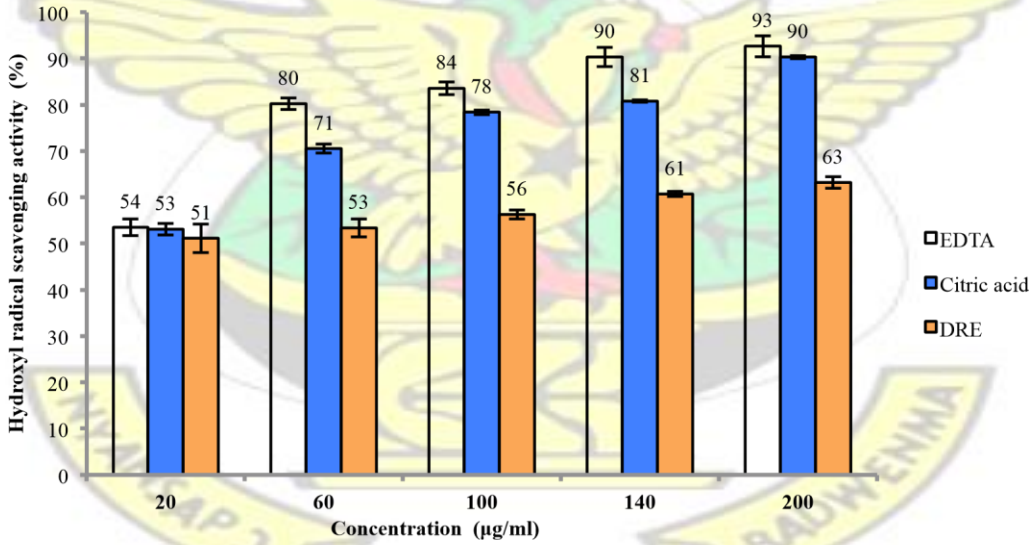


Figure 6.1. Hydroxyl radical scavenging activity of methanolic extract of *Dissotis rotundifolia* and standards at different concentrations. Each value represents mean ± SEM (n=3)

6.3.1.2 Superoxide (SO) anion-scavenging activity

Fig. 6.2 shows the superoxide anion-scavenging effect of ascorbic acid, gallic acid and DRE on the PMS/NADH–NBT system. The decrease of absorbance at 560 nm with increase in concentration of test substances observed in experimental assay (data not shown) denotes a depletion of superoxide anion in the reaction mixture to reduce NBT to a purple coloured formazan. At the concentration of 200 µg/mL, percentage inhibition of superoxide anion radical by DRE was observed to be 30.40±1.28, in comparison with ascorbic acid and gallic acid with percentage inhibition found to be 49.0±2.28 and 49.2±0.32 respectively at maximum concentration (Fig 6.2). The IC_{50} values of ascorbic acid, gallic acid and DRE were found to be 103.8±0.44, 108.8±0.76 and 103.8±0.44 µg/mL respectively.

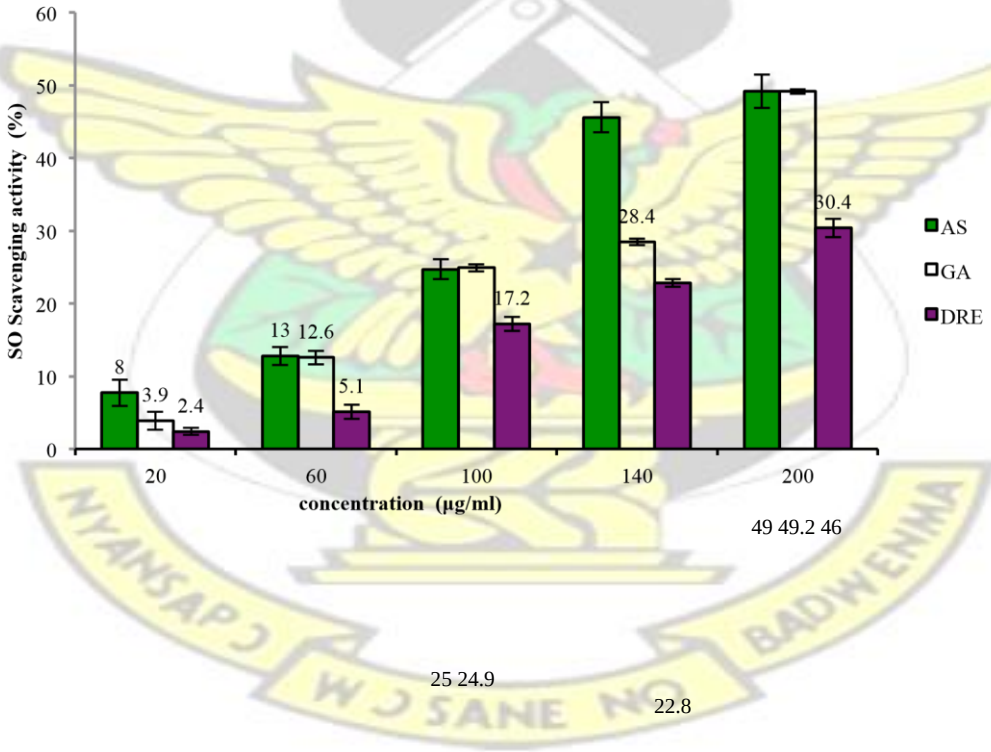


Figure 6.2. Superoxide anion scavenging activity of methanolic extract of *Dissotis rotundifolia* and standards at different concentrations. Each value represents mean ± SEM (n=3)

6.3.1.3 Nitric oxide (NO) radical-scavenging activity

Fig. 6.3 illustrates the percentage inhibition of NO generation by ascorbic acid, gallic acid and DRE. At 200 µg/mL, the percentage inhibition of ascorbic acid, gallic acid and DRE were found to be 81±0.34, 74±0.47 and 56±0.64 respectively, and their respective IC_{50} values were 56.95±0.32, 78.27±0.87 and 40.71±1.21 µg/mL.

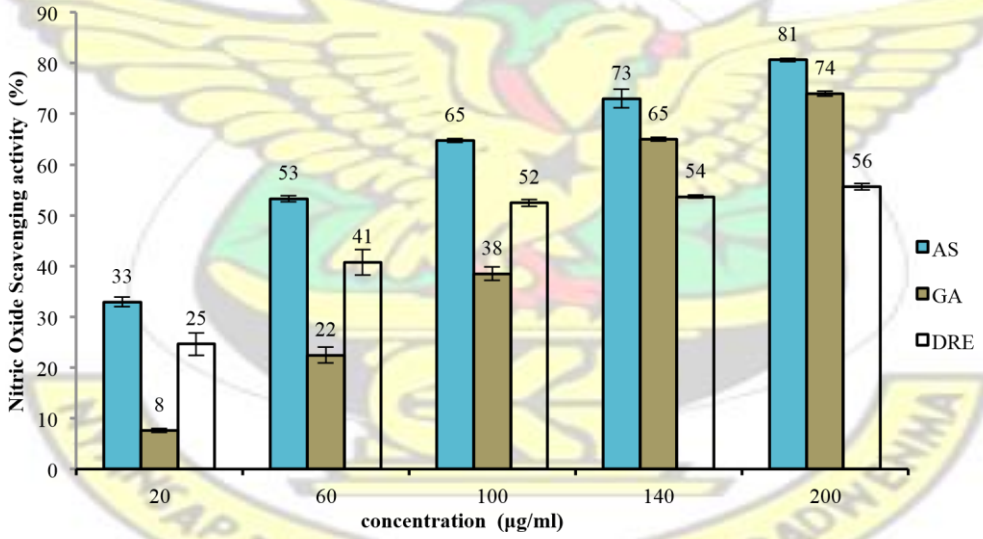


Figure 6.3. Nitric oxide scavenging activity of methanolic extract of *Dissotis rotundifolia* and standards at different concentrations. Each value represents mean ± SEM (n=3)

6.3.1.4 DPPH scavenging activity

The scavenging properties exhibited by ascorbic acid, gallic acid and DRE are shown in Fig. 6.4. At the concentration of 200 µg/mL, the scavenging property of DRE on the DPPH radical was 83±1.28 %, when compared to the 98±0.32 % and 92±2.28% for gallic and ascorbic acid respectively. The IC_{50} values of 40.07±0.48, 7.25±0.01 and 2.45±0.02 were recorded for DRE, ascorbic acid and gallic acid.

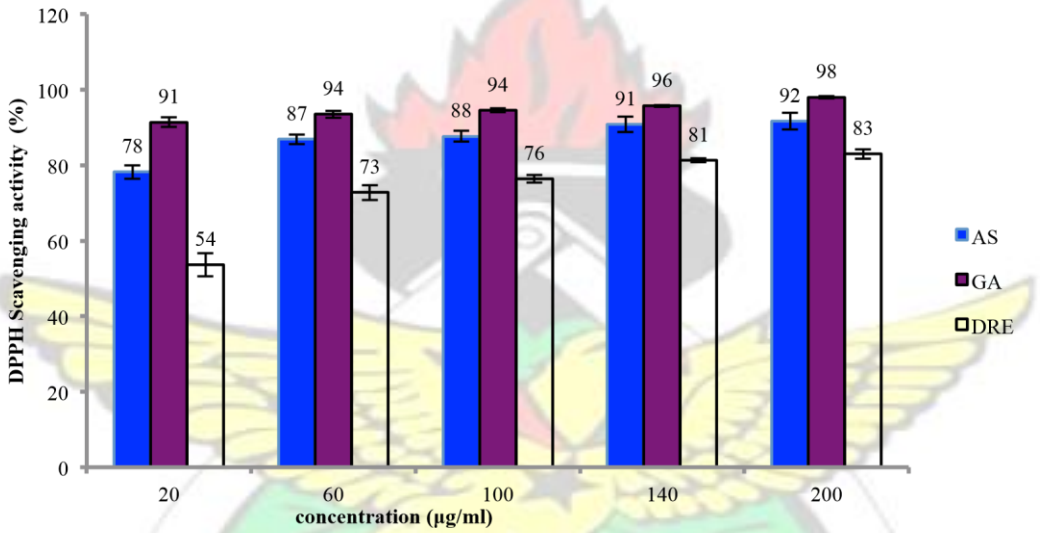


Figure 6.4. DPPH scavenging activity of methanolic extract of *Dissotis rotundifolia* and standards at different concentrations. Each value represents mean ± SEM (n=3)

6.3.1.5 Anti-lipid peroxidation

Figure 6.5 illustrates the anti-lipid peroxidation activity of the extract, ascorbic acid and gallic acid in Fe^{2+} ascorbate system by inhibiting the formation of malondialdehyde. At the concentration of 200 µg/mL the inhibitory effect of DRE extract, gallic acid and ascorbic acid in the formation of malondialdehyde were

78±1.28, 73±0.32 and 88±2.28 % respectively. The IC_{50} values of DRE, gallic acid and ascorbic acid was observed to be 49.9±2.23, 119.3±1.87 and 52.23±2.34 µg/mL respectively.

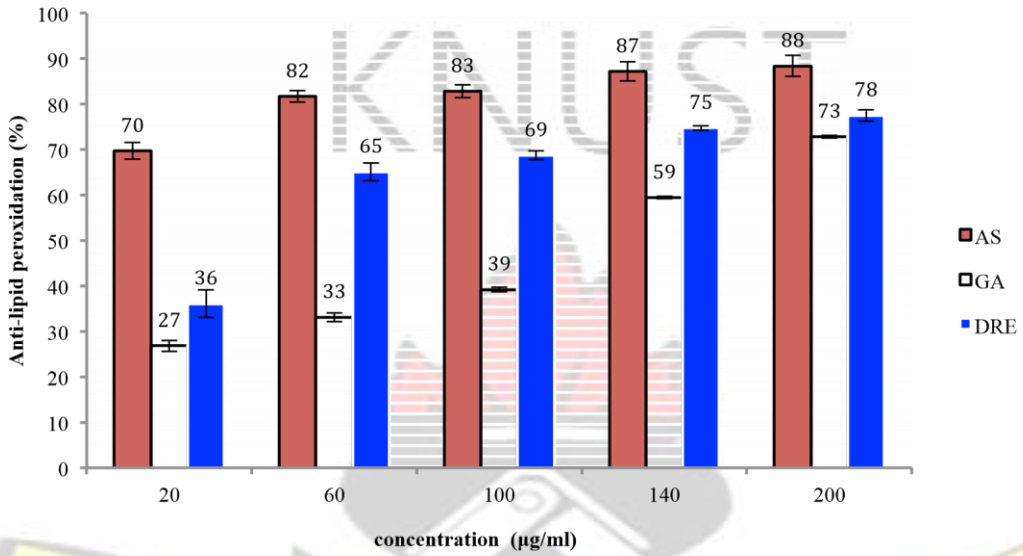


Figure 6.5. Anti-lipid peroxidation activity of methanolic extract of *Dissotis rotundifolia* and standards at different concentrations. Each value represents mean ± SEM (n=3)

6.3.2 *In vivo* antioxidant activity

The antioxidant characteristics of DRE were investigated *in vivo* using ethanol induced ulcer model. The parameters measured included MDA, CAT, SOD and GSH.

Table 6.1: Effect of whole plant extract of *Dissotis rotundifolia* (DRE) and omeprazole on the levels of MDA, CAT, SOD, and GSH in gastric mucosa of rats

Treatment (mg/kgbwt)	**MDA**	**CAT**	**SOD**	**GSH**
	(nmol/mg protein)	***(U/mg protein)***	***(U/mg protein)***	***(µM/mg protein***

1mL distilled water	2.49±0.08[a]	45.00±3.08[a]	121±3.34[a]	17.85±0.14[a]
EtOH only	4.73±0.08	22.40±1.11	61.6±0.92	10.48±0.27
DRE+EtOH	3.05±0.05[a]	34.80±1.38 [a]	100.2±4.98 [a]	14.11±0.04[a]
Omeprazole+EtOH	3.08±0.01[a]	32.20±1.78[a]	109.6±5.80[a]	13.97±0.04[a]
DRE only	2.24±0.11[a]	46.80±1.66 [ab]	97.20±1.08 [a]	16.11±0.04 [ab]
Omeprazole only	2.30±0.11[a]	44.80±1.78[ab]	95.40±2.22[a]	15.34±0.21[ab]

Values are expressed as mean ± Standard error of mean (SEM) for 5 animals in each group (a) Significant when group was compared to negative control, (b) Significant when group was compared to omeprazole and DRE pretreated group The effects of DRE on the gastric mucosa antioxidant status was examined *in vivo*. There was an observation of substantial increase in CAT and SOD activities in DRE and omeprazole pretreated group compared to negative control ethanol group ($p< 0.05$) (Table 6.1).

In addition, the effect of the DRE on the total GSH in gastric mucosal homogenates was assessed. Ethanol treatment caused a significant depletion of GSH in group that received no DRE pretreatment compared to normal control ($p< 0.05$). In contrast, drug pretreated group exhibited significant augmentation of GSH content (Table 6.1). The results in this study indicate that DRE significantly inhibited the effects of ethanol on gastric GSH depletion.

In the present study, MDA levels were measured in pretreated ethanol-induced ulcer rats, and it was found that ethanol-induced ulcer showed increased MDA. The levels of MDA in the gastric tissue of rats pretreated with DRE was significantly decreased when compared to the negative control rats ($p< 0.05$).

6.4 DISCUSSION

The preceding chapters provided evidence that DRE protects the stomach of rats from ulcerogens such as cold stress, acetylsalicylic acid and ethanol. It was demonstrated that the antiulcer effect of the extract is mediated through inhibition

of *Helicobacter pylori* and H^+ / K^+ - ATPase, increasing mucin activity and also a cholinergic receptor antagonist action.

Oxidative stress is implicated in the mechanism of acute and chronic ulceration in gastric mucosa, and scavenging free radicals is among the mechanisms reported to be involved in the healing of gastric ulcers (Salim, 1991; Hung and Wang, 2004). In this chapter, the contribution of DRE in ulcer protection as an antioxidant was investigated *in vitro* and *in vivo*. Free radical scavenging agents from natural sources are the only alternatives to synthetic antioxidants in reducing the free radical mediated diseases such as peptic ulcers. Lately, many phytochemicals and their effect on health, especially the suppression of free radicals are being studied. Several *in vitro* and *in vivo* models are used, but in this study, a few namely OH, DPPH, SO and NO test models were employed in order to assess the ability of the extract to scavenge free radicals reported to be involved in peptic ulcer development. In the case of the *in vivo* studies, only ethanol model was used since ethanol is well known to release superoxide anion, hydroxyl and hydroperoxy free radicals when it is metabolized (Glavin and Szabo, 1992 ; Schlorff *et al*., 1999).

The hydroxyl radical is a known reactive free radical formed in biological systems (Aurand, 1977). It has been implicated as a highly damaging species in free radical pathology, capable of damaging almost every molecule found in living cells (Ajitha and Rajnarayana, 2001). Amongst the oxygen radicals, the hydroxyl radical is the most reactive and provokes severe damage to adjacent biomolecules. It can conjugate nucleotides in DNA and cause strand breakage which ultimately leads to mutagenesis, carcinogenesis and cytotoxicity. The hydroxyl radical scavenging assay shows the ability of a potential drug to inhibit hydroxyl radical generated *in vitro* using ascorbic acid-iron-EDTA solution. The concentration of DRE needed for 50 % inhibition of hydroxyl radical was found to be lower than citric acid and EDTA which are standards used in hydroxyl radical assays. This result suggests

that DRE is a more effective scavenger of hydroxyl radical than citric acid and EDTA.

Superoxide anion is also very harmful to cellular components and produced from molecular oxygen due to oxidative enzyme of body as well as via non-enzymatic reaction such as autoxidation by catecholamine (Naskar *et al.,* 2010). Involvement of free radicals such as superoxide anion is well known to be involved in the pathogenesis of ischaemic injury of gastrointestinal mucosa, and also mucosal damage induced by non-steroidal anti-inflammatory drugs, ethanol, starvation stress and by *Helicobacter pylori* infection (Mutoh *et al*., 1990; Phull *et al*., 1995; Malfertheiner *et al*., 2009; Matsui *et al*., 2011). In this study the superoxide radicals generated from dissolved oxygen by PMS-NADH coupling assay was measured by their ability to reduce NBT. The decrease in absorbance at 560 nm indicates the ability of test substances to scavenge superoxide radicals in the reaction mixture. The IC_{50} obtained for DRE in this study indicate that DRE is a more potent scavenger of superoxide radicals than ascorbic acid and gallic acid.

Nitric oxide is a potent pleiotropic mediator generated from amino acid Larginine by vascular endothelial cells, phagocytes and some brain cells. It reacts with oxygen or superoxides to form highly reactive compounds such as NO_2, N_2O, N_3O and NO_3 which are involved in alteration of structural and functional characteristics of many cell components. Acute exposure to NO is directly toxic to tissues and leads to vascular collapse with septic shock, whereas chronic exposure triggers various carcinomas and inflammatory conditions such as arthritis and ulcerative colitis (Tylor *et al*., 1997). The toxicity of nitric oxide becomes adverse when it reacts with superoxide radical, producing a highly reactive peroxynitrite anion (ONOO). In the assay used in this study, the nitric oxide generated from sodium nitroprusside reacts with oxygen to form nitrite. The nitrite ions diazotize with sulphanilic acid and couple with naphthylethylenediamine forming a pink colour, whose absorbance can be measured with a spectrophotometer at 546 nm. In the

present study, the extract competes with oxygen to react with nitric oxide and thus inhibit generation of the anions. The results indicate that DRE is a more potent scavenger of NO than gallic acid and ascorbic acid.

In the DPPH test model, DPPH radical was used as substrate to evaluate the free radical scavenging capacity of the DRE extract. It involves the reaction of specific antioxidant with a stable free radical and results in a reduction in absorbance which can be measured at 517 nm. In the present study the extract at different doses demonstrated DPPH radical-scavenging activity ($p<0.05$) in comparison with the standard, indicating the ability of the extract to act as hydrogen radical scavenger. In the present investigation the extract at different doses showed some potential in scavenging hydrogen donated by DPPH though lower than both standards (gallic and ascorbic acid). This was confirmed by the EC_{50} values of 40.07, 7.25 and 2.45 for DRE, ascorbic acid and gallic acid respectively obtained from data analysis. This suggest that the extract has the potential of reducing and preventing oxidative stress related disease caused by hydrogen.

In vitro anti-lipid peroxidation was determined by means of an assay system which determines the production of malondialdehyde and related compounds in egg homogenate. Malondialdehyde (MDA) is one of the major degradative products of lipid peroxidation and is used as a marker for oxidative stress. Thiobarbituric acid reactive species (TBARS), the by-products of lipid peroxidation that happen in non-polar region of the biological membranes is involved in the free radical induced cellular damage (Halliwell *et al*., 1996) that lead to several diseases. The lower IC_{50} values obtained for DRE compared to standards indicate that DRE is a more potent scavenger of TBARS than standards (ascorbic acid gallic acid).

In assessing the effect of DRE on *in vivo* oxidants, only the ethanol induced ulcer model was used since several studies have reported on it as an appropriate model in determining *in vivo* antioxidant status (Adinortey *et al*., 2013; Megala and Geetha,

2010; Schlorff *et al*., 1999; Pihan *et al*., 1987). Ethanol-induced ulcer is a multiple mucosal defect, that is complicated by bleeding. The balance of offensive and defensive factors plays an important role in ulcer formation in the stomach. Furthermore, disturbances in antioxidant status such as SOD, CAT, GSH of rats have been seen after the administration of ethanol in a study by Salim, 1991.

Oxidative stress from free radicals have been implicated in the mechanism of gastric ulceration (Salim, 1991; Hung and Wang, 2004) This implies that scavenging these free radicals can facilitate the healing of gastric ulcers.

Enzymatic and non enzymatic defense mechanism plays a key role against gastric tissue damage and toxicity by ROS (Bradley *et al*., 1982). The enzymatic and non-enzymatic antioxidant defenses include superoxide dismutase, glutathione peroxidase, catalase, glutathione reductase, reduced glutathione β - tocopherol, vitamin C, β - carotene and vitamin A (Mates *et al*., 1999).

Lipid peroxidation (LPO) has been postulated to be one of the important factors in ulcerogenesis (Das and Banerjee, 1993). Hence effect of DRE on gastric lipid peroxidation was also investigated as an indicator of a possible defensive factor. Studies by Lucca *et al*., 2009 and Sairam *et al*., 2002 in rats have shown that ethanol induced ulcer is associated with increased lipid peroxidation (LPO) in gastric tissue. MDA which represents an end-product of the peroxidation of polyunsaturated fatty acids and related esters within cell membranes is considered as a reliable index of oxidative tissue damage (Gutteridge, 1995). In the present study, MDA levels were measured in pretreated ethanol-induced ulcer rats, and it was found that ethanol induced ulcer group without drug pretreatment showed increased MDA. In this negative control ulcer group, the MDA levels were seen to be higher than the normal control rats, denoting lipid peroxidation. The levels of MDA in the gastric mucosal tissue of rats pretreated with DRE were significantly decreased when compared to the negative control group. An increased level of LPO is due to increased generation of ROS during ethanol stress leading to oxidative damage.

This result suggests that the plant extract could improve the pathological condition of gastric ulcer disease by a reduction of lipid peroxidation.

SOD is considered as the first line of defense against the deleterious effects of oxygen radicals in the cell and it scavenges ROS by catalyzing the dismutation of superoxide to H2O2 (Okado-Matsumoto *et al*., 2001). SOD converts the reactive superoxide radical to H_2O_2, which if not scavenged by CAT, can trigger an increase LPO by the generation of hydroxyl radicals (Ajitha and Rajnarayana, 2001). A reduction in SOD, CAT and GSH levels can lead to increased accumulation of these ROS and thus increased LPO and tissue damage, whereas an increase in SOD, CAT, GSH levels can lead to decreased accumulation of these ROS and thus reduced LPO and subsequently less tissue damage in response to oxidative stress due to ethanol treatment.

In the present study it was found that ethanol-induced ulcer showed a decreased GSH, SOD and CAT levels in rats. This decrease in GSH, SOD and CAT levels may have led to an increase in accumulation of reactive oxygen species and thus, has caused increased lipid peroxidation and subsequently gastric tissue damage. This supports available evidence indicating that ethanol significantly depresses SOD, CAT activities and GSH levels (Rukkumani *et al*., 2004). According to Rukkumani *et al*., (2004) when ethanol inhibits SOD, superoxide radicals will not be converted to H2O2. The inhibition of SOD activity may result in an increased flux of superoxide radical in cellular compartments which may be the reason for the increased lipid peroxidative indices in the negative control group in this study. Meanwhile there was increase in these parameters (GSH, SOD, CAT) in DRE and omeprazole pretreated group in this study suggesting a boost in the antioxidant system after pretreatment with DRE .

GSH is a powerful nucleophilic antioxidant which plays pleiotropic roles, including maintaining cells in a reduced state, serving as an electron donor for certain

antioxidative enzyme such as glutathione peroxidase and critical for cellular protection such as detoxification of ROS, conjugation and excretion of toxic molecules and control of inflammatory cytokines (Brown *et al*., 2004). Depletion of GSH in tissues leads to impairment of the cellular defenses against ROS and may result in peroxidative injury. The significantly high levels of GSH observed in rats pretreated with DRE and omeprazole compared to negative control ulcer group depicts a boost in the antioxidant status as a result of drug administration. The findings in this study that the levels of GSH were significantly decreased in ethanol treated negative control group compared to DRE are consistent with other published reports (Videla, 1981; Mutoh *et al*., 1990; Jaya *et al*., 1993).

Many researches have provided evidence that antioxidants may play an important role in peptic ulcer management not only by protecting against gastric mucosal injury, but also by inhibiting progression of gastric ulcer (Ibrahim *et al*., 2012; Megala and Geetha, 2010; Tandon *et al*., 2004). Thus the results in this study reechoe the important relationship between SOD, CAT, GSH levels and free radical induced oxidative stress in gastric tissues.

Overall the results in this chapter suggest that aside the other mechanisms elucidated by the study in previous chapters, the methanolic extract of *Dissotis rotundifolia* possesses antioxidant property evidenced by an excellent *in vitro* hydroxyl, superoxide, nitric oxide and DPPH radical-scavenging activities and profound Fe^{2+} – ascorbate lipid peroxidation inhibitory activity. The levels of MDA, antioxidants; enzymatic (SOD, Catalase), non enzymatic; (GSH) were adversely affected by ulcer induction. However DRE and omeprazole treated rats successfully inhibited ulcer formation and alleviated the adverse effects on these parameters by endogenously scavenging ROS generated by ethanol administration. The increased levels of SOD, CAT and GSH and the significant reduction in MDA in DRE and omeprazole treated rats confirms the significant *in vitro* free radical scavenging abillity of the extract as depicted in the *in vitro* study. The results thus

suggest that DRE is acting as an antiulcer agent not only by its effect on H^+ / K^+ - ATPase and glycoproteins but also by decreasing free radicals genetrated *in vivo* thus boosting antioxidant status. Thus the results indicate that the gastroprotective effect of DRE may be related to its ability to positively effect the antioxidant system of rats. The present findings suggest that the gastric ulcer protective effects of DRE could be due to the antioxidant activity leading to changes in the gastric mucosal LPO, SOD, GSH and CAT levels as observed in this study.

6.5 CONCLUSION

The results indicate that the methanolic extract of *Dissotis rotundufolia* possesses antioxidant property by scavenging OH, SO, DPPH, NO radicals *in vitro* and *in vivo*. In addition the extract is capable of boosting antioxidant status by increasing endogenous antioxidants namely GSH, CAT SOD and reducing the levels of MDA involved in lipid peroxidation.

Chapter 7

TOXICOLOGICAL ASSESSMENT OF METHANOLIC EXTRACT OF *DISSOTIS ROTUNDIFOLIA* (MELASTOMATACEAE) WHOLE PLANT IN SPRAGUE-DAWLEY RATS

7.1 INTRODUCTION

Herbals have served as templates for the development of modern orthodox medicines and continue to be widely used in their original form (Jordan *et al*., 2010). Reports indicate that about 80% of the world's population depend on plant medicine for healthcare delivery and about 70-95% of developing countries rely on plant medicine for primary healthcare (WHO, 2011) suggesting that the practice of herbalism has become mainstream throughout the world. This may be due in part to the recognition of the value of traditional medical systems and the identification of medicinal plants from indigenous pharmacopeias that have been shown to have significant pharmacotherapeutic effects, either in their natural state or as the source of new pharmaceuticals.

In Ghana about 80% of the populace rely on plants for primary healthcare (WHO, 2011). This is an indication that plant medicine continues to play a key role in maintaining health in spite of the great advances made in modern or orthodox medicine. Phytomedicine is gaining popularity in developing countries. In Ghana the use of medicinal plants as food and in the treatment of several ailments for

decades with little or no scientific data on their safety, has rested largely on clinical experience. Moreover, plant medicine remedies are often patronized and perceived to be of low risk in comparison to synthetic drugs because they are natural (Jordan *et al*., 2010; Rai, 1994) though they are not completely free of toxic or other adverse effects (Smet, 2004).

This upsurge in popularity of the use of plant medicines coupled with scarcity of evidence-based data on their safety have raised serious concerns regarding their therapeutic usage. The evaluation of safety of plant medicines in rodents and other animals is becoming increasingly vital. Previous chapters in this study support the use of *Dissotis rotundifolia* as a potential antiulcer agent. Additionally *Dissotis rotundifolia* is reported to possess a multiplicity of effects (Abere *et al*., 2010, Mann *et al*., 2009). In this chapter the safety profile of DRE was asssessed in Sprague - Dawley rats.

Dissotis rotundifolia is a member of the family Melastomataceae and geographically native to some parts of Africa including Western and Eastern Africa (Trevithick, 1927; Wickens, 1975). The plant is among the most widely used medicinal plant in tropical Africa which has been reported to have various ethno-medicinal uses (Abere *et al*., 2010; Mann *et al*., 2009). Proof of toxicological assessment of the plant extract is necessary in order to assure consumers of its safety. Thus the study was designed to assess any potential toxic effects of *Dissotis rotundifolia* extract in Sprague Dawley rats.

7.2 MATERIALS AND METHODS

7.2.1 Experimental animals

Sprague Dawley rats of mixed sex (200-260 g) were obtained from the Centre for Plant Research, Akuapem-Mampong, Ghana. The experimental protocol was

approved by the Institutional Review Board of the University of Cape Coast and experiments were conducted according to the guidelines on the use and care of experimental animals. The study was performed in compliance with the Good Laboratory Practice (GLP) of the Organization for Economic Cooperation and Development (OECD, 1997). Animals were fed with commercial rat food supplied by GAFCO and water *ad libitum*. They were maintained in a 12 h light / dark cycle at 25 ± 2°C. All animals were acclimatized for at least a week before tha start of the experiment.

7.2.2 Determination of median lethal dose (LD50)

The LD50 values of the extracts were determined according to the method of Finney (1964). *Dissotis rotundifolia* extract at a maximum dose level 5000 mg/kg (orally) was used for acute oral toxicity study. A total of 60 rats; 10 rats (5 males and 5 females) per group were used for the experiment. Group 1 served as control (received distilled water) and the other five groups were treated with extracts at different doses (10, 100, 500, 2500 and 5000 mg/kg body weight). A single dose of the extract was administrated orally to each animal. Twice-daily cage side observations included changes in skin, fur, eyes, mucous membrane (nasal), autonomic (salivation, lacrimation, perspiration, piloerection, urinary incontinence, and defecation), and central nervous system (drowsiness, gait, tremors, and convulsions) changes. Mortality, if any, was determined over a period of 14 days.

7.2.3 Sub-acute oral toxicity experiment

In this study, animals were divided into six groups of 8 animals each (4 males and 4 females). Group 1 received 2 mL of distilled water and served as control. Groups 2, 3 and 4, received 100, 300 and 1000 mg/kgbwt orally respectively. The extract was prepared such that only 2 mL was administered orally. The extract was administered daily for 14 days at the same time 9:00 am GMT and observed at least twice daily for morbidity, mortality and toxicity signs. Body weights, biochemical,

haematological and histopathological parameters were evaluated in animals. All animals were fasted overnight prior to necropsy and blood sampling.

7.2.4 Haematological indices

Blood was collected into EDTA (ethylenediamine-tetracetate)-2K tubes and analyzed for red blood cell (RBC), haemoglobin (HGB), pack cell volume (PCV) or haematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular (MCH), mean corpuscular HGB concentration (MCHC), red cell distribution width-standard deviation (RDW-SD), platelets (PLT), mean platelet volume (MPV), platelet distribution width (PDW), white blood cell (WBC) counts, neutrophils (NE), lymphocytes (LYM) using a Sysmex-KX-2IN haematology auto analyser.

7.2.5 Biochemical indices

Blood collected into gel separator tubes was processed for plasma by centrifuging for 5 mins at 3000 rpm to ensure separation of plasma. Plasma samples were stored at −20°C until use. The plasma was analyzed for levels of total protein (TP), albumin (ALB), globulin (GLB), total bilirubin (TB), indirect bilirubin (IDB), direct bilirubin (DB), alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferases (AST), high density lipoprotein cholesterol (HDL-C), total cholesterol (TC), triglycerides (TG), low density lipoprotein cholesterol (LDL-C), , very low density lipoprotein cholesterol (VLDL-C), urea, creatinine (CRE) and lactate dehydrogenase (LDH) using an automated chemistry analyser (ATAC 800 Random Acess Chemistry system. The plasma was also analyzed for levels of sodium (Na) and potassium ions (K) using selectra junior autoanalyzer

7.2.6 Histopathology analysis

Immediately after collection of blood samples, animals were opened up and various body organs were harvested. These internal organs which include liver, spleen, kidney and stomach tissues were freed of fat and connective tissues, blotted with clean wipes, examined macroscopically and individually weighed to obtain the

organ-to-body weight ratios. For histological examinations, excised internal organ tissues of the rats were cleaned in physiological saline, fixed in Bouin's fluid, dehydrated in increasing concentrations of ethanol and embedded in paraffin wax. Thereafter, sections of tissues were cut at 5 µm with a rotary microtome, mounted on clean glass slides and stained with haematoxylin. The stained tissues were observed through an Olympus microscope and photographed by charecouple device camera at magnification of X 100.

7.2.7 Data analysis

Data collected were entered into EXCEL and later transported to GraphPad Prism, windows version 4.02 (GraphPad Software, San Diego, CA, USA) for analysis.
Data were presented as mean ± SEM. Significanat differences between means was determined using ANOVA for groups with more than two means and Bonferroni posthoc test was applied where a significant difference was recorded.
Paired t-test was also used for groups with only two means.

7.3 RESULTS

7.3.1 LD_{50} and acute toxicity studies

Treated animals in this experiment showed no signs of aggressiveness, vomiting, respiratory distress, salivation, sedation, diarrhoea or death compared to the control group. Doses of extract (10 - 5000 mg/kg) administered did not end in lethality over the 24 hr period. No latent toxicity was seen after keeping animals for extra 14 days. Thus LD_{50} was above 5000 mg/kg body weight.

7.3.2 Effect of DRE on organ and body weight

In assessing the effect of extract on organ to body weight ratio, it was noticed that the target organs including the liver, heart, kidney, lungs, stomach and spleen did not show significant differences ($p>0.05$) compared to the vehicle treated control group in ANOVA analysis (Table 7.1). There was also no significant change in the body weight of extract treated group before and after (table 7.2).

7.3.3 Effect of DRE on haematological parameters

The results indicated that most of the haematological parameters measured remained unchanged within the 2-week experimental period. There were no significant changes in RBC, HGB, HCT, MCH, MCHC, PLT, LYM, MID, RDWS, PDW and MPV values compared to control groups. Despite a significant change ($P<0.05$) observed in some parameters (WBC and MCV), there was no dose response relationship ($p>0.05$). However at doses of 1000 mg/kg, it caused a significant increase in the levels of WBC when compared to control, 100 mg/kg and 300 mg/kg ($p<0.05$) (Table 7.3). Meanwhile MCV also markedly increased ($p=0.042$) in the 1000 mg/kg treated group compared to 300 mg/kg group.

7.3.4 Effect of DRE on serum biochemical parameters

The results for the biochemical parameters in the treated and control rats are presented in Table 7.4. The effect of DRE on serum biochemical parameters after

a period of 14 days treatment showed no significant differences in levels of TP, ALB, GLB, TB, IDB, DB, ALP, ALT, AST, TC, LDL-C, HDL-C, urea, CRE, and LDH and K. (Table 7.4) ($p>0.05$). Though some parameters including VLDL-C, TAG and Na) experienced significant differences, ($p<0.05$), there were no dose dependent relationships. However at doses of 1000 mg/kg, it caused a significant increase in the levels of TAG and VLD-C when compared to 100 mg/kgbwt with P value of 0.001 for the two parameters (Table 7.4). Meanwhile Na^+ markedly decreased ($p=0.034$) in the 1000 mg/kg treated group compared to control group.

Table 7.1: Body weights (g) in rats orally treated with DRE during sub-acute studies

	Body weight (g)		
Treatment	**Initial**	**Final**	**P value**
Control	238.12±12.62	256.00±13.70	0.000
100mg/kg	252.12±13.11	239.00±8.93	0.267
300mg/kg	237.50±6.25	234.12±8.27	0.664
1000mg/kg	235±8.67	251.89±15.37	0.088

Values are mean ± S.EM. (n = 8) (14 days) Data analysed using Paired t-test

Table 7.2: Organ weights (g) in rats orally treated with DRE during sub-acute studies

Parameters	**Control**	**100mg/kg**	**300mg/kg**	**1000mg/kg**	**P value**
Liver	1.62±0.12	1.51±0.04	1.47±0.11	1.45±0.05	0.559
Kidney	1.30±0.07	1.25±0.08	1.21±0.04	1.28±0.05	0.658
Heart	0.79±0.04	0.78±0.05	0.75±0.03	0.73±0.03	0.653
Spleen	0.506±0.04	0.538±0.03	0.506±0.04	0.640±0.12	0.454
Lungs	1.23±0.05	1.21±0.03	1.25±0.034	1.24±0.04	0.859
Stomach	6.89±0.61	6.67±0.43	6.69±0.26	6.75±0.33	0.983

Data analysed using one way ANOVA. Values are mean ± S.EM. (n = 8) (14 days)

Table 7.3: Haematological indices in rats orally treated with DRE

Parameters	Haematological values				
	Control	**100mg/kg**	**300mg/kg**	**1000mg/kg**	**P value**
RBC (M/ µL)	7.86±0.18	8.05±0.18	7.54±0.16	7.79±0.16	0.235
HGB (g /dL)	14.88±0.25	15.04±0.30	14.49±0.30	14.54±0.19	0.389
HCT (%)	46.70±0.83	47.80±0.97	45.64±1.10	45.43±0.88	0.287
MCH (pg)	18.95±0.23	18.66±0.14	19.23±0.13	17.83±0.98	0.265
MCV (fL)	59.46±0.62	59.41±0.40	60.33±0.30	59.31±0.57	0.055
MCHC (g/dL)	31.86±0.11	31.50±0.13	31.76±0.20	32.05±0.24*	0.143
RDW-SD (fL)	29.16±0.46	29.55±0.25	30.16±0.36	29.64±0.50	0.379
PLTx10^2 (K / µL)	9.74±75.95	9.98±56.70	8.59±108.58	9.02±52.87	0.564
MPV (%)	6.99±0.09	7.03±0.09	7.05±0.12	6.88±0.11	0.650
PDW (fL)	8.29±0.14	8.19±0.11	8.38±0.14	7.99±0.16	0.243
WBC (K / µL)	11.93±1.22	11.79±0.99	9.35±0.38	16.05±1.12***	0.001
LYM (%)	75.33±3.03	74.55±4.58	72.55±3.01	68.44±2.81	0.501
MID (%)	7.04±0.64	6.41±0.64	7.08±0.33	8.34±0.45	0.098
NE (%)	17.71±2.45	14.14±1.19	20.04±2.68	23.23±2.42	0.054

Data analysed using one way ANOVA and poshoc test with Bonferroni. Values are mean ± S.EM. (n = 8) (14 days) *** significant difference between 1000mg/kgbwt and [control or 100mg/kgbwt or 300mg/kgbwt] * significant difference between 1000mg/kgbwt and 300mg/kgbwt

Note: Red blood cell (RBC), haemoglobin (HGB), pack cell volume (PCV) or haematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular (MCH), mean corpuscular HGB concentration (MCHC), red cell distribution width- standard deviation (RDW-SD), platelets (PLT), mean platelet volume (MPV), platelet distribution width (PDW), white blood cell (WBC) counts, neutrophils (NE), lymphocytes (LYM)

Table 7.4: Serum biochemical indices in rats orally treated with DRE

Parameters	Biochemical values at indicated dosages				
	Control	**100mg/kg**	**300mg/kg**	**1000mg/kg**	**P value**
AST (U/L)	166.48±6.06	157.39±4.17	159.92±5.10	158.96±7.78	0.716
ALT (U/L)	49.00±2.56	47.88±3.30	53.50±1.86	56.13±3.07	0.142
ALP (U/L)	513.88±82.79	483.25±71.07	521.75±41.38	589.25±36.59	0.663

TP (g/L)	133.12±6.63	140.82±7.98	136.15±4.44	143.57±9.95	0.764
ALB (g/L)	32.36±0.60	31.71±1.39	33.27±1.75	31.22±2.53	0.849
GLB (g/L)	100.76±6.34	109.11±8.10	102.88±4.10	112.35±10.02	0.672
A/G	0.33±0.02	0.30±0.03	0.33±0.02	0.30±0.04	0.786
DB (µmol/L)	2.60±0.40	1.96±0.33	2.13±0.40	2.17±0.28	0.631
IDB (µmol/L)	1.01±0.23	2.07±0.72	0.67±0.22	2.86±0.79	0.038
TB (µmol/L)	3.62±0.44	4.04±0.92	2.80±0.33	5.02±0.84	0.162
CRE (µmol/L)	21.70± 2.01	22.42±1.48	25.92±1.5	21.02±1.54	0.166
UREA (mmol/L)	10.64±0.36	10.91±0.88	9.91±0.38	10.85±0.68	0.650
K (mEq/L)	5.84±0.47	4.57±0.34	5.43±0.68	5.30±0.74	0.488
Na (mmol/L)	166.27±16.55	114.17±13.91	117.89±19.47	101.38±9.65**	0.002
LDH (U/L)	1313.1±128.73	1539.5±186.77	1832.1±163.88	1916.7±160.53	0.05
TAG (mmol/L)	1.48±0.07	1.12±0.06	1.53±0.13	1.81±0.14****	0.045
TC (mmol/L)	4.07±0.26	3.28±0.39	3.31±0.20	3.56±0.32	0.247
LDL-C (mmol/L)	3.07±0.27	2.27±0.33	2.30±0.16	2.40±0.24	0.123
HDL-C (mmol/L)	0.32±0.01	0.50±0.16	0.31±0.03	0.33±0.05	0.428
VLDL-C (mmol/L)	0.29±0.01	0.22±0.01	0.30±0.02	0.36±0.02****	0.002

Data analysed using one way ANOVA and poshoc test with Bonferroni. Values are mean ± S.EM. (n = 8) (14 days)

** significant difference between 1000mg/kgbwt and control

**** significant difference between 1000mg/kgbwt and 100mg/kgbwt

Note: Asparate amimo transferase (AST), Alanine amimo transferase (ALT), Alkaline Phosphatase (ALP), Total Protein (TP), Albumin (ALB), Globulin (GLB), Albumin globulin ration, A/G, Total bilirubin (TB), Direct bilirubin (DB), Indirect bilirubin (IDB), Creatinine (CRE), Lactate dehydrogenase (LDH), Sodium ion (Na), Potassium ion (K), Total Cholesterol (TC), Triacylglycerol (TAG), Low Density Lipoprotein- Cholesterol (LDL-C), High Density Lipoprotein-Cholesterol (HDL-C), Very Low Density Lipoprotein- Cholesterol (VLDL-C).

7.3.5 Histopathology of internal organs

7.3.5.1 Photomicrograph of the liver cells

Histology of the liver tissues confirmed no cellular damage in all the treated groups, compared to the vehicle treated control group (Plate 7.1a-d). Histopathological examination of the liver confirmed no cellular damage in all the treated groups, compared to control group. Intact liver tissue with plates of hepatocytes, sinusoids that confluence with the central vein was observed. No obvious proliferation of

Kupffer cells to remove debris nor other forms of toxicity were observed (Plate 7.1a-d). Results shown are for males, similar results were obtained for females.

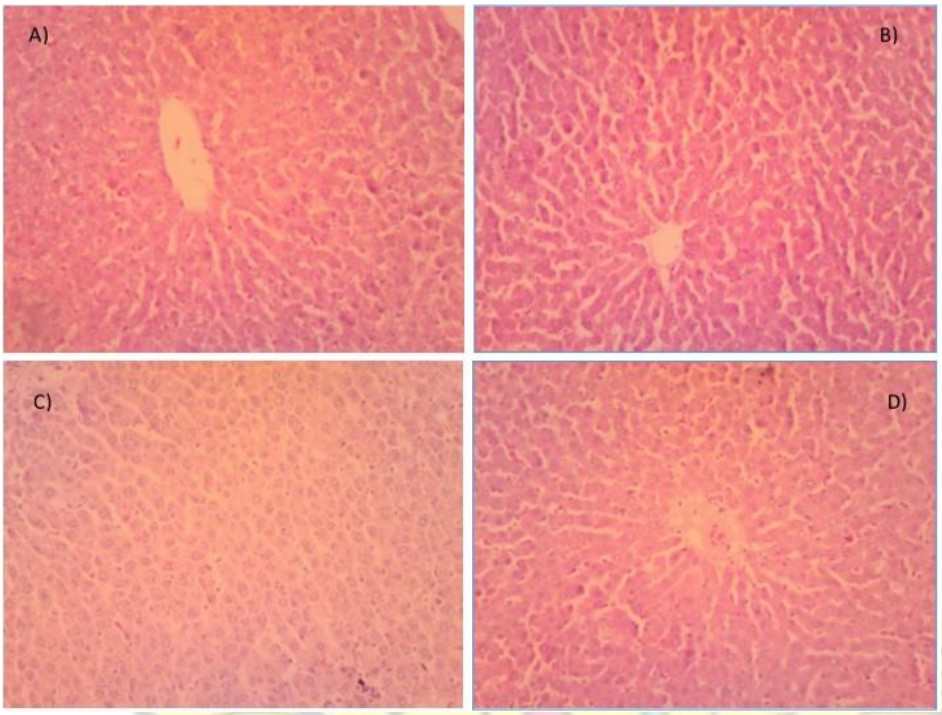


Plate (7.1a-d): Micrographs of the liver tissues for a: control; b:100 mg/kg; c: 300 mg/kg; d: 1000 mg/kg groups at the end of treatment period of 14 days with DRE.

7.3.5.2 Photomicrograph of the kidney

Histopathological examinations of the kidney revealed no significant changes in the kidney cells (Plate 7.2 a-d). Intact glomerulus in Bowman's capsule with capsular space, proximal and distal convoluted tubules with simple cuboidal epithelium, blood vessel were seen. There were no obvious signs of damage to nephrons.

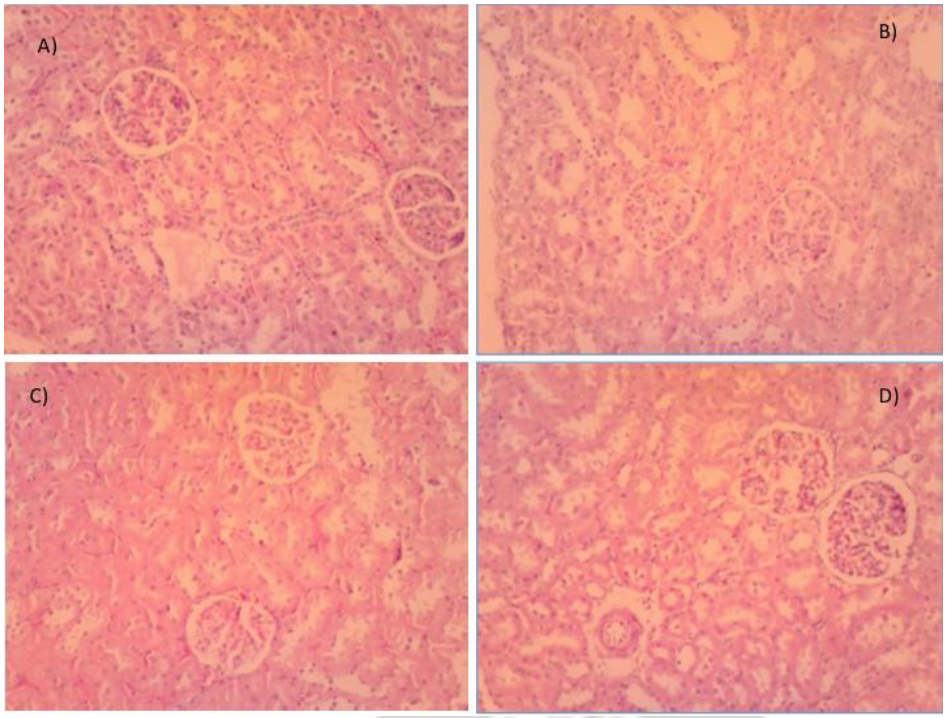


Plate (7.2 a-d): Micrographs of the kidney tissues for a: control; b:100 mg/kg; c: 300 mg/kg; d: 1000 mg/kg groups at the end of treatment period of 14 days with DRE.

7.3.5.3 Photomicrograph of the spleen

Micrographs of spleen tissues showed no significant changes in spleen between DRE treated group and control (Figure 7.3 a-d). Normal spleenic tissue with white pulp of lymphoid cells, surrounding an arteriole was observed. Surrounding the white pulp is the red pulp traversed by venous sinuses which indicated the absence of toxicity

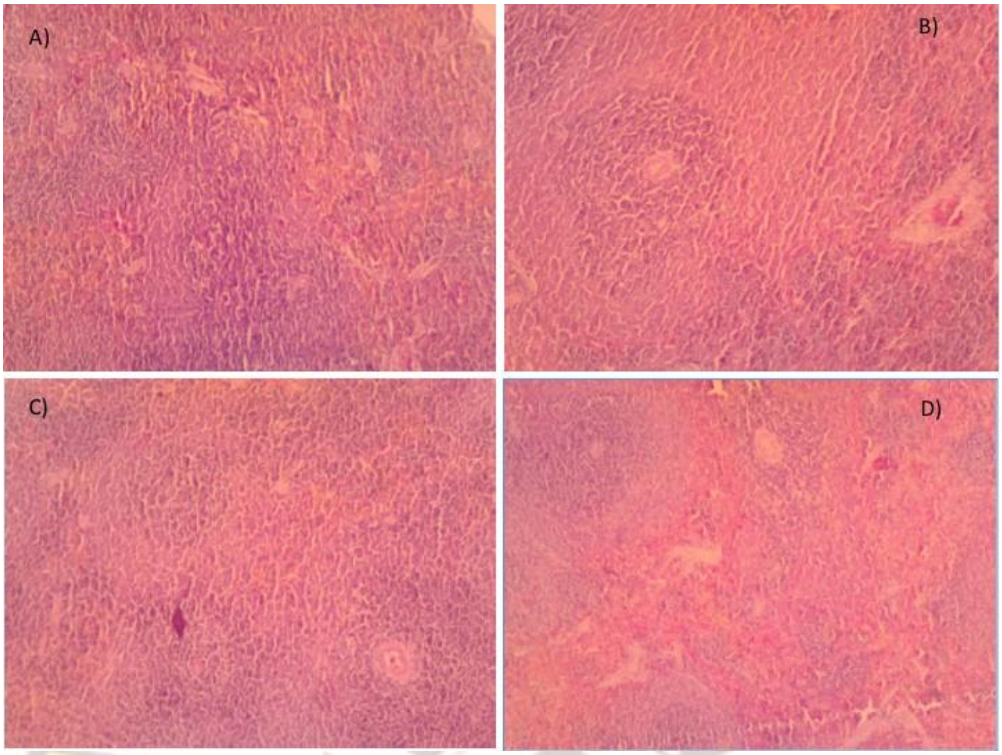


Plate (7.3 a-d): Micrographs of the splenic tissues for a: control; b:100 mg/kg; c: 300 mg/kg; d: 1000 mg/kg groups at the end of treatment period of 14 days with DRE.

7.3.5.4 Photomicrograph of the stomach

There were no salient pathological changes in the stomach tissues of extracttreated animals as compared to controls (Figure 7.4 a-d). Intact simple columnar epithelium and blood vessel in submucosa and smooth muscle were seen. Ulceration of the epithelia was not evident.

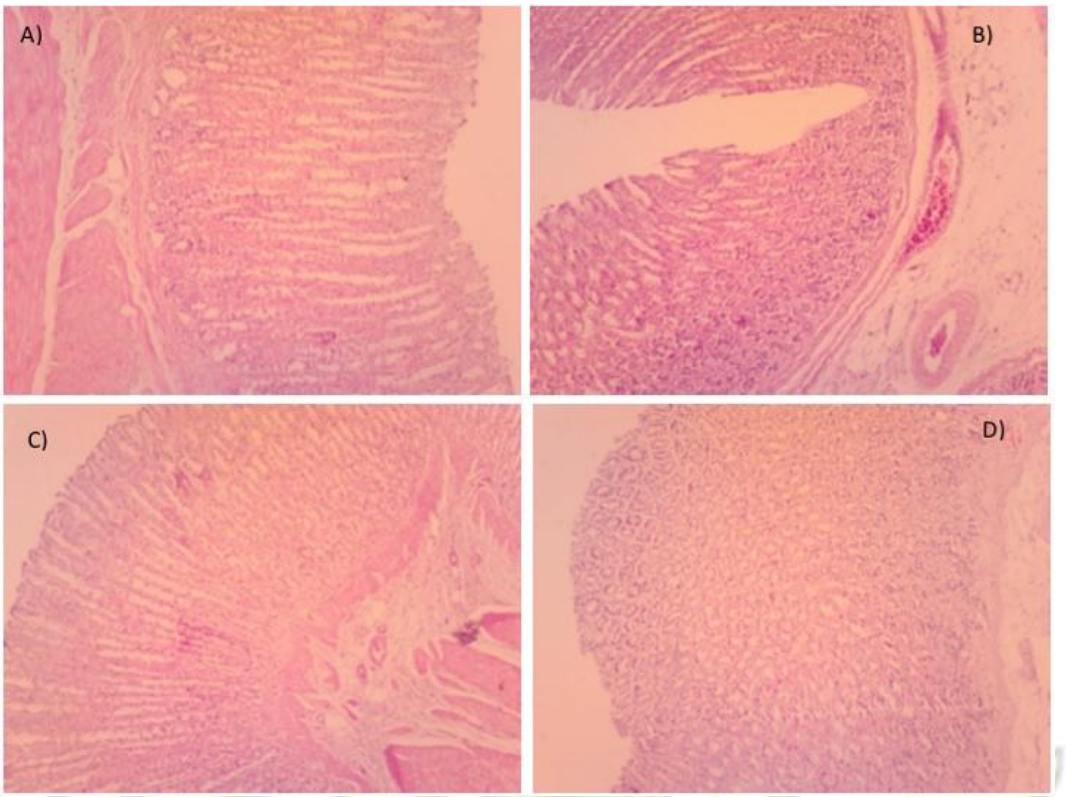


Plate (7.4 a-d): Micrographs of the stomach tissues for a: control; b:100 mg/kg; c: 300 mg/kg; d: 1000 mg/kg groups at the end of treatment period of 14 days with DRE.

7.4 DISCUSSION

In any pharmacological studies, proof of safety is necessary. No potential drug is approved for use clinically without the animal toxicity and clinical trial data. Toxicological studies assist in deciding whether a new drug should be adopted for clinical use or not (Timbrel, 2009). Establishment of safety is very critical for the assessment of herbal remedies which usually do not undergo any experimental safety assessment before use with the notion that anything natural is not toxic. Toxicity studies in appropriate animal models are generally used to assess the potential health risk of drugs to humans (Timbrel, 2009). It also provides

information on health hazards likely to arise from a repeated exposure of mammals to the test substance, as it is being used repeatedly for the management of several diseases. Depending on the duration of exposure of animals to drug agents, toxicological studies may be put into four categories; acute, sub-acute, sub-chronic and chronic studies.

In the present study, acute and sub-acute toxicity profile of the extract of whole plant of *Dissotis rotundifolia* was evaluated in Sprague-Dawley rats. Animals were subjected to extract treatment via oral gavage of the *Dissotis rotundifolia* extract. This treatment regimen and the administration method were considered the most suitable because they were similar to the form and mode of administration of *Dissotis rotundifolia* extract in humans.

There were no deaths recorded in rats tested during the acute toxicity period in this study, placing the LD_{50} above 5000 mg kg^{-1}. According to Oslon *et al*., (2000) a substance that shows LD_{50} at 1000 mg kg^{-1} body weight is considered safe or of low toxicity once administered, implicitly DRE could be said to be of low toxicity.

Organ and body weight are indices which are often used in toxicological assessments. These parameters may be altered in the presence of tumours, hyperplasia and other diseases although genotoxicity studies are mostly required to evaluate carcinogenic potentials of drug agents. The results in this study show that organ and body weight ratio indices of liver, heart, kidney, spleen and stomach were not significantly altered by the 14 day treatment regime. This suggest the absence of injuries to these internal organs.

This study has also shown that treatment with the extract did not cause any deteriorating changes in haematological parameters. Haematological changes such as anaemia often results from bone marrow toxicity among other causes (Flanagan and Dunk, 2008). Haematological indices are mostly associated with health status

and are of diagnostic importance in clinical assessment of animals and humans. Blood indices are good pointers of physiological, pathological and nutritional status. Changes in these parameters have the potential implication on therapeutic drug evaluation and toxicological factors in animals. The haematological data show that *Dissotis rotundifolia* extract has no significant adverse effect on blood suggesting that treatment did not affect the incorporation of haemoglobin into red blood cells nor the morphology and osmotic fragility of red blood cells. Haematocrit is a measure of the relative volume of red blood cells. The PCV which was unaffected by the extract in all the groups indicates that the volume of RBC in the blood remained constant. However, the Total WBC which showed an increase in the 1000 mg/kg group compared to the control, 100 and 300 mg/kg group indicates an improvement in the immune system of the extract - treated animals. The lack-of-effect on neutrophil levels indicates that the extract may not have induced any inflammatory process since these cells are usually elevated in the course of inflammation (Formela *et al*., 1995).

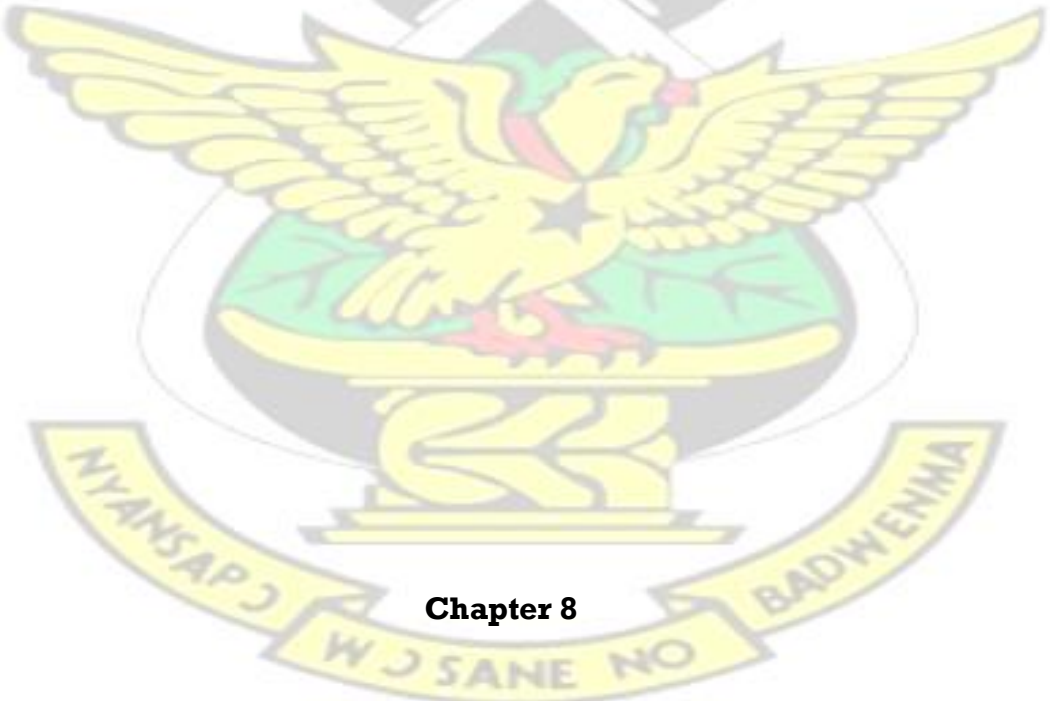
A predominance of unconjugated bilirubin is suggestive of hemolytic jaundice, whereas a predominance of conjugated bilirubin is generally associated with hepatic disease and extra-hepatic biliary obstruction (Ramaiah, 2007). The insignificant decrease in the level of the bile pigments in this study also indicates that the plant extracts may not have any adverse effects on the liver. There was also no change in the levels of urea in this study, suggesting that kidneys were not adversely affected. It is also interesting to note that the plant extract did not cause any impairment on the cardiovascular system as shown by the insignificant changes in the levels of K^+ electrolyte and lipid profile.

Additional proof that there was no liver injuries is seen in ALT, ALP and AST levels which were not significantly different between the treated and the control groups. Elevated levels of AST and ALT are often diagnostic of underlying cellular injuries (Karthikeyan *et al*., 2006).

In order to confirm the absence of damage in the internal organs, hispathological analysis was done. In all the tissues stained none showed any sign of pathology, thus the extract did not cause any effect to the liver, kidney, stomach and spleen within the 14 day administration of the extract.

7.5 CONCLUSION

The study demonstrates that there is a wide margin of safety for the use of *Dissotis rotundifolia* extract up to 1000 mg/kg within a 14 day period.



Chapter 8

GENERAL DISCUSSIONS, SUMMARY OF FINDINGS, CONCLUSIONS AND FUTURE WORK

8.1 GENERAL DISCUSSION

Phytomedicines have shown great promise in the treatment of several diseases, including peptic ulcers. Plants are natural blueprints for the development of new drugs (Njume *et al*., 2009). Of a total of 422,000 flowering plants reported in the world (Govaerts, 2001), more than 50,000 are used for medicinal purposes (Schippmann *et al*., 2002). These emphasize the need for more research into plant medicines to evaluate their potential in managing diseases as supplements to conventional medications.

Peptic ulcer, a gastrointestinal disorder, is a pathological condition in which the biological balance between aggressive and defense factors is disturbed. Peptic ulcer is a global problem with increasing incidence and prevalence attributed to many factors such as stress, excessive intake of NSAIDs, alcohol and exposure to infection from the bacteria *Helicobacter pylori*. The effect of DRE on peptic ulcers caused by these agents was studied in rat models. Interestingly the study demonstrated that, pretreatment or / and treatment with DRE markedly decreased ulcer index and also increased percentage protective and curative ratios respectively. These results suggest that DRE displays an antiulcerogenic effect

which is related to cytoprotective activity, gastrohealing potential and antisecretory effects. This is because when DRE was administered to rats in all the ulcer models used, a significant reduction in ulcer indices was observed in DRE pretreated and treated rats compared to only ulcerogen pretreated or treated group.

Evidence was established that DRE protects the stomach of rats from ulcerogens such as cold stress, acetylsalicylic acid and ethanol. It became necessary to establish the possible mechanism of action of the extract.

Antagonism at muscarinic cholinergic receptors was evaluated and the process of establishing the possible mechanism of action continued with an assessment of the effect of DRE on H^+/K^+-ATPase *in vivo.* The effects of DRE on the levels of mucosal glycoproteins was also determined in gastric mucosae.

Helicobacter pylori is regarded as the chief etiological agent of peptic ulcer. The use of plant treatment regimes have shown great promise in managing peptic ulcer diseases. One such plant currently used is *Dissotis rotundifolia*. The whole plant of *Dissotis rotundifolia* plant, also known as pink lady is popularly used for treatment of gastric disorders. This plant has been reported to be active against *Staphylococcus aureus*, *Salmonella typhi*, *E. coli*, *P. aeruginosa* and other organisms (Abere *et al*., 2010). There is currently no previous record that this plant has been evaluated scientifically for its antimicrobial activity on clinical isolates of *H. pylori.* This necessitated the need to test the effect of *Dissotis rotundifolia* extract on *H. pylori*.

Drugs with free radical scavenging properties have been shown to have beneficial effects in alleviating gastric lesions (Demir *et al*., 2003) thus the effect of DRE in *in vitro* antioxidant models and on *in vivo* antioxidant status was carried out to ascertain the significant *in vitro* free radical scavenging activity of the plant extract observed in this study.

In elucidating the possible mechanisms, it was shown that the antiulcer effect of the extract is through antagonism of cholinergic receptor, inhibition of H^+ / K^+ - ATPase and *Helicobacter pylori*, increasing mucin activity and a boost in antioxidant status.

Studies have shown that changes in gastrointestinal motility during treatment may play a role during disease management. The anticholinergic effect of DRE was also evaluated. The results showed a significant antagonist effect of DRE at cholinergic receptors. The extract decreased the propulsive movement of charcoal meal through the gastrointestinal tract (GIT). This observation depicts the involvement of the cholinergic system in the antiulcer activity of the DRE. According to Bertaccini and Scapignato (1981), the delayed gastric emptying increases the absorption of orally administered drugs such as DRE and this significant delay in gastrointestinal transit is considered to be a beneficial pharmacological property in disease management including peptic ulcer management.

Flattening of the mucosal folds in DRE treated rats in cold stress model was also observed. This suggests that the gastroprotective effect of the DRE might be due to a decrease in gastric motility. The relaxation of the muscles may shield the gastric mucosa by the flattening of the folds. This flattening will reduce the mucosal area that will be exposed to necrotizing agents and reduce the volume of the gastric irritants on the rugal crest.

The proton pump is a major step for the production of gastric acid. The proton pump is the main component responsible for the acidic environment in the stomach, and drug agents that inhibit this enzyme represent a principal pharmacological treatment target for peptic ulcer disease .

DRE was also found to inhibit the acid secreting enzyme H^+/K^+-ATPase *in vivo* in this study. Activation of cAMP pathway stimulates the H^+/K^+-ATPase on parietal cells, a high capacity proton pump, with its insertion into the apical membrane leading to the formation of secretory canaliculi. In recent years, drugs that reduce the acid secretion and H^+/K^+-ATPase activity are the preferred therapeutic choice due to their clinical efficacy.

The antisecretory effect of DRE was further supported and confirmed from biochemical results on the levels of mucosal glycoproteins and gastric wall mucus. The gastric wall mucus is known to play an important role as a defensive barrier against gastrointestinal damage (Davenport, 1967). Gastric wall mucus weakening induced by acetylsalicylic acid is also among the mechanisms responsible for gastric mucosal injury. It has been reported that certain antiulcer drugs increase the amount of gastric mucus secretion in the gastric mucosa (Ibrahim *et al*., 2012). In this study DRE was able to significantly increase gastric mucus when compared to negative control group. The preservation of adherent mucus on the glandular mucosa is one of the key factors in the prevention of gastric mucosal injury induced by chemical irritants (Thirunavukkarasu *et al*., 2009). The present results indicate that the gastroprotective effect of DRE is mediated partly by preservation of the gastric wall mucus.

Oxidative stress plays a critical role in the pathogenesis of various diseases, including gastric ulcer disease, with antioxidants reported to play a important role in the protection of the gastric mucosa against various necrotic agents. Drugs with free radical scavenging properties have been shown to have beneficial effects in alleviating gastric lesions (Demir *et al*., 2003). Free radical species are continuously produced in the body. These reactive species trigger cellular damage in many tissues. In this study, the free radical scavenging potential of the extract was assessed *in vitro*. The results showed significant high OH, DPPH, NO, SO

scavenging activities of the plant extract. To confirm the *in vitro* scavenging effect, an *in vivo* experiment was done using ethanol induced ulcer model.

MDA is the final product of lipid peroxidation and is used to determine the extent of lipid peroxidation (Johansen *et al*., 2005; Gutteridge, 1995). Lipid peroxidation has been reported to be a key pathophysiological process in many diseases, including gastric ulcer (Pratibha *et al*., 2006). A study by Lipinski, (2001) indicates that reactive oxygen species provoke cellular events such as enzyme inactivation, DNA strand cleavage and membrane lipid peroxidation. Oxidative stress is associated with the peroxidation of cellular lipids (Ajitha and Rajnarayana, 2001). In the non extract treated rats, the MDA levels were seen to be higher than the normal control rats, indicating high lipid peroxidation. The levels of MDA in the gastric tissue of rats that received DRE were significantly reduced when compared to the negative control rats. This result suggests that the DRE could reduce gastric ulceration by decreasing lipid peroxidation.

SOD, which plays an important function in protecting gastrointestinal mucosa, owes its antioxidant properties to its capacity to scavenge superoxide anions. SOD converts superoxide to hydrogen peroxide, which is transformed into water by catalase in lysosomes or by glutathione peroxidase in the mitochondria (Wohaieb *et al*., 1987). The rise in SOD level in the DRE pretreated experimental group in the present study point to an antioxidant mechanism underlying its gastroprotective action, whereas the ability of the DRE to prevent lipid peroxidation *in vitro* reinforces its prospective use as a therapeutic drug for diseases mediated by free radical. The results in this study revealed that the activity of SOD was reduced in gastric tissue homogenates of untreated ethanol induced ulcer group compared to pretreated group. The decline in the activity of SOD in tissue homogenate may be due to the increased production of reactive oxygen radicals, which can reduce the

levels of this enzyme (Ajitha, and Rajnarayana, 2001). The decrease of SOD enzyme level in gastric homogenate may lead to a number of injurious effects.

Catalase (CAT) acts as a protective antioxidant against the deleterious effects of LPO. CAT is an enzyme that converts H_2O_2 to water and oxygen. It can also form methanol, ethanol, formic acid and phenols by donating hydrogen. In the present study it was observed that DRE and omeprazole decreased CAT activity which had been provoked by ethanol. Pretreatment of rats with DRE in ethanolinduced ulcer model significantly modulated the antioxidant status in gastric tissues, suggesting the enhancing effect of DRE on cellular antioxidant defenses. Phytochemical analysis of DRE in this study showed the presence saponins, tannins, flavonoids, alkaloids, general glycosides, cardiac glycosides, reducing sugars, and terpenoids. Earlier studies by Rath *et al*., 1995 have isolated Cglycosylflavones namely isoorientin, orientin, vitexin and isovitexin from methanolic extract of *Dissotis rotundifolia* whole plant. Studies by Quílez et al. 2010 have reported the anti-*Helicobacter pylori* activity, anti-myeloperoxidase, antisecretory and H^+/ K^+ - ATPase inhibitory effects of vitexin and isovitexin isolated from the leaves of *Piper carpunya* (Quílez *et al*., 2010). Implicitly the role of vitexin and isovitexin in the pharmacological activity of DRE in this study cannot be overlooked. The presence of these phytoconstituents in the whole plant extract of *Dissotis rotundifolia* may be responsible for its medicinal properties (anticholinergic effect, H^+ / K^+ - ATPase inhibition, mucin activity preserver, anti-*Helicobacter pylori* and antioxidant activity).

The upsurge in popularity of the use of plant medicines coupled with scanty scientific evidence-based data on their safety have raised serious concerns regarding their therapeutic usage. The evaluation of toxic effects of plant medicines in rodents and other animals is indispensible in order to ensure their safety. Therefore the need for assessment of toxicity profile of medicinal plants is necessary.

Consequently this study assessed the potential toxic effects of *Dissotis rotundifolia* extract in Sprague Dawley rats for a period of 14 days. Haematological and biochemical indices measured showed no significant damage on the liver, spleen, kidney, heart and stomach. These results were confirmed in the micrographs obtained from the histopathological analysis of tissues. All together the toxicity studies suggest that DRE has a wide margin of safety.

This discussion has elaborated on the results of the study, which has provided an additional support for the use of this plant as an antiulcer remedy in Ghanaian traditional medicine. Thus development of a natural plant medicine with antiulcer activity involving improvement of mucosal protective indicators, H^+/K^+ ATPase inhibitory activity, anticholinergic effects, ability to scavenge free radicals and anti-*Helicobacter pylori* activity could be a prefered drug of choice due to its multifaceted pharmacological effects. In addition DRE could overcome the challenge of compliance experienced with the current triple therapy for peptic ulcers due to its all in one preparation.

8.2 SUMMARY OF FINDINGS

The main objective of this study was to investigate the antiulcer effects of *Dissotis rotundifolia* whole plant extract and to assess its toxicological effects in rats. Phytochemical screening tests were carried out. Three ulcer models were employed namely cold stress, acetylsalicylic acid and ethanol-induced ulcer models. Studies on the possible mechanisms of action were also carried out after the extract proved to possess antiulcer effect. The safety of the extract was determined in a 14 day study. Analysis of the results indicated that *Dissotis rotundifolia* extract:

1. possesses anticholinergic effects
2. possesses *in vivo* H^+/K^+-ATPase inhibitory activity

3. increases mucin activity
4. possesses anti-*Helicobacter pylori* activity
5. has the ability to scavenge OH, DPPH, SO free radicals *in vitro*
6. possesses an *in vitro* anti-lipid peroxidative effect
7. is able to boost antioxidant status *in vivo* by increasing SOD, CAT and GSH levels.
8. is able to decrease lipid peroxidation in the stomach of ethanol induced ulcerated rat
9. is safe within a 14 day period of usage

8.3 CONCLUSIONS

The results of this study have provided evidence to support the use of DRE as an antiulcer plant medicine. The effect may be due in part to the The H^+ / K^+- ATPase inhibitory property. It was also found that DRE acts as mucin preservative by increasing the production of glycoproteins. This direct effect exerted on the mucosa by DRE could also contribute to its gastroprotective effect.

The results from this study also indicated that DRE possesses anticholinergic effects, has the ability to scavenge OH, DPPH, NO and SO free radicals *in vitro* and also possesses an *in vitro* anti-lipid peroxidative effects. DRE is able to boost antioxidant status *in vivo* by increasing SOD, CAT and GSH levels. The toxicological findings from this study show that, methanolic extract of DRE is safe at a dose of 1000 mg/kgbwt within a 14 - day period in rats. Overall, the study indicates that DRE possesses antiulcer effects and is potentially non - toxic at the dose used in this study thus preparations obtained from DRE could be used for the development of a new phytopharmaceutical drug for the management of gastric ulcer.

8.4 FUTURE WORK

Prostaglandin contributes to mucosal blood flow and improvement in peptic ulcer healing. In this study, PGE_2 was not measured. It is recommended that future work should consider PGE_2 measurements. The effect of DRE on urease enzyme isolated from *H. pylori* should be included in future studies in determining the possible mechanism of action of the plant extract. Phytochemical studies should also be carried out on DRE using HPLC to isolate and identifiy the individual constituents for bioactivity tests.

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