GASTROPROTECTIVE EFFECT OF AQUEOUS STEM BARK EXTRACT OF *ANOGEISSUS LEIOCARPUS* AGAINST ETHANOL-INDUCED GASTRIC ULCER IN RATS

BY

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A DISSERTATION SUBMITTED TO THE SCHOOL OF POSTGRADUATE STUDIES AHMADU BELLO UNIVERSITY, ZARIA IN PARTIAL FULFILLMENTS OF THE REQUIREMENTS FOR THE AWARD OF MASTER DEGREE IN BIOCHEMISTRY (MSc. BIOCHEMISTRY).

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DECLARATION

I, Hafsat Rufa'i, hereby declare that the work in this thesis entitled "Gastroprotective effect of aqueous stem bark extract of *Anogeissus leiocarpus* against ethanol-induced gastric ulceration in rats" was performed by me in the Department of Biochemistry under the supervision of Prof. H. C. Nzelibe and Dr. M. A. Musa. The thesis was not previously presented in whatever form to any institute or organization other than Ahmadu Bello University, Zaria, Nigeria for the award of any degree. The information derived from the literature has been duly acknowledged in the text and a list of references provided.

Hafsat Rufa'i ---------- ----------

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CERTIFICATION

This thesis entitled "GASTROPROTECTIVE EFFECT OF AQUEOUS STEM BARK EXTRACT OF *ANOGEISSUS LEIOCARPUS* AGAINST ETHANOL-INDUCED GASTRIC ULCER IN RATS" carried out by Hafsat Rufa'i, meets the regulation governing the award of the degree of Master in Biochemistry, Faculty of Life science, Ahmadu Bello University Zaria and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

This work is dedicated to my parents: my father Alhaji Rufa'i Kani and my mother Prof. Hannatu Sabo Ahmad. To my husband Saleh Aminu Kani, my children Aminu, Fatima and Habiba.

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I am grateful to Almighty Allah, the beneficent the merciful, for giving me all that is required in life. All glory to Him who facilitated this accomplishment. May His blessings and mercies continue to shower on Prophet Muhammad (Sallallahu Alaihi Wasallam), members of his household, his companions and those who follow his teachings until the Day of Judgment.

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LIST OF ABBREVIATIONS

AMP…….....Adenosine monophosphate

ATP……..Adenosine triphosphate

Cag A……...Cytotoxin-associated gene protein

CAT……..Catalase

c-AMP……Cyclic Adenosine monophosphate

CCK……..Cholocytokinin

CCL4…….Carbon tetrachloride

COX…….Cyclooxygenase

ECL…….Enterochromaffin-like cell

EGF…….Epidermal growth factor

e-NOS…….endothelial nitric oxide synthase

FDA……....Food and drug agency

FGF…….Fibroblast growth factor

FTIR……..Fourier transformed infra-red spectroscopy

G cells……...…….Gastrin cells

GC-MS……..……..…...Gas chromatography-mass spectroscopy

GERD…….Gastroesophageal reflux disease

GIT…….Gastro Intestinal Tract

GPx……...Glutathione Peroxidase

GSH…….Glutathione

GSSG-R……..Glutathione reductase

H.pyroli……...Helicobacter pyroli

$H^+ K^+$-ATPase……...……Proton pump

H_2……..Histamine

H_2O……...Water

H_2O_2……Hydrogen peroxide

HCl……Hydrochloric acid

HCO_3.....Hydrogen carbonate

HO……..……Heme oxygenase

$HO^{\cdot}$.....Hydroxyl radicals

HOCl……...……Hyperchlorous acid

IFN……Interferon

IFN-γ…….....Interferon gamma

IL…….……Interleukin

iNOS…….inducible nitric oxide synthase

LD_{50}……...Median Lethal Dose

LOXs…….....Lipoxygenases

MAPK……..Mitogen activated protein kinases

MPO……...……Myeloperoxidase

MtDNA……Mitochondrial Deoxyribonucleic acid

N_2O_3……Dinitrogen trioxide

NADPH…….Nicotinamide adenine dinucleotide phosphate

NMR…….....Nuclear Magnetic Resonance

NO…….Nitric oxide

NO_2…….....Nitrogen dioxide

NPSH……..... Sulfhydryl groups

NSAID……...Nonsteroidal anti-inflammatsory drug

O_2.....Oxygen

$O_2^{\cdot -}$……..Superoxide anion radical

O_3.....Ozone

OD…….Optical density

$ONOO^-$ …… Peroxynitrate

P…….Parietal cells

PaI……..Pathogenicity island

PG……Prostaglandin

PIXE……..Particle Induced X-ray Emission

PPI…...……..Proton pump inhibitors

RNHCl……...Chloramines

RNS……...Reactive nitrogen species

ROI……...Reactive oxygen intermediates

ROOH……..…...Carboxyl

ROS……Reactive Oxygen Specie

SMS……...…….Somatostatin

SOD……...Superoxide Dismutase

TBARS……Thiobarbituric acid reactive substances

TGF-α…….....Transforming growth factor alpha

TLC……Thin layer chromatography

TNF-α……Tumor necrosis factor alpha

Trx…….…….Thioredoxin

TrxR……..Thioredoxin reductases

VEGF…….Vascular endothelial growth factor

UI…….Ulcer index

ABSTRACT

The Gastroprotective effect of aqueous stem bark extract of *Anogeissus leiocarpus* against ethanol induced gastric ulcer in albino rats was investigated. Eighty-six albino rats (weighing 160-250g) of both sexes were used in this study. Phytochemical studies revealed the presence of flavonoids, alkaloids, saponins, anthraquinones, tannins, cardiac glycosides, steroids and triterpenes. The median lethal dose (LD_{50}) of the aqueous stem bark of *A. leiocarpus* was found to be above 5000mg/kg body weight orally. Pre-treatment by oral administration of aqueous stem bark extract of *A. leiocarpus* at doses of 100, 200 and 400mg/kg b.w for 14 days, dose dependently and significantly decreased the mean ulcer score, ulcer index, percentage ulceration and preventive index ($p<0.05$) induced by 70% ethanol. The standard drug (cimetidine 100mg/kg) also decreased the ulcer scores. The severity of the reaction to ethanol on gastric mucosa and cytoprotection by aqueous *A. leiocarpus* were apparent by histological assessment of the gastric mucosa. The ulcer control showed intense ulcerated gastric mucosal epithelial cells, necrotic tissue and heavy infiltration. The section of gastric mucosa from rat pre-treated with stem bark aqueous extract of *Anogeissus leiocarpus at* 100 and 200mg/kg b. w. showed slightly eroded mucosal epithelial cells, less infilteration and haemorrhage. In the 400mg/kg b.w. stem bark aqueous extract of *Anogeissus leiocarpus* pre-treated rat, there is no observable haemorrhagic necrosis of gastric mucosa and showed protection against the histopathological changes observed in ulcer (ethanol) treated group with an intact gastric pits, maintenance of mucosa even after exposure of ethanol. Cimetidine (100mg/kg b.w.) pre-treated group demonstrates slight ulceration, less hemorrhagic necrosis and infiltration in the gastric mucosa of rat. The oxidative analysis shows significant decrease in Malondialdehyde (MDA) at 100mg/kg, 200mg and 400mg/kg b.w as well as the (100mg/kg b.w) cimetidine group ($p<0.05$) when compared with the ulcer control activity. Antioxidant capacity of animals treated with 100mg/kg, 200mg/kg and 400mg/kg b.w showed a significant increase in catalase activity as well as 100mg/kg cimetidine group ($p<0.05$) when compared with the ulcer control group. There was a significant increase ($p<0.05$) in Superoxide dismutase (SOD), at 400mg/kg b.w and 100mg/kg cimetidine groups when compared with the ulcer control group. In Glutathione Peroxidase (GPx), there was a significant increase at 200 and 400mg/kg b.w group ($p<0.05$) but there was no significant increase in 100mg/kg groups as well as (100mg/kg) cimetidine group ($p>0.05$) when compared with the ulcer control group. The aqueous extract of *A. leiocarpus* was partially purified by column chromatography. Eluents with similar Rf values were pooled together into five fractions using thin layer chromatography (TLC). The qualitative (spectrophotometrically using DPPH) and quantitative antioxidant activity of the five pooled fractions were determined to using 1, 1-Diphenyl-2-picrylhydrazyl (DPPH) spray to identify the fraction with highest activity on a TLC plate, an active spot turned from violet to yellow. Fraction A had higher DPPH percentage inhibition of (97.95%) and the lowest IC_{50} (20.88). Fourteen phytocomponents with known and unknown biological activities in the most active fraction A were characterized using Gas chromatography-Mass spectrometry (GCMS) and Fourier transformed infrared (FTIR). In conclusion these findings suggest that aqueous stem bark extract of *A. leiocarpus* possesses antioxidant properties and dose-dependent gastroprotection, these justify the ethno medicinal use of the plant in the treatment and management of gastric ulcer.

CHAPTER ONE

1.0 INTRODUCTION

Gastric ulcer is one of the major gastrointestinal disorders, which occurs due to an imbalance between the offensive (gastric acid secretion) and defensive (gastric mucosal integrity) factors (Laine *et al.,* 2008 and Shaker *et al.,* 2010). The incidence of peptic ulcer is increased due to stress, smoking, alcohol, Helicobacter pylori infection and ingestion of non-steroidal anti-inflammatory drugs (NSAID) (Vonkeman *et al.,* 2007; Ineu *et al.,* 2008; Sowndhararajan *et al.,* 2013). It has been suggested that reactive oxygen species (ROS), primarily super-oxide anions, hydroxyl radicals, and lipid peroxides, are the harmful species known to cause the gastric ulcer development (Smith *et al.,* 1996 and Sultana *et al.,* 2014). Gastric cell have several enzymatic and non-enzymatic antioxidants including catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx), endogenous glutathione (GSH) and sulfhydryl groups (NPSH), but excessive generation of ROS enhance lipid peroxidation and depletes these antioxidants enzymes (Boligon *et al.,* 2014).

The prevention or cure of peptic ulcers is one of the most important challenges confronting medicine nowadays, as it is certainly a major human illness affecting nearly 8%–10% of the global population, of which 5% suffer from gastric ulcers (Calam and Baron, 2001, Mahdy *et al.,* 2018). Gastric ulcer therapy faces a major drawback because most of the drugs currently available in the market show limited efficacy against gastric ulcers and are often associated with severe side effects such as nausea, abdominal pain, constipation, diarrhea, gynaecomastia, loss of libido. (Mahdy *et al.,* 2018 and Mota *et al.,* 2009). Most drugs used are anti-secretory drugs such as: Proton pump inhibitors (Omeprazole, Lansoprazole, etc.) and Histamine H_2-receptor blocker (cimetidine, Ranitidine etc.), which extensively control increased acid secretion and acid

related disorders (Martelli *et al.*, 1998; Wolfe and Sachs, 2000). However, there are reports of different adverse effects of these drugs besides high rate of recurrence of the disease (Radwan *et al.*, 2003).

Controlling the formation of reactive oxygen species and secretion of gastric acid are essential for the treatment of these pathologies. In this context, medicinal plants containing a wide variety of anti-oxidants, such as phenols, flavonoids, coumarins, tannins and terpenoids compounds, are some of the most attractive sources of new drugs and have been shown to produce promising results in the treatment of gastric ulcers (Boligon *et al.*, 2014). Gastroprotective substances not only effectively prevent gastric mucosa from the development of erosions and ulcerations, but also accelerate ulcer healing (Zayachkivska *et al.*, 2005), these could play a potential role in the prevention and treatment of peptic ulcer disease.

Ethanol is also known to cause gastric damage by altering protective factors, which includes decreasing mucus production and blood circulation within the mucosa (Boligon et al., 2014). In addition, the gastric damage caused by ethanol may be due to the generation of reactive species, decreased cell proliferation, and an exacerbated inflammatory response (Amaral *et al.*, 2013). The World Health Organization (WHO) has estimated that there are about 2 billion people worldwide who consume alcoholic beverages and 76.3 million with diagnosable alcohol use disorders. Alcohol consumption is an important factor to induce the gastric ulcer.

Anogeissus leiocarpus DC.Guill. & Perr. belongs to the phylum, *Tracheophyta*; Order; Myrtales and Family: *Combretaceae* commonly called 'axlewood', locally referred to in Nigeria as '"Ayin", "Orin-odan" in Yoruba, "Marke" in Hausa, "Kojoli" in Fulani, "Annum" in Kanuri, "Kukunchi" Nupe and "Atara" in Igbo'. It is an evergreen tall tree found in savannah region of

Tropical Africa, especially west and east Africa through tropical Southeast Asia (Steentoft, 1988; Odugbemi and Akinsulire, 2008). In Africa, its occurrence extends from Senegal in West Africa to Sudan and Ethiopia in East Africa. (Abdullahi, *et al.*, 2003). It is typically found growing at altitudes of 450 to 1900 m, and grow on a range of soil types including compact clay soils (Vertisols) (Moctar and Sidi, 2007).

In Nigeria, earlier workers have reported that powdered bark is applied to wounds, sores, boils and diabetic ulcers with good results (Ogunyemi, 1979). The plant, widely used in the treatment of parasitic diseases, has superior leishmonicidal activity (Shuaibu *et al.,* 2008). It is effective in the traditional treatment of trypanosomiasis (Wurochekke and Anyanwu, 2012). Many studies have demonstrated the antimicrobial; anti-carries, anti-periopathic and anti-fungal properties of both aqueous and ethanol extracts of both chewing sticks of Anogeissus leiocarpus tree (Akande and Hayashi, 1998). Many *combretaceae* species are widely distributed in Nigeria and are used in traditional medicine for the treatment of respiratory diseases (Asthma, catarrh, chronic bronchitis, cough, hay-fever, pneumonia, pulmonary disorders and tuberculosis (Mann *et al.,* 2007). *Anogeissus Leiocarpus* may serve as remedies for gonorrhea, diabetes, hypertension, general body pain, blood clotting agent, as acaricide and as antihelmentic in different communities (Abdullahi, *et al.,* 2003; Agaie *et al.,* 2007).

Anogeissus leiocarpus has been shown to contain a lot of active phytochemical constituents which may be responsible for the observed prokinetic action and antibacterial activities against *Staphylococcus aureus, Salmonella typhi* and *Klebsiella* species (Timothy *et al.,* 2015). A research in conducted to investigate in vitro Anthelmintic potential of crude ethanolic leaf extracts of *Anogeissus leiocarpus* relative to that of the commercial Anthelmintic febendazole against eggs and infective larvae of *Haemonchus controtus* indicated significant dose dependent

inhibition of egg hatch and larval motility. The potency of the plant extract was comparable to that of febendazole, the finding suggests that this plant could yield natural alternative treatment for *Haemonchus controtus* (Ademola and Eloff, 2011). Methanol and ethyl acetate extracts of the plant were investigated for their1,1-diphenyl-2- picryl hydrazyl (DPPH) free radical scavenging activity and Ferric reducing antioxidant power (FRAP). The results revealed that the plant exhibited scavenging ability and strong reducing ability (Olajide, 2011; Victor and Grace, 2013).

Methanol extract of leaves, roots and stem barks of the plant showed interesting in vitro trypanocidal activity. The aqueous butanol fractions of the methanol extract of *Anogeissus leiocarpus* were associated with in vitro trypanocidal activity against four strains of Trypanosoma species. Castalagin isolated from these fractions showed trypanocidal activity on both, the human and domestic animal pathogens causing trypansomiasis (Shuaibu, 2008). The methanolic extract of the plant stem bark has high antimalarial activities, and capable of boosting HDL level in malaria-infected organisms.

A. leiocarpus was reported to have high antimicrobial activities in many chemotherapeutic applications, hence its continued use in the treatment of bacterial infections (Mann *et al.,* 2009; Mann *et al.,* 2010; Mann, 2012). Antibacterial activity of alcohol extracts of leaf, stem bark and root bark of *Anogeissus leiocarpus* showed higher activity against *Staphylococcus aureus* than other test organisms. Invitro investigation of extracts of *A. leiocarpus* for antifungal activities against *Aspergillus niger, Penicillium species, Microsporum audouinii* and *Trichophyton rubrum* using radial growth technique displayed depression on rats (Mann *et al.,* 2008a; Mann *et al.,* 2008b).

A. leiocarpus is suitable for use in the therapeutic management of asthma, and has been developed to single tablet dosage form (Emeje *et al.,* 2011). It possesses wound healing activity in a dose-dependent manner and provides a scientific rationale for its traditional use in the management of wounds (Barku *et al.,* 2013). Studies suggest that the aqueous extract of *A. leiocarpus* could be used, with some degree of safety, by oral route (Agaie *et al.,* 2007; Olabanji *et al.,* 2007). In Nigeria, the inner bark, which possesses antibacterial activities, is used as chewing stick. It was assessed as one of sixteen medicinal plants used for cleaning teeth in southwestern Nigeria. It affects the formation, growth, development, and protection of human teeth. The PIXE (particle-induced X-ray emission) technique was used (Olabanji *et al.,* 2007).

Reports of toxicity studies on the plant extracts showed it had no toxic effect on the liver, and its consumption is safe at a dose up to 200 mg/kg body weight (Ahmad and Wudil, 2013). Castalagin was isolated from stem bark of *A. leiocarpus* along with other hydrolysable tannins (Shuaibu *et al.,* 2008). The plant contains other important classes of bioactive constituents such as glycosides, phenols, tannins, saponins, alkaloids, steroids, flavonoids, ellagic acids and anthraquinones, which may be responsible for its medicinal uses and activities (Adamu *et al.,* 2013; Barku and Abban, 2013). Studies on essential oils of *Anogeissus leiocarpus* show that acids dominate the leaf and stem bark oils (65.8% and 43.6% respectively), followed by esters (17.9% and 20.2% respectively). Root oil rather has esters as most abundant (49.9%), followed by acids (36.7%). Notable is the unique presence of sugar (di-arabinose) in stem oil. Methyl hexadecanoate and hexadecanoic acid are common to the three oils, hence are chemo-taxonomic compounds (Moronkola and Kunle, 2014). Methanol extract of the stem bark of *Anogeissus leiocarpus* possesses antioxidant, hepatoprotective and ameliorative effects on hepatocellular injury following pre-treatment or post-treatment with carbon tetrachloride (CCl_4) (Atawodi *et al.,*

2011). It was demonstrated that *Anogeissus leiocarpus (DC.) Guill. & Perr.* and *Terminalia glaucescens Planch ex Benth*. had significant antimicrobial properties including inhibition of the growth of *Helicobacter pylori* (Lawal *et al.,* 2016).

Figure 1: Anogeissus leiocarpus plant

1.1 Gastric protection by medicinal plants

The need for more effective and cheaper management and prevention of gastric ulcer has attracted an increasing interest for herbal products because of their effectiveness, less side effects and relatively low costs). The aqueous extract of *Boswellia dalzielii* showed anti-ulcer activity (Nwinyi *et al.,* 2005). *Allophylus serratus Kurz* is commonly known as Tippani, it belongs to the family Sapindaceae and used to treat elephantiasis, oedema, and inflammation, fracture of bones, dyspepsia, anorexia and diarrhoea. Phytochemical studies proved the presence of phenacetamide, betasitosterol, quercetin. The mechanism involved in production of antiulcer activity by plant leaf extract is cytoprotective mechanism as well as anti-secretory (Vinay *et al.,* 2005). Curcumin demonstrated protective effect against gastric ulcer via inhibiting gastric acid secretion, relieving oxidative stress and ameliorating apoptosis.

A number of Chinese naturally occurring phytochemicals were reported to have gastro-protective action with potent anti-*Helicobacter pylori* effects (Li *et al.,* 2005). The study of methanolic leaf extract of *Alchonea cordifolia* on HCL/Ethanol and pyrolus ligation ulcer models showed significant ulcer inhibition and decrease in gastric juice formation hence adding more credibility in ethnomedicinal use of the plant in the treatment of gastric ulcer (Nguelefack *et al.,* 2005). For long, some herbal tea constituents and food additives have been known for their gastro-protective effects. For example, liquorice has been used as gastro-protective agent. Eugenol, a compound extracted from clove oil, has also protective effect against the formation of indomethacin-induced gastric ulcer. This effect was mediated by its anti-oxidant activity, decreasing acid-pepsin secretion and increasing mucus production (Morsy and Fouad, 2008). *Carica papaya Linn*. is commonly known as Papaya. It belongs to the family *Caricaceae* and well known for

various medicinal properties. The aqueous extract of the fruits were reported to possess antiulcer activity on aspirin induced ulcer in rats (Ologundudu *et al.,* 2008).

Nigella sativa seeds were found to be equally effective in healing of gastric ulcer as is cimetidine in aspirin induced gastric ulcer which suggests the use of the drug in the therapy of gastric ulcer disease in routine practice (Khalil *et al.,* 2010). Similarly, Lysophosphatidic acid, which is a component of soybean lecithin and antyusan, has a protective effect against gastric ulcer induction in an animal model, suggesting that daily intake of lysophosphatidic acid-rich foods or Chinese medicines may be beneficial for prevention of gastric ulcer in humans (Adachi *et al.,* 2011). In the ongoing search for bioactive natural products of herbal origin that have ulcer protective activity, crude plant extracts and plant-derived compounds are tried in different experimental models (Morsy and El-Sheikh, 2011).

A study shows that *Stachys. lavandulifolia* extract protected gastric mucosa from alcohol-induced gastric ulcer. This gastroprotection may mediate via gastric mucosal nitric oxide production (Nabavizadeh *et al.,* 2011). The anti-ulcer activity of *Aloe vera* was reported in Indomethacin induced ulcer model. The mechanism involved in production of antiulcer activity of the plant is due to its antioxidant, anti-inflammatory, mucus secreting, cytoprotective or healing activities (Sai *et al.,* 2011). *Mangifera indica L.* is commonly known as Mango. It belongs to the family *Anacardiaceae.* The petroleum ether and ethanol plant leaf extracts reported antiulcer activity (Lakshmi *et al.,* 2012). Study indicates that *Flabellaria paniculata Cav*. Leaf (FPL) was more effective against experimentally induced gastric ulcer models than *Flabellaria paniculata Cav.* roots (FPR). The presence of varied phytochemical constituents probably influenced the pharmacological differences between the two extracts. This may justify

its use and validated the inclusion of the plant in folk preparations for gastrointestinal remedies in the South Western part of Nigeria (Sofidiya *et al.,* 2012).

The study of Mechanisms of gastroprotective effects of ethanolic leaf extract of *Jasminum sambac* against HCl/Ethanol-Induced gastric mucosal injury in rats provided evidence that the *J. sambac* possessed an antigastric ulcer effect, which is related partly to a preservation of gastric mucus secretion, to increased production of HSP70 protein, and to the antioxidant enzymes (AlRashdi *et al.,* 2012). A study showed that hydroethanolic extract of young seedless pods of *Acacia nilotica* has antiulcer activity in pyrolus ligation, swimming stress and NSAID induced rat ulcer models. The extract containing more amount of phenolic components showed high antiulcer activity indicating the phenolic component of the extract to be responsible for the activity of the extracts (Bansal and Goel, 2012). Pre-treatment with the leaf extract of *Nauclea latifolia* caused a beneficial effect on indomethacin-induced gastric ulcers in rats as evident by the reduction in the ulcer index. The gastroprotective effect of the leaf extract is dose dependent and this may justify its use as an anti-ulcerogenic agent (Balogun *et al.,* 2013).

The study of *Rosmarinus officinalis L*. extract (*eeRo*) deserved further attention due to its importance in the prevention of gastric ulcerations induced by ethanol through three different mechanisms: antioxidant, antiinflammatory and vasodilator. The work presented the main mechanisms of *R. officinalis L*. extract, analyzing the macroscopic, microscopic and biochemical aspects (Amaral *et al.,* 2013). The aqueous leaf extracts of *Bombax. buonopozense* has an ulcer healing property against experimentally induced ulcers in rats and this study confirms folkloric claims of the benefits of *B.buonopozense* in treatment of ulcer. The results also suggest that the anti-ulcer activity is probably due to possible involvement of mucus in anti-ulcer-effect of

extracts, or probably by its free radical scavenging effect or may be also due to its anti-secretary activity (Nwagba *et al.,* 2013).

Methanolic extract of *Balanite aegyptiaca L*. showed significant antiulcer activity at 200mg/kg by indomethacin induced ulcer rats (Sudhakar *et al.,* 2013). *Scutia buxifolia* (ceSb) demonstrated gastric mucosal protection against oxidative injuries caused by ethanol and this protection is most likely due to antioxidant properties of *S.buxifolia*. In addition, the presence of phenolic acids and flavonoids in *ceSb* certainly contribute to the anti-ulcerogenic activity described in the study (Boligon *et al.,* 2014). Study has shown that *Sida corymbosa* has constituents with the ability to reduce the severity of haemorrhagic gastric lesions, thus validates the use of the plant in traditional medicine for the treatment of stomach ulcers (John-Africa, *et al.,* 2014).

Pre-treatment of the gastric tissue with ethanolic leaf extract of *Andrographys. paniculata* significantly prevented hypothermic stress-induced gastric wall mucus depletion. These findings suggest that a significant antisecretory and cytoprotective action of *A. paniculata* can be responsible for its antiulcer activity. Ethanolic extract of leaves of *A. paniculata* produced a significant decrease in gastric secretion in pylorous ligated rats and a highly significant cytoprotective effect against 80% ethanol-0.6 M HCl, 0.2 M NaOH, and 25% NaCl-induced cyto-destruction (Sharma *et al.,* 2014).

The attenuation of gastric affronts of indomethacin by administration of aqueous leaf extracts of *Spondias mombin* and *Ficus exasperate* at 200 mg/kg b.w. regimen is indicative of their excellent gastroprotective and antioxidative potentials in rats (Sabiu *et al.,* 2015). Vitamins C and E co-administration reduced gastric acid secretion and increased gastric mucus output, a

possible mechanism for reduced gastric ulceration occasioned by thermally oxidized palm oil diet consumption (Obembe *et al.*, 2015).

A study investigated the antibacterial activity of *Anogeissus leiocarpus* (DC.) Guill. & Perr. and *Terminalia glaucescens* Planch ex Benth. (Family *Combretaceae*) on nineteen strains of *Helicobacter pylori* including *H. pylori* ATCC 43504. The aqueous extracts of both plants were the most active on the test organisms supporting the use of these plants in folklore medicine where they are most often infused in hot water to administer to the patients for the treatment of various diseases including peptic ulcer disease (Lawal *et al.,* 2016). The attenuation of gastric affronts of ethanol by administration of extracts of *Cyathula prostrata* at various concentration regimen is indicative of its excellent gastroprotective and antioxidant potential in rats, which laid credence to traditional use of hot water extracts of *Cyathula prostrata* in the treatment of ulcer (Richard *et al.*, 2017).

The folkloric use of *Indigofera pulchra* in the therapy of peptic ulcer disease was validated. The extract protects against the ethanol-induced gastric ulceration and down-regulated the basal acid secretory parameters to promote mucosal cytoprotection. The presence of phytoconstituents in the medicinal plant might be responsible for those pharmacological actions observed (Saleh *et al.*, 2017). A The wide usage of *Fagonia indica* extract in folk medicine is therefore encouraged since it is now demonstrated to possess a potent gastroprotective effect against ethanol-induced gastric ulcer. Combination of both the plant extract and honey can be advised since expected to produce more protection. Both honey and *Fagonia indica* exhibit their beneficial effects probably through antioxidant and mucus production mechanisms, owing to that they are both rich in flavonoids, thus falling in the category of cytoprotective agents (Mahdy *et al.*, 2018).

1.2 Statement of Research problem

Peptic ulcer is one of the most common diseases affecting mankind and the incidence has been estimated to range from 5 to 10% (Akimoto *et al.,* 1998 and Mahdy *et al.,* 2018). Africa has the highest pooled prevalence of *Helicobacter pylori* infection (70.1%; 95%Cl, 62.6-77) (Hooi *et al.,* 2017). More than 100,000 cases per year were reported in Nigeria (WHO 2018). In US alone, more than six million people are affected each year (Feinstein *et al.,* 2010). Predisposing factors of gastric ulcer include, stress, alcohol, cigarette smoking, non- steroidal anti-inflammatory drugs, *Helicobacter pylori* infection and chronic pancreatitis (Tariq *et al.,* 1986 and Mustafa *et al.,* 2015). Several orthodox pharmaceutical drugs such as histamine H_2-receptor antagonists, antacids, anticholinergic drugs and proton-pump inhibitors have been used in the management of peptic ulcers, but they bring about many adverse effects (Mahdy *et al.,* 2018). Presently, there has been growing interest in alternative therapies especially from plant sources due to their perceived lower side effects, ease of accessibility and affordability (Rates, 2001, Bassi *et al.,* 2014 and Strand *et al.,* 2017). Decoction from the plant has been used in folk medicine for the treatment of ulcers (Oluronti et al., 2012). There is need to validate these findings as a way to boost acceptability and hence usage of anti-ulcer therapy from stem bark of *Anogeissus leiocarpus*.

1.3 Justification

Owing to the persistent problem of recurring ulcers after treatment and the occurrence of several adverse reactions with the anti-ulcer agent, new approach is constantly being pursued. Different therapeutic agents especially plant extracts are currently undertaken in various studies primarily to evaluate its antiulcerogenic potentials through basic scientific research using experimental animal models.

1.4 Null Hypothesis

Anogeissus leiocarpus aqueous stem bark extract has no gastroprotective activity.

1.5 Aim and objectives

The aim of this study is to investigate the effect of aqueous stem bark extract of *Anogeissus leiocarpus* on ethanol- induced gastric ulcers in albino rats.

The study is designed with the following objectives:

I. Qualitative and quantitative screening of phytochemical constituents of aqueous stem bark extract of *Anogeissus leiocarpus* and the median lethal dose (LD_{50}) determination.

II. The gross (mean ulcer index, percentage ulceration and preventive index) and microscopic (histopathology) effect of aqueous stem bark extract of *Anogeissus leiocarpus* on the gastric mucosal membrane.

III. The effect of aqueous stem bark extract of *Anogeissus leiocarpus* on some antioxidant enzymes capacity (catalase, superoxide dismutase and glutathione peroxidase) and thiobarbituric acid reactive substances (TBARS) in ethanol-induced gastric ulceration.

IV. Partial purification of aqueous stem bark extract of Anogeissus leiocarpus using column chromatography, quantitative evaluation of the radical scavenging activity of each purified pooled fraction on the resolved TLC plate and qualitative DPPH scavenging activity.

V. To characterize the bioactive component(s) of the aqueous stem bark extract of *Anogeissus leiocarpus* using Gas chromatography-Mass spectrometry (GCMS) and Fourier transformed infrared (FTIR).

CHAPTER TWO

2.0 LITERATURE REVIEW

Peptic Ulcer Disease (PUD) is a disorder of the upper gastrointestinal tract. Ulcers occur when the mucosal lining of the Gastro intestinal tract breaks down, resulting in acute or chronic inflammatory response (Academy of Nutrition and Dietetics (AND), 2013). Ulcers can develop in the esophagus, stomach, duodenum, or other regions of the GI tract. The digestive and absorptive functions of the gastrointestinal system depend upon a variety of mechanism, which involve gastric acid secretion and various digestive enzymes (Boron and Boulpaep, 2005). Ulcer occurs when the stomachs or duodenums mucosal lining cannot withstand the corrosive action of gastric juice (Boligon et al., 2014). Gastric juice consisting of hydrochloric acid and enzyme pepsin that breaks down protein can digest any living tissue, including the stomach and duodenum. However protective mechanisms prevent the stomach from digesting itself (Del Valle, 2001).

The upper Gastro intestinal tract is dependent upon the equilibrium between hostile factors that damage the mucous lining, like stomach acid, and protective factors, such as prostaglandins and mucus. When the hostile factors outnumber the natural protective defenses of the mucosa, ulcers form. Hydrochloric acid and pepsin are the primary aggressive factors, while defensive factors involve all elements that constitute the gastric mucosal barrier that counters the effect of aggressive factors (Repetto and Liesuy, 2002). Studies have shown that reactive oxygen species, especially the hydroxyl radical, plays a major role in oxidative damage of gastric mucosa (Phull *et al.,* 1995; Das *et al.,* 1997).

The mucosal defense system is highly a complex biological system that provide defense from mucosal injury and repair any injury that may occur (Del Valle, 2001). The gastric mucosa is

covered by continuous layer of mucous that protects against acids and enzymes produced by the mucosa itself (Singh, 2002).

Studies on Ethanol induced mucosal damage revealed that ethanol is involved in the formation of free radicals generated intracellular and extracellular (Terano *et al.,* 1989 and Hernandez *et al.,* 2000). Tissue damage to the gastroduodenal mucosa can be induced by acute or chronic ethanol exposure which may be associated with the production of toxic reactive species, an unbalanced oxidant/ antioxidant cellular process (Repetto and Liesuy, 2002). The relationship between the gastric mucosal damage and antioxidant protection is still under investigation. Several studies of cytoprotective agents especially, herbs and plants extract for peptic ulcers is on the rise in an attempt to find a solution to problems of recurring ulcers and to prevent occurrence of the disease.

2.1 Physiological Anatomy of the stomach

The stomach is divided into the cardiac region, fundus, body or copus and pyrolic region. The cardiac region is the upper part of the stomach where esophagus opens. The opening is guarded by a sphincter called cardiac sphincter, which opens only towards stomach. This portion is known as cardiac end. Fundus is a small dome-shaped structure. It is elevated above the level of esophageal opening. Body is the largest part of stomach forming about 75 to 80% of the whole stomach. It extends from just below the fundus up to the pyrolic region (Fig 2.1). Pyrolic region has two parts namely: antrum and pyrolic canal. The body of the stomach ends in antrum. Junction between the body and antrum is marked by an angular notch called insura angularis. Antrum is continued as a narrow canal, which is called pyrolic canal or pyrolic end. The pyrolic canal opens into first part of the intestine called duodenum. The opening is guarded by a sphincter called pyrolic sphincter. It opens towards the duodenum (Moore *et al.,* 2018).

The anterior wall of the stomach has been removed to demonstrate its interior. The longitudinal gastric folds disappear on distension. Along the lesser curvature, several longitudinal mucosal folds extend from the esophagus to the pylorus, making up the gastric canal along which ingested liquids pass.

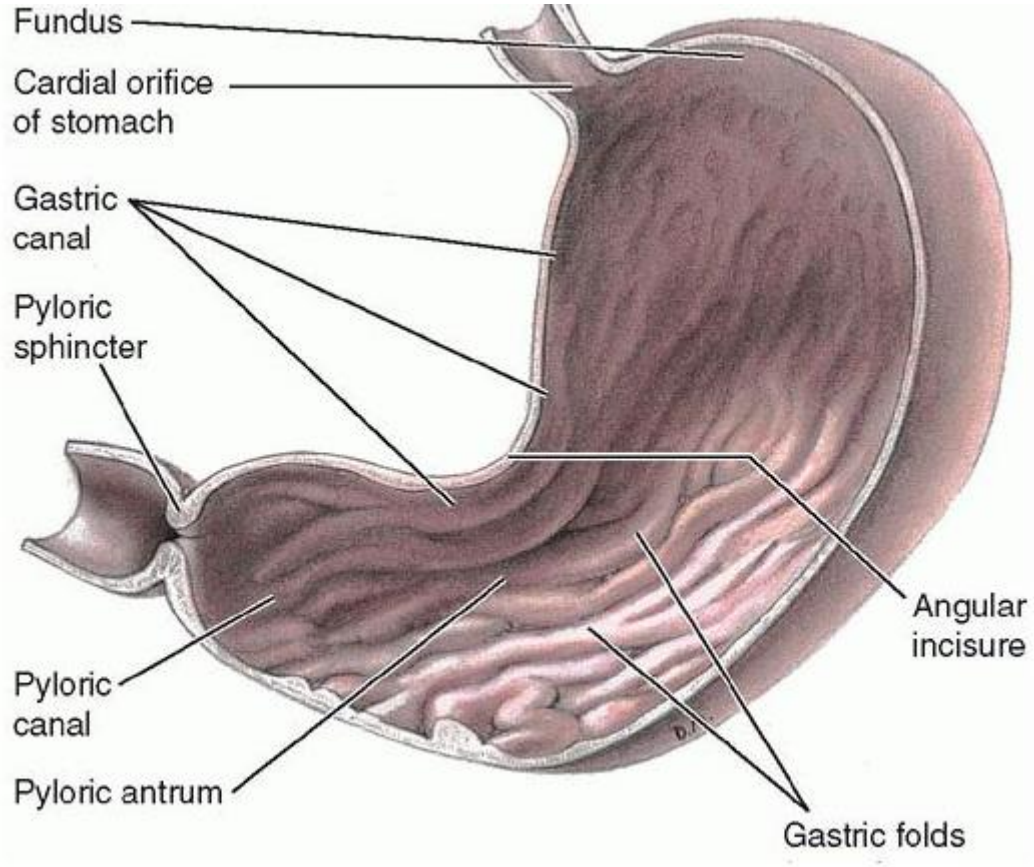


Figure 2.1: Anterior view of the stomach. (Moore *et al.*, 2018)

2.2 Histology of the stomach

The structures that make up the wall of the GIT are the same throughout its entire length, although some regional variations exist. The common histological feature of the entire gastrointestinal tract is essentially a muscular layer lined by a mucous membrane or mucosa. The tract has four distinct concentric layers from inside outward: mucosa, submucosa, muscularis and serosa (Wheater, 1993).

2.2.1 Mucosal layer

The mucosa consists of the epithelial layer, as well as an underlying layer of loose connective tissue known as the lamina propria, which contains capillaries, enteric neurons, and immune cells (e.g., mast cells), as well as a thin layer of smooth muscle known as the lamina muscularis mucosae (literally, the muscle layer of the mucosa) (Balogun *et al.*, 2013). The surface area of the epithelial layer is amplified by several mechanisms. Most cells have microvilli on their apical surfaces. In addition, the layer of epithelial cells can be evaginated to form villi or invaginated to form glands (or crypts). Finally, on a macroscopic scale, the mucosa is organized into large folds (Boron and Boulpaep, 2005).

2.2.2 Submucosal layer

The submucosal layer consists of loose collagen fibers, elastic fibers, reticular fiber and few cells of the connective tissues. Large blood vessels, lymphatic vessels and nerve plexus are present in this layer. The submucosa may also contain glands that secrete material into the gastrointestinal (GI) lumen (Singh, 2002; Sembulingam, *2014*).

2.2.3 Muscular layer

The muscle layer, the muscularis externa, includes two layers of smooth muscle fibers in the gastrointestinal (GI) track. The inner layer is circular, whereas the outer layer is longitudinal. Enteric neurons are present between these two muscle layers. The circular layer of the

muscularis continues with the circular layer of the esophagus, but absent in the fundus. The thickness of the circular layer increases in the antrum and pyloric sphincter. It does not continue into the duodenum (Wheater, 1993; Sembulingam, 2014).

2.2.4 Serosa/ Serous layer

The serosa is an enveloping layer of connective tissue that is covered with squamous epithelial cells (Singh, 2002). It is also formed by mesoepithelial cells. It covers the stomach, small intestine and large intestines. The fibrous layer is otherwise called fibrosa and it is formed by connective tissue. It covers pharynx and esophagus (Sembulingam, 2014).

2.3 Gastro duodenal mucosa mucosal glands

Gastric glands are classified into three types, on the basis of their location in the stomach:

1. Fundic glands or main gastric glands or oxyntic glands: Situated in body and fundus of stomach

2. Pyloric glands: Present in the pyloric part of the stomach

3. Cardiac glands: Located in the cardiac region of the stomach.

2.3.1 Fundic glands or main gastric glands or oxyntic glands

Fundic glands are considered as the typical gastric glands (Fig. 2.2). These glands are long and tubular. Each gland has three parts, viz. body, neck and isthmus. Consist of a pit, a neck, and a base. These glands contain several cell types, including mucous, parietal, chief, and endocrine cells; endocrine cells also present in both corpus and antrum. The surface epithelial cells, which have their own distinct structure and function, secrete HCO_3 and mucus (fig 2.2) (Boron and Boulpaep, 2005; Sembulingam, 2014).

2.3.1.1 Parietal or oxyntic cell

These are most numerous in the necks of the glands, but do not border directly onto the lumen, being separated from it by the peptic cells. They are triangular in shape, with the apex projecting towards to the lumen between the sides of the two peptic cells. These cells are intensely acidophilic (Fielding and Hallissey, 2005). It has an abundance of mitochondria, intracellular tubulovesicular membranes, and canalicular structures. It secretes acid and intrinsic factor, a glycoprotein that is required for cobalamin (vitamin B12) absorption in the ileum (Boron and Boulpaep, 2005).

2.3.1.2 Peptic or chief Cells

Peptic cells secrete pepsinogens, but not acid. These epithelial cells are substantially smaller than parietal cells. A close relationship exists among pH, pepsin secretion, and function. Pepsins are endopeptidases (i.e., they hydrolyze "interior" peptide bonds) and initiate protein digestion by hydrolyzing specific peptide linkages. The basal luminal pH of the stomach is 4 to 6; with stimulation, the pH of gastric secretions is usually reduced to less than 2. At pH values that are less than 3, pepsinogens are rapidly activated to pepsins. A low gastric pH also helps to prevent bacterial colonization of the small intestine (Arab *et al.,* 2015).

2.3.1.3 Mucous neck cells

These cells resemble the mucous cells of the cardiac and pyrolic zones. They lie between the parietal cells in the necks of the glands but are smaller than the surface mucous cells. Their mucigen granules are larger and less dense than those of the surface cells (Fielding and Hallissey, 2005) (fig 2.2). The population of cells at the fundal and pyloric gland areas is very stable and their replacement is probably accelerated only after injury. The mucigenic cells, the mucous neck cells and the surface epithelium cells all secrete a highly alkaline secretion which is

extruded to the surface forming a three dimensional jelly coat for the surface epithelial cells that are exposed to digestive juices (Del Valle, 2001).

2.3.1.4 Hormone-secreting (endocrine) cells

Endocrine cells are located near the basal part of the gastric gland which contains membrane bound neurosecretory granules. The endocrine cells include; G-cells and D-cells which secretes gastrin and somatostatin respectively. These two peptide hormones function as both endocrine and paracrine regulators of acid secretion. Gastrin stimulates gastric acid secretion and also a major trophic or growth factor for GI epithelial cell proliferation, while somatostatin inhibits gastric acid secretion (Boron and Boulpaep, 2005).

2.3.2 Pyloric Glands

Pyloric glands are short and tortuous in nature. These glands are formed by G cells, mucus cells, EC cells and ECL cells.

2.3.3. Cardiac Glands

Cardiac glands are also short and tortuous in structure, with many mucus cells. EC cells, Enterochromaffin-like cells (ECL) and chief cells are also present in the cardiac glands (Sembulingam, 2014).

Glands of the stomach or gastric glands are tubular structures made up of different types of cells. These glands open into the stomach cavity via gastric pits.

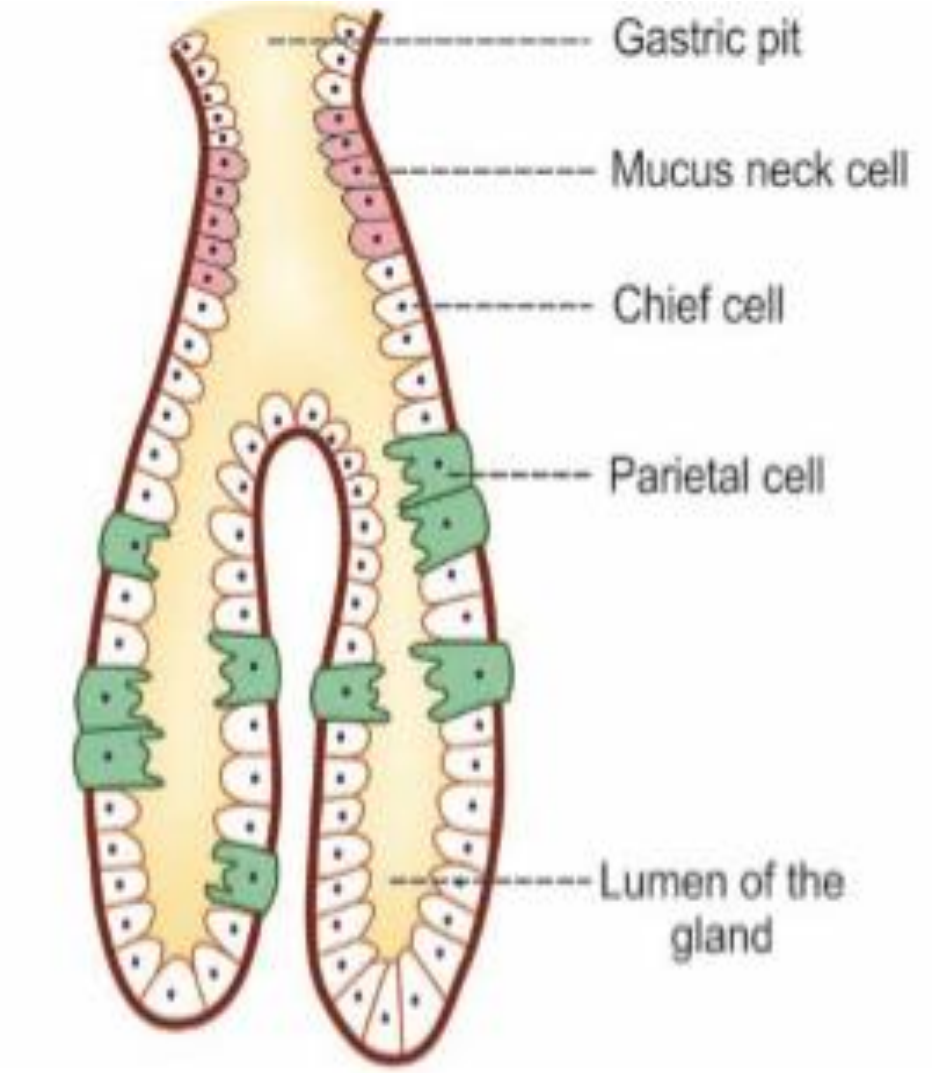


Figure 2.2 Gastric glands (Sembulingam, 2014)

2.3.4 Histology of an ulcerated mucosa

Chronic peptic ulcer (stomach) is a mucosal defect which penetrates the muscularis mucosae and muscularis propria, produced by acid-pepsin aggression. Ulcer margins are regular, slightly elevated due to gastritis (Del Valle, 2001). During the active phase the base of the ulcer shows 4 zones: inflammatory exudate, fibrinoid necrosis, granulation tissue and fibrous necrosis. The fibrous base of the ulcer may contain vessels with thickened wall or with thrombosis (Arab et al., 2015).

2.4 Gastric secretion

The cells of the gastric glands secrete about 2500 ml of gastric juice daily. This contains a variety of substances and gastric enzymes, whose role is to kill ingested bacteria, aid protein digestion, stimulate the flow of biliary and pancreatic juices and provide the necessary pH for pepsin to begin protein degradation. Contents of normal gastric juice include; Electrolytes, pepsins: I–III gelatinase, mucus, intrinsic factor, water (Fielding and Hallissey, 2005).

Hydrochloric acid and pepsinogen are the two principal gastric secretory products capable of inducing mucosal injury. Basal acid production occurs in a circadian pattern, with highest levels occurring during the night and lowest levels during the morning hours. Cholinergic input via the vagus nerve and histaminergic input from local gastric sources are the principal contributors to basal acid secretion (Del Valle, 2001).

2.4.1 Regulation of gastric acid secretion

Regulation of gastric acid secretion occurs primarily in three phases: The cephalic phase is secretion of gastric juice by the stimuli arising from head region (cephalus). This phase of gastric secretion is regulated by nervous mechanism (Del Valle, 2001). The gastric juice secreted during this phase is called appetite juice. During this phase, gastric secretion occurs even without the presence of food in stomach. The quantity of the juice is less but it is rich in enzymes and

hydrochloric acid. It stimulates gastric secretion via the vagus nerve due to sight, smell, and taste of food (Del Valle, 2001).

The gastric phase is activated once food enters the stomach. This phase is regulated by both nervous and hormonal control. Gastric juice secreted during this phase is rich in pepsinogen and hydrochloric acid. Mechanisms involved in gastric phase are:

(1). Nervous mechanism through local myenteric reflex and vagovagal reflex.

(2). Hormonal mechanism through gastrin Stimuli, which initiate these two mechanisms are: This component of secretion driven by distention of the stomach wall, nutrients (amino acids and amines) and components of food that directly stimulate the G cell to release gastrin, which in turn activates the parietal cell via direct and indirect mechanisms. As well as mechanical stimulation of gastric mucosa by bulk of food

The last phase of gastric acid secretion is initiated as food enters the intestine is mediated by luminal distention and nutrient assimilation. Additional neural (central and peripheral) and hormonal (secretin, cholecystokinin) factors play a role in counterbalancing acid secretion. Under physiologic circumstances, these phases occur simultaneously (Del Valle, 2001).

The gastrointestinal hormone somatostatin is released from endocrine cells found in the gastric mucosa (D cells) in response to Hydrochloric acid (HCl). Somatostatin can inhibit acid production by both direct (parietal cell) and indirect mechanism (decreased histamine release from enterochromaffin-like (ECL) cells and gastrin release from G cells) (Del Valle, 2001).

2.4.2 Mechanism of hydrochloric acid secretion

According to Devonport theory, hydrochloric acid secretion is an active process that takes place in the canaliculi of parietal cells in gastric glands. The energy for this process is derived from oxidation of glucose.

Carbon dioxide is derived from metabolic activities of parietal cell. Some amount of carbon dioxide is obtained from the blood also. It combines with water to form carbonic acid in the presence of carbonic anhydrase. This enzyme is present in high concentration in parietal cells. Carbonic acid is the most unstable compound and immediately splits into hydrogen ion and bicarbonate ion. The hydrogen ion is actively pumped into the canaliculus of parietal cell (Davies, 2008).

Simultaneously, the chloride ion is also pumped into canaliculus actively. The chloride is derived from sodium chloride in the blood. Now, the hydrogen ion combines with chloride ion form hydrochloric acid. To compensate the loss of chloride ion, the bicarbonate ion from parietal cell enters the blood and combines with sodium to form sodium bicarbonate. (Sembulingam, 2014). $CO_2+H_2O+NaCl \rightarrow HCl+NaHCO_3$ (Figure 2.3)

Hydrochloric acid (HCl) secretion that takes place in the parietal cells of gastric gland

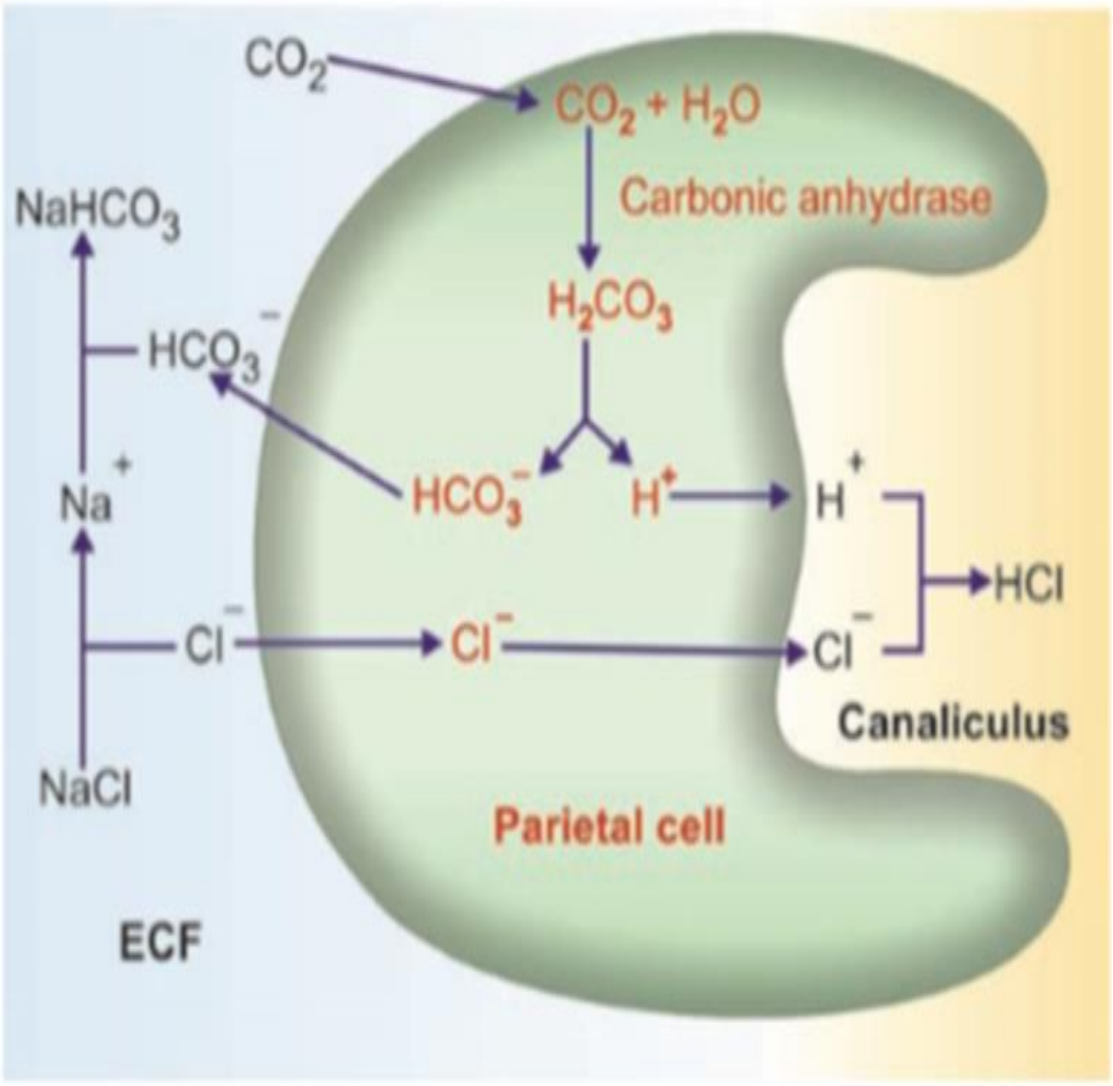


Fig 2.3: Secretion of hydrochloric acid in the parietal cell of gastric gland (Sembulingam, 2014)

2.4.3 Control of Acid Secretion

A variety of substances are capable of reducing gastric acid secretion when infused intravenously, including PGE_2 and several peptides hormones, including secretin, gastric inhibitory peptide, glucagon and somatostatin (Davies, 2008). PGE_2, secretin and somatostatin may be physiologic regulators. Somatostatin inhibits secretion of gastrin and histamine, and appears to have a direct inhibitory effect on the parietal cell (Fig 2.4) (Del Valle, 2001).

The parietal cell, also known as the oxyntic cell, is usually found in the neck, or isthmus, or in the oxyntic gland. The resting, or unstimulated, parietal cell has prominent cytoplasmic tubulovesicles and intracellular canaliculi containing short microvilli along its apical surface (Fig. 2.4). H^+, K^+-ATPase is expressed in the tubulovesicle membrane; upon cell stimulation, this membrane, along with apical membranes, transforms into a dense network of apical intracellular canaliculi containing long microvilli (Davies, 2008). Acid secretion, a process requiring high energy, occurs at the apical canalicular surface. Numerous mitochondria (30–40% of total cell volume) generate the energy required for secretion (Del Valle, 2001).

The G cell releases he hormone gastrin which enters the blood circulation. Gastrin directly stimulates the parietal cells to produce acid secretion and also indirectly by stimulating the ECL cell to release histamine. Histamine binds on receptors on the parietal cells and stimulates gastric acid secretion. Acetylcholine released by the vagus also acts as a stimulator of the parietal cells to produce acids. Somatostatin released by the D cells acts as an inhibitor of acid secretion directly by binding to receptors on the parietal cells and indirectly by binding to receptors on the ECL thereby inhibiting the release of histamine but the cells. Somatostatin also inhibits the G cell to release gastrin in into the blood circulation. (Del Valle, 2001)

Gastric acid secretion regulation at the parietal cell

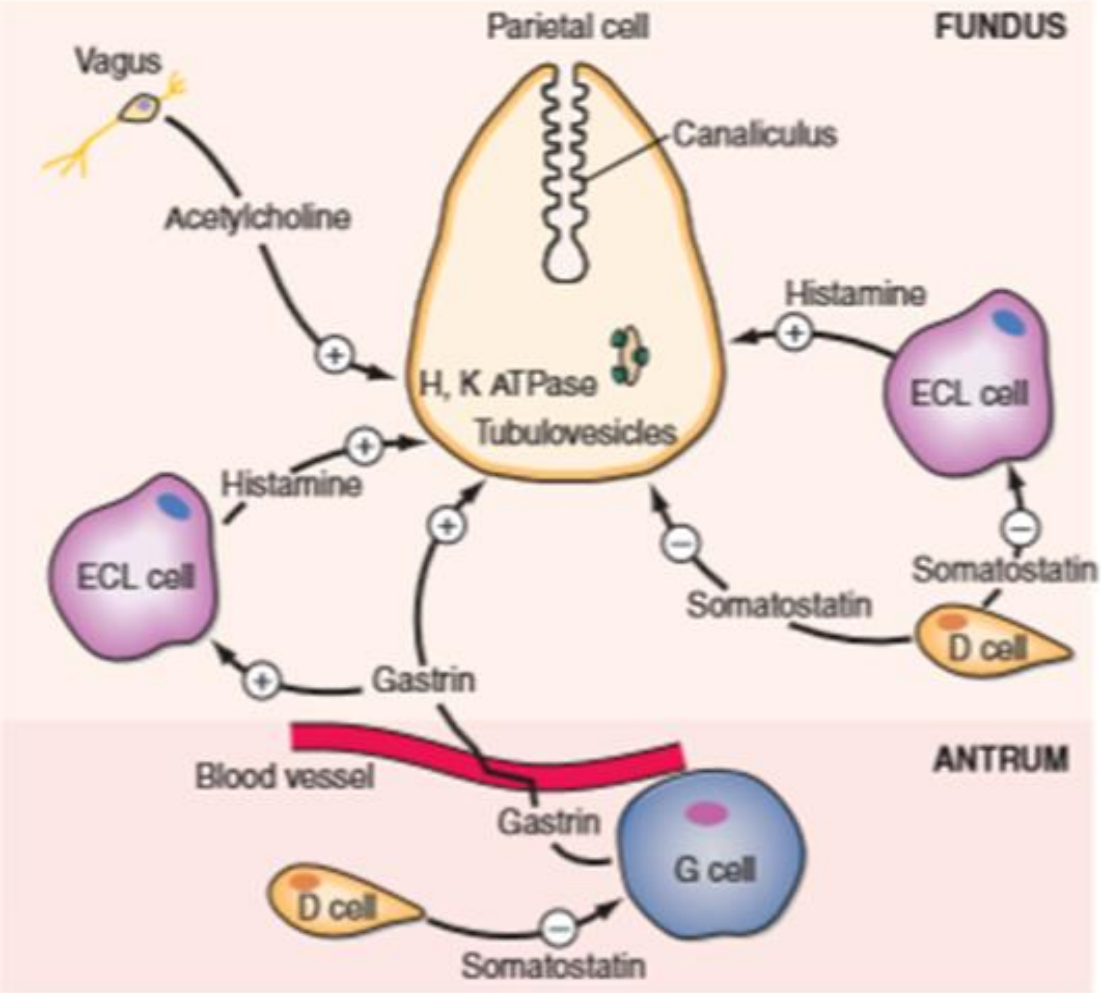


Figure 2.4: Regulation of gastric acid secretion at the cellular level. ECL cell, enterochromaffin-like cell (Del Valle, 2001).

2.4.4 Pepsinogen secretion

The chief cells in gastric glands, as well as mucous cells, secrete pepsinogens, a group of proteolytic proenyzmes (i.e., zymogens or inactive enzyme precursors) that belong to the general class of aspartic proteinases (Boron and Boulpaep, 2005). Several slightly different types of pepsinogen are secreted by the peptic and mucous cells of the gastric glands. Even so, all the pepsinogens perform the same functions. When pepsinogen is first secreted, it has no digestive activity. However, as soon as it comes in contact with hydrochloric acid, it is activated to form active pepsin by spontaneous cleavage of a small N- terminal peptide fragment (the activation peptide). Pepsin functions as an active proteolytic enzyme in a highly acid medium (optimum pH 1.8 to 3.5), but above a pH of about 5 it has almost no proteolytic activity and becomes completely inactivated in a short time (Boron and Boulpaep, 2005).

2.4.5 Pepsin activity

Pepsinogen is inactive and requires activation to a protease, pepsin, to initiate protein digestion. This activation occurs by spontaneous cleavage of a small N- terminal peptide fragment (the activation peptide), but only at a pH that is less than 5.0 (Fig. 2.5). Between pH 5.0 and 3.0, spontaneous activation of pepsinogen is slow, but it is extremely rapid at a pH that is less than 3.0. In addition, pepsinogen is also auto activated; that is, newly formed pepsin iself cleaves pepsinogen to pepsin (Boron and Boulpaep, 2005).

The activation of pepsinogen to pepsin which is pH dependent. It is the spontaneous cleavage of the N-terminal peptide which occurs at a very low pH of less than 3.0.

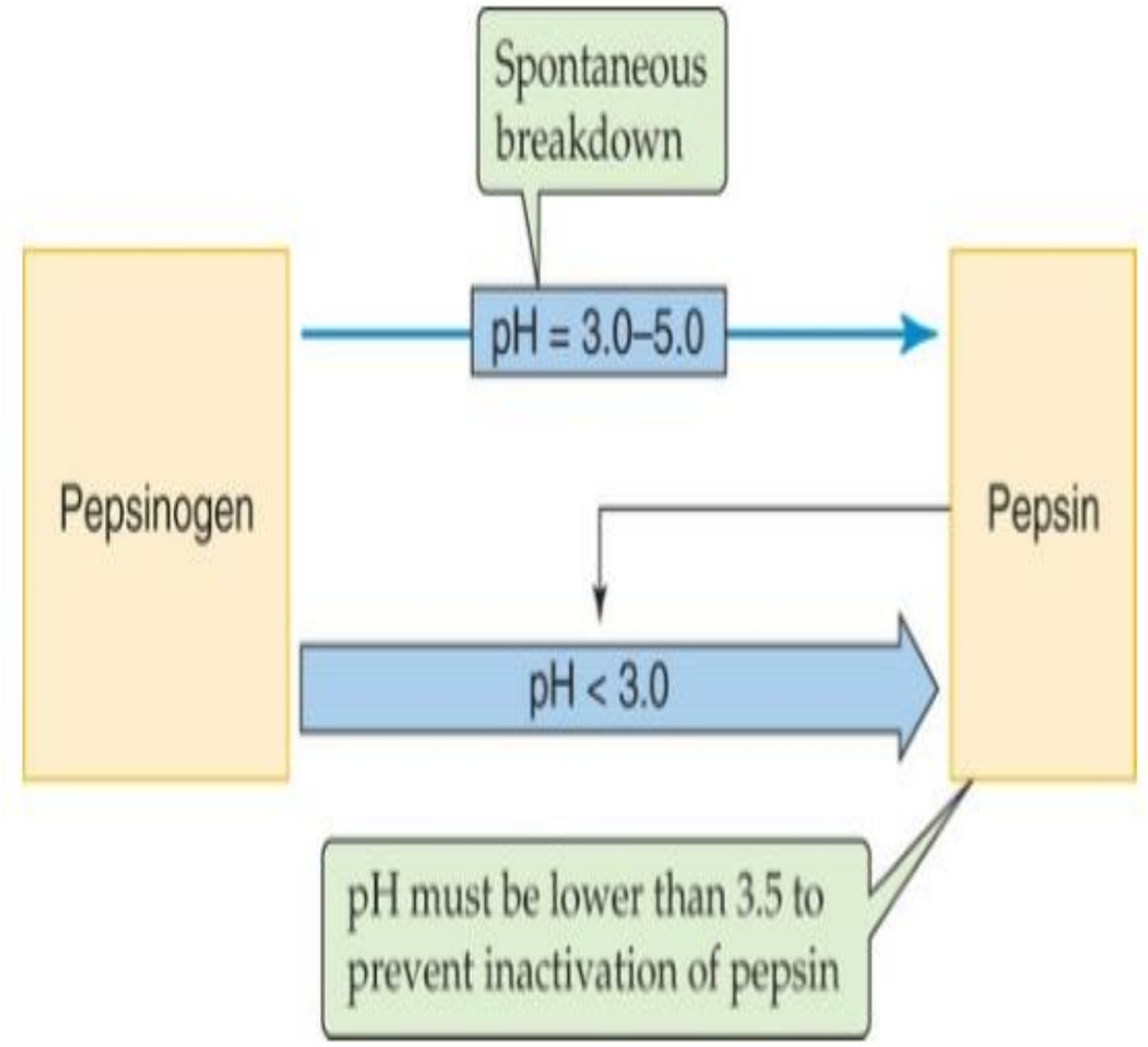


Figure 2.5: Activation of the pepsinogens to pepsins (Boron and Boulpaep, 2005).

At pH values from 5 to 3, pepsinogens spontaneously activate to pepsins by the removal of an N-terminal activation peptide. This spontaneous activation is even faster at pH values lower than 3. The newly formed pepsins themselves—which are active only at pH values lower than 3.5—also can catalyze the activation of pepsinogens (Davies, 2008).

Once pepsin is formed, its activity is also pH dependent. It has optimal activity at a pH between 1.8 and 3.5; the precise optimal pH depends on the specific pepsin, type and concentration of substrate, and osmolality of the solution. pH values higher than 3.5 reversibly inactivate pepsin, and pH values higher than 7.2 irreversibly inactivate the enzyme (Boron and Boulpaep, 2005). These considerations are sometimes useful for establishing optimal antacid treatment regimens in peptic ulcer disease. Pepsin is an endopeptidase that initiates the process of protein digestion in the stomach. Pepsin action results in the release of small peptides and amino acids (peptones) that, as noted earlier, stimulate the release of gastrin from antral G cells; these peptones also stimulate CCK release from duodenal I cells. As previously mentioned, the peptones generated by pepsin stimulate the very acid secretion required for pepsin activation and action. Thus, the peptides that pepsin releases are important in initiating a coordinated response to a meal. However, most protein entering the duodenum remains as large peptides, and nitrogen balance is not impaired after total gastrorectomy (Boron and Boulpaep, 2005).

2.5 Gastro duodenal mucosal defense mechanisms

The gastric epithelium is under constant assault by a series of endogenous noxious factors, including HCl, pepsinogen/pepsin, and bile salts. In addition, a steady flow of exogenous substances such as medications, alcohol, and bacteria encounter the gastric mucosa. A highly intricate biologic system is in place to provide defense from mucosal injury and to repair any

injury that may occur. The mucosal defense system can be envisioned as a three-level barrier, composed of pre-epithelial, epithelial, and sub-epithelial elements (Fig. 2.6) (Del Valle, 2001).

The first line of defense is a mucus-bicarbonate layer, which serves as a physicochemical barrier to multiple molecules, including hydrogen ions. Mucus is secreted in a regulated fashion by gastroduodenal surface epithelial cells. It consists primarily of water (95%) and a mixture of lipids and glycoproteins (mucin). The mucous gel functions as a nonstirred water layer impeding diffusion of ions and molecules such as pepsin. Bicarbonate, secreted in a regulated manner by surface epithelial cells of the gastroduodenal mucosa into the mucous gel, forms a pH gradient ranging from 1 to 2 at the gastric luminal surface and reaching 6 to 7 along the epithelial cell surface (Zhu and Kauntiz, 2008).

Surface epithelial cells provide the next line of defense through several factors, including mucus production, epithelial cell ionic transporters that maintain intracellular pH and bicarbonate production, and intracellular tight junctions. If the pre-epithelial barrier were breached, gastric epithelial cells bordering a site of injury can migrate to restore a damaged region (restitution) (Zhu and Kaunitz, 2008).

This process occur independent of cell division requiring an uninterrupted blood flow and an alkaline pH in the surrounding environment. Several growth factors, including epidermal growth factor (EGF), transforming growth factor (TGF) - α, and basic fibroblast growth factor (FGF), modulate the process of restitution. Larger defects that are not effectively repaired by restitution require cell proliferation. Epithelial cell regeneration is regulated by prostaglandins and growth factors such as EGF and TGF-α. In tandem with epithelial cell renewal, formation of new vessels (angiogenesis) within the injured microvascular bed occurs. Both FGF and vascular endothelial

growth factor (VEGF) are important in regulating angiogenesis in the gastric mucosa (Del Valle).

The third line of defense is composed by an elaborate microvascular system within the gastric submucosal layer which is the key component of the sub-epithelial defense/repair system. It provides HCO_3^-, which neutralizes the acid generated by parietal cell. It also provides an adequate supply of micronutrients and oxygen while removing toxic metabolic by-products (Del Valle, 2001).

Prostaglandins play a central role in gastric epithelial defense/repair (Fig. 2.6). The gastric mucosa contains abundant levels of prostaglandins that regulate the release of mucosal bicarbonate and mucus, inhibit parietal cell secretion, and are important in maintaining mucosal blood flow and epithelial cell restitution. Prostaglandins are derived from esterified arachidonic acid, which is formed from phospholipids (cell membrane) by the action of phospholipase A_2 (Zhu and Kauntiz, 2008).

A key enzyme that controls the rate-limiting step in prostaglandin synthesis is cyclooxygenase (COX), which is present in two isoforms (COX-1, COX-2), each having distinct characteristics regarding structure, tissue distribution, and expression. COX-1 is expressed in a host of tissues, including the stomach, platelets, kidneys, and endothelial cells (Del Valle, 2001). The highly COX-2–selective NSAIDs have the potential to provide the beneficial effect of decreasing tissue inflammation while minimizing toxicity in the gastrointestinal tract. Selective COX-2 inhibitors have had adverse effects on the cardiovascular system, leading to increased risk of myocardial infarction (Del Valle, 2001).

The mucosal defense system involves a three layer barrier which comprises of pre-epithelial, epithelial and sub-epithelial cells. Each layer provides a defensive layer aggressive factors that could damage the membranes.

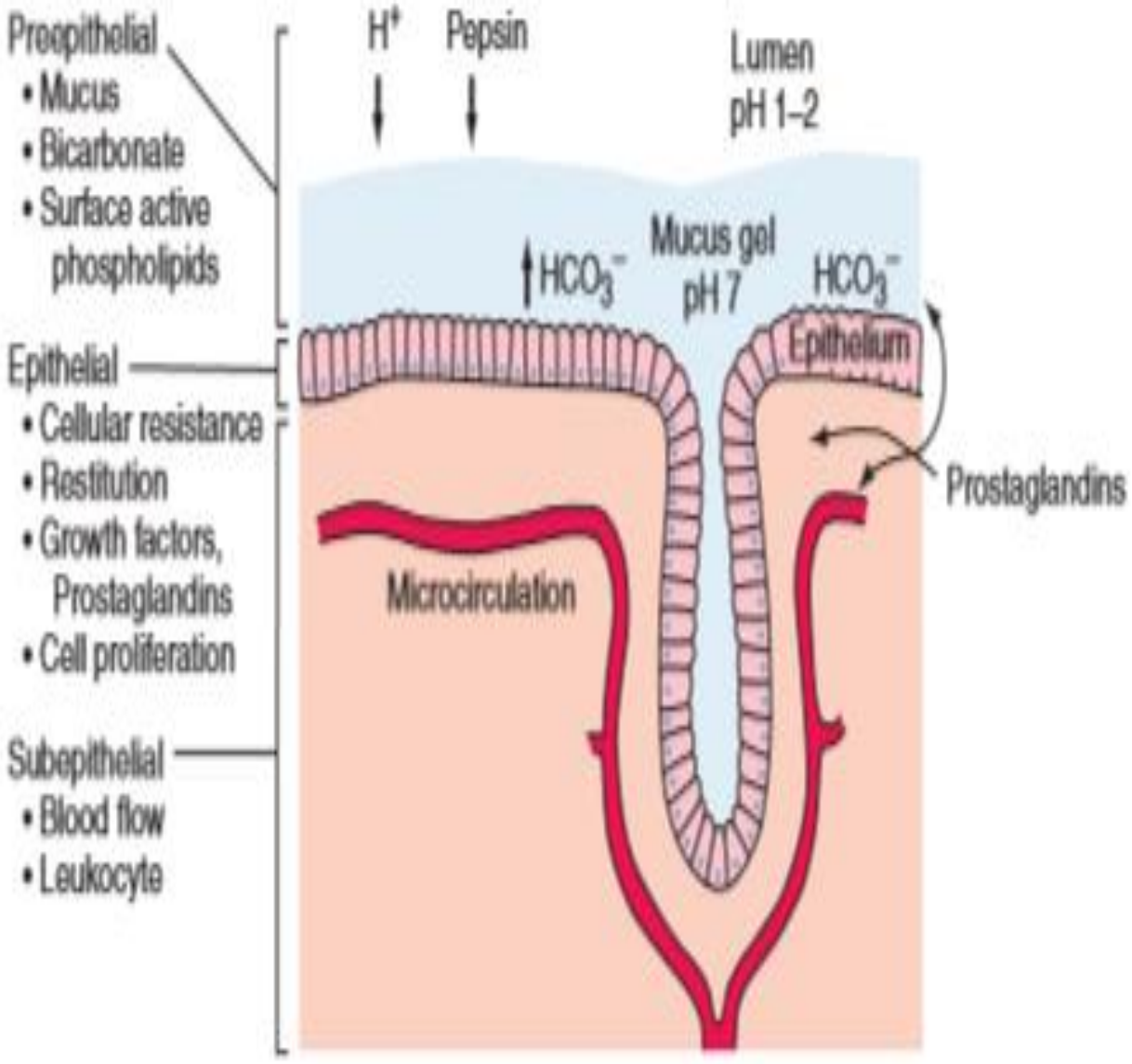


Figure 2.6: Components involved in providing gastroduodenal mucosal defense and repair (Del valle, 2001).

2.6 Pathogenesis of peptic ulcer

Peptic ulcers are defects in the gastric or duodenal mucosa that extend through the muscularis mucosa. The epithelial cells of the stomach and duodenum secrete mucus in response to irritation of the epithelial lining and as a result of cholinergic stimulation. The superficial portion of the gastric and duodenal mucosa exists in the form of a gel layer, which is impermeable to acid and pepsin. Other gastric and duodenal cells secrete bicarbonate, which aids in buffering acid that lies near the mucosa. Prostaglandins of the E type (PGE) have an important protective role, because PGE increases the production of both bicarbonate and the mucous layer (Anand, 2015).

2.6.1 Pathogeneses of alcohol induced peptic ulcer disease

The mechanism of alcohol-induced gastric lesions is varied, including the depletion of gastric mucus content, damaged mucosal blood flow and mucosal cell injury (Saleh, *et al.,* 2017). It has been documented that alcohol causes severe damage to the gastrointestinal mucosa which starts with microvascular injury resulting in increased vascular permeability, edema formation and epithelial lifting. Szabo *et al* (2010) suggested that a rapid and time dependent release of endothelin-1 into systemic circulation precedes the development of the hemorrhagic mucosal erosions by vasoconstriction after intragastric administration of ethanol (Szabo *et al.,* 2010). Decreased bicarbonate (HCO_3) secretion and mucus production produces necrotic lesions in the gastric mucosa. Ethanol has also been reported to activate TNF-α and mitogen activated protein kinases (MAPK) (Vidya *et al.,* 2001). Ethanol causes release of superoxide anion and hydroperoxy free radicals which leads to an increased lipid peroxidation (Mahmood *et al.,* 2011).

2.6.2 Physiological consequences of ethanol intoxication in the gastric mucosa.

Alcohol is absorbed rapidly through the bloodstream from the stomach and intestinal tract. High concentration of ethanol induce vascular endothelium injury of the gastric mucosa, which became edematous, and congestive, present point and scattered bleeding lesions, focal

hemorrhage, necrosis and giant deep ulcers (Pan *et al.*, 2008). Principal cells and parietal cells become swollen and diminished. These cells are rich in mitochondria which provide energy by oxidative phosphorylation, which is critical for maintaining the proper morphology and function of the gastric mucosa. The mitochondrion is an easily injured organelle, and mtDNA is the major target of ethanol-associated intracellular oxidative stress (Hoek *et al.*, 2002).

Alcohol exposure affects the mitochondrial structure which became swollen and disintegrates while the cristae cristae dissolves and disappears, giving rise to mega mitochondria which have lower ATP synthesis, oxygen consumption and ROS formation rates. It was proposed that the enlargement of the mitochondria is an adaptive process by which cells attempt to decrease the intracellular amount of ROS when they are subjected to oxidative stress (Wakabayashi, 2002).

2.6.3 Role of *Helicobacter Pylori* infection in the pathogenesis of peptic ulcer disease

About 70% of patients with peptic ulcer disease are infected by bacterium ***Helicobacter pylori***. This organism disrupts the protective coating of the stomach and duodenum and allows the digestive juices to irritate the sensitive lining below (Kang, 1994; Graham, 1998). The apparent role of *H. pylori* in peptic ulceration cannot be over-emphasized. H. *pylori* infection is present in virtually all patients with duodenal ulcers and about 70% of those with gastric ulcers. *H.pyroli*-infected gastric mucosa shows infilteration of polymorphonuclear leukocytes, lymphocytes, monocytes and plasma cells in the lamina propria, and intraepithelial severe neutrophil infilteration (Fan *et al.*, 1996).

2.6.4 Mechanism of *Helicobacter pylori* induced mucosal damage

Helicobacter pyroli is a gram-negative, spiral-shaped microorganism which has an ecological niche restricted to the stomach of man and occasionally of other mammals. Cytotoxigenicity is an important determinant of virulence in *H. pyroli* and a consistent putative factor in mucosal

damage. *H. pyroli* infection is associated with gastric mucosal damage and the infilteration of neutrophils. The key pathophysiological event in *H. pyroli* infection is initiation and continuance of an inflammatory response. Bacteria or their products trigger this inflammatory process and the main mediators are cytokines. Identification of both host and bacterial factors that mediate is an intense area of interest in current researches. Recent data indicates that the cytotoxin-associated gene protein (Cag A) plays a crucial role in *H.pyroli*-induced gastric inflammation via the activation of gene transcription (Naito and Yoshikawa, 2002). Myeloperoxidase from neutrophils produces hypochlorous acid, which yields monochloramine in the presence of ammonia produced by urease enzyme of *Helicobacter pyroli* (Silva *et al.*, 2012). Both hypochlorous acid and monochloramine are strong free radicals that can destroy mammalian cells. It makes proteases and phospholipases that break down glycoprotein lipid complex of the mucous gel, thus reducing the efficacy of the first line of mucosal defense (Del Valle, 2001).

2.6.5 Non-steroidal anti-inflammatory drugs induced peptic ulcer disease

Non-steroidal anti-inflammatory drugs (NSAIDs) which include aspirin, ibuprofen, naproxen, piroxicam, fenoprofen, indomethacin, diclofenac, tolmetin, oxaprozin, ketoprofen, sulindac, nabumeton, etodolac and salsalate are acidic and most common cause of ulcer. They block prostaglandins in the stomach which help maintain blood flow and protect it from injury (Balogun *et al.*, 2013) People suffering from Zollinger-Ellison have tumors in the pancreas and duodenum that produce gastrin, a hormone that stimulates gastric acid production which leads to ulcer production.

2.6.6 Mechanism of NSAID induced PUD

- *Oxidative Stress*.

NSAIDs mediated gastric lesions accompanied by oxidative damage. Oxidative stress refers to a situation of serious imbalance between production of reactive oxygen/reactive nitrogen species (ROS/RNS) and antioxidant defense. This is manifested from diminished antioxidants, mutations affecting antioxidant defense enzymes, or increased production of ROS/RNS and is induced by various endogenous and exogenous factors. This leads to extensive damage to key biomacromolecules leading to various diseases including gastric ulceration. It is now established that neutrophil infiltration, generation of ROS (Yoshikawa *et al.,* 1993), cytokine imbalance, and initiation of lipid peroxidation play significant roles in the pathogenesis of peptic ulcer.

- *Prostaglandin Synthesis Inhibition*.

Prostaglandins (PGs) are 20-carbon fatty acids produced by the cyclooxygenase (COX)-catalyzed reaction of arachidonic acid. Most of the NSAIDs are nonspecific cyclooxygenase blockers. They block both constitutive COX-1 and inducible COX-2, ultimately hindering the synthesis of PGs. PGs generally act in an autocrine or paracrine manner. PGs appear to exert their cytoprotective action by stimulating mucus and bicarbonate secretion, maintaining mucosal blood flow and enhancing the resistance of epithelial cells to injury induced by cytotoxins (Hawkey and Rampton, 1985). PGs can inhibit the generation of reactive oxygen metabolites produced by neutrophils there by reducing inflammation and tissue injury (Wong and Freund, 1981).

- *Nitrogen Metabolizing Enzymes*.

Nitric oxide (NO) is one of the main mediators of gastrointestinal mucosal defense but, in contradiction, it also contributes to mucosal damage depending upon the concentration of NO (Muscara and Wallace, 1999). It is seen that endothelial nitric oxide synthase (e-NOS) isoform of constitutive NOS produces low amounts of NO. In contrast, the inducible form of NOS (iNOS) produces NO in higher quantities (Wallace *et al.,* 1990). Piotrowski *et al.* (1999)showed that indomethacin induced gastric ulceration gives a 12-fold increase in gastric epithelial expression of iNOS activity compared with controls which is correlated with the damage of epithelium (Piotrowski *et al.,* 1999), whereas Wallace and Miller, 2000 showed that NO mediates a critical role in modulation of several components of mucosal defense, including increased gastric blood flow, reduced neutrophil adhesion, and increased mucus secretion (Wallace and Miller, 2000).

2.6.7 Role of reactive oxygen, lipid peroxidation, and antioxidants in the pathogenesis of peptic ulcer

Reactive oxygen species (ROS) also referred to as reactive oxygen intermediates (ROI), are by-products of normal cellular metabolism. Low and moderate amounts of ROS have beneficial effects on several physiological processes including killing of invading pathogens, wound healing, and tissue repair process. However disproportionate generation of ROS poses a serious problem to bodily homeostasis and causes oxidative tissue damage. While natural antioxidant pathways can limit the adverse effects of ROS, the levels of ROS can be stimulated by many oxidative stressors and maintained such that they contribute to tissue damage (Ahmadinejad, *et al.,* 2017*).*

Molecular oxygen (O_2) is not only essential for the survival of aerobic organisms, its reduction to H_2O via mitochondrial respiration complexes provides ATP, but paradoxically contribute to cell

death (Kulkarni, *et al.,* 2007). Partially reduced O_2, collectively named ROS, are highly reactive and are continuously produced as by-products of cellular respiration. ROS are also generated during enzymatic reactions. ROS include radical compounds such as superoxide ($O_2^{\cdot-}$), hydroxyl radicals ($HO^{\cdot}$), lipid hydroperoxides, and reactive nonradical compounds including singlet oxyden ($^1O_2$), hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), chloramines (RNHCl), and ozone (O_3) (Bedard and Krause, 2007).

Reactive radical compounds such as nitric oxide ($^{\cdot}NO$), nitrogen dioxide ($^{\cdot}NO_2$), and nonradical compounds, e.g., peroxynitrite ($ONOO^-$) and dinitrogen trioxide (N_2O_3), are collectively called reactive nitrogen species (RNS). These free radicals are unstable because of the presence of unpaired electrons in their outer electron orbit. RNS is often linked to ROS, e.g., in the formation of peroxynitrite causing nitrosative stress (Ahmadinejad, *et al.,* 2017).

The mitochondrial electron transport chain is the major site of ROS production in most mammalian cells (Poyton *et al.,* 2009). Enzymes that catalyze ROS- generating chemical reactions are peroxidases, NADPH oxidase, NADPH oxidase isoforms (NOX), xanthine oxidase (XO), lipoxygenases (LOXs), glucose oxidase, myeloperoxidase (MPO), nitric oxide synthase, and cyclooxygenases (COXs) (Kulkarni *et al.,* 2007 ; Swindle and Metcalfe, 2007)

2.6.7.1 *Lipid peroxidation*

Lipid peroxidation modifies membranes and provokes the release of unstable hydroperoxides and final secondary products, such as aldehydes, ketones, esthers and polymers, most remarkably toxic to the viability of cells, even tissues (Esterbauer, 1993; Ferrari, 1998). Lipid peroxidation is a process generated naturally in small amounts in the body, mainly by the effect of several reactive oxygen species. Enzymatic (catalase, superoxide dismutase) and nonenzymatic (Vitamins A and E) natural antioxidant defense mechanism exist; however, these mechanism

may be overcome, causing lipid peroxidation to take place. Since lipid peroxidation is a self-propagating chain reaction, the initial oxidation of only a few lipids may result in significant tissue damage (Ahmadinejad, *et al.,* 2017*).* Despite extensive research in the field of lipid peroxidation it has not yet been precisely determined if it is the cause or an effect of several pathological conditions (Mylonas and Kouretas, 1999). Studies have shown that reactive oxygen species, especially the hydroxy radical, plays a major role in oxidative damage of gastric mucosa in almost all forms of gastric ulcers (Phull *et al.,* 1995; Das *et al.,* 1997).

2.6.7.2 Antioxidants

Antioxidants are compounds that protect cells against the harmful effects of reactive oxygen species such as; singlet oxygen, superoxide, peroxyl radicals, hydroxyl radicals and peroxynitrite. An imbalance between reactive oxygen species and antioxidants results in oxidative stress, leading to cellular damage (Burler and Miranda, 2003). The major enzymatic antioxidants are superoxide dismutases, glutathione peroxidase, glutathione reductase, catalase, and superoxide reductases. Superoxide reductase is an oxidoreductase present only in the anaerobic and facultative microorganisms. SOD and catalase provide major antioxidant defenses against ROS (Bhattacharyya *et al.,* 2014). The cellular antioxidant defenses in superoxide anion 0^{2-}, hydrogen peroxide H_2O_2, hydroxyl radical OH^- is shown in fig 2.7

Superoxide dismutase (SOD) are metal ion cofactor-requiring enzymes that catalyze dismutation of $O_2^{\cdot -}$ into O_2 and H_2O_2. Three isoforms of SOD exist in human (Nozik-Grayck *et al.,* 2005): cytosolic copper and zinc- containing enzyme (Cu-Zn-SOD), manganese- requiring mitochondrial enzyme (Mn-SOD), and an extracellular Cu-Zn- containing SOD (EC-SOD). $O_2^{\cdot -}$ formed in the mitochondria is dismutated to H_2O_2 by Cu-Zn-SOD present in the mitochondrial intermembranous space and Mn- SOD present in the mitochondrial matrix (Okado-Matsumoto

and Fridovich, 2001). Gastrointestinal mucosal injury can be prevented by SOD in the gastrointestinal mucosa (Klinowski *et al.,* 1996; Koharyova and Kolarova, 2008). Reduced SOD activity in the gut causes gastric ulcer, and increased SOD activity has been associated with ulcer healing in patients (Naito *et al.,* 1992). These responses illustrate both the detrimental effects of ROS on tissue damage and the importance of antioxidant activity in promoting health (Janssen *et al.,* 2000).

Glutathione peroxidase converts (GPX) converts glutathione (GSH), a tripeptide consisting of glutamate, cysteine and glycine, into oxidized glutathione (also called glutathione disulfide, GSSG) and during this process, reduces H_2O_2 to H_2O and lipid hydroperoxides (ROOH) to corresponding stable alcohols. The GPX reaction is coupled to glutathione reductase (GSSG-R), which maintains reduced glutathione (GSH) levels (Bompart *et al.,* 1990). Neutrons are most vulnerable to free radical damage as they have very low levels of GSH. GPX serves an important role in protecting cells from the harmful effects of peroxide decomposition. Isoenzymes of GPX are found in cytoplasmic, mitochondrial, and extracellular compartments (Toppo *et al.,* 2008). Humans have eight isotypes of GPX, most of which contain selenocysteine residues at their active site (Dayer *et al.,* 2008). GPX1 is ubiquitous, but GPX2 has epithelium-specific expression. GPX2 was discovered in the gastrointestinal tract (Chu *et al.,* 1993) which protects the gut against the absorption of dietary hydroperoxides (Wringler *et al.,* 2000). GPX2 provides the first line of defense against ROS derived from inflammation associated with both pathogenic and nonpathogenic commensal bacteria in the gut (Chu *et al.,* 2004).

Catalase dismutases hydrogen peroxide tso water (H_2O_2 to H_2O) and is found mainly in the perixomes (Schrader and Fahimi, 2006). Catalases are heme enzymes, but a manganese catalase

is found in prokaryotes. In humans catalase is found largely in liver kidney and erythrocytes, although all organs express these enzymes (Zamocky and Koller, 1999).

Glutathione reductase (GR or GSR) reduces oxidized glutathione disulfide (GSSG) to GSH. GR is ubiquitously expressed except for Drosophila, Trypanosomes, and gram-negative bacteria (Kanzok *et al.,* 2001). GR protects red blood cells, hemoglobin, and cell membranes from oxidative stress by generating GSH (Chang *et al.,* 1978).

The figure 2.11 below shows several radical scavengers of hydroxyl radicals and the decomposition of hydrogen peroxide into water.

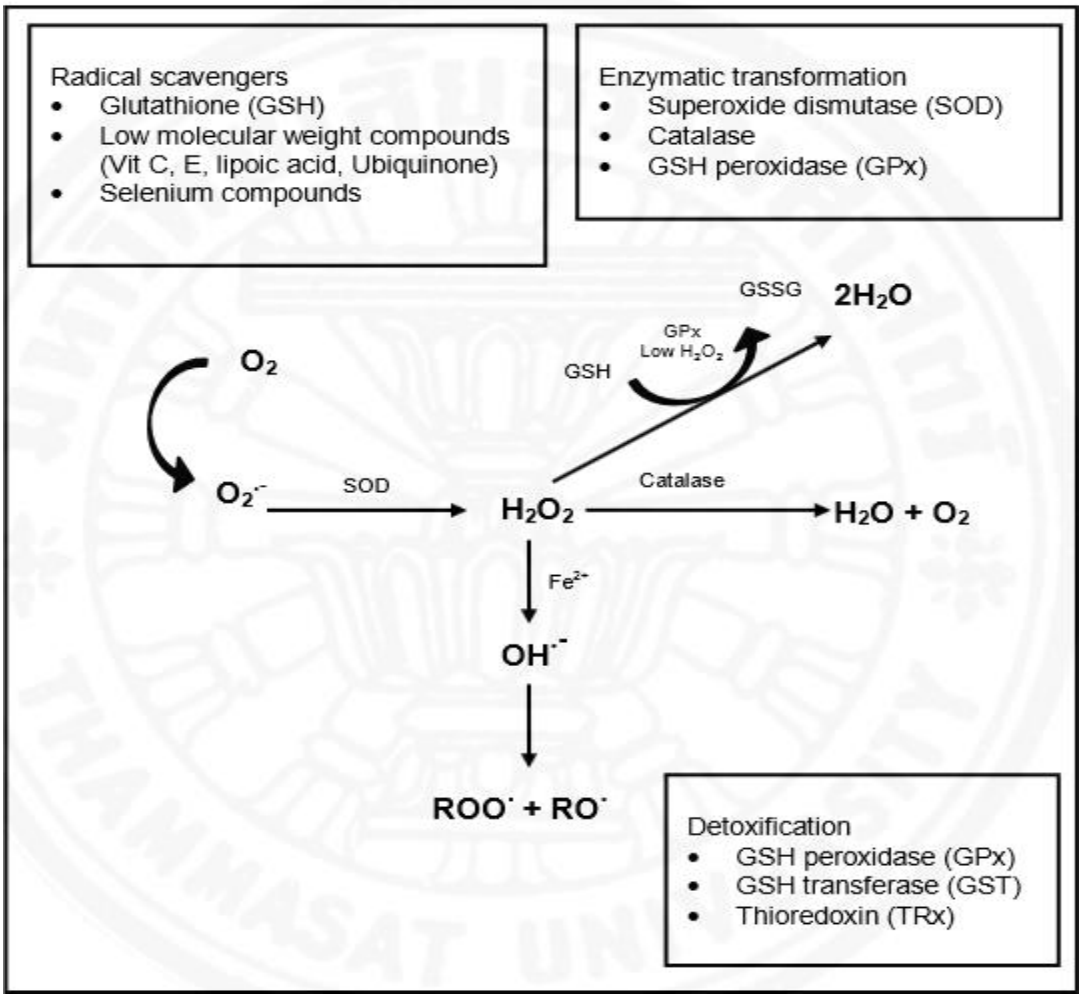


Figure: 2.7: The cellular antioxidant defenses in superoxide anion 0_2^-, hydrogen peroxide H_2O_2, hydroxyl radical OH^-

Heme oxygenase (HO) catalyzes degradation of heme and generates CO, biliverdin, and iron (Tenhunen *et al.*, 1968). Two distinct HO isoforms, HO-1 and HO-2, have been reported (Ryter *et al.*, 2006). HO -2 is constitutively expressed, and HO-1 is inducible. Although HO-1 does not

have a direct antioxidant enzymatic function HO-1 and its product CO are believed to have indirect cytoprotective responses against oxidative stress (Vile *et al.,* 1994; Otterbien and Choi, 2000). Endogenous Non-enzymatic antioxidants include Glutathione which is found in all eukaryotic cells and is one of the key non-enzyme antioxidant in the body. I t is generally present in reduced form, GSH (Meister and Anderson, 1983). Thioredoxin system is comprised of Thioredoxin (Trx) and Thioredoxin reductases (TrxR). Trx is disulfide- containing oxidoreductase that modulated activities of radox-sensitive transcription factors. Anti-ulcer drugs like geranylgeranylacetone can induce Trx production in rat hepatocyte. This drug also promotes secretion of Trx in rat gastric mucosa, suggesting that it has protective roles in at least experimental gastric ulceration (Deskigai *et al.,* 2001). Melatonin is a hormone synthesized from serotonin in the mammalian pineal gland but is also found in the retina, Lymphocytes, GI tract, and bone marrow. It is ubiquitous can be found in dietary sources such as oats, yeast and other plants. It is effective in both aqueous and lipid phases in neutralizing $HO^{\cdot}$ And peroxyl radicals, $CO_3^{\cdot -}$, $^{\cdot}NO_2$, $O_2^{\cdot -}$ and HOCl (Reiter *et al.,* 2002) and can readily cross the blood-brain barrier. As melatonin can directly cross the mitochondrial membranes, it plays a very significant role in protecting mitochondria from oxidative damage.

Exogenous Antioxidants include vitamin C, vitamin E, Minerals (zinc, copper, manganese, iron and selenium), and carotenoids. However, recent studies have demonstrated that flavonoids (Polyphenols) found in fruits and vegetables may also act as antioxidants, they contain chemical structural elements that may be responsible for their antioxidant activities (Alanko, 1999)

Vitamin C or ascorbic acid is the primary antioxidant in plasma and cells. It is synthesized from glucose in the liver of most mammalian species, but not by humans and therefore must be supplemented in diet. Vitamin C can be obtained in fresh fruits and vegetables. Vitamin C

donates electrons to other compounds and prevents their oxidation. The many relevant species reduced by Vitamin C include ROS, RNS, sulphur radicals, O_3, nitrosating compounds, and HOCl. Vitamin C reduces heavy metal ions (Fe, Cu) that can generate free radical via Fenton reaction, and thus can have pro-oxidant activity although its main function is as an antioxidant (Stohs and Bagchi, 1995). Although Vitamin E (tocopherol, Toc) is only moderately effective against singlet oxygen; it is the most effective antioxidant for terminating the chain reactions of lipid peroxidation in cell membranes. Intracellular antioxidant such as glutathione and tocopherol protect the gastric mucosa from ethanol-induced oxidative stress (Repetto and Liesuy, 2002).

Beta-carotene lycopene and cryptoxanthin are the main carotenes in food as well as in the body. Beta-carotene and other carotenoids exhibit antioxidant properties depending on the in vitro experimental system used. Antioxidant properties of biological carotenoids depend on retinol-binding proteins and other endogenous antioxidants in vivo. Beta –carotene has shown to suppress lipid peroxidation in mouse models used. Minerals (zinc, copper, manganese, iron and selenium) are key components of enzymes with antioxidant functions and are designated as antioxidant nutrients. Zn, Cu and Mn are cofactors of superoxide dismutase (Cu/Zn-SOD). Fe is a component of catalase. Selenium is a major antioxidant in the form of selenoproteins that mitigates the cytotoxic effects of ROS.

2.7 Peptic ulcer management

Specific treatment for stomach and duodenal ulcers is decided by the physician on the basis of patient's age, overall health, medical history, extent of the pathogenesis, tolerance for medications, procedures, or therapies, and expectations or preference. Besides, personal factors such as smoking, caffeine, alcohol, stress, secretion of acid and pepsin, and so forth are suspected to play a role in the development of stomach or duodenal ulcers. Hence, the simplest

treatment involving lifestyle changes namely abstinence from smoking and alcohol and over stress in many cases.

Earlier, the major approach towards therapy was targeted to reduce the secretion of gastric acids, which were considered as the sole cause of ulcer formation. Now, the treatment modality has changed to potentiating of the mucosal defense along with reduction of acid secretion (Wallace, 2005). The treatment of peptic ulcer is often designed with single or combination drugs.

2.7.1 Antiulcer drugs

- *Antisecretory agents*

Histamine H_2 receptor antagonist, block the action of histamine at the Histamine H_2 receptors of the parietal cells in the stomach thus decreasing the production of stomach acid (Saleh, *et al.*, 2017). Enhance healing by inhibition of intracellular mechanism involving calcium and/or c-AMP, protection of gastric mucosa (cytoprotective function) from chemically induced injury. In addition, these agents reduce the gastric stimulating effects of gastrin and acetylcholine. The H - receptor antagonists are: cimetidine (Tagamet®), famotidine (Pepcid AC®), nizatidine (Axid®), and ranitidine (Zantac®) (ACPE, 2003).

- *Proton Pump Inhibitors*

Proton pump inhibitors (PPIs) are drugs which irreversibly inhibit proton pump (H+/K+ ATPase) function and are the most potent gastric acid-suppressing agents in clinical use. There is now a substantial body of evidence showing improved efficacy of PPIs over the histamine H_2 receptor antagonists and other drugs in acid-related disorders (Richardson *et al.*, 1998). Inhibition of the proton pump in the parietal cells has been established as the main treatment of acid-related diseases, such as peptic ulcer and gastro-esophageal reflux. The proton pump inhibitors are

tailored for their purpose. They accumulate in the target cell, are activated by acid and bind strongly to the specific target-the proton pump (AlRashdi *et al.,* 2012. The clinically superiority of the proton pump inhibitors is due not only to the high efficacy but also to the long duration of the acid inhibition in comparison with other antisecretory drugs (Olbe, 1999).

Proton pump inhibitors includes: Omeprazole (Prilosec®), Esomeprazole (Nexium®) and Lansoprazole (Prevacid®) a newer proton pump inhibitor that is being used quite often for gastroesophageal reflux disease (GERD) (ACPE, 2003). Recent studies show that Omeprazole blocks stress-induced increased generation of OH- and associated lipid peroxidation, and protein oxidation indicating that its antioxidant role plays a major part in preventing oxidative damage (Biswas *et al.,* 2003).

- *Mucosal protectants*

Sucralfate (Carafate®) is formed from the basic compounds aluminum salt of sulfated sucrose and aluminum hydroxide. It is a non-absorbable medication (less than 3%) that binds to gastric mucosa and ulcerated tissue. These properties favor healing and provide cytoprotective effects. When exposed to gastric acid the sulfate ions bind to proteins in ulcer base and stimulate angiogenesis, delivery of growth factors and formation of granulation tissue (Mejia and Kraft, 2009).

- *Bismuth*

The commonly used salt of salicylic acid, bismuth salicylate has antacid properties. Bismuth suppresses *H. pylori* and has been approved by the US FDA for use in combination with other agents for its eradication. Other actions that may promote ulcer healing include inhibition of

pepsin activity, increase in mucosal prostaglandin production and mucus and bsicarbonate secretion as well as retards hydrogen-ion back diffusion. It is largely unabsorbed and is excreted in feces. In the colon it reacts with hydrogen sulfide and forms bismuth sulfide, which blackens the stools. It has modest efficacy in non-ulcer dyspepsia, and is presently used in *H. pylori* regimens (Lambert and Midolo, 1997).

- *Antacids*

Antacids work nearly instantaneously and find utility for rapid relief of mild or sporadic symptoms. The primary effect of antacids on the stomach is due to partial neutralization of gastric hydrochloric acid and inhibition of the proteolytic enzyme pepsin. Neutralization of acid in the gut lumen bypasses the need for systemic absorption of the drug. They are all administered orally and their potency is usually measured by the amount of acid neutralized by a given dose of the antacid. The effective time for antacids to reduce stomach acidity is relatively short on an empty stomach, but can be prolonged to 1–3 h if taken with food. Commonly used antacids contain various salts of calcium, magnesium and aluminum as active ingredients. (Pratiksha and Steve, 2018).

- *Anticholinergics*

Anticholinergics may be used as antiulcer agents due to their ability to depress gastric motility and to diminish gastric secretions. However, these muscarinic antagonists require high doses, to inhibit acid secretion. At such doses, they produce a number of side effects such as dry mouth, constipation, blurred vision, and urinary retention. These drugs are not used very often. Pirenzepine is a muscarinic antagonist that produces selective blockade of the muscarinic receptors that regulate gastric acid secretion. Because of its selectivity, pirenzepine is capable of

producing acid inhibition without causing pronounced anticholinergic adverse reactions (ACPE, 2003).

- *Antibiotics*

Several antibiotics may be used to combat *H. pylori* infection. A triple therapy utilizes a combination of proton pump inhibitors or H_2 -receptor antagonists, clarithromycin, and amoxicillin administered for 14 days. Quadruple therapy consists of a combination of bismuth compounds, antisecretory agents such as proton pump inhibitor or H_2 - receptor antagonists and two antibiotics composed of tetracycline and metronidazole (ACPE, 2003). Both therapies have resulted in eradication of *H. pylori*.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Chemicals

Ethanol, methanol, Elabscience assay kits (CAT, SOD and GPx), DPPH (Sigma-Aldrich, Germany), chloroform, ethyl acetate, silica gel 60-200 mesh, Cimetidine 200mg tablets.

3.2 Equipment

The required equipment includes, weighing balance, surgical gloves, cannula, syringes, cages, water troughs, feeders, centrifuge, water bath, spectrophotometer, ELISA micro plate reader. All other equipment and materials used were routine laboratory models

3.3 Experimental animals

Animals used in this study were purchased from the animal house of the Faculty of Pharmaceutical Science, Ahmadu Bello University, Zaria. Albino rats of both sexes weighing 160-250g were housed in environmentally controlled room (25 ± 2ºC, 12 hours light/dark cycles) for three weeks to acclimatize which they were divided randomly into five groups of six animals each coded to prevent observer bias. The animals were fed with standard feed (Vital feeds LTD Kano) and water ad *libitum*.

3.4 Collection of plant materials

The plant was obtained from Zaria city in March 2017. It was identified and authenticated in the herbarium unit of the Department of Botany, Faculty of life sciences, Ahmadu Bello University, Zaria, and Voucher number 900389 was deposited.

3.4.1 *Anogeissus leiocarpus* stem bark aqueous extraction

Stem bark of *Anogeissus leiocarpus* was thoroughly washed and dried under the shade for 7 days. The stem bark was pulverized using pestle and mortar to fine powder (250g), placed in a mechanical shaker and exhaustively macerated in cold distilled water for 24 hours. The mixture

was allowed to settle and then filtered using a filter paper (Whatmann No 1). The filtrate was transferred into a petri-dish and concentrated in water bath at 40°C and was subsequently preserved as the extract. The extract obtained was weighed, and the percentage yield was calculated in term of air dried weight of the plant material as shown below:

$$Percentage\ yield = \frac{Weight\ of\ extract\ obtained}{Weight\ of\ initial\ sample} \times \frac{100}{1}$$

3.5 Median Lethal Dose (LD_{50}) Determination in Rats.

LD_{50} determination was conducted using Lorke's method (1983). This method was carried out in two phases. In the first (initial) phase, 3 rats per group of different weights were treated with the extracts at a dose of 10 mg/kg, 100 mg/kg and 1000 mg/kg body weight orally and were observed for signs of toxicity (weakness, drowsiness, lethargy) and death for 24 hours.

In the second phase, 4 rats of different weights were administered doses of the extract at 1200mg/kg, 1600 mg/kg, 2900 mg/kg, and 5000 mg/kg body weight respectively based on the result of phase 1(i. e initial phase). The LD_{50} value was determined by calculating the geometric mean of the highest non-lethal dose (0/1) and lowest lethal dose (1/1) as shown in the formula below:-

$$LD_{50} = \sqrt{(Highest\ non\ lethal\ dose) \times (lowest\ lethal\ dose)}$$

3.6 Preliminary phytochemical screening (Qualitative) of crude aqueous stem bark extract of *Anogeissus leiocarpus*.

3.6.1 Test for Flavonoids

- Shinoda test: The extract (2.0g) was dissolved in 50% methanol by heating. Magnesium metal and 5 drops of concentrated hydrochloric acid was added. Appearance of an orange colour would indicate the presence of flavonoids (Ciulie, 1984).

- Using 10% NaOH and dilute HCl: 1.0 ml of 10% NaOH was added to 2.0 ml of extract. A yellow precipitate which turn colourless on addition of excess dilute HCl indicates presence of flavonoids (Ciulie, 1984).

3.6.2 Test for Alkaloids

The extract (2g) was weighed into a test tube and dissolved in 2.0 ml of distilled water, then it was separated into three separate test tubes, 2-3 drops of Dragendoff's, Wagner's and Mayer's reagents were added separately. An orange red precipitate/turbidity with Dragendoff's reagent, a brown precipitate with Wagner's reagent or white precipitates with Mayer's reagent denotes the presence of alkaloids (Ciulie, 1984).

3.6.3 Test for Saponins

The extract (0.5g) was taken in a test tube and 5.0 ml of water was added and vigorously shaken. A persistent froth that lasts for at least 15 minutes indicates the presence of Saponins (Brian and Turner, 1975).

3.6.4 Test for Tannins

- Using ferric chloride solution:

The extract (2mg) was transferred into a test tube and dissolved in 2.0ml of distilled water, about 3 drops of 5% ferric solution was added. A green or greenish-black colouration indicates the presence of tannins.

- Using lead sub acetate solution:

The extract was dissolved in a little amount of ethanol and 2.0ml of water. A few drops of lead sub acetate were then added. A white precipitate indicates presence of tannins (Ciulie, 1984).

3.6.5 Test for Steroid and Triterpenes (Lieberman-Burchards test)
Acetic acid anhydride (2ml) was added to the extract and then shaken. 1ml of concentrated sulphuric acid was then added down the side of the tube to form a lower layer. Purple colour indicates the presence of triterpenes only (Rojjas *et al.,* 2006).

3.6.6 Test for anthraquinones derivatives
Test for free anthraquinones (Borntrager‟s test)

Small portion of the extract was shaken with 10 ml of benzene and then filtered. About 5 ml of 10% of ammonia solution was added to the filtrate and stirred for few minutes. Colourless ammonia layer indicate presence of anthraquinones (Rahman, 2010)

3.6.7 Test for cardiac glycosides (Kella-Killiani)

The extract (0.5 g) was dissolved in 4 ml of glacia acetic acid containing traces of ferric chloride. This was left for 1 min and then transferred into a dry test tube. The test tube was held at an angle of 45^0C and 5 drops of concentrated sulphuric acid was added down the side of the test tube. On standing, a purple ring colour at the interface indicates the presence of cardiac glycosides (Evans, 2002).

3.7 Quantitative phytochemical determinations of the Crude aqueous stem bark extract of *Anogeissus leiocarpus*
The detected phytochemical constituents were quantified as described:

3.7.1 Determination of Alkaloids (Harborne, 1973)
The stem bark extract (1g) was weighed into a 250 ml beaker and 8ml of 10% acetic acid in ethanol was added and covered and allowed to stand for 4 h. It was filtered and the extract was concentrated on a water bath to one quarter of the original volume. Concentrated NH_4OH was added drop wise to the extract until the precipitation was completed and washed with dilute

ammonium hydroxide and then filtered. The residue is the alkaloid, which was dried and weighed. The percentage alkaloid was calculated using the formula of Kumar and Bhardwaj et al. (2012).

$$\%\ Alkaloid = \frac{mass\ of\ alkaloid\ residue}{mass\ of\ sample} \times 100$$

3.7.2 Determination of Tannin (Van-Buren and Robinson)

The extract (0.5g) was weighed into a 50 ml plastic sample bottle, and 50 ml of distilled water was added and shaken for 60 min in a mechanical shaker. This was filtered into 50 ml volumetric flask and made up to the mark. Then 5ml of the filtrate was pipetted out into a test tube and mixed with 2ml of 0.1M $FeCl_3$ in 0.1MHCl and 0.008M potassium ferrocyanide $[K_3(Fe(CN)_6]$. The absorbance was measured with spectrophotometer at 720 nm within 10 min.

$$Tannins = \frac{A \times DF \times GF}{W}$$

A=Absorbance at 720nm

DF=Dilution factor $\frac{Total\ volume}{Aliquot\ volume}$

GF=Gradient factor (Slope of standard tannic acid curve)

W=weight of sample/extract used

3.7.3 Determination of Flavonoids (Bohm and Kocipal- Abyazan, 1994)

The extract (1g) was repeatedly extracted with 10ml of 80% aqueous methanol of room temperature. The mixture was filtered using Whatman No 1 filter paper. The filtrate was transfer into 250ml beaker and was put into a water bath and allowed to evaporate to dryness and weighed. The percentage flavonoid was calculated using the formula.

$$\% \, Flavonoid = \frac{mass \; of \; flavonoid \; residue}{mass \; of \; plant \; sample} \times 100$$

3.7.4 Determination of Saponins (Obadoni and Ochuko, 2001)

The plant sample (1g) was weighed into a 250 ml conical flask. 10 ml of 20% ethanol (C_2H_5OH) was added. The mixture was heated over a hot water bath for 4 hours with continuous stirring at about 55^oC. The mixture is then filtered and the residue re-extracted with another 20 ml of 20% ethanol (C_2H_5OH). The combined extract was concentrated to 16 ml over a water bath at about 90^oC. The concentrated extract was then transferred into 250 ml separating funnel and 20 ml of diethyl ether $(CH_3CH_2)_2O$ was added to the extract and shaken vigorously. The aqueous layer was recovered while the diethyl ether $(CH_3CH_2)_2O$ layer was discarded and the purification process was repeated. About 60ml of n-butanol (C_4H_9OH) was added and the combined n-butanol was washed with 10ml 5%NaCl. The remaining solution was heated on a water bath to evaporate to dryness and the residue was weighed. The percentage Saponins was calculated as:

$$\% \, Saponin = \frac{mass \; of \; saponin \; residue}{mass \; of \; plant \; sample} \times 100$$

3.7.5 Determination of phenols (Edeoga *et al.*, 2005)

The sample (0.5g) was boiled with 50ml of ether for the extraction of the phenolic component for 15 minutes. About 5ml of the extract was pipetted into a 50ml flask then 10ml of distilled water was added. About 2ml of ammonium hydroxide solution and 5ml of concentrated amylalcohol was added also. The sample was left to react for 30 minutes for colour development. This was measured at 505nm.

$$Total \; phenols = \frac{A \times DF \times GF}{W}$$

A=Absorbance at 505nm

DF=Dilution factor $\frac{Total\ volume}{Aliquot\ volume}$

GF=Gradient factor (Slope of standard tannic acid curve)

W=weight of sample/extract used

3.8 The experimental protocol and ethanol-induced gastric lesions method

Group A–control: Received only distilled water (0.5mL/100g body weight).

Group B–-Treated with 70% ethanol: Received only 70% ethanol 1hr before sacrifice (0.5mL/100g body weight).

Group C–100mg/kg of the extract+ ethanol: Received 70% ethanol (0.5mL/100g body weight), 2 weeks after daily administration of stem bark extract of *A. leiocarpus* (100mg/kg body weight).

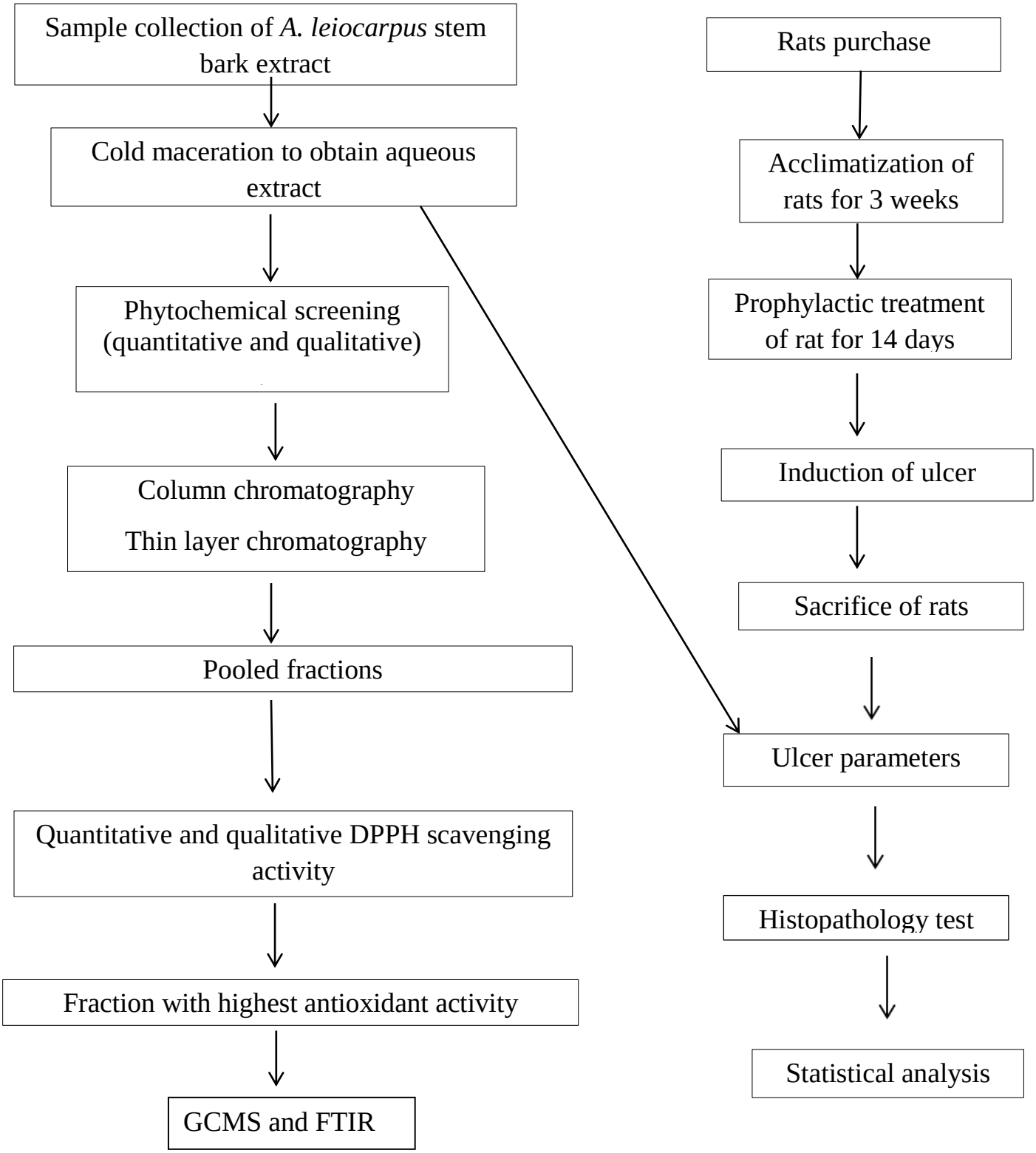
Group D–200mg/kg of the extract+ ethanol: Received 70% ethanol (0.5mL/100gbodyweight), 2 weeks after daily administration of stem bark extract of *A. leiocarpus* (200mg/kg body weight).

Group E –400mg/kg of the extract+ ethanol: Received 70% ethanol (0.5mL/100g body weight), 2 weeks after daily administration of stem bark extract of *A. leiocarpus* (400mg/kg body weight).

Group F–cimetidine+ ethanol: Received 70% ethanol (0.5mL/100g body weight), 2 weeks after daily administration of cimetidine (100mg/kg body weight).

Total number of animals in a group= 5

Figure 3.1: EXPERIMENTAL DESIGN



3.9 Gastric ulcer inducing dose of ethanol

To induce ulcers with ethanol, animals were fasted for 24–36 hours following which 70% ethanol was administered at a dose of 1mL/200g body weight to each animal and after 1hr the animals were sacrificed. It is recommended that for every study, a preliminary assessment be done to determine the effective dose required for optimum induction of ulcers (Hollander et al., 1985). An hour after the ethanol administration the animals were sacrificed by cervical dislocation, by applying a firm pressure on the skull along with twisting of the thumb and fore finger. This severs the spinal cord at the base of the brain. It is followed by an abdominal incision were stomach was removed and afterwards incised along the greater curvature. It was washed gently in running tap water and gastric mucosa spread on a filter paper for gastric lesions assessment. A 2x hand lens was used to locate the ulcers. Stomach ulceration was expressed in terms of: ulcer score, ulcer index, preventive index and % of ulceration.

3.9.1 Determination of Ulcer parameters

Ulcer score was assessed using the method of Takagi and Okabe (1968). Severity of mucosal damage was assessed on a score grade as follows;

0=no lesion,

1=mucosal oedema and petechiae,

2= one to five small lesions (1-2mm),

3=more than five small lesions or one intermediate lesion (3-4mm),

4=two to more intermediate lesions or one gross lesion (>4mm),

5=perforated ulcers.

Mean Ulcer index:

It is calculated by dividing total number of ulcers in animals in a group by number of animals in that group (Robert *et al.,* 1968).

Preventive index:

The preventive effect on the severity of ulceration was calculated according to the method of Hano et al., (1976). Percentage preventive index was calculated by:

Preventive index = $\frac{U.I\ control - U.I\ Treated}{U.I\ Control} \times \frac{100}{1}$

Percentage of ulceration:

The ulcer incidence (%) = $\frac{Number\ of\ animals\ with\ ulcer}{Number\ of\ animals\ in\ a\ group} \times \frac{100}{1}$

3.10 Histopathology studies

The stomach tissue samples were fixed in 10% buffered formalin overnight and then processed in an automated tissue processor. Stomach tissues were embedded in paraffin, sectioned by a microscope and stained with haematoxylin and eosin stain. Each section was examined by light microscope with magnification of ×400

3.11 Biochemical Assay

The stomach was homogenized in ice-cold phosphate buffer (pH7.2) so as to provide a 10% homogenate solution. These homogenates were centrifuged at 3000×g for 10 minutes to obtain supernatants that were immediately stored in deep freezer at -20^{o}C until required for analysis. The level of protein in the homogenate was determined using the Biuret method. Oxidative stress markers namely; catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase and thiobarbituric acid reactive substances (TBARS) were determined.

3.11.1 Thiobarbituric acid reactive substances (TBARS)

Stomach tissue lipoperoxidation (LPO) estimation was performed using the LPO product malondialdehyde (MDA) which thiobarbituric acid (TBA) is quantified. The concentration of TBA reactive substances was measured at 532 nm using a standard curve of MDA, and the results was expressed as nmol MDA/ mg protein (Ohkawa *et al.,* 1979).

Procedure

About100 µL of the supernatant was deproteinized by adding 2 ml of 14% trichloroacetic acid (TCA) and 2 ml of 0.67% thiobarbituric acid solution. The mixture was heated in a water bath at 80^0C for 30 minutes to complete the reaction and then cooled rapidly on ice for 5 minutes. After centrifugation at 2000rpm for 10 minutes, the absorbance of the colored product (TBARS) was measured at 532 nm with a UV spectrophotometer. The concentrations of TBARS were determined in triplicate and calculated using the molar extinction coefficient of malondialdehyde -1.56×10^5mol/L/cm (Yavuz *et al.,* 2004).

3.11.2 Catalase activity (CAT) Assay

Principle

This ELISA kit uses Sandwich-ELISA as the method. The micro ELISA plate provided in this kit has been pre-coated with an antibody specific to CAT. Standards or samples were added to the appropriate micro ELISA plate wells and combined with the specific antibody. Then a biotinylated detection antibody specific for CAT and Avidin-Horseradish Peroxidase (HRP) conjugate was added to each micro plate well successively and incubated. Free components are washed away. The substrate solution was added to each well. Only those wells that contain CAT, biotinylated detection antibody and Avidin-HRP conjugate appeared blue in color. The enzyme-substrate reaction was terminated by the addition of a sulphuric acid solution and the color turns

yellow. The optical density (OD) was measured spectrophotometrically at a wavelength of 450 nm ± 2 nm. The OD value is proportional to the concentration of CAT. The concentration of CAT in the samples was calculated by comparing the OD of the samples to the standard curve.

Assay procedure

All reagents and samples were brought to room temperature before use. The sample was centrifuged again at 3000g after thawing before the assay. All the reagents were mixed thoroughly by gently swirling before pipetting, foaming was avoided. It's recommended that all samples and standards be assayed in duplicate.

About 50µl of the standard, blank, and tissue homogenate were added per well. The blank well was added with reference standard & sample diluent. About 50 µl of biotinylated detection Ab working solution was added immediately to each well. The plate was covered with the plate sealer provided. The plate was gently tapped to ensure thorough mixing. The plate was incubated for 45 minutes at 37°C. (Solutions were added to the bottom of micro ELISA plates well, inside wall touching and foaming was avoided as possible.)

Each well was aspirated and washed, the process was repeated three times by filling each well with Wash Buffer (approximately 350µl) using an automated washer. Complete removal of liquid at each step is essential to good performance. After the last wash, remaining Wash Buffer was removed by aspirating or decanting. The plate was inverted and pat against thick clean absorbent paper.

About 100µl of HRP Conjugate working solution was added to each well. Covered with a new Plate sealer and incubated for 30 minutes at 37°C. Aspiration/wash process was repeated for five times.

About 90µl of Substrate Solution was added to each well. Covered with a new Plate sealer and incubated for about 15 minutes at 37°C protected from light. The reaction time was shortened or extended according to the actual color change, but not more than 30minutes. When apparent gradient appeared in standard wells, the reaction was terminated.

About 50µl of Stop Solution was added to each well. Yellow colour appeared immediately. The adding order of stop solution was as the same as the substrate solution. The optical density (OD value) of each well was determined at once, using a microplate reader set to 450 nm. The microplate reader is opened ahead, preheated the instrument, and set the testing parameters.

After experiment, all the unused reagents were put back into the refrigerator according to the specified storage temperature respectively until their expiry.

3.11.3 Superoxide dismutase activity (SOD) assay
Principle

Superoxide dismutase plays a vital role in body balancing status of oxidation and antioxidation. This enzyme can remove superoxide anion free radical, protect cells from damage. The activity of SOD was measured by WST-1 method in this kit. Water-soluble tetrazolium, the sodium salt of 4-[3-(4iodophenyl)-2-(4-nitrophenyl)- 2H-5-tetrazolio]-1,3-benzene disulfonate (WST-1), was used as a detector of superoxide radical generated by xanthine oxidase and hypoxanthine. The rate of the reduction with a superoxide anion is linearly related to the xanthine oxidase (XO) activity, and is inhibited by SOD. Therefore, the inhibition activity of SOD can be determined by a colorimetric method.

Superoxide dismutase activity (SOD) assay procedure

Four set of test tubes were labeled blank 1, blank 2, tissue homogenate and blank 3 respectively. About 20µl of tissue homogenate was added to the sample and blank 3 test tubes. About 20µl DdH_2O was added to blank 1 and blank 2 test tubes. About 20µl of the enzyme working solution was added o blank 1 and tissue homogenate test tube. About 20µl of dilution buffer was added to blank 2 and blank 3. About 200µl of substrate working solution was added o he four set of test tubes respectively. The tubes were mixed and incubated for 20 minutes a 37°C. Optical densities were measured spectrophotometrically at a wavelength of 450nm

Computational formulas

SOD activity (Inhibition ratio %) = $((A_{blank1} - A_{blank2}) - (A_{tissue\ homogenate} - A_{blank3}) / A_{blank1} - A_{blank2}) \times 100\%$

Tissue sample use protein to indicate result

SOD activity (U/mgprot) = Inhibition ratio of SOD (%) ÷ 50%

× Dilution multiple of reaction system ($\frac{0.24ml}{0.02ml}$)

÷ Concentration of protein sample under test (mgprot/ml)

SOD was expressed as unit per milligram of protein (U mg^{-1} protein).

3.11.4 Glutathione peroxidase
Principle

Glutathione peroxidase (GSH-PX) is an important catalytic enzyme that widely exists in the body which catalyzes the decomposition of hydrogen peroxide. GSH specifically catalyze the reaction between GSH and hydrogen peroxide protecting cell membrane structure and keeping membrane function integrity. Se-cysteine is the active center of the GSH-PX. Determination of GSH-PX activity in organism can be an indicator of selenium level as Se is an essential section of GSH-PX.

Table 3.2: Glutathione peroxidase activity assay procedure (1)

(1) Enzymatic reaction (Reagent 1 application solution were pre-warmed in advance at 37^{o}C)

Two set of test tubes were labeled non-enzymatic and enzymatic tubes. 0.2ml of 1mmol/L GSH was added into the test tubes respectively. 0.2ml of the tissue homogenate was added into the enzymatic tube only. The tubes were warmed in a water bath at 37^{o}C for 5 minutes. 0.1ml of Reagent 1 application solution was added into the test tubes respectively. The test tubes were warmed in a water bath at 37^{o}C for 5 minutes. About 2ml of reagent 2 application solution was added to the test tubes respectively. About 0.2ml of the tissue homogenate was added to the non-enzymatic tube only. The tubes were mixed and centrifuged for 10 minutes at 3500-4000rpm. 1ml supernatant was taken for chromogenic reaction.

(2) Chromogenic reaction

Four set of test tubes labeled blank, standard, enzymatic and non-enzymatic tubes. About 1ml GSH standard application solution was added in the blank tube. About 1ml of 20μmol/L GSH standard solution was added into the standard tube. About 1ml of supernatant was added into the

enzymatic and non-enzymatic tube. About 1ml of reagent 3 application solution was added to the set of test tubes each. About 0.25ml of reagent 4 application solution was added to the set of test tubes each. About 0.05ml of reagent 5 application solution was added to the set of tubes respectively. The tubes were mixed and allowed to stand for 15 minutes. Optical densities were measured at 412nm wavelength using a 1cm diameter cuvette, set to zero with double-distilled water (ddH_2O)

Calculation formula

GSH-PX activity = $\frac{(OD\ non-enzyme-ODenzyme)}{OD\ standard-ODblank}$×(20μmol/L)×reaction time ÷(protein concentration of sample ×sample volume)

The GPx activity was expressed as milliunit per milligramm of protein (mU mg^{-1} protein).

3.12 Partial purification of crude stem bark aqueous extract of *Anogeissus leiocarpus* using column chromatography and thin layer chromatography

The crude extract was partially purified using Column chromatography and thin layer chromatography. Silica gel was used as a stationary phase. The slurry (the first eluent) was prepared by mixing silica gel 60-200 mesh and ethyl acetate. The bottom of the column was plugged with little cotton to prevent the adsorbent pass out, and then the silica gel suspension was poured into the column, set aside for 10 minutes and used. 4g of crude extract was mixed with silica gel to become more porous and subjected to column chromatography to separate the extract into its component fractions, methanol and ethyl acetate were added at various ratios (Ethyl acetate 100%, ethyl acetate: methanol 2:1, ethyl acetate: methanol 1:1, ethyl acetate: methanol 1:2 and methanol 100%). The column was eluted gradually with solvents of increasing

polarity and effluents of 50mls was collected in labeled beakers and concentrated at room temperature. The flow rate was 5ml per minutes. Thin layer was used to monitor the column and the eluents were pooled together into five fractions. The column was eluted with solvents of increasing polarity (Appendix 10).

Thin layer chromatography

Thin layer chromatography (TLC) provides partial separation of both organic and inorganic materials using thin-layered chromatographic plates especially useful for checking the purity of fractions. Each fraction is applied on activated TLC plates with the help of capillary tube at a 1/2 inch apart from the lower edge of TLC plate, and plate is kept in a developing chamber containing suitable solvent system until the developing solvent reaches top of the upper edge of TLC plate. Plate was taken out from developing chamber, dried and solvent front was marked by lead pencil. Compound bands/spots visualized on TLC chromatogram were detected by visual detection, under UV light (254 nm), in iodine chamber and by using spray reagent (vanillin-sulfuric acid) for the presence of specific compounds. The visualized spots of the components in the chromatoplate were marked and the Rf value of each spot was calculated by the formula: Rf = distance travelled by the sample (cm)/distance travelled by the solvent (cm) (Appendix 11).

3.12 Quantitative antioxidant activity

Evaluation of the radical scavenging activity of each purified pooled fraction on the resolved TLC plate was carried out in quantitative terms only of strong, moderate, weak or no activity. The method reported by Braca *et al.* (2002) was followed. The TLC plates were sprayed with 0.2% (w/v) 1, 1-Diphenyl-2-picrylhydrazyl (DPPH) in methanol and left. If free radicals have been scavenged an active spot with antioxidant activity would turn from violet to yellow.

3.13 Quantitative antioxidant activity

This is the most widely reported method for determining the antioxidant activity of many plant drugs. DPPH is a stable free radical with violet colour. If free radicals have been scavenged, DPPH will change its colour from violet to pale yellow or colourless. This property allows visual monitoring at 517 nm. The 50% inhibitory concentration (IC_{50}) of each sample was calculated from the regression equation for each curve by substituting 50 for y and obtaining the unknown x in the equation $x = \frac{y+c}{m}$.

The method of Liyana- Pathiranan and Shahidi (2005) was used for the determination of scavenging activity of DPPH free radical in the extract solution. A solution of 0.135mM DPPH in ethanol was prepared and 1.0ml of this solution was mixed with 1.0ml of extract prepared in methanol containing 0.025-0.5mg of the plant extract and standard drug respectively. The reaction mixture was vortexed thoroughly and left in the dark at room temperature for 30 minutes. The absorbance of the mixture was measured spectrophotometrically at 517nm. The ability of the plant extract to scavenge DPPH radical was calculated by the equation:

$$DPPH\ radical\ scavenging\ activity = \left\{\frac{(Abs\ \text{control} - \text{Abs sample})}{(Abs\ control)}\right\} \times 100$$

Where; Abs control is the absorbance of DPPH radical+ methanol;

Abs sample is the absorbance of DPPH radical+ sample extract or standard.

3.14 Gas chromatography-Mass spectroscopy (GC-MS)

GC-MS analysis of sample Components of the most active fraction A was analyzed using a n Agilent technology 7890B GC/ 5977A MSD with PAL RSI 85 autosampler. GC equipped with a HP-5 MS ultra-inert column (30m in lenght×250µm in diameter×0.25µm in thickness of film)

and coupled to a Mass spectrometer available at the Multi-User Science Research Laboratory Department of Chemistry Ahmadu Bello University Zaria. The sample was dissolved in methanol prior to analysis. Pure helium gas was used as a carrier (1ml/min). Initial temperature was set at 50^{o}C with increasing rate of 3^{o}C/min and holding time of about 10min. Finally the temperature was increased to 300^{o}C at 10^{o}/min.

3.15 Infrared Spectrum

Fourier transform infra-red spectroscopy analysis was carried out at the Multi-User Science Research Laboratory Department of Chemistry Ahmadu Bello University Zaria. The sample was evaporated onto a KBr salt plate and acquisition of the spectrum from the thin film. The instrument was turned on making sure that N_2 is flowing through the chamber so that excessive CO_2 and H_2O are flushed from the chamber and from inside the instrument. Scan was performed using IR blank in the instrument. The sample plate was placed in the FIR and waited for N_2 to purge out the air. The display spectrum automatically subtracted the stored background and the spectrum was displayed. He data was printed. Absorption is written in terms of wavenumbers (units cm^{-1}).

3.16 Statistical Analysis

The results were expressed as Mean ±standard error of mean (SEM). Statistical comparisons were performed by one-way analysis of variance (ANOVA), followed Dunnets post-hoc test. The data was analyzed by using Statistical Package for the Social Sciences (SPSS, version20.0). A P-value less than 0.05 were considered to be significantly different.

CHAPTER FOUR

4.0 RESULTS

4.1 Qualitative and Quantitative analysis of phytochemical constituents of aqueous stem bark extract of *Anogeissus leiocarpus*.

The results of quantitative analysis of phytochemical constituents of aqueous stem bark extract of *Anogeissus leiocarpus* shows the presence of flavonoids, alkaloids, saponins, Anthraquinones, tannins cardiac glycosides, steroids and triterpenes (Table 4.1).

The results of the qualitative analysis of phytochemical constituents of aqueous stem bark extract of *Anogeissus leiocarpus* shows the degree of abundance of these phytochemicals in mg/100g of the extract is as follows; 87.67 ± 0.09 of flavonoids, 51.00±0.12 of alkaloids, 36.33±0.09 of saponins and mg/g 11.23±0.15 of tannin, and 15.53±0.03 of total protein (Table 4.2).

4.2 Medium lethal dose LD_{50}

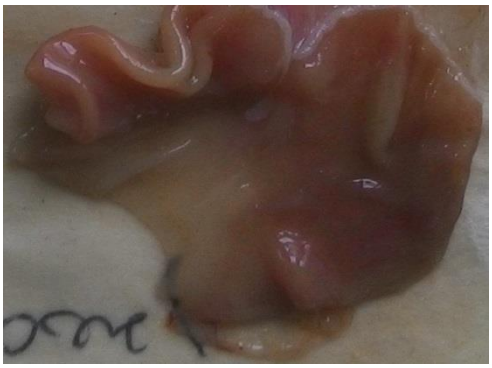
Oral administration of aqueous stem bark extract of *Anogeissus leiocarpus* for 24hrs showed no symptoms of toxicity which include fatigue, loss of appetite and drowsiness, and none of the rats died up to a dose of 5000 mg/kg b.w.

4.3 Effect of aqueous stem bark extract of *Anogeissus leiocarpus* and cimetidine on mean ulcer index

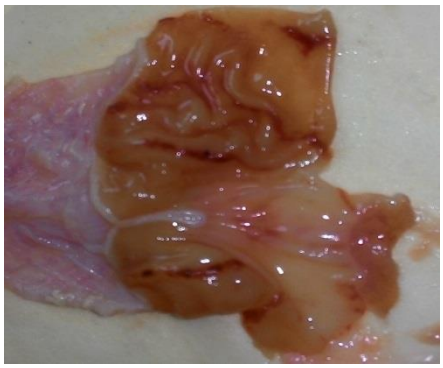
Mean ulcer score expressed as mean ulcer index expresses the degree of ulceration on mucosal membrane. It was determined by naked eye using a hand lens as described in 3.9.1. Pre-treatment of the gastric mucosa with oral administration of aqueous stem bark extract of *Anogeissus leiocarpus* at various concentrations of 100, 200 and 400mg/kg body weight, for 14 days showed a significant dose-dependent decrease in mean ulcer score, in rats induced by 70% ethanol (1ml/200 gm b.w.) for 1hour. Pre-treatment with a reference drug cimetidine (100 mg/kg b.w) for 14 days, also significantly reduced gastric ulcers with mean ulcer score of 2.6 ± 0.68 SEM

(Table 4.3), when compared to 400 mg/kg b.w of aqueous *Anogeissus leiocarpus* stem bark extract with mean ulcer score of 1.4±0.87 SEM (Appendix 3).

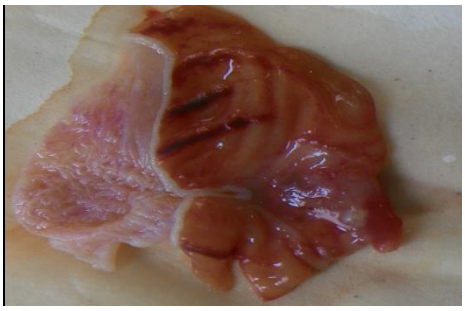
The preventive index was calculated from ulcer index of control and treated group. Pretreatment with aqueous *Anogeissus leiocarpus* stem bark extract at various concentrations 100, 200 & 400 mg/kg b. w, produced a significant dose-dependent protective effect against gastric ulcers induced by 70% EtOH (1 ml/200g b. w.), (Table 4.3). Pretreatment with aqueous *Anogeissus leiocarpus* stem bark extract at a dose 100 mg/kg b. w for 14 days, has a preventive index of 48%, 200 mg/kg b.w of 59% and 400 mg/kg b.w. of 74%. Cimetidine (standard) at a dose of 100 mg/kg b.w has a preventive index of 52% which falls in between the preventive indices of 200 and 400 mg/kg b. w of the aqueous *Anogeissus leiocarpus* stem bark extract (Appendix 4 and 5).



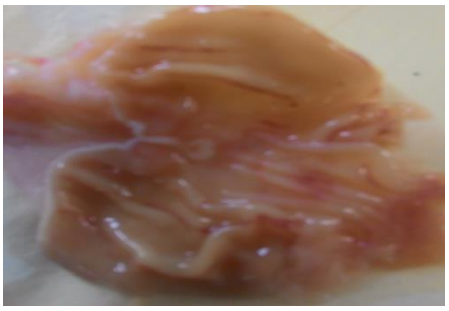
Normal control group A



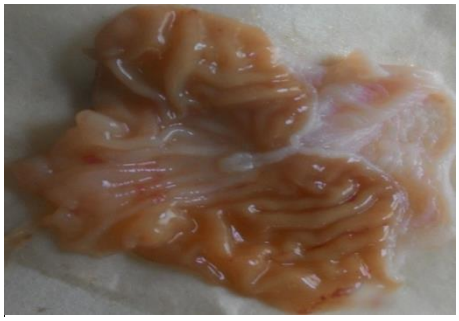
Ulcer control group B



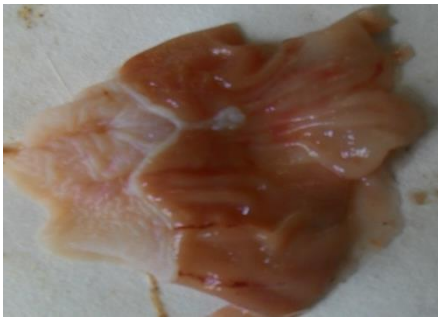
100 mg/kg+ ethanol group C



200 mg/kg +ethanol group D



400 mg/kg+ ethanol group E



Cimetidine-100 mg/kg + ethanol group F

Effects of pre-treatment with aqueous stem bark extract of *Anogeissus leiocarpus* for 14 days on stomach lesions induced by 70% ethanol in rats A: normal stomach mucosa. B: untreated group receiving only ethanol ulcer control. C: 100 mg/kg + ethanol group. D: 200 mg/kg + ethanol group. E: 400 mg/kg + ethanol group. F: Cimetidine-100 mg/kg + ethanol group.

Table 4.1: Quantitative analysis of phytochemical constituents of crude aqueous stem bark extract of *Anogeissus leiocarpus*.

Constituent	Remark
Flavonoids	+
Alkaloids	+
Saponins	+
Anthraquinones	+
Tannins	+
Cardiac glycosides	+
Steroids and triterpenes	+

+= Present

Table 4.2: Quantitative analysis of phytochemical constituents of the crude aqueous stem bark extract of *Anogeissus leiocarpus*

Flavonoids (mg/100g)	Alkaloids (mg/100g)	Saponins (mg/100)	Tannins (mg/100g)	Phenols (mg/100g)
87.67±0.09	51.00±-0.12	36.33±0.09	11.23±0.15	15.53±0.03

Values are expressed as Mean ± SEM (n=3)

Table 4.3: Effect of aqueous stem bark extract of *Anogeissus leiocarpus* on gastric mucosal membrane of ethanol-induced ulceration in albino rats.

Groups	Mean ulcer index	% Ulceration	Preventive index
Normal control	0	0	0
Ulcer control	5.4 ± 0.40^{a}	100	0
100 mg/kg extract + ethanol	2.8 ± 0.37^{b}	100	48
200 mg/kg extract + ethanol	2.2 ± 0.68^{c}	80	59
400 mg/kg extract+ ethanol	1.4 ± 0.87^{d}	40	74
100 mg/kg cimetidine+ ethanol	2.6 ± 0.68^{e}	100	52

Data represent the Mean± SEM. (n=5)

Different superscripts along the column are significantly different at $p<0.05$

4.4 Effect of aqueous stem bark extract of *Anogeissus leiocarpus* on the histopathology of stomach tissue, in 70% ethanol induced ulceration in rats.

Histological evaluations on the effect of crude aqueous stem bark extract of *Anogeissus leiocarpus* and cimetidine on ethanol induced gastric lesions in rats (hematoxylin and eosin, 400×). Photomicrograph of gastric mucosa from positive control (normal) rats demonstrates an intact surface mucosal cell and gastric pits (Plate I). The ulcer control group (70% ethanol) had intense ulcerated gastric mucosal epithelial cells, necrotic tissue and heavy infiltration (Plate II). There were differences in the histopathology of the stomach in the different groups, where 100 mg/kg, 200 mg/kg and 400 mg/kg of the crude aqueous stem bark extract of *Anogeissus leiocarpus* were administered per kg body weight of the animals for 14 days with administration of 70% ethanol (1ml/200g b. w.) as shown in the photomicrograph (Plate III-IV) as compared with the group treated with standard drug Cimetidine (Plate VI). The section of gastric mucosa from rat pre-treated with stem bark aqueous extract of *Anogeissus leiocarpus* at 100 and 200mg/kg b. w. had slightly eroded mucosal epithelial cells, less infilteration and haemorrhage, as shown in Plates III and V respectively. In the 400mg/kg b.w. stem bark aqueous extract of *Anogeissus leiocarpus* pre-treated rat, there is no observable haemorrhagic necrosis of gastric mucosa and showed protection against the histopathological changes observed in ethanol treated group with an intact gastric pits, maintenance of mucosa even after exposure of ethanol (Plate V). Cimetidine (100mg/kg b.w.) pre-treated group demonstrates slight ulceration, less hemorrhagic necrosis, infiltration in the gastric mucosa of rat (Plate VI)

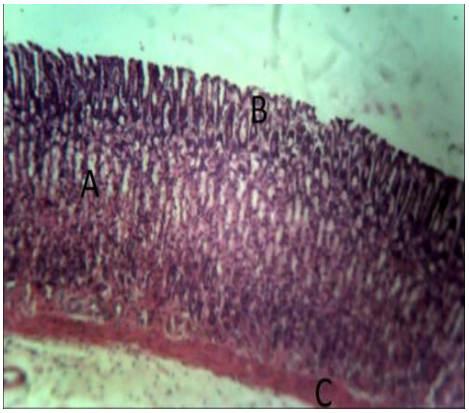


Plate I: Normal control group A

A= Gastric pits, B= Gastric glands, C= Muscularis mucosae

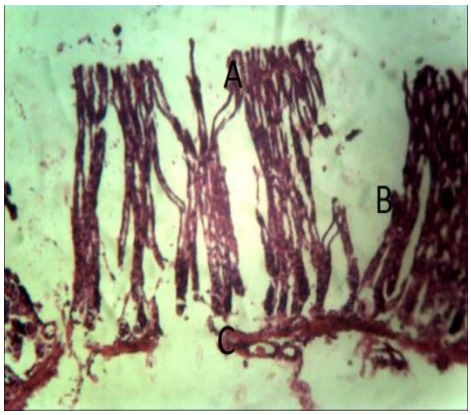


Plate II: Ulcer control group B

A= Gastric glands, B= Gastric pits, C= Muscularis mucosae

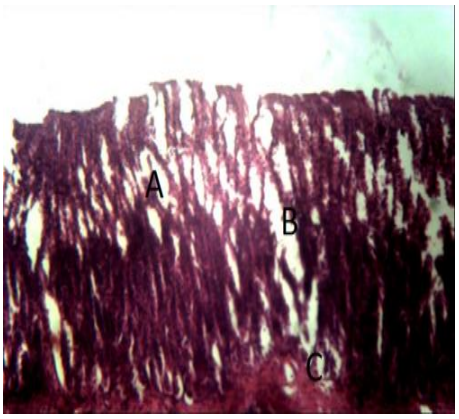


Plate III: 100 mg/kg+ ethanol group C

A=Gastric pit, B= Gastric gland, C= Muscularis mucosae

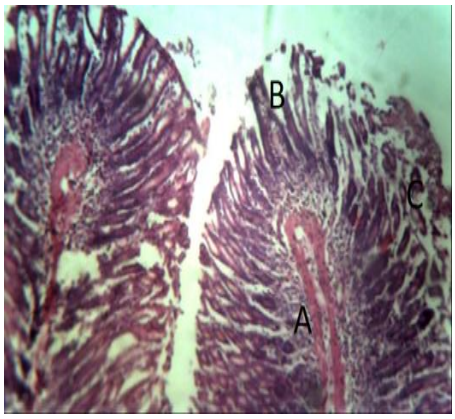


Plate IV: 200 mg/kg +ethanol group D

A=Muscularis mucosae, B=Gastric pits, C=Gastric glands

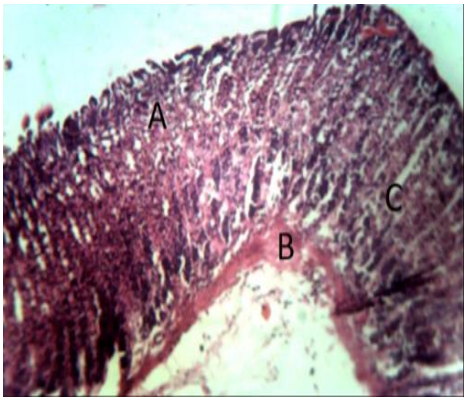


Plate V: 400 mg/kg+ ethanol group E

A=, Gastric pits B=Muscularis mucosa, C=Gastric gland

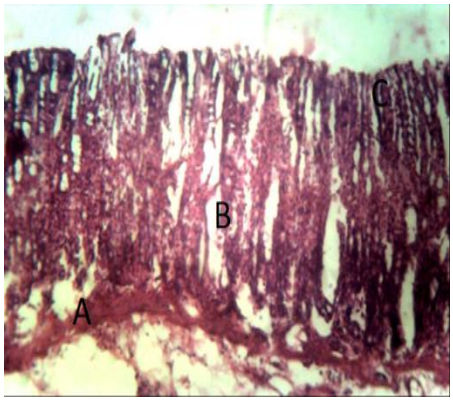


Plate VI: Cimetidine-100 mg/kg + ethanol group F

A=Muscularis mucosae, B=Gastric gland, C=Gastric pit

4.5 Effect of aqueous stem bark extract of *Anogeissus leiocarpus* on antioxidative parameters in 70% ethanol induced ulcer in rats.

Normal control rats showed; MDA of 0.06±0.03 nmolmg^{-1}protein, catalase 7.87±0.63 Umg^{-1}protein, SOD of 1.50±0.36 Umg^{-1}protein and GP_x of 2.85±0.72 mUmg^{-1}proteins (Table 4.4). Pre-treatment with aqueous extracts of *Anogeissus leiocarpus* 100, 200 and 400mg/kg b. w given orally for 14 days (0.11±0.00, 0.04±0.01 and 0.05±0.03 nmolmg^{-1}protein) , as well as standard cimetidine (0.10±0.03 nmolmg^{-1}protein) shown no significant difference in MDA when compared to normal group. There was also a significant increase in ($p<0.05$) catalase in the group administered 400mg/kg (12.25±1.30Umg^{-1}protein) when compared to normal group (7.87±0.63 Umg^{-1}protein) however there was no significant difference in the group administered 100mg/kg b.w. (6.56±0.85Umg^{-1}protein), 200mg/kg b.w (8.98±1.39Umg^{-1} protein) and positive cimetidine group (8.51±1.22Umg^{-1} protein) as compared with the normal control (7.87±0.63 Umg^{-1}protein). There was a significant decrease in SOD of 100mg/kg b. w. group (0.72±0.08Umg^{-1} protein) when compared to normal group (1.50±0.36 Umg^{-1}protein) but no significant difference at a dose of 200mg/kg b.w (0.98±0.12 Umg^{-1} protein), 400mg/kg b.w (1.20±0.08 Umg^{-1} protein) and cimetidine group (1.01±0.10 Umg^{-1} protein) when compared to normal group (1.50±0.36 Umg^{-1}pro). There was also no significant difference in GPx at a dose of 200 and 400mg/kg b. w. (1.59±0.32 and 2.33±0.29 mUmg^{-1} protein) when compared to normal group (2.85±0.72 mUmg^{-1}protein). However, there was a significant decrease in GPx of 100mg/kg b.w (1.32±0.31 mUmg^{-1}protein) and cimetidine group (1.05±0.27 mUmg^{-1}protein) when compared with normal group (2.85±0.72 mUmg^{-1}protein) (Table 4.4)

Table 4.4: Effect of aqueous stem bark extract of *Anogeissus leiocarpus* on some antioxidative enzymes and thiobarbituric acid reactive substances (TBARS) in ethanol- induced ulceration in albino rats (compared with positive (normal) control and negative (ulcer) control).

Treatment	MDA	Catalase	SOD	GPx
Unit	$nmolml^{-1}prot$	$Umg^{-1}prot$	Umg^{-1} prot	$mUmg^{-1}prot$
Normal control	$0.06\pm0.03^{a,b}$	$7.87\pm0.63^{a,b}$	$1.50\pm0.36^{a,b}$	$2.85\pm0.72^{a,b}$
Ulcer control	$0.27\pm0.03^{b,a}$	$1.87\pm0.62^{b,a}$	$0.35\pm0.72^{b,a}$	$0.36\pm0.06^{b,a}$
100 mg/kg extract+ ethanol	$0.11\pm0.00^{a,c}$	$6.56\pm0.58^{a,c}$	$0.72\pm0.08^{c,a}$	$1.32\pm0.31^{c,a}$
200 mg/kg extract + ethanol	$0.04\pm0.01^{a,d}$	$8.98\pm1.39^{a,d}$	$0.98\pm0.12^{a,a}$	$1.59\pm0.32^{a,c}$
400 mg/kg extract+ ethanol	$0.05\pm0.03^{a,e}$	$12.25\pm1.30^{c,e}$	$1.20\pm0.08^{a,c}$	$2.33\pm0.29^{a,d}$
100 mg/kg cimetidine+ ethanol	$0.10\pm0.03^{a,f}$	$8.51\pm1.23^{a,f}$	$1.01\pm0.10^{a,d}$	$1.05\pm0.27^{d,a}$

Data represent the Mean± SEM. (n=5)

Different superscripts along the column are significantly different at $p<0.05$ when compared with normal control and ulcer control

.

The ulcer control group (70% EtOH) administered orally for 1hr showed; MDA of 0.27±0.03 nmolmg^{-1}protein, catalase of 1.87±0.62 Umg^{-1}protein, SOD of 0.35±0.07 Umg^{-1}protein and GPx of 0.36±0.06 mUmg^{-1}protein (Table 4.4). Pre-treatment with aqueous stem bark extract of *Anogeissus leiocarpus* at 100, 200 and 400mg/kg b.w. orally for 14 days (0.11±0.00, 0.04±0.01 and 0.05±0.03nmolmg^{-1}protein), as well as standard cimetidine at 100mg/kg (0.10±0.03 nmolmg^{-1}prot) shown significant decrease ($p<0.05$) in MDA when compared with the ulcer control (ethanol) group (0.27±0.03 nmolmg^{-1}prot). There was a significant increase ($p<0.05$) in catalase of groups administered 100, 200, 400mg/kg (6.56±0.58, 8.98±1.39 and 12.22±1.30 Umg^{-1} protein) as well as standard cimetidine at 100mg/kg (8.51±1.23 Umg^{-1} protein) when compared with ulcer control (ethanol) group (1.87±0.62 Umg^{-1}protein). There was no significant difference in SOD of groups administered 100 and 200 mg/kg (0.72±0.08 and 0.98±0.12 Umg^{-1} protein) when compared with ulcer control (ethanol) group (0.35±0.07 Umg^{-1}protein). However there was a significant increase ($p<0.05$) in groups administered 400mg/kg extract and standard cimetidine at 100mg/kg (1.20±0.08 and 1.01±0.10 Umg^{-1}protein) when compared with ulcer control (ethanol) group (0.35±0.07 Umg^{-1}protein). There was a significant increase ($p<0.05$) in GPx of groups administered 200 and 400mg/kg of extract (1.59±0.32 and 2.33±0.29mUmg^{-1}protein) when compared with ulcer control (ethanol) group (0.36±0.06 mUmg^{-1}protein). However here was no significant difference in groups administered 100mg/kg extract and standard cimetidine at 100mg/kg (1.32±0.3 and 1.05±0.27 mUmg^{-1}protein) when compared with ulcer control (ethanol) group (0.36±0.06 mUmg^{-1}protein). (Table 4.4)

4.6 Column Chromatography the crude aqueous stem bark extract of *Anogeissus leiocarpus*.

Chromatogram of the five pooled fractions from column chromatography resolved using solvent system ethyl acetate: methanol 9:2 sprayed with p-anisaldehyde. The plates were labeled A-E (Plate VIII).

A 1-4

B 5-12

C 13-22

D 23-33

E 34-50

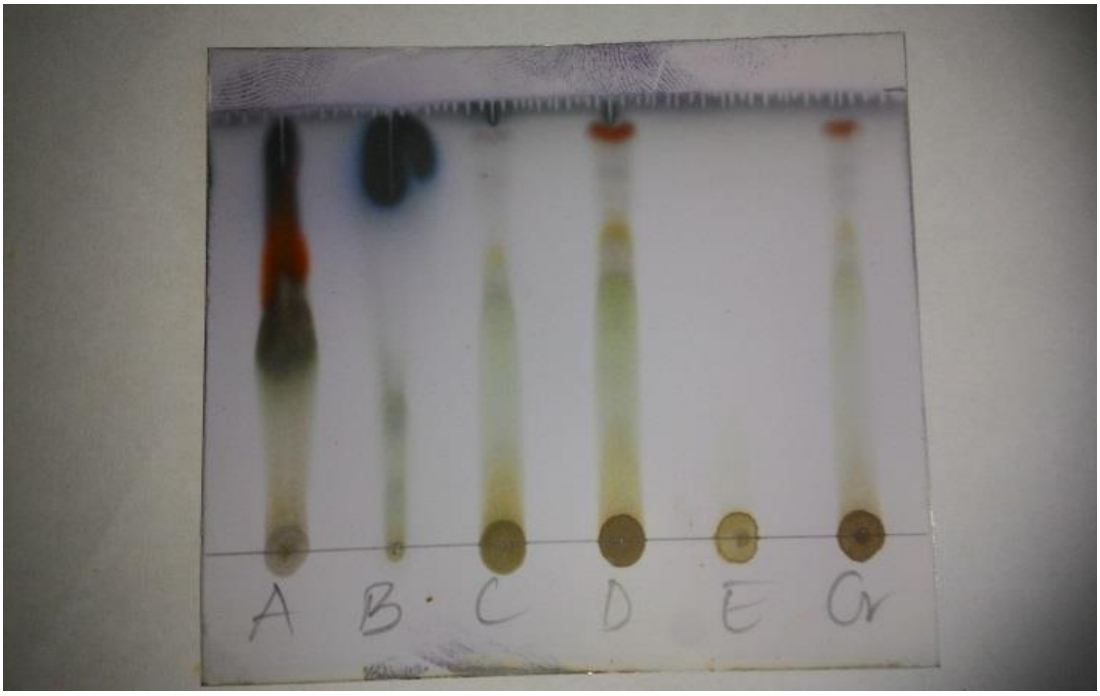


Plate VII: TLC plate of partially purified pooled fractions of crude aqueous stem bark of *Anogeissus leiocarpus*.

4.6.1 Quantitative antioxidant activity of partially purified aqueous stem bark extract of *Anogeissus leiocarpus*.

The quantitative DPPH scavenging activities of the five pooled fractions using DPPH spray labeled A-E revealed fraction A to have the highest antioxidant activity. (Plate IX).

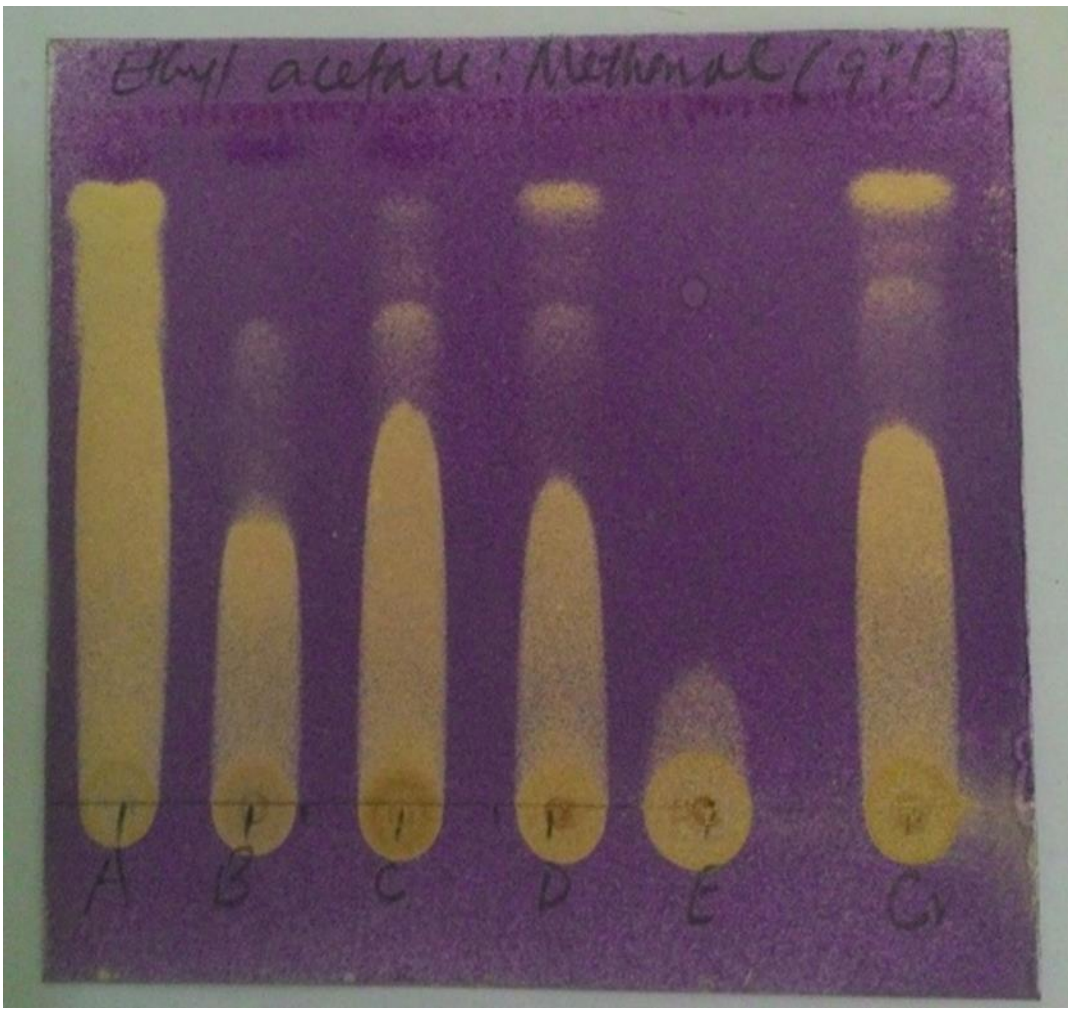


Plate VIII: TLC plate of the five partially purified pooled fractions of crude aqueous stem bark of *Anogeissus leiocarpus* resolved by ethyl acetate methanol 9:1 sprayed with DPPH solution

4.6.2 Qualitative antioxidant activity partially purified pooled fractions of crude aqueous stem bark of *Anogeissus leiocarpus*.

A scavenging activity in % inhibition and IC_{50} of the five pooled fractions from column chromatography is given in Table 4.6. Fraction A has an IC_{50} of 20.88, fraction B 53.89, fraction C 35.37, fraction C 35.37, fraction D 60.97, fraction E 197.95 and ascorbic acid 24.32. Fraction A had the highest percentage inhibition at various concentrations (52.60±0.10, 66.35±0.15, 92.20±0.10, 94.40±0.20, 97.95±0.35) as well as a lower IC_{50} (20.88) compared to Ascorbic acid with IC_{50} (24.32) and percentage inhibition (52.80±0.75, 63.40±0.75, 79.05±0.75, 91.35±0.15, 94.05±0.35).

Table 4.5: DPPH Free radical scavenging activity % and IC_{50} of the five pooled fractions

Concentration	Free radical scavenging activity (%)					
(µg/ml)	Ascorbic acid	Fraction A	Fraction B	Fraction C	Fraction D	Fraction E
31.25	52.80±0.75[a]	52.60±0.10[a]	22.95±0.15[b]	35.20±0.10[c]	39.00±0.10[d]	15.70±0.00[e]
62.5	63.40±0.75[a]	66.35±0.15[a]	67.00±0.10[a]	73.05±0.75[b]	54.00±0.10[c]	19.80±0.10[d]
125	79.05±0.75[a]	92.20±0.10[b]	77.00±0.10[a]	79.05 ±0.75[a]	59.40±0.10[c]	29.70 ±0.70[d]
250	91.35±0.15[a]	94.40±0.20[a]	84.75±0.05[b]	91.35±0.15[a]	66.25±0.05[c]	49.60±1.60[d]
500	94.05±0.35[a]	97.95±0.35[a]	92.25±0.50[a]	92.85±0.05[a]	78.75±0.55[b]	81.65±2.95[c]
IC_{50}	24.32	20.88	53.89	35.37	60.97	197.95

x= y+c/m = Straight line equation obtained from graph

Free radical scavenging activity (%) expressed as Mean ± SEM. (n=3)

Different superscripts along the rows are significantly different ($P<0.05$) when compared with ascorbic acid

4.7 Gas chromatography- mass spectroscopy of the most active partially purified fraction A of the aqueous stem bark of Anogeissus leiocarpus.

Gas chromatography – mass spectrometry (GC-MS) is a method that combines the features of gas- liquid chromatography and mass spectrometry to identify different substances within a test sample (Gnanasundaram and Balakrishnan, 2017). In the last few years, GC-MS has become firmly established as a key technological platform for secondary metabolite profiling in both plant and non-plant species. Interpretation on mass spectrum GC-MS was conducted using the database of National Institute Standard and Technology (NIST) having more than 62,000 patterns. The spectrum of the unknown component was compared with the spectrum of the known components stored in the NIST library. The name, molecular weight and structure of the components of the test materials were ascertained. The GCMS profile of fraction 'A' revealed 14 compounds. The active principles with their retention time (RT), molecular formula, molecular weight (MW) and concentration (%) are presented in (Table 4.6 and Fig 4.1). The compounds were: (1) 2-Hydroxy ethyl vinyl sulfide, (2) 2-Furanmethanol, (3) Propanamide, (4) Isobutylamine, (5) Phenol, (6) 4H-pyran-4-one, 2,3 dihydro-3,5-dihydroxy methyl-, (7) 2-phenyl propylamine, (8) Phenol, 2-4-Bis (1,1-dimethyl ethyl)-, (9) Inositol-1-deoxy, (10) 2-methoxy-4-vinylphenol, (11) n-hexadecanoic acid, (12) [1,2,3,4] Tetrazolo [1,5-a]pyridin-8 –amine, (13) Ethane, methoxy, (14) Acetic acid (aminooxy)-. Some of the reviewed compounds have been known to possess antioxidant activities in literature these are: (1) 2-Furanmethanol, (2) Phenol, (3) 4H-pyran-4-one, 2, 3 dihydro-3, 5-dihydroxy methyl-, (4) Phenol, 2-4-Bis (1,1-dimethyl ethyl)-, (5) 2-methoxy-4-vinylphenol, (6) n-hexadecanoic acid. Other compounds that possess other biological activities which include antiparasitic, antibacterial, antimicrobial, anti-inflammatory, antiproliferative, and anti-convulsant are: (1) 2-Hydroxy ethyl vinyl sulfide, (2)

Phenol , (3) 4H-pyran-4-one, 2,3 dihydro-3,5-dihydroxy methyl-, (4) 2-phenyl propylamine, (5) Phenol, 2-4-Bis (1,1-dimethyl ethyl)-, (6) n-hexadecanoic acid, (7) [1,2,3,4] Tetrazolo [1,5-a]pyridin-8 –amine, (8) Acetic acid (aminooxy)-. Compounds with unknown biological activities have also been reviewed vis: (1) Propanamide, (2) Isobutylamine, (3) Inositol-1-deoxy, (4) Ethane, methoxy. By comparative inspection, the major phytocomponents present in aqueous stem bark extract of *Anogeissus leiocarpus* in terms of their relative abundance were [1,2,3,4] Tetrazolo [1,5-a] pyridin-8 –amine, Inositol-1-deoxy, 2 phenyl propylamine and Phenol 2-4-Bis (1,1-dimethyl ethyl)-, which corresponds to 17.53%,10.57%, 9.03%, 8.75% respectively. In the same context the minor phytocomponents are in the range of 0.97%-7.99%

Table 4.6: Phytochemical compounds identified in aqueous stem bark extract of *Anogeissus leiocarpus* most active fraction A.

S/N	Compound name	Retention time	Peak area %	Molecular formular/ molecular weight(g/mol)	Activity
1	2-Hydroxy ethyl vinyl sulfide	67.600	3.48	C_4H_8OS/104.71	Antiparasitic
2	2-Furanmethanol	72.113, 72.113	0.97	$C_5H_6O_2$/98	Antioxidant
3	Propanamide	73.817, 84.792, 88.297, 92.013	7.23	C_3H_7NO/73.09	Unknown
4	Isobutylamine	79.968, 80.334, 82.754, 83.226, 83.463, 95.574	7.93	$C_4H_{11}N$/73.14	Unknown
5	Phenol	83.826, 86.290	5.13	C_6H_6O/94	Antioxidant/Antibacterial
6	4H-pyran-4-one, 2,3	84.123	1.88	$C_6H_8O_2$/144	Antimicrobial, Anti-

	dihydro-3,5-dihydroxy methyl-				inflamatory, Antiproliferative, Antioxidant.
7	2-phenyl propylamine	84.452, 85.016, 86.585, 93.190, 96.803	9.03	$C_9H_{13}N$/135.21	Anti inflammatory
8	Phenol, 2-4-Bis (1,1-dimethyl ethyl)-	85.420, 93,501	8.75	$C_{14}H_{22}O$/206	Antioxidant, Antibacterial
9	Inositol-1-deoxy	85.905, 87.202, 87.747, 88.588, 89.439	10.57		Unknown
10	2-methoxy-4-vinylphenol	86.585, 86.967	7.01	$C_9H_{10}O_2$/150	Antioxidant
11	n-hexadecanoic acid	87.365, 90.280	6.33	$C_{16}H_{32}O_2$/256	Antioxidant, Anti Inflammatory
12	[1,2,3,4]Tetrazolo[1,5-a]pyridin-8 –amine	87.531, 88.017, 88.852,	17.53	$C_5H_5N_5$/135.13	Antimicrobial, Anti-inflammatory

		89.156, 89.622, 89.859, 90.840, 91.245			
13	Ethane, methoxy	91.593, 93.816, 94.446, 94.825, 95.254, 96.148	7.99	C_3H_8O/60.09	Unknown
14	Acetic acid (aminooxy)	97.383, 97.661, 98.156, 98.714	6.17	$C_2H_5O_3$/91.07	Anti convulsant

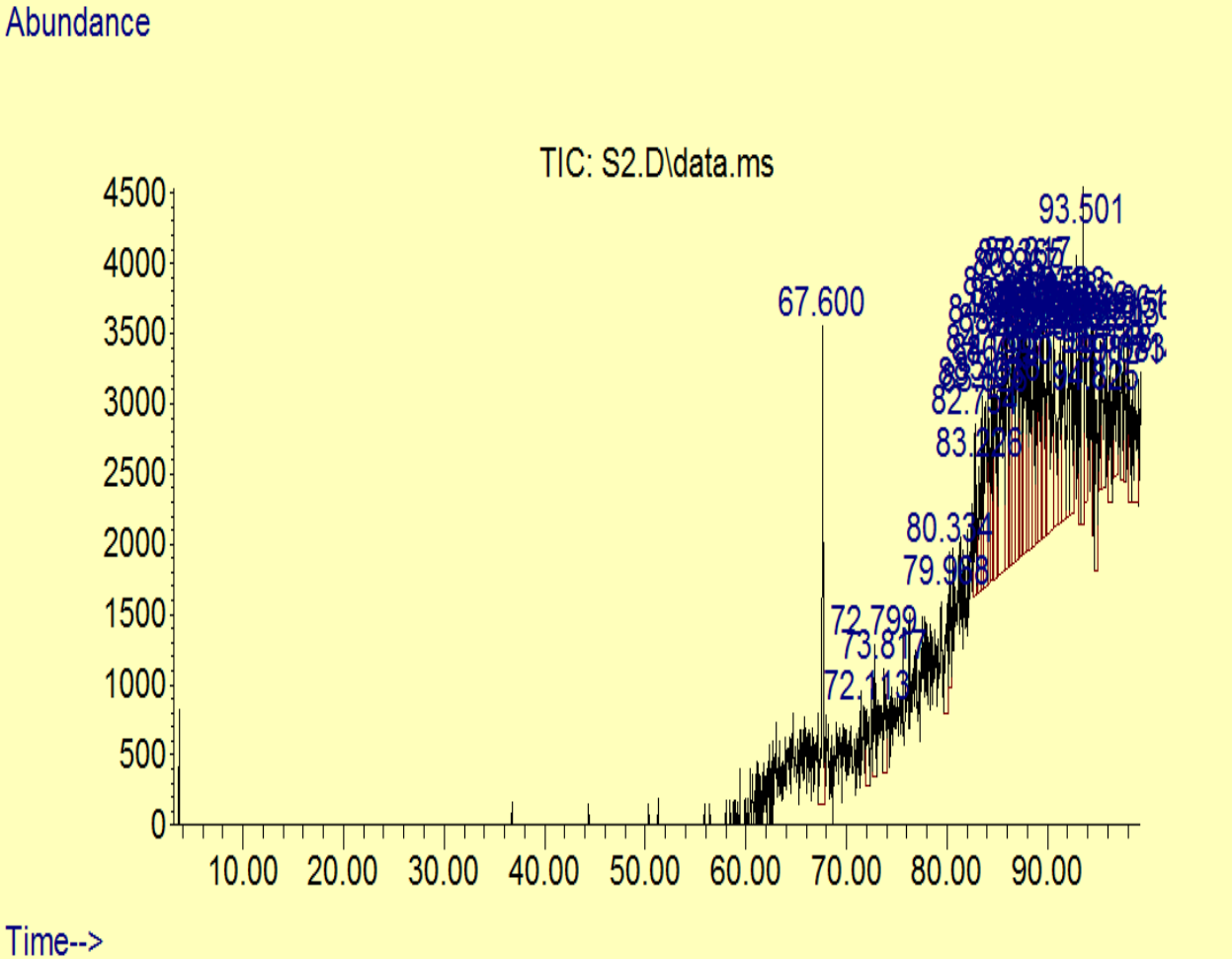


Figure 4.1: GC-MS chromatogram of aqueous stem bark extract *Anogeissus leiocarpus* most active fraction A.

4.8 Fourier transformed infrared radiation (FTIR) analysis of the most active fraction A

FT-IR spectral data revealed presence of various functional groups in fraction A as shown in Figure 4.2. Spectral data in Table 4.7 gives the interpretation of the frequencies, vibration bonds and the functional groups present in the compound. The compound shows hydrogen bonded –OH stretching, C-H stretching, N-H bending, C-C stretching in ring, C-O stretching and C-N stretching in common.

Table 4.7: FT-IR peak values of aqueous stem bark extract of *Anogeissus leiocarpus* most active fraction A.

S/N	IR υ_{max} (cm^{-1})	(Transmission mode)	Functional group
1	3377.0	O-H(H-bonded): N-H stretch	Alcohols, phenols: Amines
2	2926.0	C-H stretch	Alkanes
3	2855.1	C-H stretch	Alkanes
4	1740.7	C=O stretch	Ketones
5	1613.9	N-H	Amines
6	1481.1	C-C stretch (in ring)	Aromatics
ft7	1375.4	O-H bend	Aldehyde
8	1237.5	N-O stretch	
9	1118.2	C-O stretch	Alcohols, carboxylic acids, esters, ethers
10	1047.4	CH_2X: C-H wag: =C-H bend	Alkyl halides
11	913.2	=C-H:=CH_2	Alkenes
12	846.1	=C-H bend: C-Cl stretch: C-C stretch (ring)	Alkenes: alkyl halides: aromatics
13	775.3	=C-H bend: C-Cl stretch: C-C stretch (ring)	Alkenes: alkyl halides: aromatics
14	723.1	=C-H bend: C-Cl stretch: C-C stretch (ring): C-H rock, methyl	Alkenes: alkyl halides: aromatics

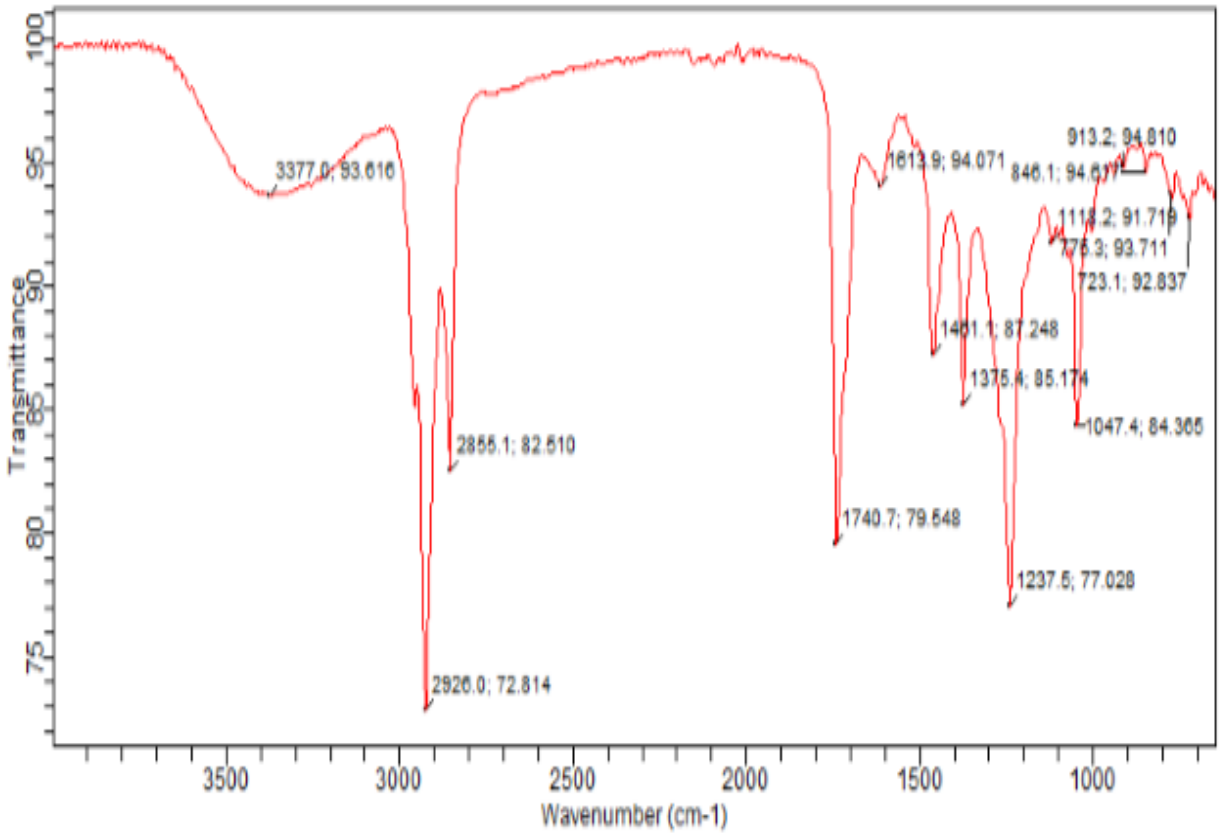


Figure 4.2: FT-IR peak values of aqueous stem bark extract of *Anogeissus leiocarpus* most active fraction A.

CHAPTER FIVE

5.0 DISCUSSION

The increased frequency of occurrence of gastric ulcers in humans, severe side effects and cost of some available synthetic drugs, arises the use of natural products an important alternative treatment (Bassi, *et al.,* 2014 and Strand *et al.,* 2017). In this sense aqueous stem bark of *Anogeissus leiocarpus*, have proven to be advantageous in the treatment of various diseases in lab animals and patients (Shuaibu *et al.,* 2005, Atawodi *et al.,* 2011, Victor and Grace, 2013, Timothy *et al.,* 2015).

Toxicity studies LD_{50} of aqueous stem bark extract of *Anogeissus leiocarpus* in rats indicated a no lethal effect up to a dose of 5000mg/kg body weight for 24 hours suggesting that the extract has a wide margin of safety.

Plants are known to contain a variety of secondary metabolites. These secondary metabolites or bioactive compounds produce definite physiological actions on the human system. Many of these phytochemicals have been discovered and even isolated from a variety of medicinal plants. Unfortunately, however, not many of them have been exploited for clinical use (Ekwueme *et al.,* 2014). Phytochemical analysis of plants is predicated by the need for drug alternatives of plant origin, made imperative by the high cost of synthetic drugs as in the case of anti-ulcers. These secondary plant metabolites extractable by various solvents exhibit varied biochemical and pharmacological actions in animals when ingested (Nwogu *et al.,* 2008). The aqueous stem bark extract of *Anogeissus leiocarpus* contains alkaloids, flavonoids saponins, tannin, steroid and triterpenes, anthraquinones and cardiac glycosides (Table 1). The results of the qualitative analysis of phytochemical constituents of aqueous stem bark extract of *Anogeissus leiocarpus* shows the degree of abundance of these phytochemicals in mg/100g of the extract is as follows;

87.67 of flavonoids, 51.00 of alkaloids, 36.33 of saponins, 11.23 of tannin, and 15.53 of phenol (Table 2). The result of the quantitative phytochemical analysis in this study varies from the result of Ahmad and Wudil were concentration of saponins (89.5 mg/100g) was found higher than the other phytochemicals (alkaloids-26.7 mg/100g, tannins-29.9 mg/100g, steroids-10.6 mg/100g, flavonoids-27.3 mg/100g, phenols-5.2 mg/100g, glycosides-1.7mg/100g) in the aqueous stem bark extract (Ahmad and Wudil, 2013). Flavonoids were found to be higher in concentration than other phytochemicals. The difference in the concentration of the phytochemical then those in literature might be due to geographical variations, nutrients, sunlight, irrigation, time of collection, age of plant among others.

The presence of flavonoids in the aqueous stem bark extract of *Anogeissus leiocarpus* could account for its use as an antinflammatory agent (Ekwueme *et al.*, 2014). It also means that the plant could be used to prevent damage caused by free radicals in the body (Dweck and Mitchell, 2002). Oxidative stress induced by ethanol was suppressed in the treatment group which proves the radical scavenging activity of the extract. Flavonoids exhibit dramatic effects on immune and inflammatory cells; these can be either immunosuppressant or immune stimulatory (Huang et al., 2010). This was evident from the histopathological assessment of the gastric tissues. Tannins are known to possess immuno stimulating activites. This suggests the possible potential of *Anogeissus leiocarpus* in the treatment of dysentery, diarrhoea, bacterial infection and in wound healing.

In the present study, oral administration of 70% ethanol to rats produced gastric mucosal lesions which correspond to score grade 3 (more than five small lesions or one intermediate lesion 3-4mm) by the method of Takagi and Okabe (1968) of severity of mucosal damage assessment. Ulcers caused by ethanol may possibly arise from direct damage of gastric mucosal cells,

abnormal elevation of reactive species which correspond to one of the main aggressive mechanism of ethanol mediated ulceration (Amaral *et al.*, 2013).

In this study ethanol induced oxidative stress in stomach tissue causes inhibition of antioxidant enzymes SOD, catalase (CAT) and glutathione peroxidase (GPX) has been corroborated and is directly involved in increased lipid peroxidation observed in ethanol- treated rats (negative control group) when compared with positive (normal) control group. Study carried out by Boligon et al. reported similar results relating to antioxidant enzyme activity (Boligon *et al.*, 2014). In addition lipid peroxidation measured as a biomarker as MDA caused by ethanol plays an important role in the pathogenesis of ethanol induced gastric lesions in gastric mucosa of rats. Pre-treatment with aqueous stem bark extract of *Anogeissus leiocarpus* exhibited antioxidant properties by decreasing the levels of MDA, suggesting its potential to protect against ethanol-induced lipid peroxidation in rats. Furthermore, aqueous stem bark extract of *Anogeissus leiocarpus* preserved the antioxidant activity of GPx, CAT, and SOD enzymes after ethanol administration, thus protecting the gastric mucosa.

The histology of the stomach tissue in accordance with previous studies of the negative (ethanol) control group showed typical histological damage 1 h after ethanol administration. The damage was characterized by intense ulcerated gastric mucosal epithelial cells, necrotic tissue and heavy infiltration (Gomez-Guzman *et al.*, 2018). The aggregation of neutrophils plays a fundamental role in the process of injury and inflammation in the gastric mucosa due to their release of tissue-disruptive substances like proteases, leukotrienes B4 (LTB4), and reactive oxygen species Via NADPH oxidase, neutrophils release superoxide anions, and these in turn are metabolized into the hydroxyl radical. The latter can mediate lipid peroxidation of polyunsaturated fatty acids and cause damage to cell membranes, leading to an alteration in the structural integrity and

biochemical function of membranes (Naito *et al.,* 1995 and Kobayashi, *et al.,* 2001). Pre-treatment with 100 and 200 mg/kg b.w of aqueous stem bark extract of *Anogeissus leiocarpus* showed slightly erosion, less infilteration and hemorrhage to the gastric mucosa cells, intact gastric pits and surface mucosal cells. 400mg/kg b.w. of aqueous stem bark extract of *A. leiocarpus* conferred a high protection of the histological structures with no observable hemorrhagic necrosis of the gastric mucosa as well as intact gastric pits and muscularis mucosae. Pre-treatment with cimetidine was protective however there were some histological injuries in the gastric mucosa of the stomach. The result showed that the gastroprotection from the aqueous stem bark extract of *Anogeissus leiocarpus* used was dose dependent on the dosage as also showed from the gastric ulcer score result.

Aqueous stem bark extract of *Anogeissus leiocarpus* restored in a dose dependent manner the oxidative stress induced by ethanol. The antioxidant properties of *Anogeissus leiocarpus* were demonstrated by decreased levels of Malondialdehyde MDA and increase of antioxidant defenses (catalase, superoxide dismutase and Glutathione peroxidase). These protective effects described for the crude aqueous stem bark extract of *Anogeissus leiocarpus* could be associated with the presence of flavonoids, alkaloids, total phenols, tannins and saponins which was similarly reported in study by Shuaibu et al. where castalagin, flavogallonic acid, bislactone ellagic acids, flavonoids and phenolic diterpenes were found present (Shuaibu *et al.,* 2008). The free radical scavenging activity of the aqueous stem bark extract of *Anogeissus leiocarpus* might be considered as one of the possible mechanisms of its gastroprotective effects observed. Oxygen derived radicals and agents with antioxidant properties have been implicated in the pathogenesis of ethanol induced ulcers (Boligon *et al.,* 2014). Alcohol rapidly penetrates the gastric mucosa apparently causing cell and plasma membrane damage leading to increased intracellular

membrane permeability to sodium and water. The massive intracellular accumulation of calcium represents a major step in the pathogenesis of gastric mucosal injury (Raju *et al.,* 2009). Ethanol-induced gastric ulcer can arise as a result of direct damage to mucosal cells, development of free radicals and hyper oxidation of lipid (Terano *et al.,* 1989). But scavenging these free radicals can play a role in healing of these ulcers (Halliwell and Gutteridge, 1992). Oxidative stress has been showed to play a role in alcohol-induced gastric mucosal damage (Gomez-Guzman, *et al.,* 2018).

SOD represents the first line of defense against ROS by catalyzing the conversion of O_2—to oxygen and H_2O_2, the latter of which is catalyzed to H_2O by CAT or GPx (Nozik-Grayck *et al.,* 2005, Dayer *et al.,* 2008 and Schrader and Fahimi, 2006). The possibility of this protective effect being fostered by aqueous stem bark extract of *Anogeissus leiocarpus* is consistent with previous findings that *Anogeissus leiocarpus* engender a significant decrease in oxidative stress by increasing the antioxidant defense system and reducing the levels of lipid peroxidation in different pathologic conditions. (Atawodi et al., 2011)

The quantitative antioxidant activity of the partially purified pooled fraction using 1, 1-Diphenyl-2-picrylhydrazyl (DPPH) spray as well as qualitative antioxidant activity using DPPH spectrophotometrically using ascorbic acid as a standard showed fraction A to possess the most activity. The DPPH test showed the reactivity of the test compound with a stable free radical. The DPPH gives a strong absorption band at 517 nm in vissible region. The degree of reduction in absorbance measurement is an indication that the extract had a radical scavenging property hence its antioxidant power. The results are either expressed as IC_{50} that is the concentration of the antioxidant causing 50% DPPH scavenging or as % scavenging of DPPH at a fixed antioxidant concentration for all the samples.

The aqueous stem bark extract of *Anogeissus leiocarpus* exhibited a significant concentration dependent inhibition of DPPH activity with IC_{50} of 20.88 when compared with ascorbic acid IC_{50} 24.32. The lower the IC_{50} the higher the antioxidant potential of the sample. The extract had shown to be potent as compared with vitamin C with a maximum inhibition percentage of 97.95 % at 500 µg/ml and 94% for vitamin C at 500 µg/ml. The other fractions had higher IC_{50} and DPPH lower percentage inhibitions. This correlates with the research finding of Olutaye et al. where antioxidant activity of five traditionally used medicinal plants were evaluated including *Anogeissus leiocarpus* (Olutayo et al., 2011). Results of this study suggest that the plant extract contain phytochemical constituents that are capable of donating hydrogen to a free radical to scavenge the potential (antioxidant activities), which can counteract the oxidative stress/damage induced by ethanol on the gastric mucosa.

GC-MS profile results showed the presence of 4H-pyran-4-one, 2,3 dihydro-3,5-dihydroxy methyl- in most active fraction A of aqueous stem bark extract of *Anogeissus leiocarpus*, which is similarly present in aqueous extract of unripe fruit of *Carica papaya,* methanolic leaf extract of *Lawsonia inermis* and *C.viscosum* as well as in methanolic dry fruit extract of *Prunus armenicus* (apricot). Evidence revealed that methanolic dry fruit extract of apricot conferred antimicrobial and antioxidant activities (Ezekwe and Chikezie, 2017).

Previous studies showed the presence of n-hexadecanoic acid in methanolic leaf extracts of *Clerodendrum viscosum* and *Justicia adhatoda* (Ghosh *et al.,* 2015; Jayapriya and Shoba, 2015). The n-hexadecanoic acid, like its ester derivative, exhibits antioxidant activity and may serve as anti-cancer, anti-microbial, anti-haemolytic, anti-diabetic agents in addition to causing pesticidal inhibitory action to 5-α reductase activity (Sheeba and Viswanathan, 2016). Aqueous stem bark extract of *Anogeissus leiocarpus* contained relative abundance of n-hexadecanoic acid

The compound phenol 2,4-bis (1,1-dimethylethyl)- is known for its antibacterial and antioxidant activity (Teresa *et al.,* 2014; Manorenjitha *et al.,* 2013). The compound was relatively available in the aqueous stem bark extract of *Anogeissus leiocarpus.*

2-Methoxy-4-vinylphenol was found in GC-MS analysis of *Tinospora cordifolia, Gymnema sylvestre, Pterocarpus marsupium and Acacia Arabica* (Yadav *et al.,* 2015). 2-Methoxy-4-vinylphenol is a natural occurring phenolic compound, exerts potent anti-inflammatory effects by inhibiting, prostaglandins, COX-2 and iNOS in RAW264.7 cells. 2- Methoxy-4-vinylphenol showed a strong anti- inflammatory activity through the elimination of NF-κB and MAPK activation (Jeong *et al.,* 2011). The compound was found in relative abundance in the most active fraction A of aqueous stem bark extract of *Anogeissus leiocarpus.*

Phenolic extracts from the olive leaf extract is a source of potential antioxidant and antimicrobial agents. Research has confirmed that phenolic compounds and polyphenols are secondary plant metabolites, which are considered the best scavengers to prevent the production of free radicals (Altemimi *et al.,* 2017). The aqueous stem bark extract of *Anogeissus leiocarpus* has a considerable abundance of phenols.

For FTIR, the analysis time was less than five minutes and it required a minute quantity of the sample. The spectrum revealed the presence of functional groups such as aromatics, ethers, esters, alcohol, alkanes, ketones, alkenes, alkynes, amines and carboxylic acid. The presence of keto carboxylate elicits antioxidant effect by scavenging hydrogen peroxide; compounds with alcohol groups and aromatic rings as seen in phenols have been known to modulate inflammation at different levels by decreasing the production of reactive oxygen and nitrogen species, limiting

the activity of COX and iNOX, suppressing inflammatory chemokines and cytokines synthesis as well as controlling pathways for NF-κβ signaling (Owolabi *et al.*, 2018).

CHAPTER SIX

6.0 CONCLUSION, RECOMMENDATION AND CONTRIBUTION TO KNOWLEDGE

6.1 CONCLUSION

The results from the study shown that aqueous stem bark extract of *Anogeissus leiocarpus* (100, 200, 400mg/kg), demonstrated a gastroprotective effect against ethanol-induced gastric ulceration in rats. The protection might be due to the antioxidant properties which are evident on their inductive effect on antioxidant enzymes (Superoxide dismutase, catalase and glutathione peroxidase) that make up endogenous scavengers of reactive oxygen species (ROS) evaluated in the study. Diminishing lipid peroxidation and improving defenses against the erosive lesions that characterize the development of gastric ulcer produced by ethanol. The GC-MS study has also revealed diverse phytochemical constituents that are known to be biologically active. This justifies the ethno medicinal use of the plant in the management of gastric ulcer.

6.2 RECOMMENDATION

Further research should be carried out on the anti-secretary activity of the plant. The bioactive components of the plant with anti-ulcer activity need to be isolated and characterized.

6.3 CONTRIBUTION TO KNOWLEDGE

- The use of aqueous stem bark extract of *Anogeissus leiocarpus* in traditional medicine is relatively safe.
- Antioxidant properties of aqueous stem bark extract of *Anogeissus leiocarpus* has been established
- Gastroprotective properties of aqueous stem bark extract of *Anogeissus leiocarpus* has been established.
- Fourteen pytocomponents with known and unknown biological activities have been identified.

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APPENDIX

Appendix 1: Calculation of Percentage yield of the aqueous stem bark extract of *Anogeissus leiocarpus*.

The crude plant powder of 250g was extracted using 2.5 liters of distilled water.

Weight of crude drug that was extracted = 250g

Weight of the crude extract obtained = 80.6g

Percentage yield=$\frac{Weight\ of\ the\ crude\ extract}{Weight\ of\ the\ crude\ plant\ powder} \times 100$

=$\frac{80.6}{250} \times 100 = 32.2\%$

Appendix 2: Lethal dose (LD_{50}) determination of aqueous stem bark extract of *Anogeissus leiocarpus*.

		First phase (n=3)		Second phase (n=1)		LD50 (mg/ml)
Species	Route of Admin.	Doses used (mg/kg)	Quantal mortality	Doses used (mg/kg)	Quantal mortality	
Rats	Oral	10	0/3	1600	0/1	
		100	0/3	2900	0/1	>5000
		1000	0/3	5000	0/1	

$LD_{50}\sqrt{(Highest\ non\ lethal\ dose) \times (lowest\ lethal\ dose)}$

Appendix 3: Mean ulcer score

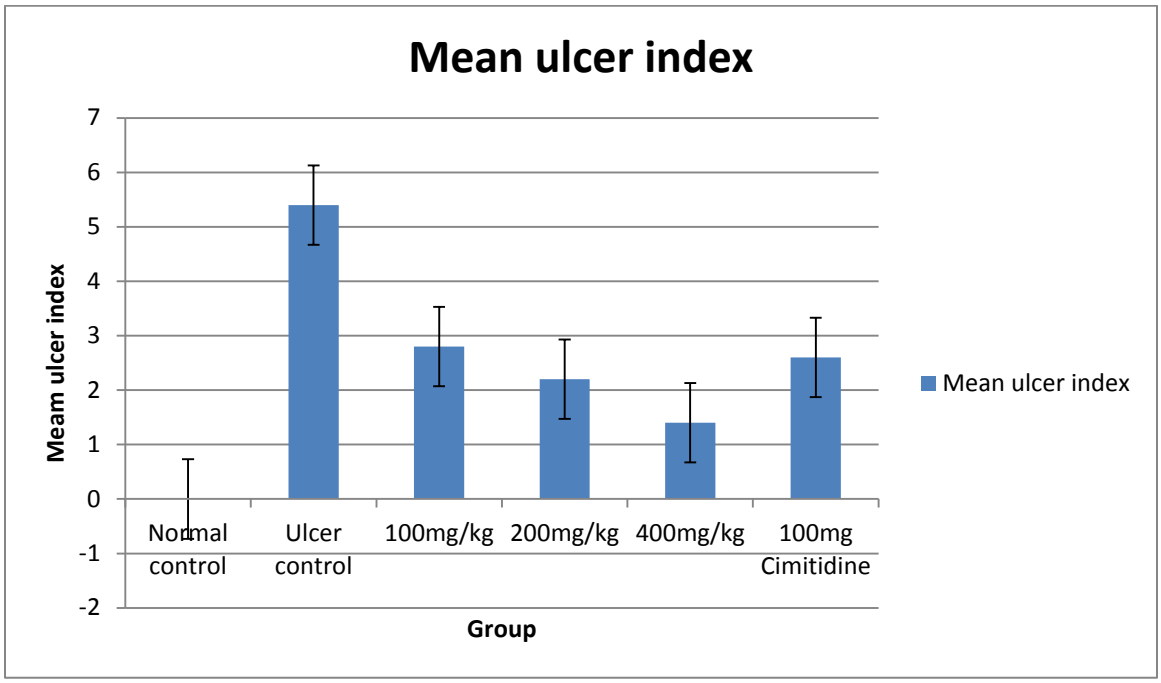


Fig 4.1: Effect of aqueous stem bark extract of *Anogeissus leiocarpus* and cimetidine on mean ulcer score in albino Wister rats.

Appendix 4: Ulceration (%)

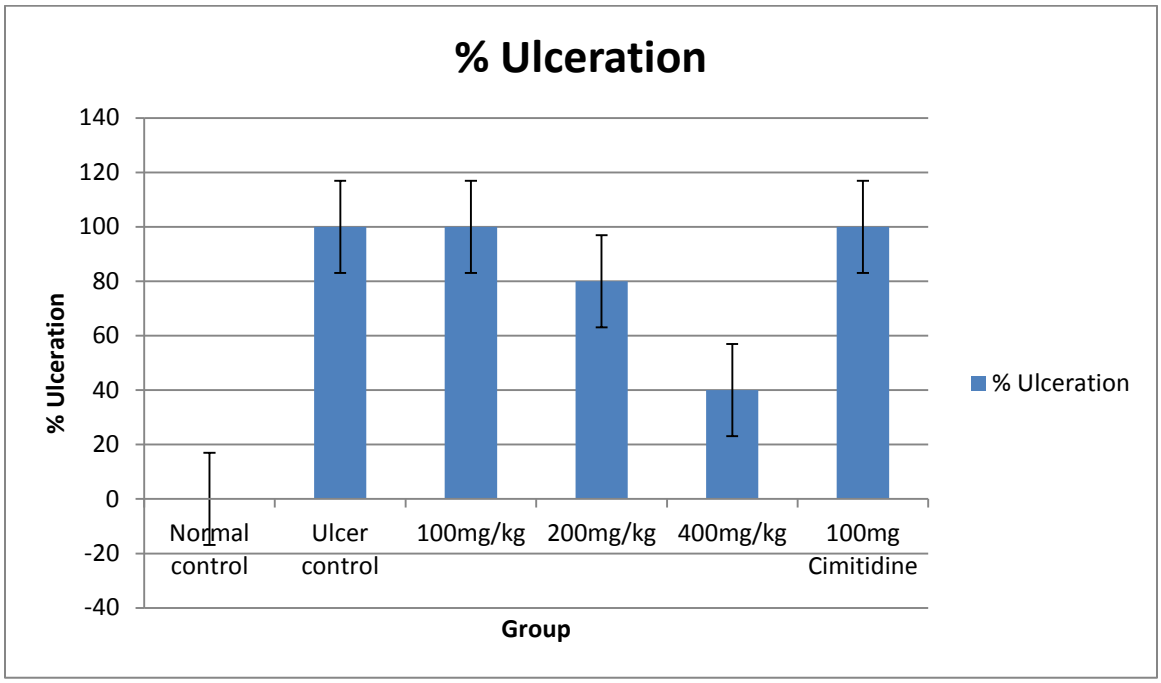


Fig 4.2: Effect aqueous stem bark extract of *Anogeissus leiocarpus* and cimetidine on percentage ulceration in albino Wister rats.

Appendix 5: Preventive index

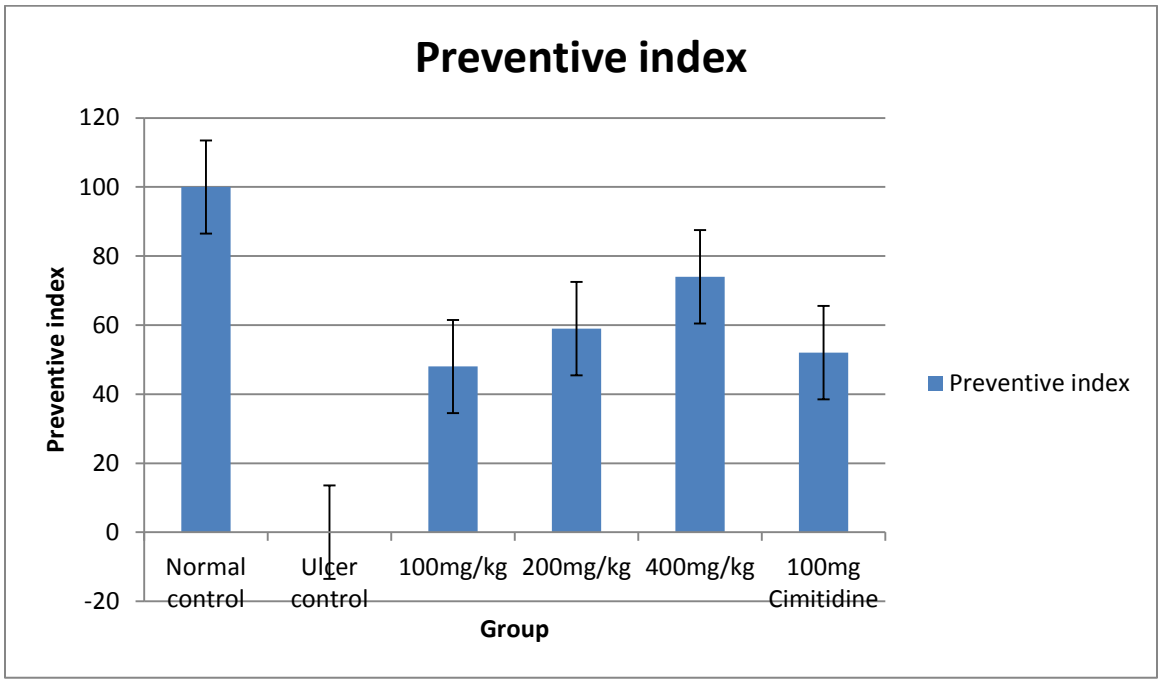


Fig 4.3: Effect aqueous stem bark extract of *Anogeissus leiocarpus* extract and cimetidine on preventive index in albino Wister rats.

Appendix 6: MDA concentration

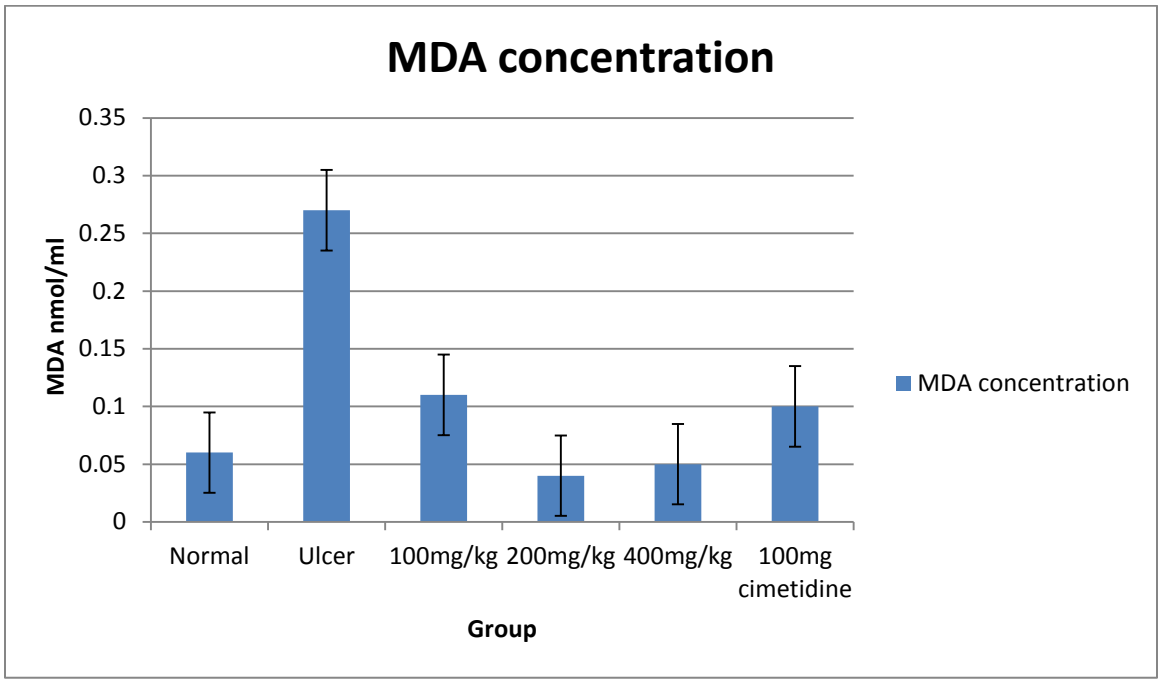


Fig 4.4 Effect of aqueous stem bark extract of *Anogeissus leiocarpus* and cimetidine on MDA in ethanol- induced ulceration in albino rats

Appendix 7: Catalase concentration

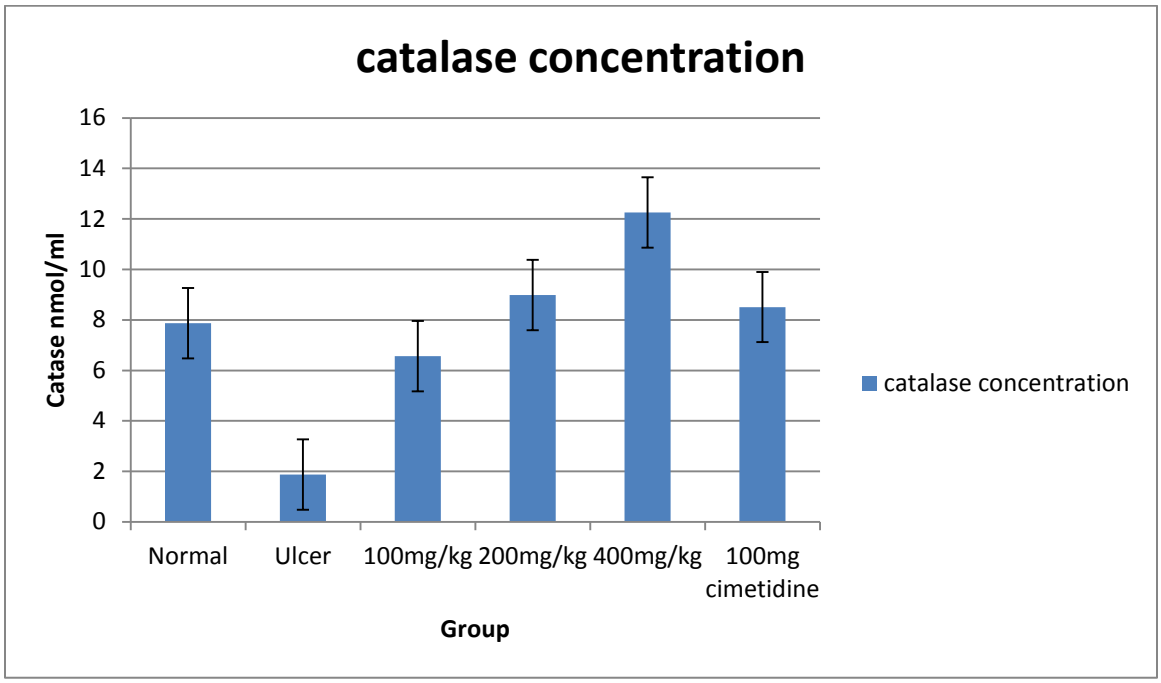


Fig 4.5 Effect of aqueous stem bark extract of *Anogeissus leiocarpus* and cimetidine on catalase in ethanol- induced ulceration in albino rats.

Appendix 8: SOD Concentration

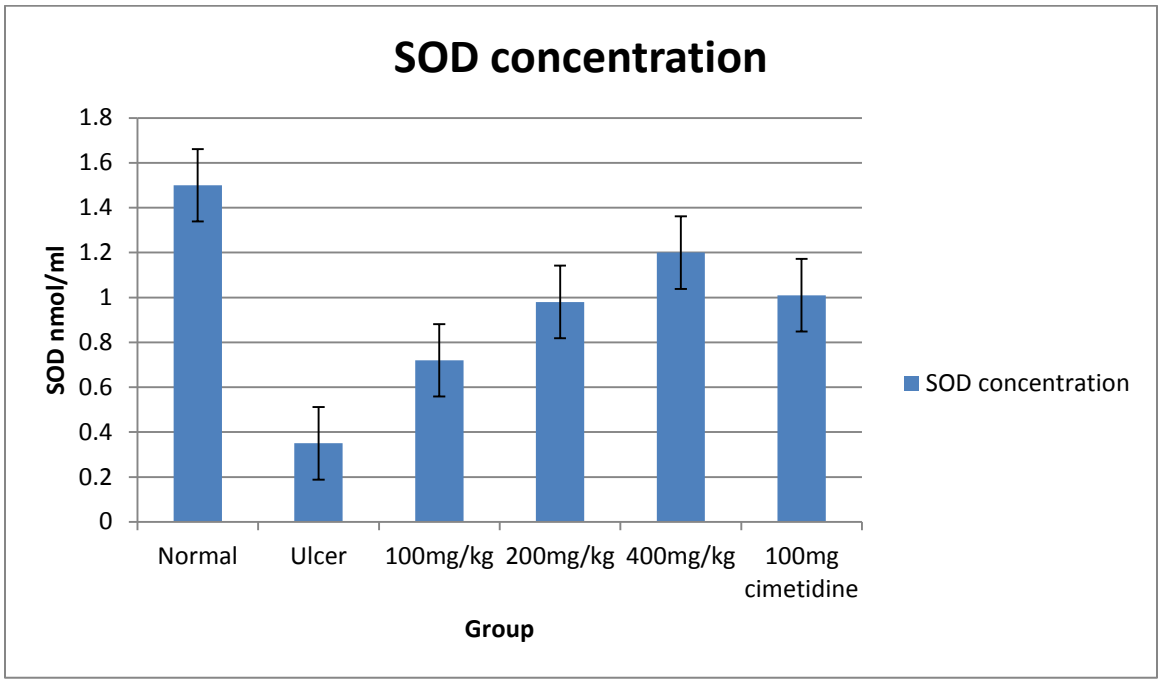


Fig 4.6 Effect of aqueous stem bark extract of *Anogeissus leiocarpus* and cimetidine on catalase in ethanol- induce ulceration in albino rats.

Appendix 9: GPx concentration

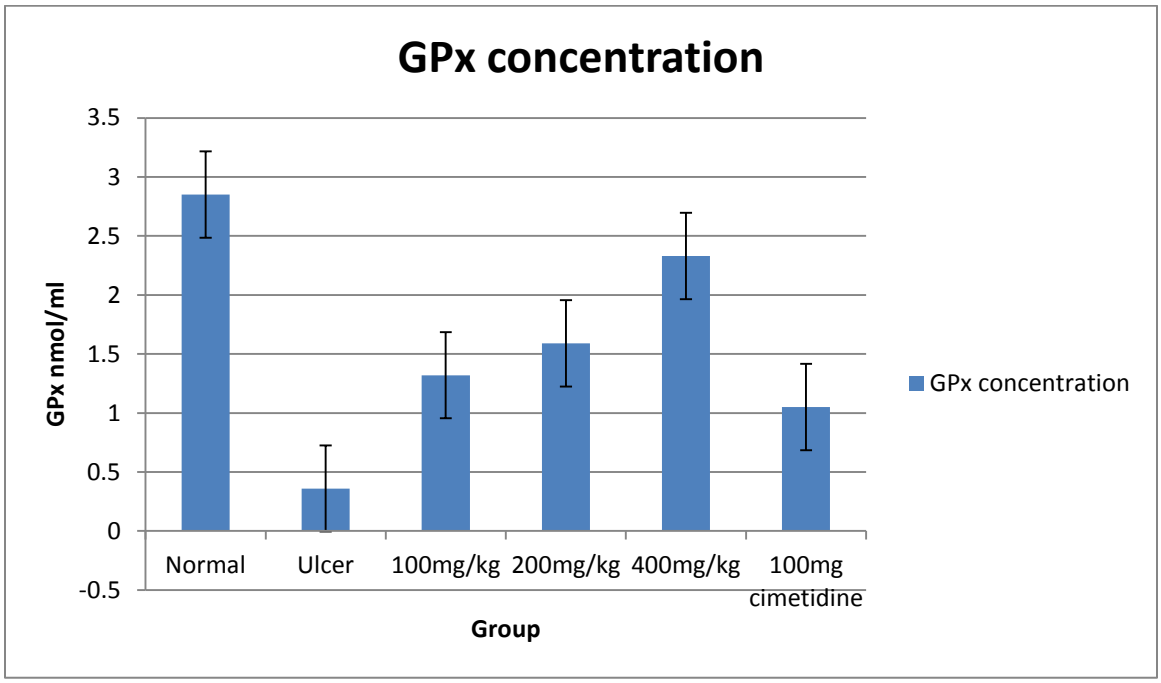
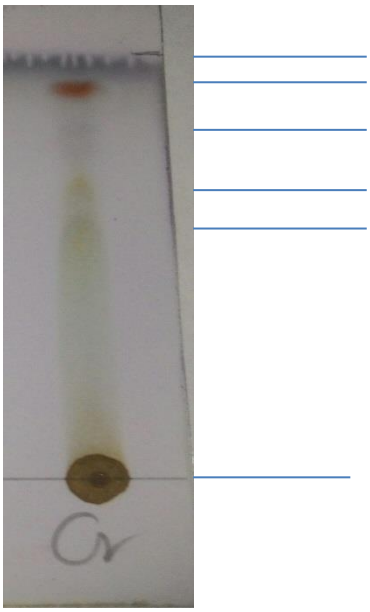


Fig 4.7 Effect of aqueous stem bark extract of *Anogeissus leiocarpus* and cimetidine on Glutathione peroxidase (GPx) in ethanol- induced ulceration in albino rats.

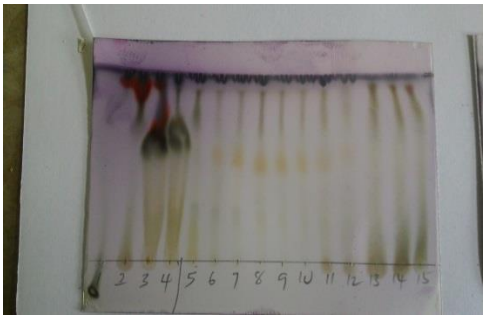
Appendix 10: Column chromatography solvent system.

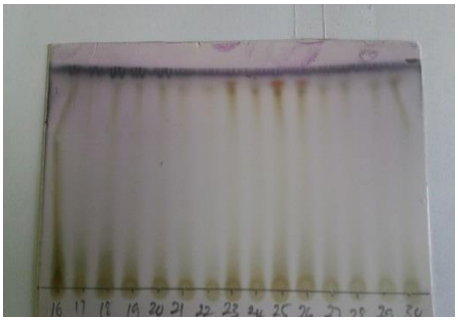
Solvent system	Ratio
Ethyl Acetate	100%
Ethyl Acetate: Methanol	2:1
Ethyl Acetate: Methanol	1:1
Ethyl Acetate: Methanol	1:2
Methanol	100%

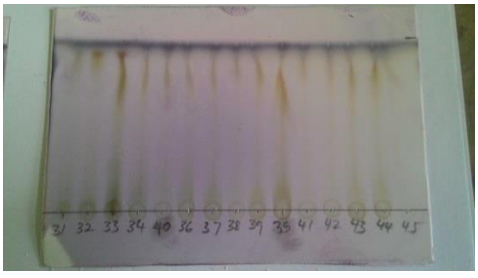
Appendix 11: Chromatographic plates

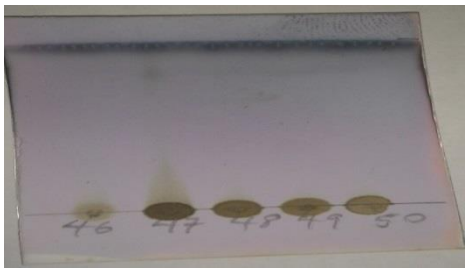


Chromatographic plate of the crude extract (solvent system Ethyl acetate: Methanol 9:1) sprayed with p-anisaldehyde









50 eluents collected in a beaker resolved on a TLC plate using solvent system ethyl acetate: methanol 9:

Appendix 12: DPPH Free radical scavenging activity %