PROTON PUMP (H^+/K^+ - ATPase) INHIBITORY POTENTIAL AND ANTISECRETORY ACTIVITY OF THE LEAVES EXTRACTS OF *Saccharum spontaneum* Linn. IN ASPRIN-INDUCED GASTRIC LESIONS

BY

USMAN BELLO

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DEPARTMENT OF BIOCHEMISTRY

FACULTY OF LIFE SCIENCES

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USMAN BELLO, B.Sc. (ABU) 2011

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ZARIA, NIGERIA

JANUARY, 2018

DECLARATION

I hereby declare that the work in this dissertation entitled "Proton pump (H^+/K^+ - ATPase) inhibitory potential and antisecretory activity of the leaves extracts of *Saccharum spontaneum* linn. in asprin-induced gastric lesions" is an authentic record of my own work carried out in the Department of Biochemistry under the supervision of Prof. Sani Ibrahim and Dr. I. A Aimola Idowu. The information derived from literature has been quoted and duly acknowledged in the text and a list of references provided. No part of this dissertation was previously presented for another degree or diploma at this or any other institution.

USMAN BELLO

Name of Student	Signature	Date

CERTIFICATION

This dissertation titled "PROTON PUMP (H^+/K^+ - ATPASE) INHIBITORY POTENTIAL AND ANTISECRETORY ACTIVITY OF THE LEAVES EXTRACTS OF *SACCHARUM SPONTANEUM* LINN. IN ASPRIN-INDUCED GASTRIC LESIONS" by USMAN BELLO meets the regulations governing the award of a master degree in Biochemistry of Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

Prof. S. Ibrahim (Signature) _____

Chairman, Supervisory Committee

Date _____

Dr. I. A Aimola (Signature) _____

Member, Supervisory Committee Date _____

(Signature) _____

Member, Supervisory Committee Date _____

Prof. M.N Shuaibu (Signature) _____

Head of Department

Date _____

Prof. A. Z Abubakar (Signature) _____

Dean, School of Postgraduate Studies Date _____

DEDICATION

This research work is dedicated to my uncle Alhaji Abubakar Umar, who by all means stood by me vividly to the completion of this work and to the entire family of Late Alhaji Usman Laushi.

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ABSTRACT

Gastric acid has been a major pathogenic factor in peptic ulcer diseases thus, the suppression of acid secretions is a key therapeutic target for gastric ulcers, which includes the use of proton pump inhibitors, antacids, targeting gastrin receptors and histamine receptors antagonists. In this study, the acute toxicity, phytochemistry, antisecretory activity, histopathology and proton pump (H^+/K^+-ATPase) inhibitory potential of the aqueous, methanol and hexane leaves extracts of *Saccharum spontaneum Linn.* were evaluated on the stomach of Wister albino rats. Aspirin induced gastric ulcer generated as lesions, from pin-point craters to larger eruptions and the dose-dependent effectiveness of the extracts were assessed and methanol extract was found to be significantly most effective ($P<0.05$). Acute oral toxicity for the aqueous, methanol and hexane extracts of the leaves of *S. spontaneum* showed that the extracts were relatively safe with an LD_{50} value greater than 5000 mg/kg body weight with no lethal effect in rats. Preliminary Phytochemical studies of the extracts revealed the presence of alkaloids, triterpenes, glycosides, saponins, steroids, flavonoids, tannins and phenols. All the extracts (200 mg/kg and 400 mg/kg) exhibited a high antisecretory effect as it significantly ($P<0.05$) lowered the lesion index induced by aspirin, reduced the volume of gastric juice, free and total acidity with a consequent rise in gastric pH as compared to the ulcer group. The methanol extract showed the highest antiulcer activity with a percentage inhibition of 65.22% when compared to the standard control (73.91%). Histopathological examination of rats gastric mucosa showed mild abrasion on the lining epithelium with no mucosal necrosis and hemorrhage, with the methanol extract exhibiting a better cytoprotection against gastric mucosal damage. The methanol extract also inhibited the H^+/K^+- ATPase activity with IC_{50} value of 116.41 ± 41.98 μg/ml compared with omeprazole with

IC_{50} value of 37.79 ± 0.10 μg/ml. These results suggest that *S. spontaneum* has antiulcer effect which is exerted by suppression of H^+/K^+-ATPase activity in gastric parietal cells.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF ABBREVIATIONS

NSAIDs	Non-steroidal Anti-inflammatory Drugs
PPIs	Proton Pump Inhibitors
AESS	Aqueous Extract of *Saccharum spontaneum*
MESS	Methanol Extract of *Saccharum spontaneum*
HESS	Hexane Extract of S*accharum spontaneum*
LD_{50}	Median Lethal Dose
MUC	Mucin Glycoproteins
TFF	Trefoil Factor Family
ITF	Intestinal Trefoil Factor
EGF	Epidermal Growth Factor
EGF-R	Epidermal Growth Factor Receptor
TGF- α	Transforming Growth Factor-α
IGF-1	Insulin-like Growth Factor-1
TNF- α	Tumor Necrosis Factor- α
CGRP	Calcitonin Gene-related Peptide
PGE	Prostaglandin
bFGF	Basic Fibroblast Growth Factor
PDGF	Platelet Derived Growth Factor
VEGF	Vascular Endothelial Growth Factor
TGF- β	Transforming Growth Factor- B

HGF	Hepatocyte Growth Factor
HGF-R	Hepatocyte Growth Factor Rececptor
Tris	Hydroxymethyl aminomethane
HEPES	2-(4-hydroxyethyl)-1-piperazinylethanesulfonic Acid
EDTA	Ethylenediaminetetraacetic Acid
OECD	Organization for Economic Development
p.o	Per os
COX	Cycloxygenase
COX-1	Cycloxygenase-1
COX-2	Cycloxygenase-2
MEq/L	Milliequivalent per litre
ROS	Reactive Oxygen Specie
DMRT	Duncan Multiple Range Test
SPSS	Statistical Package for Social Sciences
TCA	Trichloroacetic Acid
DMSO	Dimethyl Solfoxide
IC_{50}	Inhibitory Concentration

CHAPTER ONE

1.0 INTRODUCTION

Acid-related disorders are highly prevalent in both developed and developing world, which have a significant impact on patient quality of life and cause heavy burden on health care systems (Cylwik *et al*., 2005). Gastric acid has been demonstrated to be a major pathogenic factor in peptic ulcer diseases such as gastric and duodenal ulcer as well as gastroesophageal reflux disease (Shin *et al*., 2008). Peptic ulcer embracing both gastric and duodenal ulcers has been a major threat to the world's population over the past two centuries, with a high morbidity and substantial mortality (Pahwa *et al*., 2010). An estimated 15,000 deaths occur each year as a consequence of peptic ulcer disease (Valle, 2005). Peptic ulcer is a gastro-intestinal disorder due to an imbalance between the aggressive factors like acids, pepsin, non-steroidal anti-inflamatoery drugs (NSAIDs), *Helicobacter pylori* infection and defensive factors such as bicarbonate secretions, prostaglandins, gastric mucus, and innate resistance of the mucosal cell factors (Dashputre and Naikwade, 20011). Factors related with lifestyle such as smoking, alcohol, spicy foods and stress are also associated with peptic ulcer formation. Peptic ulcer usually develops when aggressive factors overcome the defensive factors (Izzo and Borelli, 2000). Ulceration of the gastrointestinal mucosa is caused by disruption of normal balance of the corrosive effect of gastric juice and the protective effect of mucus on gastric epithelial cells (Waugh *et al.*, 2006). The gastric proton potassium (H^+/K^+)-ATPase is the proton pump responsible for the final step of acid secretion in the stomach, which locates in gastric membrane vesicles and catalyzes the electro-neutral exchange of intracellular H^+ and extracellular K^+ coupled with the hydrolysis of cytoplasmic ATP (Shin *et al*., 2009; Weidemüller and Hauser, 2009). Drugs for treatment of

acid-related diseases are ultimately implemented through inhibiting H^+/K^+-ATPase activity (Shin *et al*., 2008; Suzuki *et al*., 2008; Palmer *et al*., 2010). A variety of heterocyclic structures has been described as the gastric proton pump inhibitors (PPIs), for example omeprazole, esomeprazole, lansoprazole and pantoprazole, bind to the H^+/K^+-ATPase and have been available as therapeutic agents for a long time (Vagin, 2002; Bamford 2009). Thus, the suppression of acid secretions is a key therapeutic target for gastric ulcers, which includes the use of antacids, specific muscarinic receptor antagonists, targeting gastrin receptors and histamine receptors antagonists, and the use of proton pump inhibitors (Jain *et al*., 2007). However, these drugs are not completely effective and confer simpler to severe side effects like arrhythmia, gynecomastia, cell hyperplasia and hematopoietic changes (Akhtar *et al*., 1992). Hence, the adverse effect of long-term use of these drugs caused strong desire for investigators to develop new alternative medicine (Li, 2009).

Natural products have been used for combating human diseases since they possess a wide range of compounds with significant biological activities that can be exploited for medical applications and other therapeutic purposes (Gupta *et al.*, 2012). Plants are recognized as important sources of natural compounds that protect from diseases and infections (Babincova *et al*., 2008). In developing countries, herbal preparations have played an important role side by side with modern medicine especially in the primary health care system of the poor and rural areas, and are increasingly utilized to treat a wide variety of clinical diseases including ulcer (Ezike *et al*., 2009). Many plants have been shown to possess anti-ulcerogenic property attributed to their flavonoids, tannins, alkaloids and phenolic contents (Alanko *et al*., 1999). They also prevent gastric lesions and protect the mucosa against necrotic agents (Alanko *et al*., 1999). In the process of wound healing, burns and inflammation, tannins stimulate gastric mucosa formation,

diminish acid secretion, inhibit production of pepsinogen and decrease ulcerogenic lesions (La Casa *et al*., 2000).

Saccharum spontaneum Linn is a perennial plant that belongs to the family Poaceae. It is commonly called wild sugar cane in English and *Kibiya* in Hausa. It is also widely distributed in Africa, Malaysia and Polynesia. It is use traditionally in the treatment of several disorders including mental disturbances, dyspnoea, anaemia, diuretic, obesity, lithotriptic, purgative, tonic, aphrodisiac, dyspepsia, (Chopra *et al*., 1992; Yoganarashimhan, 2002), gynecological troubles respiratory troubles (Muhammad and Hefazat. 2011). The whole plant including the stems, leaves and roots are used medicinally in Nigeria. The bark and leaves have been used to treat ulcer (Aiyeloja and Bello, 2006; Celestina *et al*., 2012). Although many benefits were claimed, very few scientific report were available on the phytochemistry and pharmacology of *Saccharum spontaneum.* Thus, this study is aimed at investigating the effects of *Saccharum spontaneum* leaves extracts on gastric ulcer in order to provide a scientific support or otherwise for use of the plant.

1.1 Statement of Research Problem

Peptic ulcer have become one of the major human and animal illness throughout the world, affecting over 10% of the global population (Baron and Calam, 2001), and of these number, 5% suffer from gastric ulcers (Bandyopadyay *et al*., 2001). The incidence of gastric ulcer has increased during the last 30 years. Approximately 11-14% of men and 8-11% of women will develop peptic ulcer disease in their life time. The mortality due to gastric ulcer is approximately one death per 10,000 cases. In Nigeria, its prevalence is estimated to cause death of 5090 people in a year (WHO, 2009).

Although, proton pump inhibitors have improved ulcer therapy, the problems associated with their use (side actions), tolerance and possible incidence of relapse are still a great concern. There has been continuing interest in the development of reversible gastric H^+/K^+-ATPase inhibitors as anti-ulcer agents to overcome the potential side effects of irreversible inhibitors (Pope and Parsons, 1993). Thus, the adverse effect of long-term use of these drugs caused strong desire for researchers to develop new alternative medicine (Li, 2009).

1.2 Justification

Medicinal plants are of great importance to the health of individuals and communities as they have equally contributed tremendously in the management of diseases and maintenance of health. WHO estimates that 60% of the people in sub-Saharan Africa use traditional medicine to alleviate their spiritual, psychosocial and physical problems (WHO, 2001). This could be as a result of their: availability, effectiveness, higher safety margin as well as cheaper cost. In Nigeria, many indigenous plants are used in herbal medicine to cure diseases and heal injuries. *Saccharum spontaneum Linn* is used in treatment of peptic ulcers in the northern part of Nigeria (Celestina *et al*., 2012). To the best of my knowledge, the anti-ulcer activity of this plant has not been subjected to scientific evaluation. Investigation on the phytotherapy of medicinal plants that are highly valued and widely used in the traditional systems of medicine might provide efficient formulation for better management. Thus, development of a drug with antiulcerogenic property from plant sources without compromising the efficacy and safety would be expected to benefit millions of suffering humanity.

1.3 Aim and Objectives

1.3.1 Aim

The aim of this research work was to investigate the proton pump (H^+/K^+ - ATPase) inhibitory potential and antisecretory activity of the leaf extracts of *Saccharum spontaneum* linn in asprin induced gastric lesions in rats.

1.3.2 Specific Objectives

This research has the following specific objectives;

I. To evaluate the median lethal dose (LD_{50}) of the aqueous, methanol and hexane leaf extract of *S. spontaneum.*

II. To evaluate *in vivo* antisecretory effect of the aqueous, methanol and hexane leaf extracts of *S. spontaneum* in Aspirin- induced ulcer rats.

III. To investigate the effects of the aqueous, methanol and hexane leaf extracts of *S. spontaneum* on the histology of the gastric mucosa of Wister rats.

IV. To determine the *in vitro* proton potassium (H^+/K^+) ATPase inhibitory activity of the most potent extract of *S. spontaneum*

1.4 Null hypothesis

The extracts of the leaves of *Saccharum spontaneum* do not have proton pump (H^+/K^+ - ATPase) inhibitory potential and antisecretory activity in asprin induced gastric lesions in rats.

CHAPTER TWO

2.0 LITERATURE REVIEW

Gastric ulcer is a benign lesion that occurs at the stomach where mucosal epithelium is exposed to acid and pepsin (John, 2006). It is one of the common disorders of human gastrointestinal system, affecting 10% of the world population and characterized by gastrointestinal bleeding, perforation and erosion of the mucosal wall due to excess acid secretion (Schubert, 2008).

The most common sign and symptoms of gastric ulcer includes abdominal and epigastric pain, nausea, bloating, fullness, vomiting, anemia, gassiness, loss of appetite and loss of weight. A number of factors are responsible for this gastric ulcer disease of which 70% to 80% are due to infection of *Helicobactor pylori*, a spiral shaped, gram negative bacteria (Chatterjee *et al*., 2012). The use of NSAIDs accounts for approximately 25% of gastric ulcer cases with an upward trend (Tarnawski and Jones, 2003). The NSAIDs are one of the most widely prescribed drugs in the world and are extensively used to alleviate clinical cases especially for pain and inflammation (Brooks *et al*., 1999). However, these drugs are well-known to induce stomach ulceration and delay ulcer healing (Lanas *et al*., 2005). Despite recent advances, an adequate remedy for the NSAID- induced gastropathy remains elusive. The NSAIDs have an effect on the gastroduodenal barrier at the epithelial level, intraepithelial level, and sub epithelial level. At the epithelial level, it reduces the synthesis and the secretion of mucus and makes the proteolytic action of pepsins, which changes the viscosity and electric capacity of the mucus, favoring the back diffusion of ions. At the intraepithelial level, it causes epithelial denudation due to direct cellular damage, since ionized NSAIDs stay trapped inside the epithelium. At the sub epithelial level, it leads to thrombosis in the microcirculation and vasoconstriction of the arterioles of the sub mucosa (Ananya and Sandip, 2014).

Today, there are two main approaches for treating peptic ulcer. The first deals with reducing the production of gastric acid and second with reinforcing gastric mucosal protection (Hoogerwerf and Pasricha, 2001; Valle, 2005).

2.1 Pathogenesis of gastric ulcer

The stomach plays a pivotal role in the digestion of foods eaten. With the exception of rare cases, this organ can resist to a large variety of noxious factors, including hydrochloric acid, refluxed bile salts and alcohol, with a wide range of temperatures and osmolality. This high resistance to injuries depends on a number of physiological responses elicited by the mucosal lining against potentially harmful luminal agents, as well as to the ability of rapidly repairing the mucosal damage when it does occur (Laine *et al*., 2008). Thus, the integrity of the upper gastrointestinal tract is dependent upon the balance between "hostile" factors such as gastric acid, H. pylori, NSAIDs and pepsin, and "protective" factors such as prostaglandins, mucus, bicarbonate, and blood flow to mucosa affecting gastrointestinal mucosa (Figure 2.1).

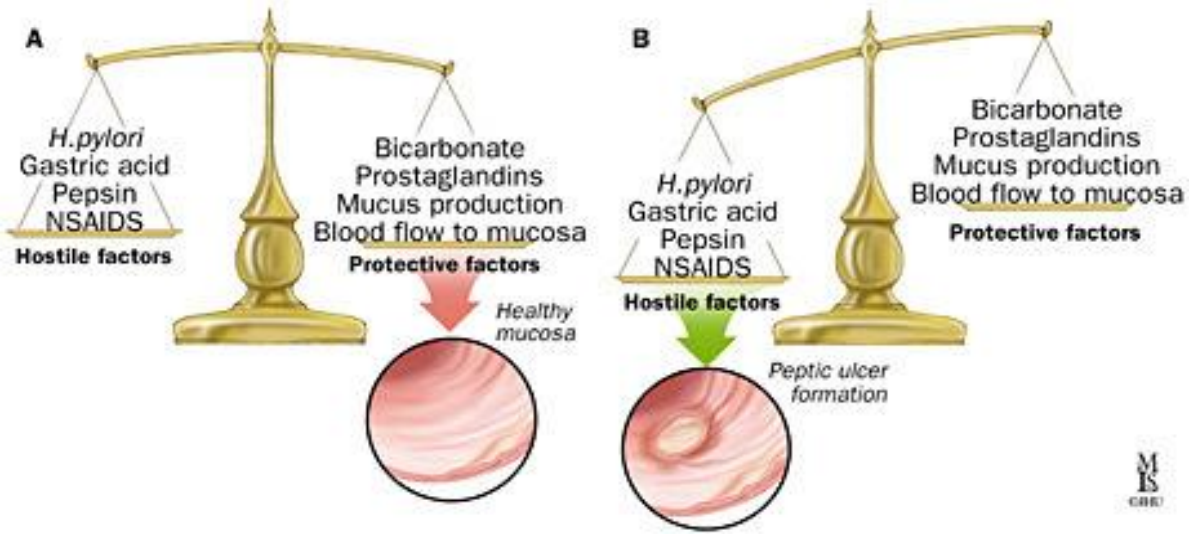


Figure 2.1. A. Protective factors; B. hostile factors (**www.halstedsurgery.org. 2017**).

Injury to gastric and duodenal mucosa develops when deleterious effects of gastric acid overwhelm the defensive properties of the mucosa. Inhibition of endogenous prostaglandin synthesis leads to a decrease in epithelial mucus, bicarbonate secretion, mucosal blood flow, epithelial proliferation, and mucosal resistance to injury. Lower mucosal resistance increases the incidence of injury by endogenous factors such as acid, pepsin, and bile salts as well as exogenous factors such as NSAIDs, ethanol and other noxious agents (Figure 2.2).

Since the discovery that prostaglandin biosynthesis could be inhibited by NSAIDs through the blockade of cyclooxygenase enzymes, there has been a great interest in the contribution of prostaglandins to the mechanisms of gastric mucosal defense. Thus, it has been appreciated that these lipidic mediators are able to modulate virtually every factor involved in mucosal protection, and the importance of this contribution is made evident by the increased susceptibility of the stomach to injury following the intake of NSAIDs. Indeed, chronic treatments with these drugs can be associated with the development of ulcers in the stomach, and research over the past two decades has helped to identify some of the key events, triggered by cyclooxygenase blockade, which are involve in ulcer formation and/or impairment of ulcer healing. Since many years, it has been recognized that NSAIDs can interfere with gastric mucosal physiology also through injuring mechanisms unrelated to the inhibition of prostaglandin biosynthesis, such as oxidative stress and changes in epithelial cell proliferation/apoptosis balance (Fornai *et al.*, 2011).

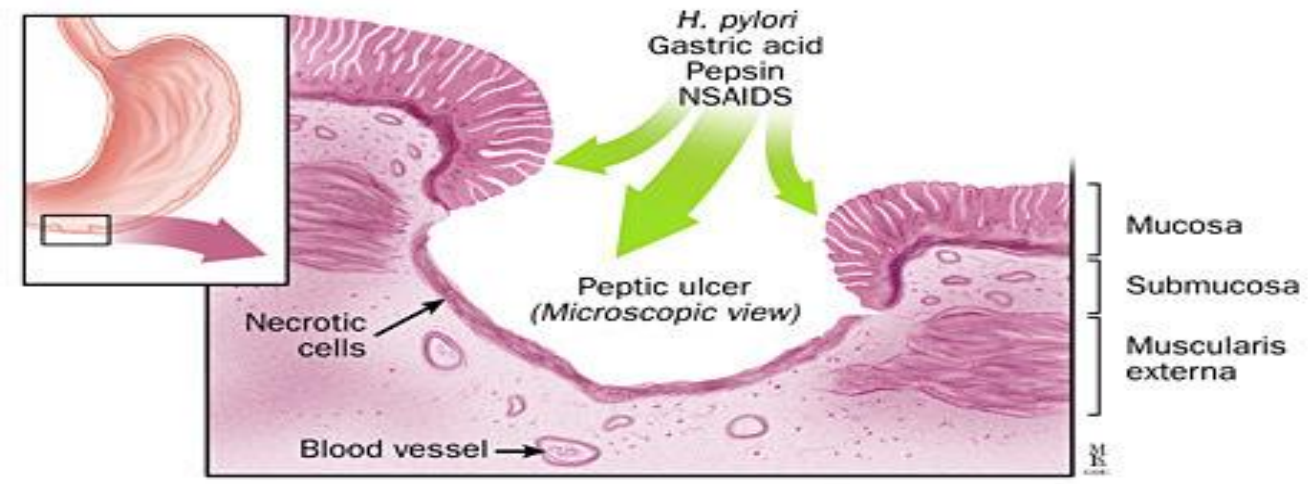


Figure 2.2. Pathogenesis of Peptic ulcer disease (**www.halstedsurgery.org. 2007**).

2.1.1 Classification of Peptic ulcer

2.1.1.1 Region/Location

- Duodenum (called duodenal ulcer)
- Esophagus (called esophageal ulcer)
- Stomach (called gastric ulcer)
- Meckel's diverticulum (called Meckel's diverticulum ulcer; is very tender with palpation)

2.1.1.2 Modified Johnson Classification of Peptic Ulcers:

- Type I: Ulcer along the body of the stomach, most often along the lesser curve at incisuraangularis along the locus minorisresistantiae.
- Type II: Ulcer in the body in combination with duodenal ulcers. Associated with acid over secretion.
- Type III: In the pyloric channel within 3 cm of pylorus. Associated with acid over secretion.
- Type IV: Proximal gastroesophageal ulcer
- Type V: Can occur throughout the stomach. Associated with chronic NSAID use (such as aspirin) (Venkateswararao, 2013).

2.1.2 Causes of Peptic Ulcer

A major causative factor (60% of gastric and up to 90% of duodenal ulcers) is chronic inflammation due to *Helicobacter pylori* that colonizes the antral mucosa (SciLife Pharma, 2010). The immune system is unable to clear the infection, despite the appearance of antibodies. Thus, the bacterium can cause a chronic active gastritis (type B gastritis), resulting in a defect in the regulation of gastrin production by that part of the stomach, and gastrin secretion can either be increased, or as in most cases, decreased, resulting in hypo- or achlorhydria. Gastrin stimulates the production of gastric acid by parietal cells. In *H. pylori* colonization responses to

increased gastrin, the increase in acid can contribute to the erosion of the mucosa and therefore ulcer formation (SciLife Pharma, 2010).

Another major cause is the use of NSAIDs. The gastric mucosa protects itself from gastric acid with a layer of mucus, the secretion of which is stimulated by certain prostaglandins. NSAIDs block the function of cyclooxygenase 1 (COX-1), which is essential for the production of these prostaglandins. COX-2 selective antiinflammatories (such as celecoxib or the since withdrawn rofecoxib) preferentially inhibit Cox-2, which is less essential in the gastric mucosa, and roughly halve the risk of NSAID-related gastric ulceration. The incidence of duodenal ulcers has dropped significantly during the last 30 years, while the incidence of gastric ulcers has shown a small increase, mainly caused by the widespread use of NSAIDs (SciLife Pharma, 2010).

Studies in the varying occurrence of ulcers in third world countries despite high *H. pylori* colonization rates suggest dietary factors such as spice consumption, caffeine and coffee play a minor important role in the development of peptic ulcers (Kurata *et al*., 1997). Although some studies have found correlations between smoking and ulcer formation, others have been more specific in exploring the risks involved and have found that smoking by itself may not be much of a risk factor unless associated with *H. pylori* infection (Martin *et al.*, 2008). Similarly, alcohol consumption cause ulcers when coupled with *H. pylori* infection with a modest increase in comparison to the primary risk factor.

2.1.3 Mechanisms of Gastric Mucosal Defense

The molecular mechanisms underlying the pathophysiology of gastric mucosal defense includes several local and neurohormonal protective factors that allow the mucosa to resist against frequent exposures to damaging factors (Laine *et al*., 2008). These mechanisms can be described as follows:

2.1.3.1 Local Mechanisms of Gastric Mucosal Defense:

2.1.3.2 Mucus-Bicarbonate-Phospholipid Barrier

The first line of gastric mucosal defense is represented by the mucus-bicarbonatephospholipid barrier (Lichtenberger, 1999). The surface of gastric mucosa is covered by a layer formed by mucus gel, bicarbonate anions and surfactant phospholipids. This unstirred layer is capable of retaining the bicarbonate ions secreted by surface epithelial cells and maintaining a microenvironment with a pH near to 7 at the mucus-mucosa interface (Fornai *et al.,* 2011). The mucus layer is also capable of preventing the penetration of pepsin, thus avoiding the proteolytic digestion of epithelium (Allen and Flemstrom, 2005). In addition, the luminal surface of mucus gel is covered by a film of surfactant phospholipids which confers hydrophobic properties to the mucus layer (Lichtenberger, 2007).

The mucus gel is secreted by surface epithelial cells and is formed by a large amount of water (about 95%) and various kinds of mucin glycoproteins (i.e., MUC2, MUC5AC, MUC5B and MUC6), the production of which may vary in different regions of the gastric mucosa (Ho *et al*., 2004; Allen and Flemstrom, 2005). Gel-forming mucin units polymerize into large mucin multimers, which are essential for gel formation. The mucus gel is secreted along with low-molecular weight trefoil factor family (TFF) peptides, which play a relevant role in the formation of the mucus layer (Newton *et al*., 2000). For example, TFF2 is known to increase the viscosity of gastric mucin and stabilize the gel network (Thim *et al*., 2002). The secretion of gastric mucus is regulated also by various gastrointestinal hormones, including gastrin and secretin, as well as prostaglandins and acetylcholine (Allen and Flemstrom, 2005).

The secretion of bicarbonate into the mucus gel layer is essential to maintain a pH gradient at the epithelial surface, which represents a first line of defense against gastric acid (Allen and Flemstrom, 2005).

Bicarbonate secretion from the apical membrane of surface epithelial cells is mediated by a Cl-/HCO3-anion exchanger, and it is stimulated by various factors, including prostaglandins (via EP1receptors), luminal acid, corticotrophin-releasing factor, melatonin, uroguanylin and orexin A (Allen and Flemstrom, 2005; Montrose *et al*., 2006).

The mucus-bicarbonate barrier is the only system which segregates the epithelium from the gastric lumen. Therefore, when this protective barrier breaks down during pathological events or upon detrimental actions by injuring agents, a second line of protective mechanisms comes into play. These include intracellular acid neutralization, rapid epithelial repair, and maintenance of mucosal blood flow (Fornai *et al.*, 2011).

2.1.3.3 Epithelial Cells

The continuous layer of surface epithelial cells represents the next line of mucosal defense.

This epithelial tissue is responsible for the production of mucus, bicarbonate and other components of the gastric mucosal barrier. These cells are hydrophobic in nature, being able to repel acid- and water-soluble injuring agents, owing to the presence of phospholipids on their surface (Lichtenberger, 1999). Surface epithelial cells are also closely interconnected by tight junctions, forming a continuous barrier, which prevents back diffusion of acid and pepsin (Allen and Flemstrom, 2005). Another relevant protective factor, available in the epithelial cells, is represented by heat shock proteins, which are activated in response to stress, including temperature increments, oxidative stress and cytotoxic agents (Tanaka *et al*., 2007). These proteins can prevent protein denaturation and protect cells against injury.

Cathelicidin and beta-defensin are cationic peptides which play an important role in the innate defensive system at the mucosal surface, preventing bacterial colonization (Yang *et al*., 2006). In addition, TFFs secreted by epithelial cells regulate the re-epithelization process and exert mucosal protective actions (Taupin and Podolsky, 2003).

2.1.3.4 Mucosal Cell Renewal

The integrity of gastric epithelium is maintained by a continuous process of cell renewal ensured by mucosal progenitor cells. These cells are subjected to a continuous, well-coordinated and controlled proliferation, which ensures the replacement of damaged or aged cells on the epithelial surface. The process of complete epithelial renewal takes about 3-7 days, while the overall glandular cell replacement requires months. However, the restitution of surface epithelium after damage occurs very quickly (i.e., few minutes) and results by migration of preserved cells located in the neck area of gastric glands (Laine *et al*., 2008). The process of cell turnover is regulated by growth factors. In particular, a marked expression of epidermal growth factor receptor (EGF-R) has been detected in gastric progenitor cells. Such a receptor can be activated by mitogenic growth factors, such as transforming growth factor-α (TGF- α) and insulin-like growth factor-1 (IGF-1) (Nguyen *et al*., 2007). In addition, PGE2 and gastrin are able to transactivate the EGF-R and promote the activation of mitogen-activated protein kinase (MAPK) pathway, with consequent stimulation of cell proliferation (Pai *et al*., 2002). Notably, the presence of EGF has not been detected in the normal mucosa, although it is contained in the gastric juice, as a product of salivary and esophageal glands, and can stimulate mucosal cell proliferation in case of injury (Milani and Calabrò, 2001). In addition, mucosal progenitor cells do express survivin, an antiapoptotic factor, which inhibits apoptotic cell death (Chiou *et al*., 2005).

2.1.3.5 Mucosal Blood Flow

Mucosal blood flow is essential to deliver oxygen and nutrients and to remove toxic metabolites from gastric mucosa. When the gastric mucosa is exposed to irritants or acid back-diffusion, a massive and rapid increase in mucosal blood flow occurs. This process allows removal and dilution of back diffusing acid or noxious agents. The increase in blood flow is regarded as a pivotal mechanism for preventing gastric mucosal cell injury, and its decrease results in the development of tissue necrosis. The increase in mucosal blood flow is mediated by NO release, and there is experimental evidence demonstrating that nitric oxide (NO) protects the gastric mucosa against injury induced by ethanol or endothelin 1, while the inhibition of NO synthase enhances mucosal injury (Holzer, 2006). It has been also observed that another endogenous compound, H_2S, can exert protective actions against gastric mucosal injury. In particular, this compound has been shown to reduce the expression of tumor necrosis factor- α (TNF- α), to decrease leukocyte adhesion to vascular endothelium, and to prevent NSAID induced gastric mucosal damage (Fiorucci *et al*., 2006).

2.1.3.6 Sensory Innervation

Sensory innervation plays a prominent role in the protection of gastric mucosa from injury, as demonstrated by studies where the ablation of sensory transmission (i.e., with capsaicin) impaired the vasodilatatory response and increased the sensitivity of gastric mucosa to injuring agents (Holzer, 2007).The vasculature of gastric mucosa and submucosa is innervated by extrinsic primary afferent sensory neurons, which are arranged in a plexus at the base of the mucosal layer (Holzer, 2007). These nerves can detect luminal acidity or back-diffusing of acid through acid-sensing channels, as such their activation modulates the contractile tone of submucosal arterioles, thereby regulating the mucosal blood flow. In particular, the stimulation

of sensory nerves leads to the release of calcitonin gene-related peptide (CGRP) and substance P from nerve terminals surrounding large submucosal vessels (Holzer, 2007). CGRP then contributes to the maintenance of mucosal integrity through the vasodilation of submucosal vessels mediated by NO release.

2.1.3.7 Prostaglandins

The gastric mucosa represents a source of continuous prostaglandin production, such as PGE2 and PGI2, which are regarded as crucial factors for the maintenance of mucosal integrity and protection against injuring factors (Halter *et al*. 2001; Brzozowski *et al*., 2005). It has been demonstrated that prostaglandins have the potential to stimulate almost all the mucosal defense mechanisms. In particular, they reduce acid output, stimulate mucus, bicarbonate and phospholipids production, increase mucosal blood flow, and accelerate epithelial restitution and mucosal healing (Brzozowski *et al*., 2005). Prostaglandins are also known to inhibit mast cell activation as well as leukocyte and platelet adhesion to the vascular endothelium (Halter *et al*., 2001; Brzozowski *et al*., 2005a). The beneficial actions exerted by PGE2 have been shown to be mediated by activation of specific prostaglandin receptor (EP) subtypes. In particular, the activation of EP1receptors mediates the most important protective effects of prostaglandins, through an increase in bicarbonate secretion and mucosal blood flow in the damaged mucosa and a decrease in gastric motility (Takeuchi *et al*., 2002). Other EP receptor subtypes are also involved in the protective actions of PGE2. For example, EP3 receptors inhibit the gastric acid secretion, while EP4 receptors stimulate the secretion of mucus (Kato *et al*., 2005).

2.1.4 Neurohormonal Mechanisms

Gastric mucosal defense is supported by mechanisms activated, at least in part, by the central nervous system and hormonal factors (Laine *et al*., 2008). Experimental studies have

demonstrated that central vagal activation stimulates mucus secretion and increases intracellular pH in the surface epithelial cells of the stomach. In addition, while the CRF pathway is involved in endocrine responses tostress (Chatzaki *et al*., 2006). In addition, peripheral CRF contributes significantly to the regulation of gastric defense mechanisms, in particular, the CRF2 receptor is known to mediate antiapoptotic effects in gastric epithelial cells as well as to inhibit gastric emptying and motility (Chatzaki *et al*., 2006). Other hormone mediators, including gastrin-17, cholecystokinin, thyrotropin-releasinghormone, bombesin, EGF, peptide YY and neurokinin A, play significant roles in the regulation of gastric protective mechanisms, which can be blunted by afferent nerve ablation, CGRP receptor blockade, and inhibition of NO synthase (Peskar, 2001; Moszik *et al*., 2001). Ghrelin, a hormone peptide produced by gastric A-like cells in rodents and P/D1 cells in humans, is involved in the regulation of growth hormone secretion and appetite stimulation (Brzozowski *et al*., 2005b). Moreover, It is also able to exert significant protective effects at gastric level, including the enhancement of mucosal blood flow via stimulation of NO and CGRP release from sensory afferent nerves (Brzozowski *et al*., 2005b).

Glucocorticoids have been shown to support the mechanisms of protection at gastric level. These hormones are involved in the response to stress, and represent potent gastroprotective factors against injury (Filaretova *et al*., 1998). Consistently with this contention, glucocorticoid antagonists enhanced the severity of stress-induced erosions, further supporting a protective role of these hormones during stress (Filaretova *et al*., 2001).

The mechanisms through which glucocorticoids exert their protective effects include the maintenance of glucose homeostasis, the increase in mucosal blood flow and mucus secretion, and the attenuation of both enhanced gastric motility and microvascular permeability (Filaretova *et al*., 2007).

2.1.5 Mechanisms of Gastric Mucosal Damage

Gastric mucosal injury may occur as a consequence of various conditions, including alcohol intake, refluxed bile salts, stress, aging and *Helicobacter pylori* infection, although the most important agents known to impair the mechanisms of gastric mucosal defense are represented by NSAIDs. For this reason, the mechanisms of NSAID-induced gastric injury can be described as follows;

2.1.5.1 Effects of NSAIDs on Gastric Mucosa

The pathophysiology of gastric injury associated with NSAID administration depends partly on cyclooxygenase inhibition and partly on cyclooxygenase-independent mechanisms, which result mainly from local direct actions (Scarpignato and Hunt, 2010). Cyclooxygenase blockade has been shown to increase the susceptibility of gastric mucosa to NSAID-induced lesion by suppression of a number of prostaglandin-mediated protective functions. For instance, prostaglandins reduce the activation of neutrophils and the local release of reactive oxygen species (ROS) (Fornai *et al.*, 2011).

. The production of prostacyclin by the endothelium of mucosal microcirculation is also highly relevant in ensuring a tonic inhibition of neutrophil adhesion. Therefore, NSAIDs can shift the mucosal balance toward the recruitment and endothelial adhesion of circulating neutrophils through the inhibition of prostaglandin biosynthesis (Whittle, 2002). Once adhered, neutrophils clog the microvasculature causing a local decrease in mucosal blood flow and a marked release of tissue damaging factors, including proteolytic enzymes and leukotrienes, which enhance the vascular tone, exacerbate tissue ischaemia, stimulate the production of ROS, and promote the destruction of intestinal matrix, leading to a severe degree of focal tissue necrosis, particularly in the presence of a low luminal pH (Whittle, 2002; Jimenez *et al*., 2004).

As anticipated above, cyclooxygenase-dependent inhibition of bicarbonate secretion contributes also to the gastric mucosal injury elicited by NSAIDs. The secretion of bicarbonate ions in the mucus gel layer generates a pH gradient on the mucosal surface, thus providing a first line defense against luminal acid (Allen and Flemstrom, 2005). A number of studies have demonstrated the expression of bicarbonate/chloride ion exchangers in the apical membranes of gastric surface epithelial cells, and shown that cyclooxygenase-derived prostaglandins stimulate bicarbonate secretion via activation of EP1 receptors (Takeuchi *et al*., 1997; Rossmann *et al*., 1999). Most NSAIDs are weakly acidic in nature and this property accounts for their local cyclooxygenase-independent injuring actions on the gastric mucosa. In the presence of gastric acidity, the undissociated lipophilic form of acidic NSAIDs can impair the hydrophobic surface barrier of the stomach (Fornai *et al.,* 2011).

This transformation of the gastric mucosal surface from a non-wettable to a wettable state appears to be linked with the ability of acidic NSAIDs to destabilize the extracellular lining of zwitterionic phospholipids, particularly phosphatidylcholine, which are present within and on surface of the mucus gel layer (Lichtenberger *et al*., 2007). Previous studies have demonstrated that such an effect contributes significantly to NSAID-induced gastric injury in experimental models, and that it can persist for prolonged periods after discontinuation of NSAID administration (Lichtenberger, 2001). There is also consistent evidence that the protonophore actions of aspirin and other acidic NSAIDs take a significant part in the topical damage to gastric mucosa (Fornai *et al.,* 2011). In particular, upon exposure to the acidic environment of gastric lumen, the undissociated lipid-soluble form of aspirin is able to penetrate cell membranes and accumulate into epithelial cells, where the inner pH is at a physiological level of 7.4. At this pH value, aspirin dissociates and remains segregated within cells. This accumulation enhances the

inhibition of prostaglandin biosynthesis, and it brings also into play other properties of aspirin, such as the uncoupling of mitochondrial oxidative phosphorylation. The consequences of such mitochondrial dysfunction are a decrease in ATP production and an increase in AMP and ADP levels, which are then responsible for increments of intracellular calcium concentration. These changes are followed by mitochondrial injury, increased generation of ROS and alterations in the Na^+/K^+ balance, which lead to weakening of the mucosal barrier and cellular necrosis (Wallace, 2001; Bjarnason *et al*., 2007).

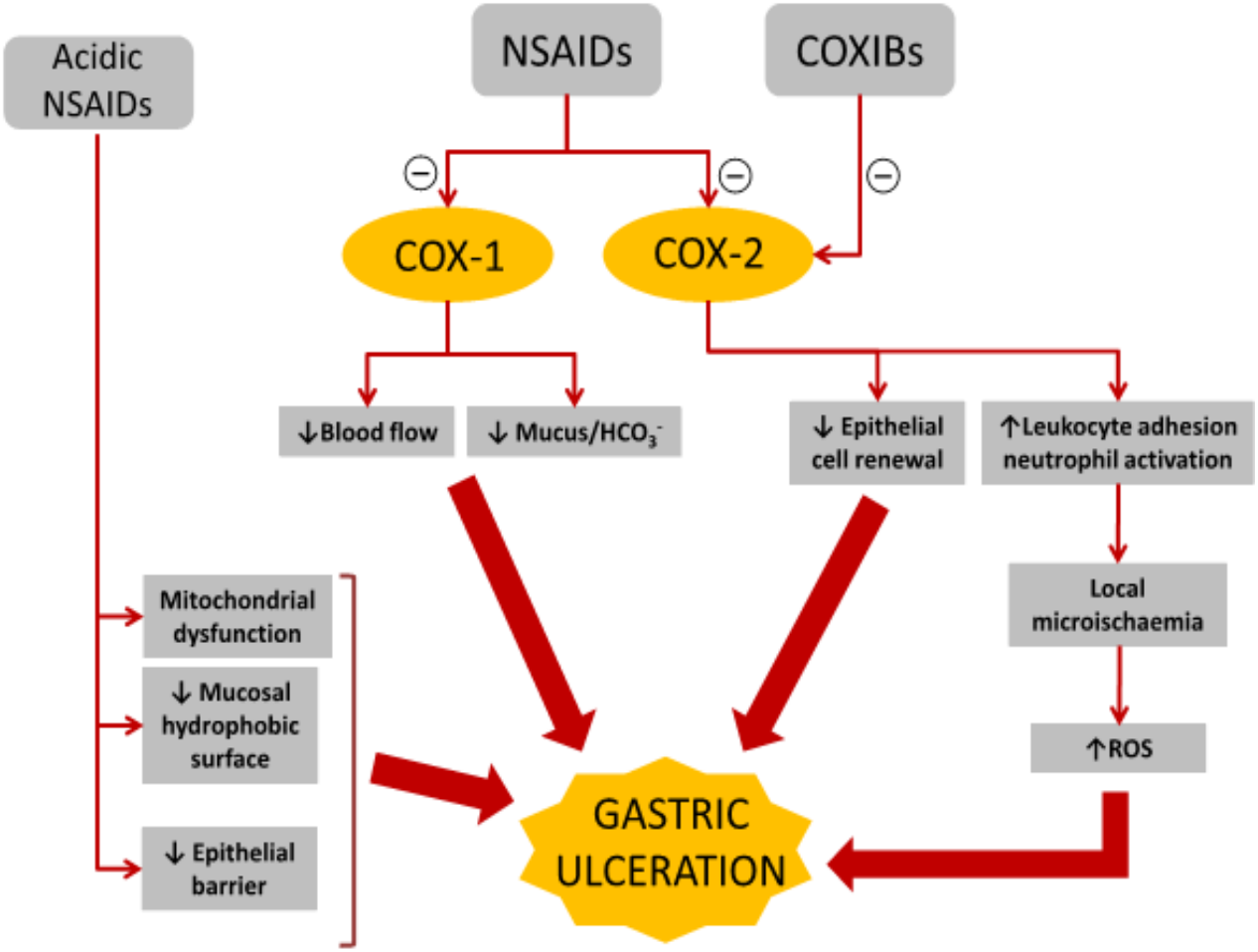


Figure 2.3. Pathophysiology of gastric injury induced by non-selective NSAIDs (Fornai *et al.*, 2011).

2.1.6 Mechanisms of Gastric Ulcer Healing

Gastric ulcer results from mucosal tissue necrosis triggered primarily by ischemia, with cessation of nutrient delivery and ROS formation (Fornai *et al.,* 2011). Tissue necrosis and subsequent release of arachidonic acid metabolites from injured cells, including leukotrienes B, attract leukocytes and macrophages, which then phagocitize the necrotic tissue and release pro-inflammatory cytokines, which in turn activate local fibroblasts, endothelial cells and epithelial cells to attempt a tissue restoration (Cotran *et al*., 1999; Tarnawski, 2005). Morphologically, gastric ulcer consists of two components: the margin, sorrounded by adjacent non-necrotic mucosa, and the base, consisting of granulation tissue, which is a connective tissue rich in macrophages, fibroblasts and proliferating microvessels (Cotran *et al*., 1999). Ulcer healing is a complex process, in which the tissue repairs itself after injury, attempting a restitution towards integrity. It has been proposed that such a process can be distinguished in sequential, partly overlapping, phases, haemostasis, inflammation, proliferation and remodeling (Stadelmann *et al*., 1998).

The phases and time course of ulcer healing can be described according to Schmassmann (1998) as follows: ulcer development phase (within 3 days from injury), characterized by tissue necrosis, inflammatory infiltration, formation of ulcer margin (de-differentiation) and development of granulation tissue; healing phase (after 3-10 days from injury), which includes an early healing (rapid migration of epithelial cells and contraction of ulcer base) followed by a late healing (angiogenesis in ulcer bed, remodeling of granulation tissue and complete re-epithelialization of ulcer crater); reconstruction phase (day 20-40 after ulceration) consisting of the reconstruction of glands, muscularis mucosae and muscularispropria; maturation phase (40-

150 days after ulceration), characterized by maturation and differentiation of specialized cells (Schmassmann, 1998).

In general, following the ulcerative injury, a set of complex biochemical events takes place to provide support for cellular migration from ulcer margin and attachment to the ulcer base, with subsequent cellular proliferation and restoration of the epithelial layer. Ulcer healing is initiated by formation of the 'healing zone', consisting of dilated glands, whose cells undergo de-differentiation, express epidermal growth factor receptor (EGF-R) and starts to actively proliferate. At this stage, inflammatory infiltration occurs closely to the necrotic tissue and ulcer crater. In response to growth factors, the ulcer margin is formed, cells adjacent to the margin de-differentiate, and granulation tissue develops at the ulcer base.

During healing, the granulation tissue undergoes continuous remodeling, contraction and changes in cellular composition, whereby the inflammatory cells appearing in the early phase of healing, are replaced by fibroblasts and microvessels in the late healing phase (Cotran *et al.*, 1999). Wong *et al.* (2000), analyzed the sequential expression of various genes during ulcer healing and were able to distinguish the following arrays: genes involved in early response (EGF-R, C-FOS, C-JUN, EGR-1, SP-1, trefoil factor-2/spasmolytic peptide [TFF-2/SP]), which are all activated shortly after ulcer formation (i.e., within 30 minutes-2 hours); intermediate response genes (EGF, basic fibroblast growth factor [bFGF], platelet derived growth factor [PDGF] and vascular endothelial growth factor [VEGF]), which become activated within 6 hours-2 days; late response genes (hepatocyte growth factor [HGF], intestinal trefoil factor [ITF], c-met/hepatocyte growth factor receptor [HGF-R]), which are activated within 14 days. (Wong *et al*., 2000). The subsequent proliferation step is initiated within 3 days from ulceration, and it is essential for the healing process, since it supplies the epithelial cells needed for re-

epithelialization mucosal surface and gland reconstruction of (Cotran *et al*., 1999). There is evidence that mucosal ulceration leads to the development of a novel cell lineage designated as ulcer associated-cell lineage, which stems from the base of surviving crypts (Cotran *et al*., 1999). These cells, which express EGF-R and initiate the synthesis of EGF, HGF, trefoil peptides and other growth factors, promote epithelial tube formation, migration and invasion of granulation tissue, and ultimately drive gland reconstruction within the ulcer scar (Tarnawski, 2005). Time-sequence analysis has shown that trefoil peptides are expressed much earlier than EGF following the induction of tissue ulceration. Furthermore, receptor analysis, using radio ligand binding assays and immunohistochemistry, has shown a rapid increase in EGF-R expression and a rapid decrease in somatostatin receptor density in the ulcer margin (Reubi *et al*., 1994).

The major stimuli for cell migration and ulcer re-epithelialization are mediated by growth factors which are produced by platelets, injured tissue and macrophages. The migration of epithelial cells from the ulcer margin, to restore the continuity of epithelial lining, is essential for ulcer healing, and it is subjected to a fine regulation, since it generates a barrier protecting the granulation tissue from any mechanical and chemical damage (Fornai *et al.,* 2011). Notably, cell migration requires complex cytoskeletal rearrangements. In particular, it has been appreciated that cytoplasmic microfilaments, consisting of G-actin, polymerize into F-actin and the latter, together with myosin II, provides contractile bundles through which cell motility can take place (Chai *et al*., 2004). A schematic diagram showing the main factors involved in gastric ulcer healing is provided in Figure 2.4

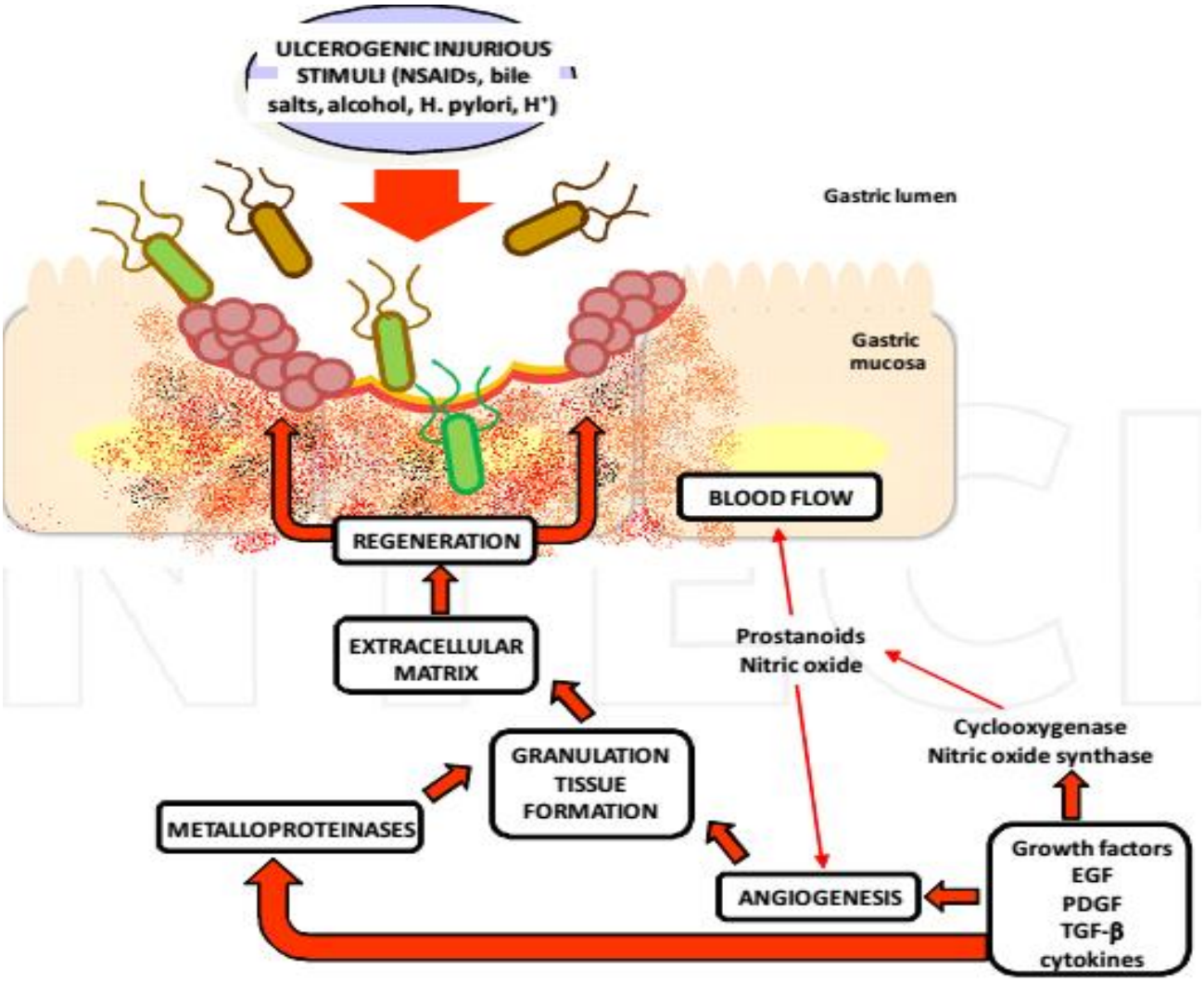


Figure 2.4, Mechanisms involved in gastric ulcer healing. EGF: epidermal growth factor; PDGF: platelet-derived growth factor; TGF-β: transforming growth factor- β (Fornai *et al.*, 2011).

2.2 Proton Pump (H+/K+ - ATPase)

2.2.1 General Properties

The gastric H^+/K^+-ATPase is a member of the P2-type ATPase family and is responsible for gastric acid secretion (Munson, 2000). The enzyme undergoes a cycle of phosphorylation and dephosphorylation coupled to the outward movement of H^+ and the inward movement of K^+ in a net electroneutral fashion. Earlier research on these ATPases, prior to the availability of cDNA-derived sequences, concentrated on the coupling between transport and catalysis, and vital concepts were derived from these studies, particularly those relating to phosphorylation/dephosphorylation and ion transport (Forte *et al*., 1974; Sachs *et al*., 1976). The enzyme transports ions in either direction as a function of conformational changes in the cytoplasmic domain. The data derived for these ATPases seem all to conform to a general model in which the binding of ATP and the outward transported ion results in phosphorylation of the pump at a particular region of the protein, the phosphorylation signature sequence, DKTGTLT, where the aspartyl at position 386 is phosphorylated in the case of the gastric ATPase (Walderhaug *et al*., 1985). When this phosphorylation occurs, the inward facing ion-binding site with high affinity is altered to form an outward-facing ion-binding site of lower affinity. This transition is generally referred to as the E1 to E2 transition, these numerals implying a major conformational change. Between the inward E1 and outward E2 ion-binding states, yet a third ion-binding site state has been inferred, in which the ion is shielded from either side of the membrane, the occluded state. This state can be defined experimentally in terms of ion binding. Occlusion can be demonstrated even after cleavage of the connections between the cytoplasm and the membrane segments. Hence, this property resides within the membrane domain of the pump.

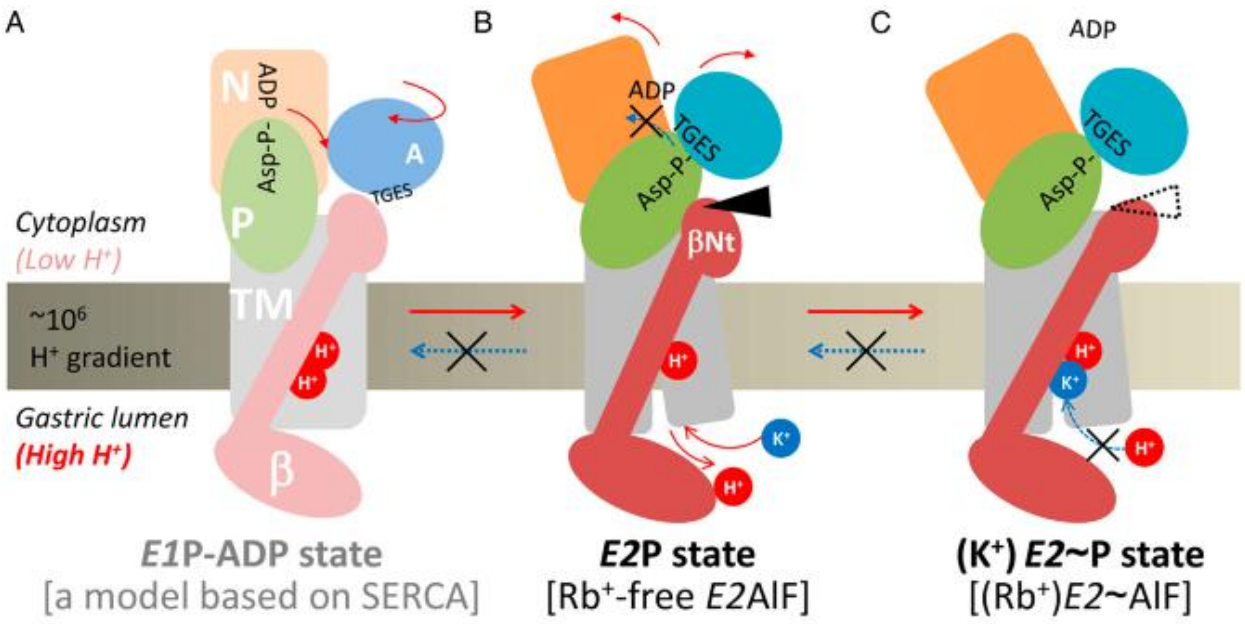


Figure 2.5. Model for the vectorial transport by H^+/K^+-ATPase. In the H+-occluded E1P-ADP state (A), the P domain is in close proximity to the ADP-bound N domain to form the ADP-aspartylphosphate (Asp–P-ADP) complex. In the proton-transporting step (E1P-ADP to E2P), the P domain is inclined to the ßNt, and the Asp-P is covered with the conserved TGES-motif of the A domain to form the E2P state (B). Now, the ßNt is tethering the P domain and stabilizes its position (black arrowhead), thus counteracting the reformation of E1P (indicated as a blue dotted arrow in B) and keeping the H-ATPase resistant to the steep proton gradient, which acts as a strong pressure to drive the transport cycle into the backward direction. Binding of K from the luminal side of the membrane, in turn, induces segregation of the ßNt-P domain interaction (dotted arrowhead) in the K+-bound E2P transition state (C), releases the P domain, and allows H+,K+-ATPase to proceed with the following transport cycle. Red and blue dotted arrows indicate the forward and reverse reactions of the transport cycle, respectively, and several of important molecular events that accompany the corresponding reaction substeps (Abe *et al.*, 2012).

The H^+/K^+- ATPases are classified into two subfamilies, gastric and non-gastric (latter also called colonic), coded by ATP4A and ATP12A. The gastric H^+/K^+-ATPase is expressed in stomach parietal cells, kidney distal nephrons (Gumz *et al*., 2010) and cochlea (Shibata *et al.*, 2006), where they are responsible for H^+ secretion, K^+ absorption and K^+ recirculation, respectively. The non-gastric H^+/K^+ -ATPase is present in several epithelial tissues including colon, kidney, skin, placenta, and prostate, and here it is associated with acid-base or K^+ and Na^+ homeostasis (Pestov *et al*., 2006). Each pump is composed of two catalytic α-subunits and two regulatory β-subunits.

The gastric proton pump which accumulates at the apical surface and subapical storage vesicles in the acid-secreting parietal cells of the stomach is responsible for such fundamental manifestations of cellular homeostasis as the maintenance of osmotic balance and intracellular ionic composition. P-type ATPase family, whose members include the Na^+-, H^+- , K^+-, and Ca^{2+}-ATPases, comprises a nearly ubiquitous collection of ion pumps with catalytic activities that drive myriad physiologic processes (Pedersen and Carafoli, 1987). Almost every transport operation performed by the cells of epithelial tissues is coupled in some fashion to the action of a P-type ATPase (Caplan, 1997). The enzymatic activities of these pumps constitute some of the principal means through which all animal cells convert the energy embodied in ATP into electrochemical gradients that can be exploited by all manner of metabolic pathways (Dunbar and Caplan, 2001).

Gastric H^+/K^+-ATPase is responsible for gastric acid secretion. ATP driven H^+ uptake into the stomach is efficiently accomplished by the exchange of an equal amount of K^+, resulting in a luminal pH close to 1. Because of the limited free energy available for ATP hydrolysis, the stoichiometry of transported cations is thought to vary from $2H^+/2K^+$ to $1H^+/1K^+$ per hydrolysis

of one ATP molecule as the luminal pH decreases, although direct evidence for this hypothesis has remained elusive. The reported free energy for ATP hydrolysis of the gastric secretory membrane, −13 kcal/mol (Reenstra *et al*., 1981) provides sufficient energy to achieve a maximum change in pH (ΔpH) of 4.7 units when two H+ ions are transported per hydrolysis of one ATP molecule.

2.2.2 Structure and Function of the Gastric H^+/K^+-ATPase

The primary structure of the gastric H^+/K^+-ATPase subunits containing the catalytic site was first elucidated in rat (Shull and Lingrel, 1986) and then in hog (Maeda *et al*., 1988), rabbit (Bamberg *et al*., 1992), dog (Song *et al*., 1993) and human (Maeda *et al*., 1990). This catalytic subunit consists of 1,033 or 1,034 amino acids in length in all species with 98% homology and a ß subunit consisting of 291 amino acids having six or seven N-linked glycosylation sites (Reuben *et al*., 1990). Examination of the catalytic subunit revealed that it was highly homologous to the Na^+/K^+-ATPase (~63%) and less so to the SERCA Ca-ATPase (25%) (Swarts *et al*., 2005). The gastric H^+/K^+-ATPase is fully assembled during biosynthesis in the endoplasmic reticulum and delivered to the apical membrane as a hetero-dimeric oligomer. In the gastric H^+/K^+-ATPase, there is also a lysine 791 located in the fifth transmembrane segment that replaces a serine present in the Na^+/K^+-ATPase isoforms. This lysine of the H^+/K^+-ATPase seems to characterize the H,K-enzyme specificity for outward transport of the hydronium ion (Shin *et al*., 2009).

2.2.3 Targets for Inhibiting Acid Secretion by the Parietal Cell

The basis for success of modern methods of therapy of acid secretion has been the identification of unique expression of target proteins by cells like the gastric parietal cell. For example, the major functional location of both H_2-receptor and the H^+/K^+-ATPase is the parietal cell. Histamine binds to the H2 receptor and stimulates acid secretion through the cAMP-stimulated

morphological changes of the parietal cell from the resting status to the stimulated state and activation of the KCl pathway (Shin *et al*., 2009). The gastric H^+/K^+-ATPase is located in the canaliculus of the stimulated state and secretes gastric acid by an electroneutral ATP dependent hydrogen–potassium exchange (Sachs *et al*., 1976). The H^+/K^+-ATPase is the final step of acid secretion, which suggested that an inhibitor of the pump would be more effective in suppressing gastric acid secretion than a receptor antagonist (Fellenius *et al.,* 1981). Accordingly, if inhibition of the pump is effective, hormonal stimulation of the parietal cell could not increase gastric acid secretion.

The secretion of acid by the parietal cell is controlled through food-stimulated and neuroendocrine pathways involving the activity of gastrin, histamine, pituitary adenylate cyclase-activating peptide and acetylcholine. There are, therefore, several potential ways in which gastric acid secretion might be modified (Sachs, 2003). Targeting the muscarinic receptors through which acetylcholine stimulates gastric acid secretion is one possible approach, but muscarinic antagonists (e.g. atropine) are not specific to the gastrointestinal system and have adverse effects such as dry mouth and blurred vision (Sachs *et al*., 2006). Competitive antagonists such as cimetidine and ranitidine can be used to block the binding of histamine to H receptors, but the parietal cell can still respond to other activating signals such as acetylcholine. Although histamine antagonists have reasonable efficacy at night, all patients quickly develop tolerance, perhaps as a result of upregulation of other pathways (Hatlebakk and Berstad, 1996).

Given the redundancy inherent in the physiological control of gastric acid secretion, targeting the final effector in the secretion pathway – the gastric H^+/K^+- ATPase – is likely the most effective pharmacological approach. The potassium-competitive acid pump antagonists (APAs), which inhibit the gastric H^+/K^+-ATPase via K+-competitive binding, are a promising new class of agent but their efficacy has yet to be demonstrated in clinical trials (Andersson and Carlsson, 2005). At present, proton pump inhibitors (PPIs) remain the most effective available therapy.

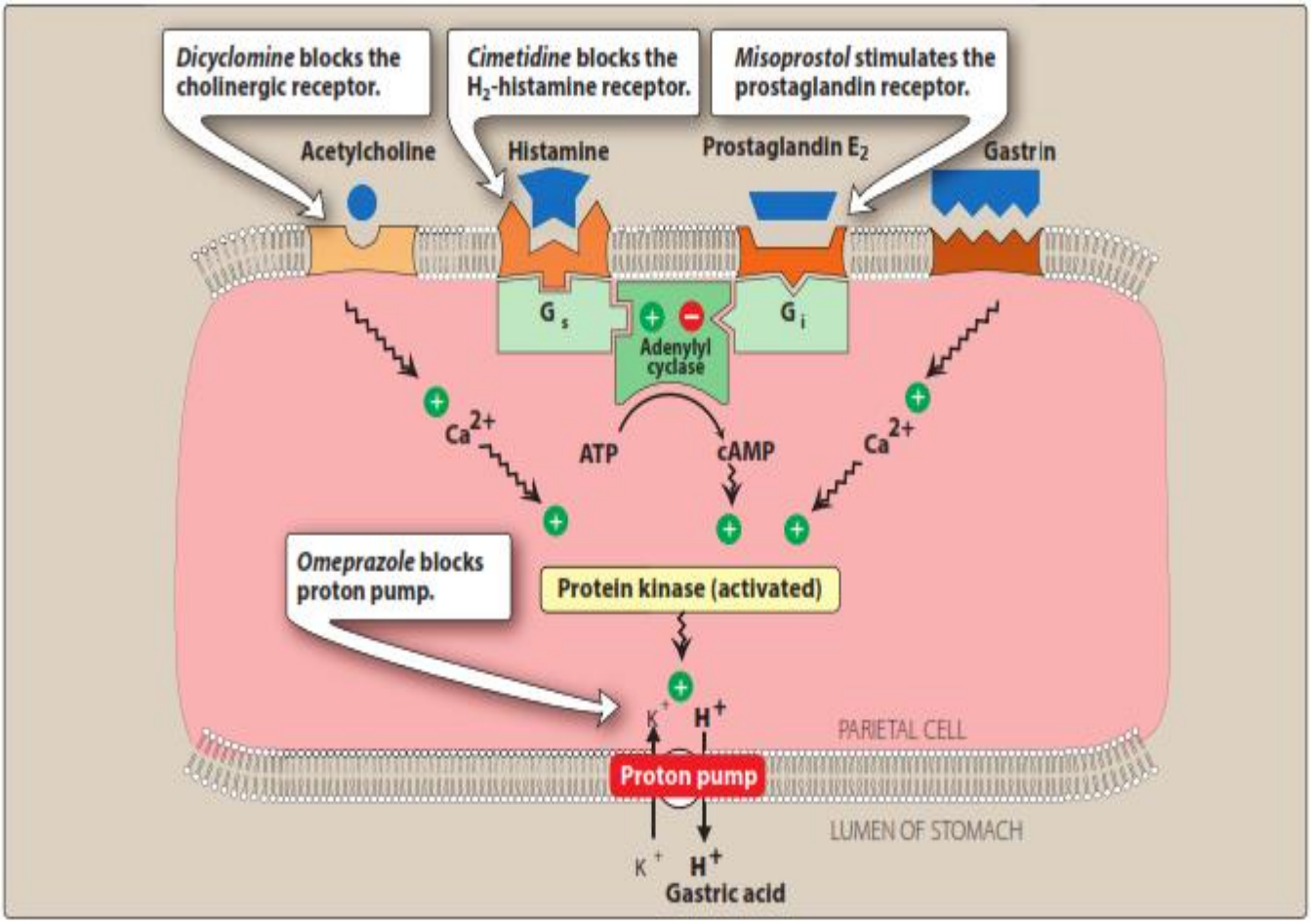


Figure 2.6. Effects of acetylcholine, histamine, prostaglandin E_2, and gastrin on gastric acid secretion by the parietal cells of stomach. Gs and Gi are membrane proteins that mediate the stimulatory or inhibitory effect of receptor coupling to adenylyl cyclase (Lippincott Illustrated Reviews: Pharmacology; Sixth Edition).

2.3 Therapeutic Drugs Used in the Management of Ulcer

Several therapeutic interventions have being described to be effective in the treatment and prevention of ulcer. Conventional medications employed in the management of ulcers includes; histamine (H_2) receptor antagonist, proton pump inhibitors, antacids, Anti *H.pylori* drugs, broad spectrum antibiotics, prostaglandine analogues, anticholinergics, cytoprotectives drugs such as mistoprostol and sucralfate.

2.3.1 Histamine Receptor Antagonist

Histamine is an organic nitrogenous compound derived from amino acid histidine in a reaction catalyzed by decarboxylase. It is produced by basophils and mast cells and involved in local immune responses and inflammatory response (Marieb, 2001). Histamine 2 receptor antagonists are widely used in the treatment of acid-peptic diseases. They are less potent in inhibiting acid production than proton pump inhibitors but suppress 24 hour gastric acid secretion by 70% (Wallace and Sharkey, 2011). Examples of H_2 receptor antagonists are cimetidine, ranitidine, famotidine and nizatidine.

2.3.1.1 Mechanism of Action

The H_2 receptor antagonists inhibit acid production by reversibly competing with histamine for binding to H_2 receptors on the basolateral membrane of parietal cells (Nwamarah and Uche2, 2014). They block histamine from stimulating the acid-secreting parietal cells of the stomach. H_2 antagonists are mainly basal, psychic and neurogenic, thus suppressing/ inhibiting gastric secretion and other stimuli such as acetylcholine, gastrin, alcohol and food (Goodman and Gilman, 2008).

During gastric acid release in the stomach, enterochromaffin-like cells located in the gastric glands of stomach release histamine that stimulates parietal cells by binding to apical H_2

receptors. Stimulation of parietal cells induces the uptake of carbondioxide and water from the blood which is then converted to carbonic acid by carbonic anhydrase. In the cytoplasm of the parietal cells, the carbonic acid readily dissociates in to hydrogen and bicarbonate ions. The bicarbonate ions diffuse through the basilar membrane into the blood stream, while the hydrogen ions are pumped into the lumen of the stomach by H^+/K^+ ATPase pump (Alvarez, 2007).

Adverse effect of H_2 rceptor antagonists include diarrhea, constipation, fatigue, drowsiness, headache and muscle pain in some patients (Alvarez, 2007). Cimetidine causes gynecomastia, galactorrhea (as it is antiandrogenic and increases prolactin level).

2.3.2 Proton Pump Inhibitors

PPIs are substituted benzimidazole derivatives (Figure 2.7) endowed with potent inhibitory effects on gastric acid secretion. They are antisecretory agents as well as prodrugs requiring activation in an acid environment. PPIs are inactive at neutral pH, but accumulates in an acid environment and are activated at a pH lower than 3 by protonation (John, 2005). Several preclinical and clinic lines of evidence have demonstrated that PPIs are highly effective in promoting the healing of gastric damage induced by NSAIDs, even in the presence of a continued NSAID administration, through the activation of both acid-dependent and -independent mechanisms (Blandizzi *et al*., 2008).

2.3.2.1 Mechanism of Action

These drugs act primarily through the blockade of the enzyme H^+/K^+-adenosine triphosphatase (proton pump), which is activated during the final step of acid secretion by the parietal cells of the stomach. PPIs are weak basic compounds, with acid dissociation constant (pKa) values ranging from 3.9 to 5.0. For this reason, they accumulate massively in the highly-acidic secretory canalicula of parietal cells,

where they are rapidly converted into their active cyclic sulfenamide form (Fornai *et al*., 2011). This highly reactive sulfenamide derivative binds sulfidryl groups of cysteine residue of the H^+/K^+-ATPase, leading to permanent enzyme inhibition and subsequent potent reduction of acid secretion (Boparai *et al*., 2008). Some studies have suggested that the beneficial effects of PPIs on ulcer healing could be ascribed to a marked inhibition of acid secretion, which can lead to a consistent increase in plasma gastrin levels, a peptide actively involved in the regulation of mucosal cell proliferation (Koh and Chen, 2000).

Besides the marked inhibition of gastric acid secretion, increasing evidence indicates that the beneficial effects of PPIs against NSAID-induced gastric injury could depend on acid-independent mechanisms. For instance, it has been shown that these drugs are able to counteract tissue oxidative damage in a direct or indirect manner (Lapenna *et al*., 1996; Natale *et al*., 2004). In particular, several *In Vitro* experiments demonstrated a direct antioxidant activity of PPIs, showing that pantoprazole (Fornai *et al*., 2005) and lansoprazole (Blandizzi *et al*., 2005) concentration-dependently reduced copper-induced oxidation of human native low density lipoproteins (LDLs), while omeprazole behaved as a scavenger of hypochlorous acid (an oxidant compound generated by phagocytes) (Lapenna *et al*., 1996). Side effect of PPIs include hypomagnesemia, Diarrhea, Clostridium difficile colitis and an increased incidence of pneumonia. Prolonged acid suppression with PPIs (and H_2 antagonists) may result in low vitamin B because acid is required for its absorption in a complex with intrinsic factor (Karen Whalen. 2015).

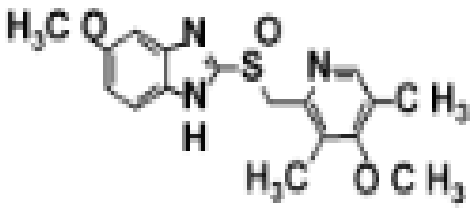


Figure 2.7. Chemical structure of Omeprazole (proton pump inhibitor) (Fornai *et al*., 2011).

2.3.3 Muscarinic Receptor Antagonist (Anticholinergics)

The main effects of parasympathetic stimulation on the gastrointestinal tract are an increase in gastric motility and increased in secretory activity. Anticholinergics are used to abolish gastric secretion provoked by parasympathetic agents but have a partial effect on histamine-induced secretion and decrease gastric motility (Rang, 2003).

2.3.3.1 Mechanism of Action

Anticholinergics such as pirenzepine, a strongly hydrophilic drug, with poor penetration in to the central nervous system, binds effectively to the sub-class of muscarinic cholinergic receptors of the gastric mucosa, resulting in effective inhibition of acid and pepsin secretion without significant anticholinergic effects elsewhere. Reported side effects include blurred vision, photophobia, constipation, urine retention, oxcanal impotence (John, 2005). Other examples includes, propantheline and oxyphenonium.

2.3.4 Antacids

These are basic substances which act by neutralizing gastric acid (hyperchloridia) and therefore raise the gastric pH, which has the effect of inhibiting peptic activity and produce prompt gastric relief in patients. Antacids can be classified as either systemic or non systemic acid. The systemic antacids are water soluble potent neutralizers with short intravenous duration of action which may raises the pH of the extracellular fluid above 7 and subsequently produces carbon dioxide. An example is sodium bicarbonate and sodium citrate hydroxide. Non systemic (Local) antacids have a slow action due to their insolubility and are poorly absorbed basic compounds (Nwamarah and Uche, 2014). They react in stomach with acid to form respective chloride salts. This again reacts with bicarbonate and is not spared for absorption, hence no acid –base disturbance. These forms of antacids may produce diarrhoea and their aluminium salts are

constipating. They include magnesiumtrisilicate, aluminium hydroxide gel, and calcium carbonate.

2.4 Medicinal Plants

Natural products from plants are a rich resource used for centuries to cure various ailments.

Hence, the use of plant parts for medicinal purposes has long been in existence and has been widely documented (Pomulo and Ierece, 2007). These ancient indigenous practices were discovered by a series of "trial and error" which then could not be substantiated by proven scientific theories. However, these practices have produced results of proven efficacies (Chopra *et al*., 1956). In recent times herbal medicines have become indispensable and are forming an integral part of the primary health care system of many nations (Fajimi and Taiwo, 2005). Study carried out by Gupta *et al.* (2012) reported that 75% of people still rely on plant-based traditional medicines for primary health care globally.

Plants have been used as valuable resources in the development of novel drugs, and many have shown potentials to be used in the treatment of gastric ulcers (Jainu *et al*., 2006). Such plant derived medicines are widely used because they are relatively safer than the synthetic medicines, readily available and considerably cheap (Yisa, 2009). Therefore, the use of natural medicine in the treatment of various diseases like peptic ulcer is an absolute requirement of our time (Sasmal *et al*., 2007). The increasing discovery of more medicinal plants has demanded for increased scientific scrutiny of their bioactivity so as to provide data that will help physician and patients make wise decision before using them.

2.4.1. Botany and Geographical Distribution of *Saccharum spontaneum*

Saccharum spontaneum is a coarse, erect, rhizomatous perennial plant, usually more or less tufted, with stout underground rootstock, growing to a height of 1 to 3.5 meters. Leaves are harsh and linear, 0.5 to 1 meter long; 6 to 15 millimeters wide. Pannicles are white and erect, measuring 15 to 30 centimeters long, with slender and whorled branches, the joints covered with soft white hair. Spikelets are about 3.5 millimeters long, much shorter than the copious, long, white hairs at the base. It is widely distributed in most parts of India, Africa, Afghanistan, China, Malaysia, Polynesia and Australia (Parrotta, 2001).

2.4.2. Scientific Classification of *Saccharum spontaneum*

Kingdom	Plantae
Division	Tracheophyta
Subdivision	Angiosperm
Class	Liliopsida
Order	Poales
Family	Poaceae
Subfamily	Panicoideae
Genus	*Saccharum*
Species	*Spontaneum*
Binomial Nomenclature	*Saccharum spontaneum* Linn. (Indiabiodiversity.org).

2.4.3. Ethnomedicinal Uses of *Saccharum spontaneum*

Saccharum spontaneum Linn is considered as a valuable medicinal herb in the traditional systems of medicine. The roots are sweet, astringent, emollient and refrigerant. In India, the roots are useful in treatment of dyspepsia, burning sensation, piles, sexual weakness, gynecological troubles, respiratory troubles etc. The stems (culm) are useful in vitiated conditions of burning sensation, renal and vesicol calculi dyspepsia, haemorrhoids, menorrhagia dysentery, agalactia phthisis and general debility (Suresh *et al.*, 2009).

2.4.4. Biological Activity of *Saccharum spontaneum*

Studies by Ghani (2010) on evaluation of phytochemicals in root extract of *S. spontaneum* revealed the presence of quinones, terpenes, alkaloids, saponins, tanins, coumarins, and other phenolic compounds. The study has also revealed the use of the plant as galactagogue and diuretic. The aerial parts possess laxative and aphrodisiac properties, and are useful in burning sensations, strangury, phthisis, vesical calculi, blood diseases, biliousness and haemorrhagic diathesis (Chopra *et al*., 1992). Anti-diarrhoel and CNS depressant activity of the whole plant was reported by Vhuiyan *et al*. (2008), and the roots of the plant have been reported to have anti-urolithiatic activity (Sathya *et al*., 2013).

The acute and sub acute toxicological assessment of the ethanolic root extract of *S. spontaneum* in male wistar albino rats was reported by Sathya and Kokilavani, (2012)

The *In Vitro* antimicrobial, cytotoxic and antioxidant activity of flower extract of *S. spontaneum* was also documented by Farhana *et al*., (2009).

Psychopharmacological studies on the stem of *S. spontaneum* showed an antipsychotic activity and CNS depressant activity on wistar rats in the queous and ethanol extracts at the dose of 1 gm/kg, as reported by Suresh *et al*., (2010).

Plate 2. 1 A view of *Saccharum spontaneum* plant. (**discoverlife.org**, 2017).

2.5 Secondary Metabolites of Plants that Protect Against Ulcer

Phytochemicals are bioactive substances derived from plants and are associated with the protection of human health against chronic degenerative diseases (Florence *et al*., 2015). The efficacies of these medicinal plants in the treatment of diseases have been attributed to the presence of bioactive compounds, which have diverse physiological and pharmacological responses (Ekanem and Udo, 2009).

The use of phyto-constituents as drug therapy to treat major ailments has proved to be clinically effective and relatively less toxic than the existing drugs and also reduces the offensive factors serving as a tool in the prevention of peptic ulcer (Jainu *et al.,* 2006). The chemical constituents present in the herbal medicine or plants are a part of the physiological functions of living flora and hence they are believed to have better compatibility with the human body (Kamboj *et al*., 2000). Many studies indicate that plant products are potential agents for healing ulcers and largely preferred because of the absence of unwanted side effects and their effectiveness (Jhansi *et al.*, 2010). Many secondary metabolites have been found to have antiulcer properties, these includes;

2.5.1 Alkaloids

Alkaloids are a group of natural products with diverse group of low molecular weight nitrogen-containing compounds derived mostly from amino acids (Ziegler *et al*., 2008). These secondary metabolites are found in 20% of plant species and are classified as true alkaloids, which have the nitrogen atoms in heterocyclic rings and pseudoalkaloids, which are not derive from amino acids but may have nitrogen atoms in heterocyclic rings (Henriques *et al*., 2004).

Different classes of alkaloids are being utilized in therapeutic and as pharmacological tools. The reported biological activities are anticholinergics, antitumor, diuretic, sympathomimetic,

antimicrobial, antiviral, antihypertensive, antidepressant, hypoanalgesic, emetic (Henriques *et al*., 2004). Several studies revealed the gastroprotective and ulcer healing activity of alkaloids by inhibition of acid secretion and gastric motility, stimulation of alkali/mucus secretion, increase mucosal blood flow which all help in prevention and healing of ulcers against injury caused by aggressive agents. Studies by Wang, (2000) showed that alkaloids are useful pharmacological tools for the discovery of new active principles that can protect the gastric mucosa against damaging effects induced in rats by indomethacin, reserpine, stress, pylorus ligature, absolute ethanol (Wang, 2000).

Piperidine alkaloid was found to protect gastric mucosal damage caused by stress, indomethacin, ethanol, pylorus ligation by decreasing volume of gastric juice, gastric acidity and pepsin activity (Bai and Xu, 2000). In the stomach, capsaicin alkaloid have been found to protect stomach by inhibiting acid secretion, stimulating the alkali/mucus secretions and increase the gastric mucosal blood flow which help in prevention and healing of ulcers against injury caused by aggressive agents (Konturek *et al.*, 2006).

Moreover, alkaloids of the indole class have been found to posses antiulcer activity or protect the gastric mucosa from the damage caused by ischemia-reperfusion and absolute ethanol through attenuation of the gastric blood flow and scavenging of free radicals (Konturek *et al.* 2006).The pyrrolidine alkaloids were also found to demonstrate significant activity in acute and chronic gastric ulcers. These alkaloids increased free mucus and prostaglandins in the gastric mucosa. Also, they showed a reduction of exfoliation of superficial cells, hemorrhages and blood cell infiltration mediated by increase in gastrin secretion and mRNA expression of epidermal growth factors (Toma *et al.*, 2004).

2.5.2 Tannins

Tannins are polyphenols found in plants, foods and beverages, water soluble with molecular weight from between 500 and 3000 daltons, and of great economic and ecological interest, that form complexes with water-soluble proteins, alkaloids and gelatin. They are responsible for the astringent taste of many fruits and vegetables, causing precipitation of salivary glycoproteins and reducing oral lubrication (Albuquerque *et al*., 2005).

In several experimental models of gastric ulcer, purified tannins have been shown to be involved with gastrointestinal tract anti-inflammatory actions, promotion of tissue repair, acid secretion inhibition, and both antioxidant and anti-*Helicobacter pylori* activity. Being phenolic compounds, tannins are chemically reactive and form inter and intra-molecular hydrogen bonds. They are easily oxidized by specific plant enzymes and influenced by metals such as ferric chloride (which causes darkening solution). Classically, the chemical structures of tannins are divided into two groups: hydrolysable and condensed. The hydrolysable tannins consist of gallic acid esters, and ellagic acid glycosides, formed from shikimate where the hydroxyl sugars are esterified with phenolic acids. In the process of wound healing, burns and inflammations, tannins help by forming protective layer (tanning-protein complex/ tannin-carbohydrate complex), over the injured epithelial tissues permitting the healing process below to occur naturally (Simoes *et al*., 2003).

Many phenolic compounds including catecholamines have been shown to modulate prostaglandins synthase and 5-lipoxygenase pathways of arachidonic acid (Alanko *et al.*, 1999). Phenols have two effects on prostaglandin biosynthesis, with low concentrations stimulating the synthesis of prostaglandins and high concentrations inhibiting prostaglandin synthase. They stimulate the synthesis by acting as reducing substrates for the oxidized intermediates of

prostaglandin synthase, thereby accelerating the peroxidase cycle and by functioning as electron-donating cosubstrates for the peroxidase components of prostaglandin synthase. Phenols inhibit cyclooxygenase-2 activity of prostaglandins by competing for the arachidonic acid-binding site and by competitive reduction of prostaglandins. The modulation of hydroperoxide tone by phenols is probably the key element explaining suppression of arachidonic acid metabolism by prostaglandins. The stimulatory effect of phenols on prostaglandin E_2 formation may be based on their action as co-substrates for the peroxidase reaction (Alanko *et al.,* 1999).

2.5.3 Flavonoids

Flavonoids are diphenylpropanes that commonly occur in plants (more than 4000 flavonoids have been found) and are frequently components of human diet. Family members include flavones (apigenin, tangeritin, tangerine, and luteolin), isoflavones (genistein, daidzein, and glycitein), isoflavonoids (rotenone and genistin), flavonols (quercetin, gingerol, kaempferol, myricetin, and rutin), flavanones (hesperetin, naringenin, and eriodictyol), anthocyanidins (cyanidin, delphinidin, malvidin, and petundin), and anthocyanins (haematien). These flavonoids with multiple OH substitution have strong antioxidant activities against peroxyl radicals (Alanko *et al.,* 1999).

The principal properties that account for the potential health benefits of flavonoids is their antioxidant activity. Several in vitro antioxidant studies have demonstrated that flavonoids can scavenge superoxide, hydroxyl and peroxyl radicals that affect various steps in the arachidonate cascade through cyclooxygenase-2 or lipoxygenase (Alanko *et al.,* 1999). They also increase the stability of membrane, and affect some processes of intermediary metabolism and inhibit lipid peroxidation in different system.

Some flavonoids have been shown to increase the mucosal contents of prostaglandins and mucus in gastric mucosa, showing cytoprotective effects. Several of these flavonoids prevent gastric mucosal lesions produced by various experimental models and protect gastric mucosa against necrotic agents (Alanko *et al*., 1999).

In addition to radical scavenging activities, flavonoids exhibit several mechanisms for their antioxidant activity such as chelating of transition metals ions, inhibition of oxidant enzymes, production of free radicals by cells, and regeneration of tocopherol radicals. They also promote gastric mucosa formation, diminish acid secretion, inhibit production of pepsinogen and decrease ulcerogenic lesions (Bansal and Goel, 2012).

2.5.4 Saponin

The saponins are naturally occurring surface-active glycosides which are composed of carbohydrate and non carbohydrate or aglycone portion. The aglycones are often referred to as sapogenins. The sapogenin nucleus may be either of steroid or triterpenoid structure. They are mainly produced by plants, but also by lower marine animals and some bacteria (Riguera, 1997; Yoshiki *et al*., 1998). They derive their name from their ability to form stable, soap-like foams in aqueous solutions. This easily observable character has attracted human interest from ancient times. Saponins consist of a sugar moiety usually containing glucose, galactose, glucuronic acid, xylose, rhamnose or methylpentose, glycosidically linked to a hydrophobic aglycone (sapogenin) which may be triterpenoid or steroid in nature. The aglycone may contain one or more unsaturated C–C bonds.

A mixture of saponins (Aescin) has been shown to posses anti-ulcer activity in various ulcer models (cold restraint and pylorus-ligated) (Marhuenda et al., 1993), an effect which is, in part,

due to inhibition of gastric acid and pepsinogen secretion. Studies by Jeong *et al*., (2003) and Lee *et al*., (2005) have also shown the antiulcer properties of Ginsenoside Rb1 and Araloside saponins respectively.

2.5.5 Triterpenes

Triterpenes are a class of chemical compounds composed of three terpene units with the molecular formula $C_{30}H_{48}$. Triterpenes exist in a huge variety of structures with nearly 200 different skeletons known from natural sources or enzymatic reactions (Xu *et al.*, 2004). Study by Andrikopoulos *et al*., (2003) revealed the antiulcer activity of various triterpenes such as β amyrin, lupeol, ursolic acid, glycerrhetinic acid and sterols like β sitosterol which is due to their ability to strengthen the defensive factors such as stimulation of mucus synthesis or maintenance of prostaglandin contents of gastric mucosa at high levels.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals/ Reagents

All the chemicals used were of analytical and pharmaceutical grade which include: ATP; EDTA; HEPES; Tris-HCl; (BDH laboratories); methanol, n-Hexane (Guangdong Guanghua Scientific Company Ltd China); Omeprazole (Brussels laboratories Pvt. Ltd India). All other chemicals and reagents used were also of analytical grade.

3.1.2 Experimental animals

A total of eighty (84) healthy adult albino rats (Wistar strain) of both sexes weighing between 150-200g were obtained from animal house, Department of Biochemistry, Faculty of Life Sciences, Kaduna State University. Animals were housed in cages with raised floors of wide wire mesh to prevent coprophagy and were kept in a clean environment with free access to water and fed with standard pellet diet. They were conditioned to the laboratory environment for two weeks to acclimatize before the commencement of the experiment.

3.1.3 Plant Material Collection and Identification

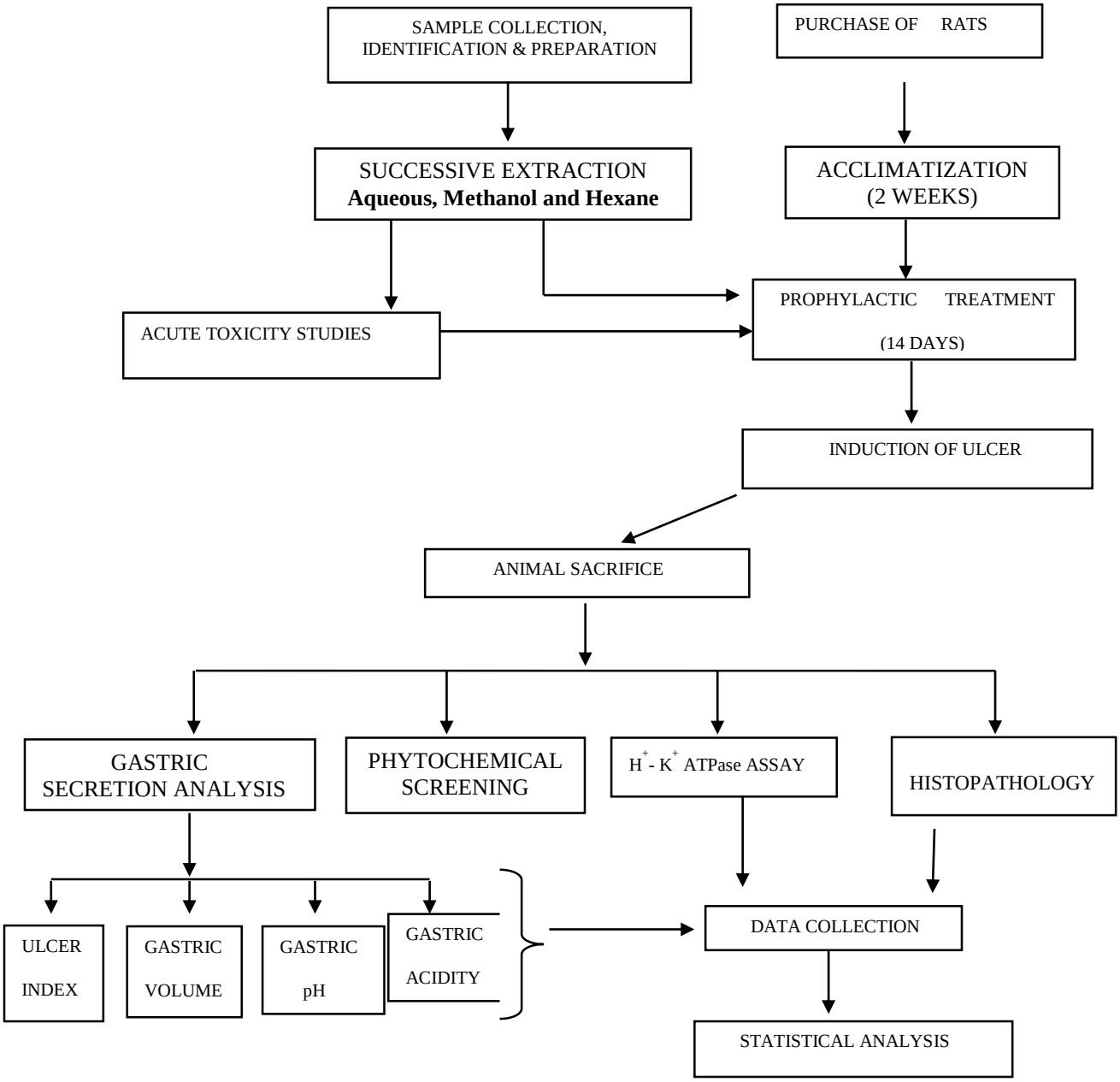
The fresh leaves of *Saccharum spontaneum* were collected from Samaru, Sabongari Local Government area, Kaduna State, Nigeria on the 4th of December, 2015. The plant was authenticated by Malam Sunusi Namadi at the herbarium unit of Botany Department, Ahmadu Bello University, Zaria, Nigeria where a voucher number of V/No: **900111** was obtained. The leaves were washed and air dried under shade for two weeks, ground into fine powder using a wooden mortar and pestle and stored in sealed plastic containers until required.

3.2 METHODS

3.2.1 Preparation of Plant Extracts

A successive extraction of the dried powdered leaves of *Saccharum spontaneum* was carried out for 48 hours using three different solvents; n-hexane, 95% methanol and cold maceration (Farnsworth, 1988). Water was chosen as a solvent so as to mimic the traditional style, since most of these plant parts are administered as either infusions or decoctions. Exactly 600g of the powdered leaves was soaked in 2.5 litre distilled water for 48 hr and then sieved using muslin cloth to obtain the aqueous extracts. The resulting extracts were filtered with Whatman No. 1 filter paper. Soxhlet extraction was done for hexane and methanol extracts. All extracts were concentrated using water bath maintained at a temperature of 40-45^{o}C and stored separately in labeled, sterile capped bottles at 4^{o}C for pharmacological evaluations.

3.2.2 EXPERIMENTAL DESIGN



3.3 Acute Toxicity Studies

Acute oral toxicity of the three different extracts of *Saccharum spontaneum* was tested according to the method of Lorke (1983). This was conducted in two phases using a total of sixteen male rats. In the initial phase, in the first phase, nine rats were randomly divided into 3 groups of 3 rats each. Groups 1, 2 and 3 animals were administered 10, 100 and 1000 mg/kg body weight (b.w.) of the extracts respectively. Administration was done orally by gavage to possibly establish the range of doses producing any toxic effect and the rats were observed for autonomic or behavioral responses such as spontaneous activity, irritability, urination and salivation as well as mortality for 24 hours. In the second phase, further specific doses of 1600, 2900 and 5000mg/kg b.w. of the extracts were administered to three rats (one rat per dose) to further determine the correct lethal dose (LD_{50}) value. The extracts were dissolved in distilled water and Tween 80. All animals were observed frequently on the day of treatment and surviving animals were monitored for 48 hours for signs of acute toxicity. Recovery and weight gain were seen as indications of having survived the acute toxicity.

3.4 Phytochemical Analysis

Chemical constituents of the different leaf extracts of *Saccharum spontaneum* were analyzed to detect the presence of bioactive compounds using standard procedures as described by Evans and Trease, (1996).

3.4.1 Qualitative Phytochemical Analysis

3.4.1.1 Test for Alkaloids (Dragendoff's Test)

To 0.5g of the extract, few drops of Dragendoff's reagent (solution of potassium bismuth iodide) were added. Formation of reddish brown precipitate indicates the presence of alkaloid (Evans, 1996).

3.4.1.2 Test for Flavonoids (Sodium Hydroxide Test)

Few drops of 10% sodium hydroxide were added to 0.5g of the extract. Yellow coloration indicates the presence of flavonoid (Evans, 1996).

3.4.1.3 Test for Tannins (Ferric Chloride Test)

About 0.5g of the extract was boiled in 10ml of water in a test tube and then filtered. A few drops of 0.1% ferric chloride were added and the solution was observed for brownish green or a blue-black coloration which indicate the presence of tannins (Evans, 1996).

3.4.1.4 Test for Carbohydrates (Benedict's test)

To a 0.5 ml of filtrate, 0.5ml of Benedict's reagent was added and the mixture was heated on boiling water bath for 2 minutes. A characteristic colored filtrate indicates the presence of sugar (Evans, 1996).

3.4.1.5 Test for Glycosides (Fehling's Test)

About 0.5g of the extract was dissolved in 5ml distilled water and filtered. The filtrate was hydrolyzed with dilute HCl, neutralized with alkali (NaOH) and heated with Fehling`s A and B solutions. Formation of red precipitate indicated the presence of reducing sugars as a result of hydrolysis of glycosides (Evans, 1996).

3.4.1.6 Test for Triterpenoids (Liberman Bucchard Test)

About 0.5g of the extract was dissolved in 1ml of chloroform. 1ml of acetic anhydride was added, followed by the addition of 2ml of concentrated sulphuric acid. Formation of reddish violet color indicates the presence of triterpenoids.

3.4.1.7 Test for Anthraquinones (Bontrager's Test)

About 0.5g of the extract was boiled with 10ml of H_2SO_4 and filtered while hot. The filtrate was shaken with 5ml of chloroform, the chloroform layer was pipetted into another test tube and 1ml of dilute ammonia was added. The resulting solution was observed for color changes. Bright pink color indicates the presence of free anthraquinones (Evans, 1996).

3.4.1.8 Test for Saponins (Frothing Test)

About 0.5g of extract was added to 5ml of distilled water in a test tube and the solution was shaken vigorously and observed for a stable persistent froth. The frothing was mixed with 3 drops of olive oil and shaken vigorously after which it was observed for the formation of an emulsion (Evans, 1996).

3.4.1.9 Test for Phenolics

About 0.5g of extract was boiled with 10 ml of distilled water for 5mins and filtered while hot. Then 1 ml of ferric chloride solution was added. Formation of blue-black or brown coloration indicated the presence of phenol (Evans, 1996).

3.4.1.10 Test for Steroids

About 0.2g of extract was dissolved in 2ml of chloroform. 0.2ml of concentrated H_2SO_4 was carefully added to form a layer. A reddish-brown color at the interphase between the layers indicates the deoxy-sugar characteristics of cardenolides which indicates the presence of steroid (Evans, 1996).

3.4.2 Quantitative Phytochemical Analysis

3.4.2.1 Estimation of Alkaloids

One gram of the extract was weighed into a 250ml beaker and 200ml of 10% acetic acid in ethanol was added, covered and allowed to stand for 4 hours. The mixture was filtered and the

extract was concentrated on a water bath to one quarter of the original volume. Concentrated NH_4OH was added by drop wise to the extract until the precipitation was complete. The whole solution was allowed to settle and the precipitate was collected and washed with dilute NH_4OH and then filtered. The residue is the alkaloid, which was dried and weighed (Harborne**,** 1973**).**

3.4.2.2 Estimation of Flavonoids

About 10g of plant sample was repeatedly extracted with 100ml of 80% aqueous methanol at room temperature. The whole solution was filtered through Whatman No. 42 (125mm) filter paper into a pre-weighed 250ml beaker. The filtrate was transferred into a water bath and allowed to evaporate to dryness and weighed to a constant weight (Bohm and Kocipal- Abyazan, 1994).

3.4.2.3 Estimation of Saponins

Twenty grams of the plant material was put into a conical flask and 100ml of 20% aqueous ethanol was added. The sample was heated over a hot water bath for 4 hours with continuous stirring at about 55^{o}C. The mixture was filtered and the residue re-extracted with another 200ml, 20% ethanol. The combined extract was reduced to 40 ml over water bath at about 90^{o}C. The concentrate was transferred into a 250 ml separating funnel and 20ml of diethyl ether was added and shaken vigorously. The aqueous layer was recovered while the other layer was discarded. The process was repeated and 60 ml of n-butanol was added. The combined n-butanol extracts was washed twice with 10 ml of 5% aqueous sodium chloride. The remaining solution was heated in water bath. After evaporation the samples was dried in the oven to a constant weight; the saponin content was calculated in percentage (Obadoni and Ochuko, 2001).

3.4.2.4 Estimation of Tannins

About 500 mg of the sample was weighed into a 50 ml plastic bottle and 50 ml of distilled water was added and shaken for 1 hour on a mechanical shaker. This will be filtered into a 50 ml volumetric flask and made up to the mark. Then 5ml of the filtrate is pipetted into a test tube and mixed with 2 ml of 0.1M $FeCl_3$ in 0.1N HCl and 0.008M potassium ferrocyanide. The absorbance was measured at 120 nm within 10 min (Van-Burden and Robinson, 1981).

3.4.2.5 Estimation of Total Phenols by Spectrophotometeric Method

The leaf sample was boiled with 50 ml of ether for the extraction of the phenolic component for 15 min. Five ml of the extract was pipetted out into a 50 ml flask, then 10 ml of distilled water was added. Two ml of NH_4OH solution and 5 ml of concentrated amyl alcohol were also added. The samples were made up to mark and left to react for 30 mins for colour development. This was read at 505 nm.

3.5 In Vivo Gastro-Protective Effect of Aqueous, Methanol and Hexane Leaf Extracts of Saccharum spontaneum

3.5.1 Animal Grouping

Animals were treated with the extracts and standard drug following the dose and mode of administration of Berenguer *et al.,* (2006) for a period of two weeks and fasted 48 hours prior to ulcer induction. Forty five (45) adult male albino rats (150-200g) were randomly divided into nine (9) groups, each consisting of five rats.

Group 1: (normal control): Rats were treated with Vehicle (water). This group served as normal control group.

Group 2: (negative control): Rats were administered Aspirin (200mg/kg b.w, p.o). This group served as ulcer control group.

Group 3: (positive control): Rats were pretreated with the reference drug omeprazole (10mg/kg b.w, p.o.) and administered Aspirin (200mg/kg b.w, p.o). This group served as standard control group.

Group 4: Rats were pretreated with aqueous extract *(*200mg/kg b.w, p. o.) and administered Aspirin (200mg/kg b.w, p.o).

Group 5: Rats were pretreated with aqueous extract (400mg/kg b.w, p. o.) and administered Aspirin (200mg/kg b.w, p.o).

Group 6: Rats were pretreated with methanol extract (200mg/kg b.w, p. o.) and administered Aspirin (200mg/kg b.w, p.o).

Group 7: Rats were pretreated with methanol extract (400mg/kg b.w, p. o.) and administered Aspirin (200mg/kg b.w, p.o).

Group 8: Rats were pretreated with hexane extract (200mg/kg b.w, p. o.) and administered Aspirin (200mg/kg b.w, p.o).

Group 9: Rats will be pretreated with hexane extract (400tmg/kg b.w, p. o.) a and administered Aspirin (200mg/kg b.w, p.o).

3.5.2 Induction of Gastric Ulcer

The non-steroidal anti-inflammatory drug, Aspirin was used as the ulcerogenic agent at the dose of 200mg/kg body weight (Rao *et al*., 2004). Four hours after Aspirin was administered, rats were anesthesized with chloroform and sacrificed by cervical dislocation using clean razor blade. The stomach of each rat was cut open along the greater curvature and the gastric portion was

removed and washed in physiological saline solution. Ulcers were assessed in the glandular portion of the stomach using the modified method of Magistretti *et al.* (1988).

3.5.3 Collection of Gastric Juice

The stomach was carefully opened along the greater curvature, keeping the oesophagus closed and the gastric contents were collected in plain tubes. The mucosa was flushed with saline and observed for gastric lesions using magnifying lens. Gastric tissues were also harvested for further studies.

3.5.4 Estimation of Ulcer Index

Ulcer scoring was done by viewing ulcers with a magnifying glass. Gastric lesions in the glandular region of the stomach were located and an arbitrary scoring system of 0-6 point scale was used to grade the incidence and severity of the lesions as described by the modified Magistretti *et al.* (1988).

0 = No lesion, 1 = 1 - 3 small lesions, 2 = 1 - 3 large lesions, 3 = 1 - 3 thick lesions, 4 = More than 3 small lesions, 5 = More than 3 large lesions, 6 = More than 3 thick lesions. The sum of the total severity of scores in each group of rats divided by the number of animals was expressed as the mean ulcer index (UI).

3.5.5 Percentage Inhibition (PI)

The percentage protection index was calculated according to the method described by (Hano *et al*., 1976) as follows:

$$\%\ Inhibition = \frac{Mean\ ulcer\ index\ (control)\ -\ Mean\ ulcer\ index\ (treated\ group}{Mean\ ulcer\ index\ (control)} \times 100$$

3.5.6 Biochemical Determination of Gastric Secretions.

3.5.6.1 Determination of Gastric Juice Volume

Gastric juice obtained was centrifuged at 3000rpm for 10mins and supernatant. The resulting supernatant was decanted and volume determined and expressed in milliliter/100g b.w (Telesphore *et al.,* 2005).

3.5.6.2 Estimation of Gastric Juice pH

An aliquot of 1ml of the supernatant liquid was pipetted out from each test tube. The pH of the supernatant was determined with the aid of a digital pH meter.

3.5.6.3 Estimation of Free Acidity

One milliliter (1ml) of the supernatant liquid was pipetted and diluted with 1ml distilled water. Two (2) drops of Topfer's reagent was added as an indicator. Free acidity of gastric juice was estimated by titration with 0.01N sodium hydroxide until canary yellow colour is observed. The volume of 0.01N NaOH consumed was noted and free acidity, expressed in mEq/liter of body weight was calculated by the following formula (Prino *et al*., 1971).

$$Free\ acidity = \frac{Volume\ of\ NaOH \times N}{0.1} \times 100$$

3.5.6.4 Determination of Total Acid Output

An aliquot of 1ml gastric juice was diluted with 1ml distilled water into a 50ml conical flask. Two drops of phenolphthalein indicator were added to it and titrated with 0.01N NaOH until a permanent pink colour is observed. The volume of 0.01N NaOH consumed was noted. Total acid output was calculated by the same formula for the determination of free acidity. (Oser, 1965).

3.6 *In vitro* Proton-potassium (H^+-K^+) ATPase Inhibitory Activity of Methanol Extract of *Saccharum spontaneum*

3.6.1 Preparation of Gastric Microsomes

Proton-potassium ATPase was prepared from mucosal scrapings of rats according to the method reported by Reyes-Chilpa *et al*., (2006) with necessary modifications. Immediately after opening the stomach, the mucosal layer of the fundus was scrapped and homogenized in 2ml of Tris–HCl (20mM, pH 7.4). The homogenate obtained was centrifuged for 10min at 14500xg and the resulting supernatant was subsequently centrifuged at 20000xg for 60min. The microsomal pellets were re-suspended in homogenization buffer. The parietal cell extracts obtained were used to determine the H^+/K^+-ATPase activity.

3.6.2 Determination of Protein Concentration

The protein concentration of the preparation (parietal cell extract) was determined by the method of Biuret using albumin as standard.

Principle; **Copper + Protein** $\xrightarrow{\text{Alkaline pH}}$ **Copper-protein complex**

Procedure; Biuret reagent was prepared by adding 1.5g of copper (II) sulphate pentahydrate and 6g of sodium potassium tartrate to 500 ml water, followed by the addition of 300 ml 10% (w/v) NaOH. The resulting solution was then brought to a total volume of 1 litre with water and 1g of potassium iodide was added to inhibit reduction of copper. A series of protein standards (albumin) ranging in concentration from 0.1 to 1 mg/ml was prepared and dilutions were made to 1ml solution. To 1 ml of each standard and parietal cell extract, 4 ml of Biuret reagent was added, the solution was mixed well and incubated at room temperature for 30 minutes. Absorbances of the samples were measured at 540 nm. A Standard curve was plotted, from which the protein concentration of the microsomal extracts was extrapolated.

3.6.3 Assay of H^+/K^+-ATPase

The substrate (20 ml) was prepared by weighing 220 mg of ATP into a 20 ml volumetric flask and making up the volume to the 20 ml mark with distilled water. Stock solutions of 1000 µg/ml of both methanolic extract and standard drug (omeprazole) was prepared, from which serial dilutions were made to obtain varying concentrations (10, 30, 50, 100 and 200 µg/ml) respectively. The enzyme source of 100 µL was added to test tubes containing 50 µL of different concentrations of the methanolic extract or standard drug (omeprazole) in water and pre-incubated at 37ºC for 60 minutes. After incubation, the reaction mixture was added with 200 µL of Tris–HCl buffer (20 mM, pH 7.4) containing ; 2 mM HEPES; 1 mM EDTA; 0.1 mM Li_2CO_3; 2 mM $MgCl_2$; 2 mM KCl; 2 mM ATP and incubated at 37ºC for 30 min. The reaction was terminated by the addition of 1 ml of 10% TCA followed by centrifugation at 2000xg for 10mins. An aliquot of the supernant was pipetted into 96 well plates and absorbance readings (A) were recorded at 405 nm by micro-plate reader and compared to a control which had the methanolic extract replaced by DMSO and distilled water mixture in equal volume. The assay was performed in triplicates and results were averaged. Percentage inhibition was calculated as:

% Inhibition = [(Absorbance control-Absorbance extract)/Absorbance control] x 100

The concentrations of test compounds (extract and standard drug) which inhibited the hydrolysis of ATP by 50 % (IC_{50}) were determined graphically using the concentration/inhibition plots.

3.7 Histopathological Studies

Hisotological examination of the gastric mucosa was carried out according to the method described by Luna (1960). Gastric tissue samples from each group were fixed in 10 per cent formalin. Tissue sections of the stomach were routinely processed in alcohol (70, 95, and 100%) using a tissue processor. The processed tissues were embedded in paraffin blocks, sectioned at

5μm using a rotary microtome and stained with hematoxylin and eosin. The histological sections were examined under microscope and then photographed at magnification of 100.

3.8 Statistical Analysis

Data obtained was expressed as mean ± standard deviation. Significance difference between the control and treated groups was tested using one-way analysis of variance (ANOVA), followed by Duncan Multiple Range Test (DMRT) for multiple comparisons using SPSS version 20 software (Statistical Package for Social Sciences, Inc., Chicago, IL, USA). Values of $p<0.05$ were regarded as statistically significant.

CHAPTER FOUR

4.0 RESULTS

4.1 Extract yield

The yields of crude extracts of *S. spontaneum* leaves are shown in Table 4.1. Aqueous extraction gave the highest yield of 19.83% followed by methanol (5.43%) and then hexane which gave the lowest yield of 2.70%.

Table 4. 1. Percentage Yields of Crude Extracts of *Saccharum spontaneum* Leaves.

Extract	Percentage Yield % (w/w)
Aqueous	19.83
Methanol	5.43
Hexane	2.70

4.2 Acute Toxicity of Aqueous, Methanol and Hexane Extracts of *S. Spontaneum* Leaves:

The result of oral acute toxicity of the aqueous, methanol and hexane leaf extract of *S. spontaneum* in rats showed no mortality recorded in the 24hrs and 48hrs period for both phases of the study (Table 4.2). However, a slight increased in activity, loss of appetite, reduced reaction to noise and resting at the corner of cages was observed after administration of the extracts at higher doses. Thus, The LD_{50} of the aqueous, methanol and hexane extracts of *S. spontaneum* were found to be greater than 5000 mg/kg b.w, indicating the margin of safety of the plant at a high dose as shown in Table 4.3.

Table 4. 2. Signs of Toxicity in Wistar Rats Following Administration of Different doses of *S. Spontaneum* Leaves Extracts.

Parameter	Dose (mg/kg bw)					
	10	100	1000	1600	2900	5000
Activity	+	+	+	+	++	+ +
Loss of appetite	+	+	+	+	++	++
Social interaction	+	+	+	+	+	+
Reaction to noise	+	+	+	+	-	-
Reaction to touch	+	+	+	+	+	+
Erect tail	+	+	+	+	+	+
Salivation	+	+	+	+	+	+
State of excrement	G	G	G	G	G	G

−= Slight Decrease; + = Normal; ++ = Slight Increase; G = Granular

Table 4. 3. Median Lethal Dose (LD_{50}) of Aqueous, Methanol and Hexane Leaves Extracts of *S. spontaneum*.

Experiment	Dose (mg/kg bw)	No. of rat	No. of dead rat after 24hrs	No. of treated rats after 24hrs	Percentage Lethality (%)
Phase I	10	3	0/3	0/3	0
	100	3	0/3	0/3	0
	1000	3	0/3	0/3	0
Phase II	1600	3	0/1	0/1	0
	2900	3	0/1	0/1	0
	5000	3	0/1	0/1	0

LD_{50} > 5000mg/kg b.w

4.3 Qualitative Phytochemical Screening of *S. spontaneum* Leaves Extracts

The preliminary qualitative phytochemical analysis of the leaves of aqueous, methanol and hexane extracts of *S. spontaneum* showed the presence of alkaloids, triterpenes, glycosides and carbohydrates. Athraquinones and proteins were not found in all the solvent extracts (Table4.4). Also, steroids, flavonoids, saponins, tannins and phenols were detected in the aqueous and methanol extracts only but not the hexane extract.

Quantitative estimations of the phytoconstituents revealed alkaloids, flavonoids, saponins, tannins and phenols in considerable percentages in the aqueous, methanol and hexane leaf extracts (Table 4.5)

Table 4. 4. Preliminary Phytochemical Contents of *S. spontaneum* Leaves Extracts.

S/N	Phytoconstituents	Solvent extracts		
		Aqueous	Methanol	Hexane
1	Alkaloids	+	+	+
2	Triterpenes	+	+	+
3	Athraquinones	-	-	-
4	Saponins	+	+	-
5	Glycosides	+	+	+
6	Carbohydrates	+	+	+
7	Steroids	+	+	-
8	Flavonoids	+	+	-
9	Tannins	+	+	-
10	Phenols	+	+	-

+ = Present; - = Absent

Table 4. 5. Phytochemical Estimations of *S. spontaneum* Leaves Extracts.

S/N	Phytochemicals	Aqueous (%)	Methanol (%)	Hexane (%)
1	Alkaloids	4.19	5.01	1.14
2	Flavonoids	7.17	10.13	2.38
3	Saponins	8.24	5.78	NIL
4	Tannins	4.12	12.07	1.26
5	Phenols	3.91	13.89	NIL

4.4 *In Vivo* Gastroprotective Studies of *Saccharum spontaneum* Leaves Extracts.

4.4.1 Effect of Different Doses of *S. spontaneum* Leaves Extracts on Gastric Ulcer Index.

Pretreatment of rats with the aqueous, methanol and hexane extracts at 200 and 400 mg/kg body weight for fourteen days reduced the severity of gastric lesions caused by aspirin in a dose dependent manner. Methanol extract at 400 mg/kg body weight showed a significant effect ($P<0.05$) similar to that of the standard (omeprazole) drug as shown in Table 4.6. Lower doses however produced an insignificant gastroprotective effect with hemorrhages and few ulcerative lesions in some rats when compared with rats that were pretreated with omeprazole. Hence, methanol extract had the highest protective effect.

Table 4. 6. Protective Effects of Different Doses of *S. spontaneum* Leaves Extracts on Gastric Lesion Induced by Aspirin.

Groups	Treatment	Dose (mg/kg bw)	Mean Ulcer index (mm)
I	Water		NIL
II	Aspirin	200	5.75 ± 0.50^{a}
III	Omeprazole + Aspirin	10	1.5 ± 0.58^{b}
IV	AESS + Aspirin	200	3.00 ± 1.41^{b}
V		400	2.50 ± 1.29^{b}
VI	MESS + Aspirin	200	2.75 ± 0.50^{b}
VII		400	2.00 ± 0.96^{b}
VIII	HESS + Aspirin	200	2.75 ± 0.96^{b}
XI		400	2.25 ± 0.82^{b}

Values are mean ± SD of 4 rats in each group. Different superscript in the same column are Significant at ($P<0.05$) using one way analysis of variance (ANOVA) followed by Duncan Multiple Range Test (DMRT). AESS = Aqueous extract of *S. spontaneum*, MESS = Methanol extract of *S. spontaneum* and HESS = Hexane extract of *S. spontaneum.*

NB; Animals were treated with extracts for 14 days before administration of Aspirin.
a = No statistical significance with Aspirin treated group at $p<0.05$.
b = Significantly different from Aspirin group but no statistical significance with omeprazole and extract treated group at $p<0.05$.

4.4.2 Percentage Inhibition of Ulceration of *S. spontaneum* Leaves Extracts**.**

Comparison of ulcer protection of the leaf extracts of *S. spontaneum* with that of positive control (Omeprazole) is shown in Figure 4.1. Based on the result obtained, *S. spontaneum* inhibited gastric acid secretion with the maximum protection (65.22%) of gastric erosion of the test groups observed in rats pretreated with 400mg/kg b.w of the methanol extract when compared to omeprazole (73.91%) at dose of 10mg/kg body weight. This is followed by hexane extract at 400 mg/kg with percentage inhibition of 60.87%, with no statistical significant ($P<0.05$) difference between the two. Also, the aqueous (400 mg/kg b.w), methanol (200 mg/kg b.w) and hexane (200 mg/kg b.w) extracts showed a remarkable percentage protection of 56.52, 52.17 and 52.17% respectively. The least percentage inhibition of ulceration (47.83%) was observed with the aqueous extract at dose of 200 mg/kg bw.

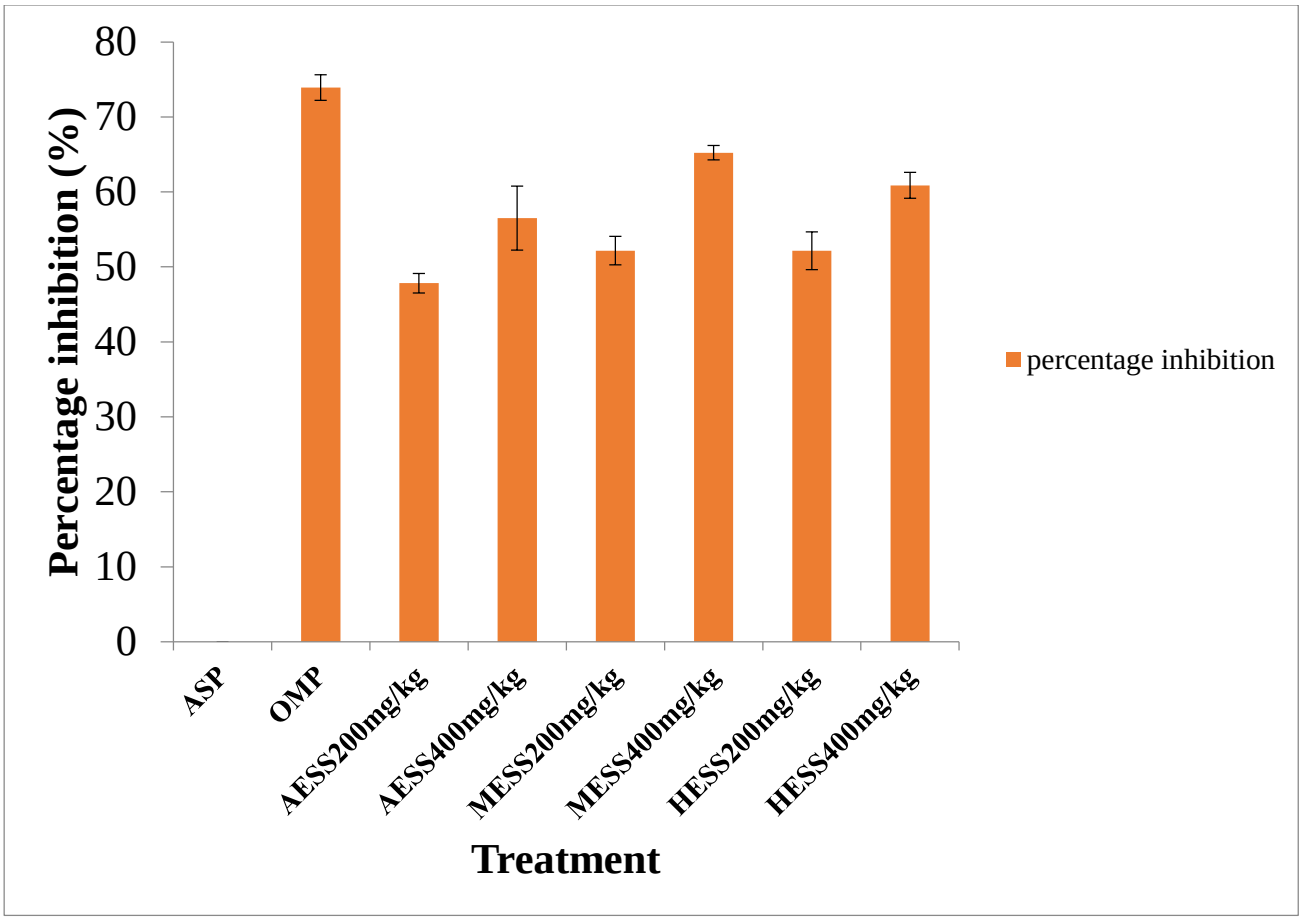


Fig 1; Percentage Inhibition of Different Doses of *S. spontaneum* Leaf Extracts on Aspirin induced Gastric Lesion in Wister Rats

4.4.3. Antisecretory effects of *S. spontaneum* Leaves Extracts in Aspirin Induced Gastric Lesion

The gastroprotective effect of *S. spontaneum* leaf extracts was observed on gastric secretions in aspirin induced gastric damage in rats. An increased in gastric volume of (3.15 ± 0.13) was observed in rats administered with aspirin (200 mg/kg b.w). Administration of *S. spontaneum* extracts showed a diminution of the gastric secretion by a decrease in volume of gastric fluid when compared to the values obtained in the aspirin induced ulcer group (Table 4.7). The decrease in the volume of gastric juice produced by methanol (200 and 400 mg/kg b.w) and aqueous (400 mg/kg b.w) extracts were found to be 2.31 ± 0.70, 2.06 ± 0.62 and 2.48 ± 0.41 respectively and were comparable to the effect exerted by omeparazole treated group (2.43 ± 0.43), all showing a significant difference at $P<0.05$ with respect to the aspirin induced ulcer group.

Tabale 4.8 shows the effect of varying doses of *S. spontaneum* extracts on gastric pH in aspirin induced ulceric rats. From the Table, All the extracts of *S. spontaneum* showed a dose dependant significant ($P<0.05$) increase in gastric pH against aspirin induced gastric ulcer. An increase in gastric pH more than the omeprazole treated group was observed with the methanol extract at dose of 400 mg/kg b.w.

Table 4. 7. Protective Effects of Different Doses of *S. spontaneum* Leaves Extracts on Gastric Volume of Wistar Rats in Aspirin Induced Gastric Lesions.

Groups	Treatment	Dose (mg/kg bw)	Gastric Volume (ml)
I	Water		1.78 ± 0.28^{a}
II	Aspirin	200	3.15 ± 0.30^{d}
III	Omeprazole + Aspirin	10	2.43 ± 0.43^{bc}
IV	AESS + Aspirin	200	2.57 ± 0.32^{bcd}
V		400	2.48 ± 0.41^{bc}
VI	MESS + Aspirin	200	$2.31 \pm 0.\ 70^{abc}$
VII		400	2.06 ± 0.62^{ab}
VIII	HESS + Aspirin	200	2.83 ± 0.13^{cd}
XI		400	2.75 ± 0.21^{cd}

Values are mean ± SD of 4 rats in each group. Different superscript in the same column are Significant at ($P<0.05$) using one way analysis of variance (ANOVA) followed by Duncan Multiple Range Test (DMRT). AESS = Aqueous extract of *S. spontaneum*, MESS = Methanol extract of *S. spontaneum* and HESS = Hexane extract of *S. spontaneum.*

NB; Animals were treated with extracts for 14 days before administration of Aspirin.
a = No statistical significance with normal control at $p<0.05$
b = Significant different from Aspirin but no statistical significance with Omeprazole and other groups at $p<0.05$.
c = No statistical significance between extract treated groups at $p<0.05$
d = No statistical significance with Aspirin treated group at $p<0.05$

Table 4. 8. Protective Effects of Different Doses of *S. spontaneum* Leaves Extracts on Gastric pH of Wistar Rats in Aspirin Induced Gastric Lesions.

Groups	Treatment	Dose (mg/kg bw)	Gastric Ph
I	Water		5.05 ± 0.55^{cd}
II	Aspirin	200	2.01 ± 0.14^{a}
III	Omeprazole + Aspirin	10	5.17 ± 0.34^{d}
IV	AESS + Aspirin	200	3.75 ± 0.55^{b}
V		400	4.84 ± 0.75^{cd}
VI	MESS + Aspirin	200	4.45 ± 0.62^{bcd}
VII		400	5.24 ± 0.59^{d}
VIII	HESS + Aspirin	200	3.66 ± 0.79^{b}
XI		400	4.20 ± 0.52^{bc}

Values are mean ± SD of 4 rats in each group. Different superscript in the same column are Significant at ($P<0.05$) using one way analysis of variance (ANOVA) followed by Duncan Multiple Range Test (DMRT). AESS = Aqueous extract of *S. spontaneum*, MESS = Methanol extract of *S. spontaneum* and HESS = Hexane extract of *S. spontaneum.*

NB; Animals were treated with extracts for 14 days before administration of Aspirin.
a = No statistical significance with Aspirin group at $p<0.05$
b = Significant different from Aspirin group at $p<0.05$.
c = No statistical significance between extract treated groups at $p<0.05$
d = No statistical significance with Omeprazole, Aqueous (400mg/kg b.w) and Methanol (200 & 400mg/kg. b.w) treated groups at $p<0.05$

The antisecretory effects of *S. spontaneum* leaf extracts on free and total acidity in aspirin induced gastric mucosal damage are shown in Figure 4.2 and 4.3 respectively. Pretreatment of the extracts showed a significant ($P<0.05$) decrease in the above parameters as compared to the aspirin induced ulceric rats. The decrease in acidity was at its maximum level for the reference standard (omeprazole) group followed by methanol extract (400mg/kb b.w) treatment group. The least decrease in acidity was observed by the aqueous extract at 200mg/kg dose. These results showed that all doses of *S. spontaneum* extracts had a protective effect against gastric mucosal lesions caused by aspirin but the higher doses had a better antisecretory effect and were comparable to the standard group.

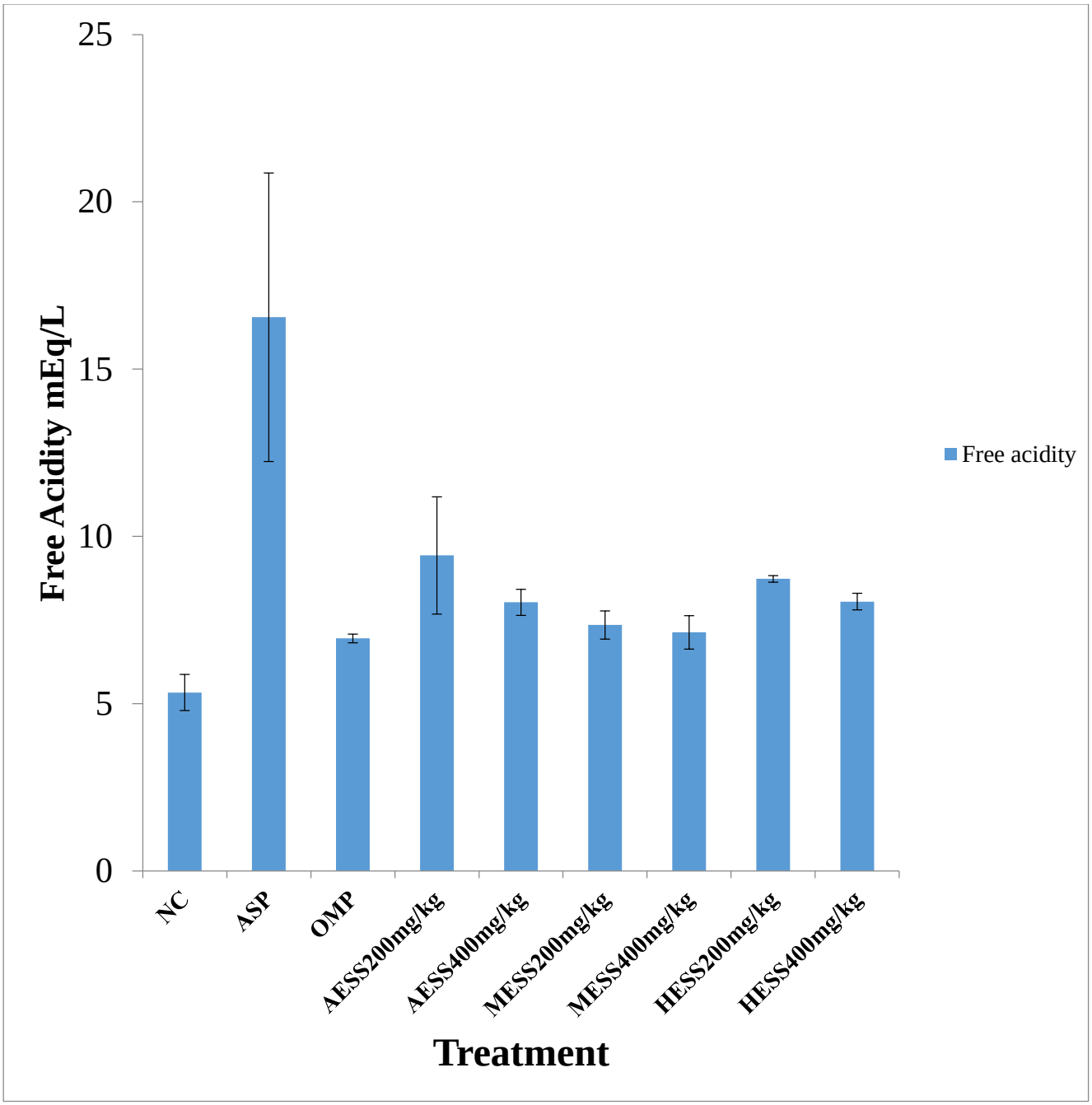


Figure 4.2. Antiacid Secretory Effect of Different Doses of *S. spontaneum* on Aspirin Induced Gastric Lesion in Wister Rats.

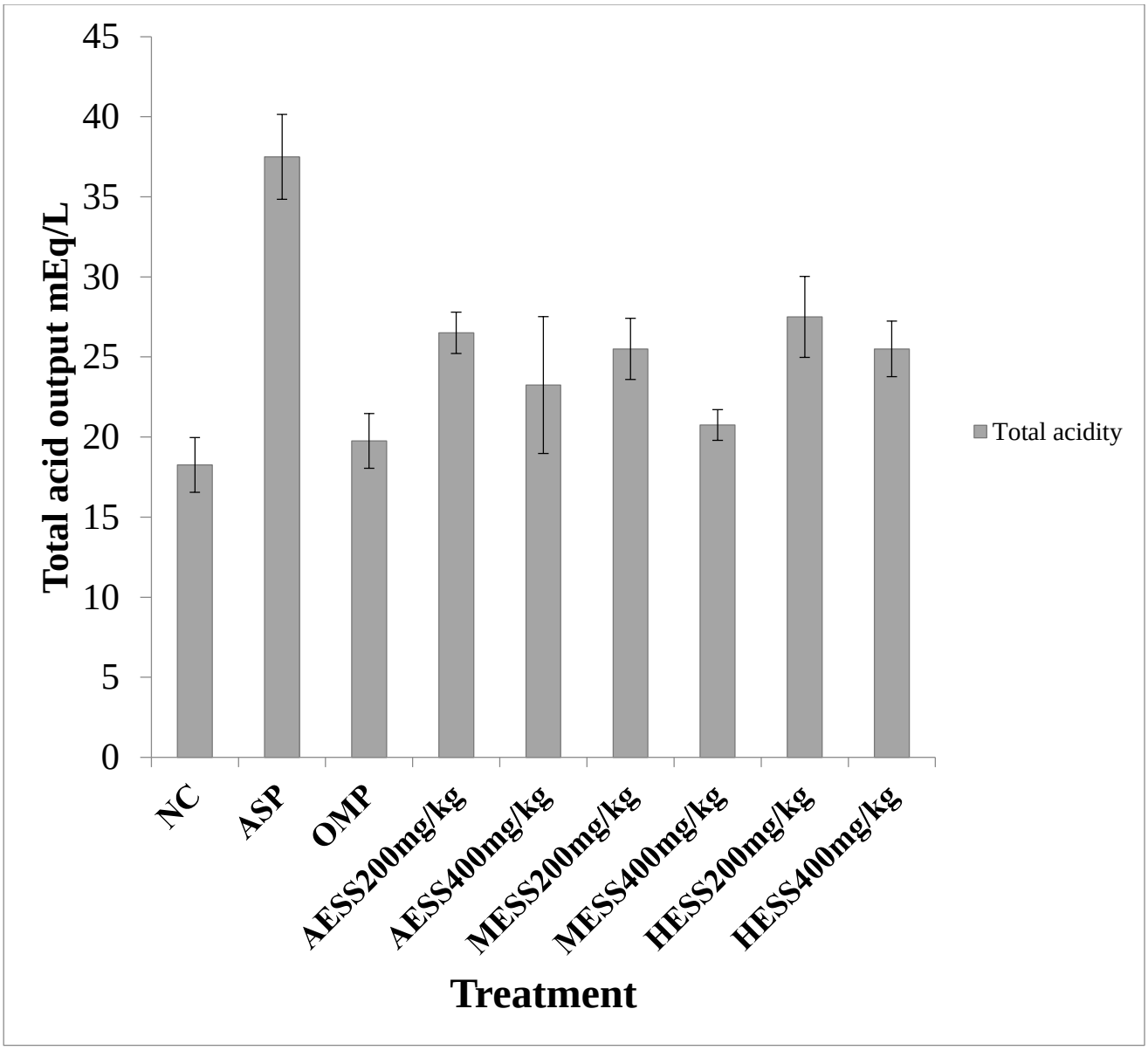


Figure 4.3. Antiacid Secretory Effect of Different Doses of *S. spontaneum* on Aspirin Induced Gastric Lesion in Wister Rats.

4.5 Histopathological Examination

The gastroprotective effect of *S. spontaneum* was evaluated by microcsopic examination of rats gastric mucosa. Plate 4.1a shows a normal histological structure of the gastric mucosa. Oral administration of aspirin (200mg/kg b.w) resulted in severe mucosal necrosis, congestion of blood vessels, hemorrhages, loss of lining epithelium and multiple ulcers along with inflammatory process characterized by neutrophils infiltration (Plate 4.1b). Administration of omeprazole (10mg/kg b.w) showed a better protection of gastric mucosa as seen by mild blood vessels congestion and mild tissue infiltrations with no hemorrhage (Plate 4.1ci and ii). Similarly, mild superficial excoriation on lining epithelium with slight apical necrosis and no hemorrhage was observed in rats pretreated with methanol (400mg/kg b.w) and hexane (200 and 400mg/kg b.w) extracts of *S. spontaneum* (Plate 4.1g, h, and i). Changes in the gastric wall as well as discontinuity of the epithelial lining in the glandular region with no ulcer craters was also observed in rats administered lower doses of the extracts (Plate 4.1d, e and f).

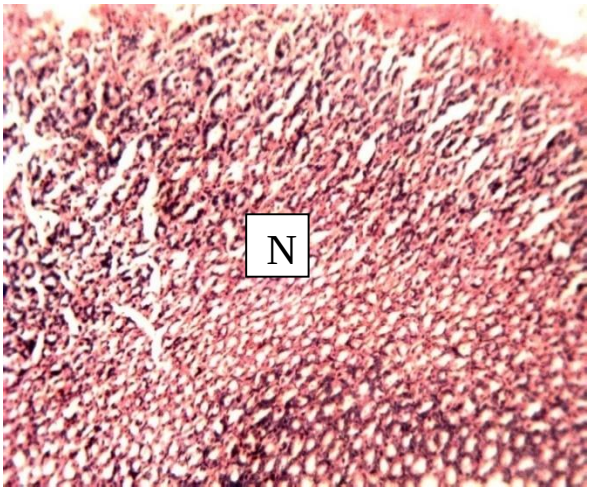


Plate 4.1a; Normal features of gastric mucosa. H & E. x100

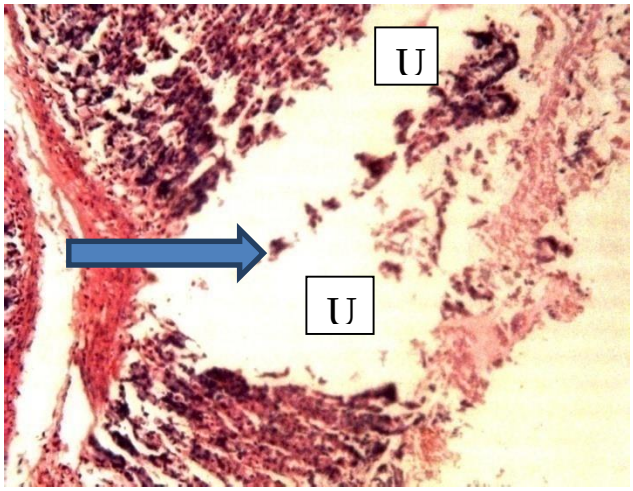


Plate 4.1b; **Aspirin 200mg/kg.** Intense mucosal necrosis, loss of lining epithelium, hemorrhages, infiltration and deep ulceration. H & E. x100

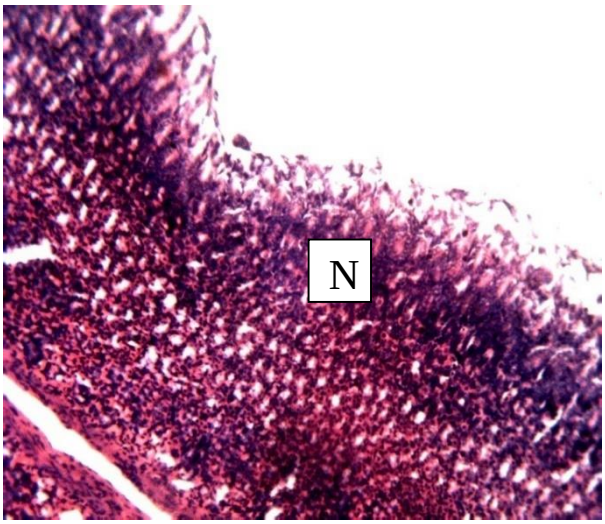


Plate 4.1c i. **Omeprazole 10mg/kg + Aspirin.** Mild blood vessels congestion and tissue infiltrations with no ulcer. H & E. x100

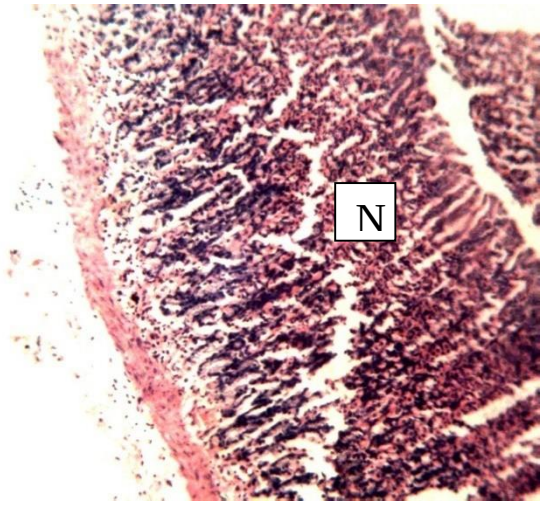


Plate 4.1c ii. **Omeprazole 10mg/kg + Aspirin.** Mild blood vessels congestion and tissue infiltrations with no ulcer. H & E. x100

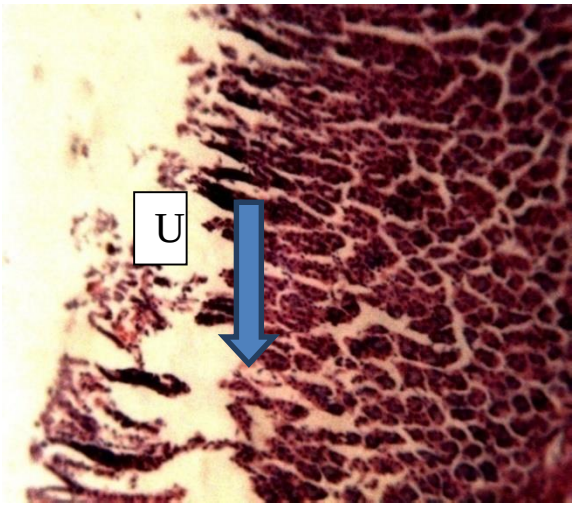


Plate 4.1d; **AESS 200mg/kg + Aspirin.** Slight apical necrosis and moderate ulceration of mucosa lining. H & E. x100

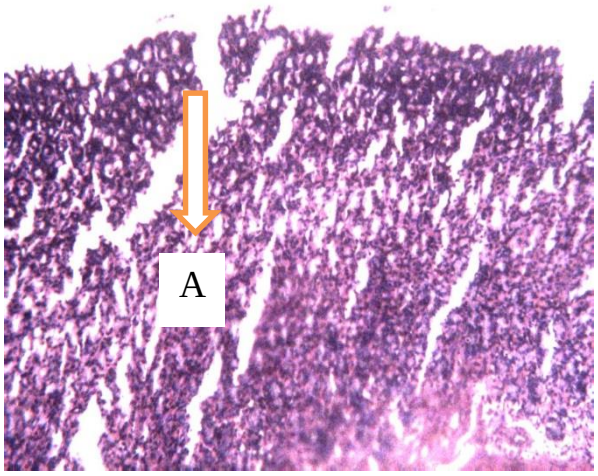


Plate 4.1e; **AESS 400mg/kg + Aspirin.** Slight mucosa epithelial changes. H & E. x100

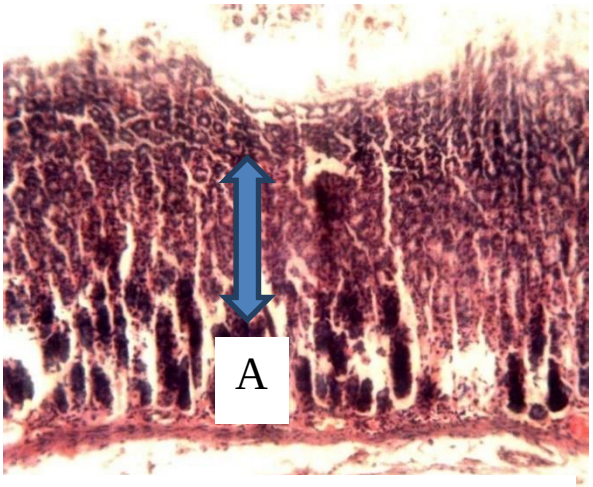


Plate 4.1f; **MESS 200mg/kg + Aspirin.** Mild superficial excoriation on lining epithelium, slight apical necrosis and mild haemorrhage. H & E. x100

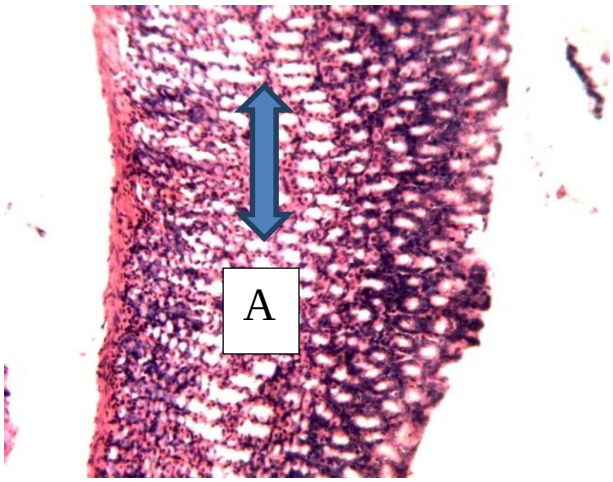


Plate 4.1g; **MESS 400mg/kg + Aspirin.** Mild superficial excoriation on lining epithelium, slight apical necrosis with no haemorrhage. H & E. x100

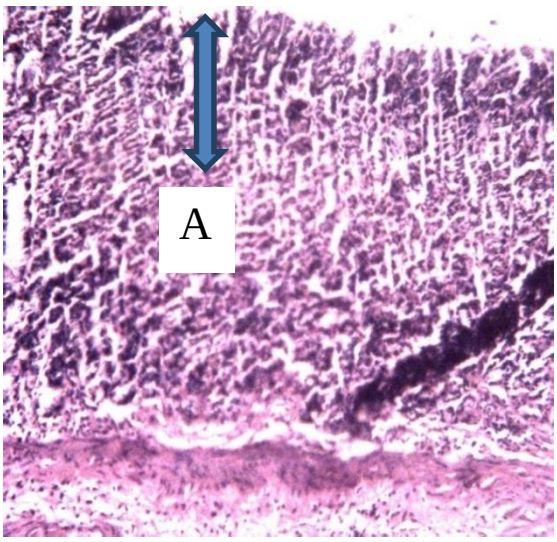


Plate 4.1h; **HESS 200mg/kg + Aspirin.**Slight mucosa epithelial change with no ulceration. H & E.

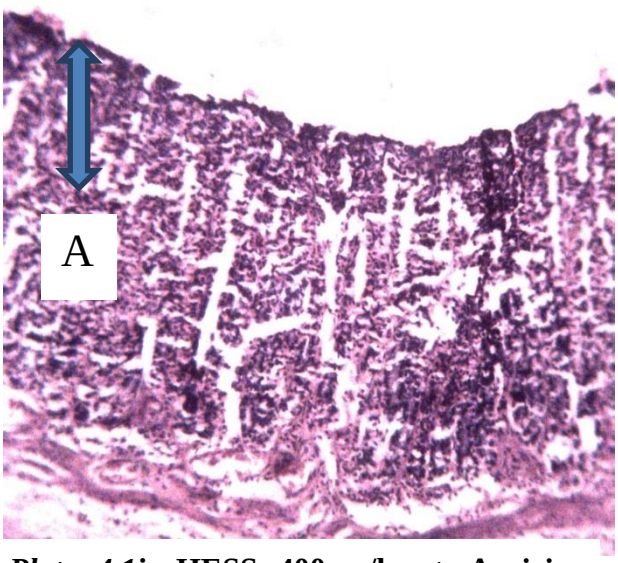


Plate 4.1i; **HESS 400mg/kg + Aspirin.** Moderate superficial abrasion of of submucosal layer. H & E. x100

Plate 4. 1; Photomicrographs of control and experimental animal showing the protective effect of 200 and 400mg/kg body weight of *S. spontaneum* leaf extracts on gastric mucosa of Wister rats. Normal control group (*4.1a*); ulcer control group (*4.1b*); omeprazole pretreated group (*4.1ci & ii*); aqueous extract pretreated group (*4.1d & e)*; methanol extract pretreated group (*4.1f & g*); hexane extract pretreated group (*4.1h & i*); (H & E x100).

N = normal; U = ulceration; A = Area

4.6. Inhibitory Effect of Methanol Extract of *S. spontaneum* on Gastric Proton Pump (H^+/K^+-ATPase) Activity.

The inhibitory effect of methanol extract of *S. spontaneum* and omeprazole on the H^+/K^+-ATPase isolated from Wister rat gastric microsomes when compared with the control group is shown in Fig. 4.5. The incubation of varying concentrations of the extract (10–200µg/ml) with the microsomes, showed a significant ($P<0.05$) inhibitory effect on the enzyme activity. The inhibitory activity was concentration-dependent and no inhibition was observed at 10µg/ml of the extract. Omeprazole (10-200µg/ml) used as positive control showed a higher inhibitory effect (86.67%) at 200µg/ml which was significant ($P<0.05$) when compared with the extract (61.05%) at the same concentration. The minimum inhibitory concentrations (IC_{50}) of the methanol extract and omeprazole on the enzyme was 116.41 ± 41.9810 µg/ml and 37.79 ± 0.10 µg/ml respectively (Table 4.9). The protein concentration of the isolated gastric mucosal homogenate was 31.501 µM/mg protein.

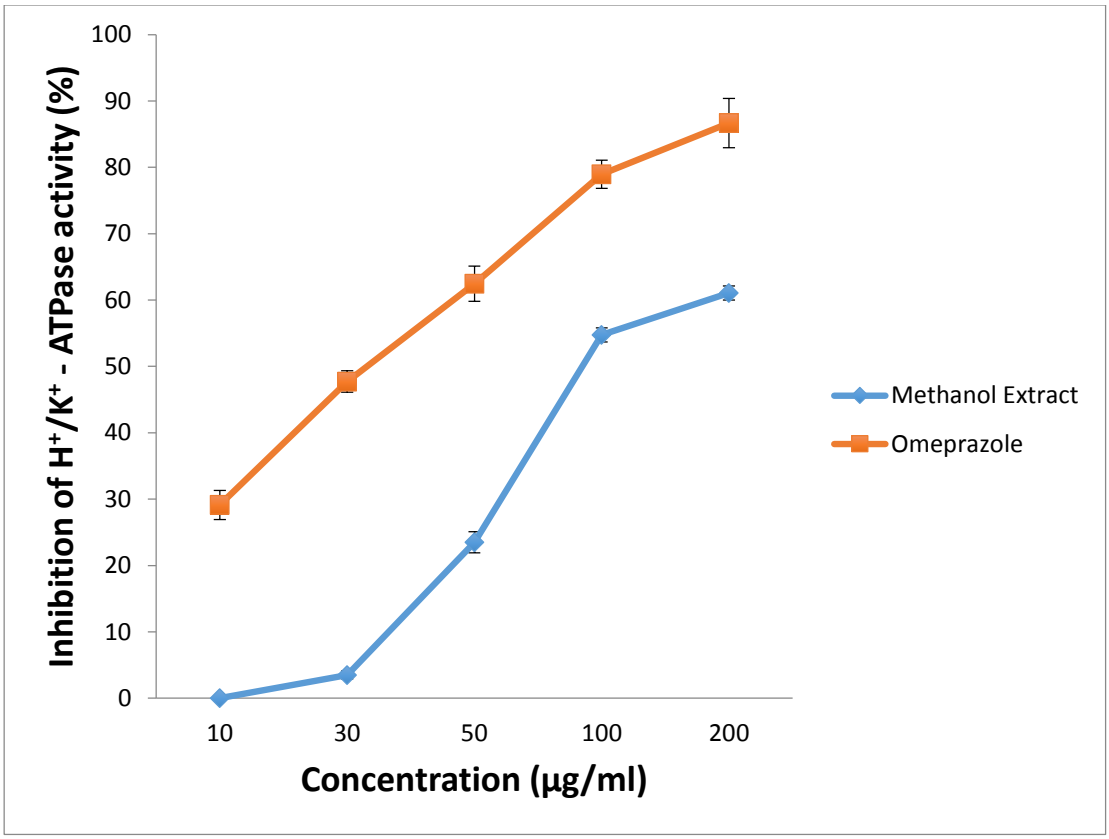


Fig. 4.4. Inhibitory Effect of Different Concentrations of Methanol Extract of *S.spontsneum* and Omeprazole on H^+/K^+ - ATPase activity in Wistar Rats Gastric Microsomes.

Table 4. 9. IC_{50} Values for the Inhibition of H^+/K^+- ATPase Activity by Methanol Extract of *S. spontaneum* Leaves.

Treatment	IC_{50} (μg/ml)
Methanol Extract	116.41 ± 41.98
Omeprazole (Standard drug)	37.79 ± 0.10

Values are Mean ± SD of Triplicate Determination.

CHAPTER FIVE

3.1 DISCUSSION

In this study, the acute toxicity, phytochemistry, anti-ulcer properties and proton pump (H^+/K^+ - ATPase) inhibitory potential of *S. spontaneum* extracts were evaluated. Histological examination of gastric mucosa was also carried out to ascertain the level of mucosal damage in rats administered with aspirin (200mg/kg b.w). The findings of this study revealed that the tested extracts have a dose-dependent antisecretory and antiulcer activity against aspirin induced gastric lesions in rats.

Acute lethal effect of *S. spontaneum* shows that all the rats in each of the solvent extract groups survived within the 48 hours after treatment with the extracts. The major signs of toxicity noticed within 24 hours included slight increase in activity, loss of appetite as well as a reduced reaction to noise (Table 4.2). These signs were not seen at lower doses of the extracts (10, 100 & 100mg/kg b.w.) but progressed and became increasingly pronounced as the dose increased towards 5000 mg/kg b.w. The LD_{50} being greater than 5000 mg/kg is considered to be relatively safe as suggested by Lorke (1983). This also is in accordance with the toxicity classification/scale of toxic substances described by Hodge and Sterner (2005) who stated that a test drug administered orally is considered to be extremely toxic at a dose ≤ 1mg/kg, highly toxic at a dose 1-50mg/kg, moderately toxic at 50-500mg/kg, slightly toxic at 500-5000mg/kg, practically non toxic at 5000-15,000mg/kg and relatively harmless at a dose ≥15000mg/kg. The absence of death among rats in all the dose groups throughout the 48 hours of the experiment seems to support this claim. Therefore *S. spontaneum* could be said to be practically non toxic. This result corresponds with the work of Bulus *et al*. (2011).

The gastroprotective effect of *S. spontaneum* in aspirin induced gastric lesions is evident from its significant ($P<0.05$) reduction in the number of ulcers and ulcer index with an increased percentage protection (65.22%) as observed in the methanol (400mg/kg b.w) treated group (Figure 4.1).

Volume of gastric secretion is an important factor in the formation of gastric ulcer due to exposure of unprotected lumen of the stomach to the accumulating acid (Sakat and Juvekar. 2009). A significant ($P<0.05$) reduction in the mean gastric volume was observed in rats that were pretreated with the aqueous and methanol extracts of *S. spontaneum* when compared with ulcerative rats (Table 4.7). The decrease in gastric secretory volume could be as a result of disruption of chemical binding in stomach cells to reduce acid production, lessening irritation and allowing the healing of ulcers (Scanlon and Tina, 2007). Thus, the decrease in volume of gastric juice by *S. spontaneum* may be attributed to its antisecretory potential.

Maintenance of a pH above 4 has been correlated with the healing of both erosive oesophagitis (Bell *et al*., 1992) and gastric ulcer (Howden and Hunt, 1988*).* A significant ($p<0.05$) increase in pH values were observed in all the extracts treated (200 and 400mg/kg body weight) groups when compared with ulcer control (Table 4.8). As a measurement of free hydrogen ion, pH represents the hydrochloric acid concentration in the stomach. Increase in pH is usually affected by either the reduction of the acid secreted in the stomach or the increase in the volume of alkaline and neutral fluids (mucus). The variation in the pH level among the groups shows tendency of protective effects of them towards gastric ulceration. This agrees with the findings of Ashoka *et al.,* (2009).

Inhibition of gastric acidity is one of the important protective factors against gastric ulcer. Hydrochloric acid is known to produce ulceration and digestion of the stomach tissues as well as

to reduce the neutralizing capability of the stomach mucus secretions (Dragstedt *et al.*, 1917; Walpole *et al.,* 1940; Hay *et al.,* 1942). Administration of aspirin significantly ($P<0.05$) increased the gastric free acidity and total acid output. The increase in gastric acidity may be due to the back-diffusion of HCl through the broken barrier, inhibition of mucosal blood flow and acute inflammation. Pretreatment with *S. spontaneum* extracts showed a significant ($P<0.05$) reduction in the mean free acidity in a dose dependent manner. The same trend was also observed with the mean total acidity in all the groups administered 200 & 400 mg/kg b.w of the aqueous, methanol and hexane extracts. Although the reduction in acidity of *S. spontaneum* was less than omeprazole (10 mg/kg). This could imply a possible cytoprotective action and inhibition of gastric acid secretion. These findings confirm the cytoprotective nature of *S spontaneum* which is in accordance with the findings of Kore *et al., (*2011) and Jainu *et al.,* (2006).

Histological studies also confirmed the cytoprotective efficacy of *S. spontaneum* in preventing aspirin induced hemorrhage and necrosis in the superficial layer of the gastric mucosa as evinced by maintenance of the functional cytoarchitecture of the mucosa as well as apparent epithelialization (Plate 4.1). The protection against these histopathological changes is in conformity with the findings of Sancer *et al.,* (2009) who demonstrated a significant protection by *Glycyrrhiza glabra* against gastric mucosal defect produced by aspirin administration.

Gastric H^+/K^+-ATPase is responsible for gastric acid secretion. The enzyme is unique to the parietal cells and transports proton (H^+) against a concentration gradient. Given the redundancy inherent in the physiological control of gastric acid secretion, targeting the final effector in the secretion pathway is likely the most effective pharmacological approach and proton pump inhibitors have being the most potent agents in reducing gastric acid production (Anderson and

Carlsson, 2005). Parietal cells are rich of mitochondria and consume more of ATP, thereby generating inorganic phosphate which acts as indirect measure of proton pump (H^+/K^+-ATPase) activity in the formation and transport of H^+ for gastric acid formation. The result of this study showed an inhibitory potential of *S. spontaneum* on the proton pump activity which may be related to the conception that *S. spontaneum* has interaction with enzyme at the ATP sites. However, the degree of inhibition by the extract was weaker compared to that of omeprazole. The inhibitory activity of *S. spontaneum* on the H^+/K^+-ATPase observed in this study correspond with the findings of Samuel *et al*., (2010).

Another possible mechanism of the antiulcer properties of *S. spontaneum* could be as a result of the combined effects of bioactive components from the different extracts. The phytochemical analysis of the aqueous, methanol and hexane extracts revealed many bioactive substances including triterpenes, alkaloids, tannins, saponins, glycosides, carbohydrates, steroids, flavonoids, and phenols. Some of these phytoconstituents have been reported to have antiulcer properties as well as a pronounce effect on the integrity of the mucous membrane (Oliver, 1960). Alkaloids have been demonstrated to have gastroprotective and ulcer healing activity by inhibition of acid secretion and gastric motility, stimulation of alkali/mucus secretions as well as increase mucosal blood flow (Pandit *et al.,* 2000). Tannins have vasoconstrictory effects and react with the proteins of tissue layers which they come in contact with (Aguwa and Nwako, 1988). Tannins being astringent, can precipitate micro proteins on the site of a lesion thereby forming an impermeable protective cuticle over the lining to prevent absorption of toxic substances and resist the attack of proteolytic enzyme and consequently promote tissue repair, inhibit acid secretion, antioxidant and anti *Helicobacter pylori* activity (Vasconcelos *et al.,* 2008). A review on antiulcer drugs of plant origin revealed that triterpenes such as β amyrin,

lupeol, ursolic acid, glycerrhetinic acid and sterols like β sitosterol exert their antiulcer effect by strengthening defensive factors such as stimulation of mucus synthesis or maintenance of prostaglandin contents of gastric mucosa at high levels (Andrikopoulos *et al.*, 2003). A number of mechanisms have been proposed to explain the gastroprotective effect of flavonoids; these includes; increase in mucosal prostaglandin contents (Alcaraz and Hoult, 1985), reduction in secretion of histamine from mast cells by inhibition of histidine decarboxylase (Bronner and Landry, 1985) and inhibition of *Helicobacter pylori* growth (Beil *et al.*, 1995). In addition, flavonoids have been found to be free radical scavengers (Cavallini et *al.*, 1978; Salvayre et al., 1982). Thus, preventing the gastric mucosal surface epithelium against the attack by physical, chemical or microbiological agents which are involved in multiple pathologies, such as gastritis and gastric ulcer, with plant-originated flavonoid substances could effectively prevent gastric mucosa from the development of erosions and ulcerations (Zayachkivska *et al.*, 2005). The presence of these chemical constituents in the leaf extracts of *S. spontaneum* has thus suggested their therapeutic benefits to mankind as they might have the ability to protect against gastric ulceration induced by aspirin. This is due to the fact that therapeutic benefits of plants are dependent on the presence of their active components (Aguoru *et al.*, 2014). Therefore, the phytoconstituents present in *S. spontaneum* extracts if properly screened would yield drugs of plant origin with pharmacological significance. This is better supported by the fact that, the plant is known to be involved in ethnomedicine in the management of certain diseases notably anti-diarrhoeal and CNS depressant activity (Vhuiyan, 2008), dyspnea, anaemia, diuretic, obesity, lithotriptic, purgative, tonic, aphrodisiac, dyspepsia (Yoganarashimhan, 2002), gynecological troubles, respiratory troubles (Muhammad and Hefazat. 2011) and anepilepsy (Mathias, 1982). The antiurolithiatic activity was also reported by Satya *et al.*, (2013).

The significant reduction in basal gastric acid secretion, acidic content and formation of ulcers by *S. spontaneum* after administration of aspirin suggests that the cytoprotective mechanism of the extract on gastric mucosa may involve direct reduction of gastric secretion. Therefore, it is considered that the preventive effect of *S. spontaneum* may be partially mediated by its antisecretory effect which might be due to a possible relationship between protection of mucosal injury and inhibition of acid secretion. Thus, *S. spontaneum* extract possess gastroprotective, antisecretory and proton pump inhibition mechanism. Therefore, the results of the present study suggest that the aqueous, methanol and hexane extract of *S. spontaneum* leaves may be beneficial in the treatment of gastric lesions.

CHAPTER SIX

6.0 SUMMARY, CONCLUSION AND RECOMMENDATION

6.1 Summary

- Acute oral toxicity for the aqueous, methanol and hexane extracts of the leaves of *S. spontaneum* showed that the extract was relatively safe with an LD_{50} value greater than 5000mg/kg body weight with no lethal effect in the studied subjects.
- Quantitative Phytochemical analysis of the plant revealed the presence of alkaloids, flavanoids, saponins, tannins and phenols in a considerable amount which are bioactive metabolites that have been documented to possess antiulcer activities.
- The leaf extracts of *S. spontaneum* exhibited a potent antisecretory effects and it significantly ($P<0.05$) prevented gastric mucosal lesions with the methanol extract having the highest antiulcer activity as it considerably lowered the lesion index, reduced the volume of gastric juice, free and total acidity with a consequent rise in gastric pH.
- Histopathological examination of rat's gastric mucosa supported the cytoprotective nature of the leaf extracts of *S. spontaneum* with methanol extract showing a better protection against gastric mucosal damage as demonstrated by mild abrasion on the lining epithelium with no mucosal necrosis and hemorrhage.
- The methanol extract of *S. spontaneum* leaves also prevented acid secretion through Proton pump inhibition (H^+/K^+- ATPase) with an IC_{50} value of 116.41±41.98µg/ml.

6.2 Conclusion

The findings of this study indicated that the leaf extracts of *S. spontaneum* exhibited anti-ulcerogenic property against characteristic gastric mucosal lesions caused by administration of aspirin probably due to the modulation of defensive factors through an improvement of gastric cytoprotection and partly due to inhibition of gastric acid secretion. On the basis of the present results and available reports, it can be concluded that the anti-ulcer activity elucidated by *S. spontaneum* extracts may be used beneficially and safely in the treatment of gastric ulcer. These findings, therefore validates the use of the plant in herbal medicine in northern Nigeria for ulcer therapy.

6.3 Recommendation

- *S. Spontaneum* extracts can be safely used against hypergastric acidosis condition.
- Further studies should be explored on different ulcer models to ascertain the efficacy of *Saccharum spontaneum* as an antiulcer agent.
- The methanol extract of the leaves of *Saccharum spontaneum* should be subjected to a bioassay guided fractionation in order to isolate the specific active compound(s) that is responsible for the inhibition of the proton pump (H^+/K^+- ATPase) and the mode of interaction of the compound with the enzyme should also be studied.
- The antioxidant property of *Saccharum spontaneum* should be evaluated to determine the level of antioxidant enzymes as there is a strong correlation between free radical formation and ulcerogenesis.

CONTRIBUTION TO KNOWLEDGE

In an attempt to combat the menace of acid-related diseases, the following findings were reported for the first time;

- *In vivo* antiulcerogenic activities of the leaves of aqueous, methanol and hexane extracts of *Saccharum spontaneum* in aspirin induced ulcer rat model.
- *In vitro* proton pump inhibitory (H^+/K^+- ATPase) property of the methanol leaf extracts of *Saccharum spontaneum.*
- The present investigation has also opened avenues for further research especially with reference to the development of potent phytomedicine for treatment of ulcer and its complication from *Saccharum spontaneum.*

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APPENDICES

APPENDIX I; Calculation of Percentage Yield of Plant Extracts

$$\text{Percentage Yield} = \frac{\text{weight of extract}}{\text{Total weight of Plant}} \times 100$$

$$\text{Aqueous extract (\%)} = \frac{118.98}{600} \times 100 = 19.83$$

$$\text{Methanol extract (\%)} = \frac{32.58}{600} \times 100 = 5.43$$

$$\text{Hexane extract (\%)} = \frac{16.20}{600} \times 100 = 2.70$$

APPENDIX II; Preparation of Extracts for Acute Toxicity Studies

Stock Concentration = 500mg/ml.

$$\frac{\text{Stock conc} \times \text{Volume}}{1000} = \frac{500 \times 3}{1000} = 1.5\text{g of extract dissolved in 3mls of distilled water.}$$

APPENDIX III; Administration of Different Doses (10, 100, 1000, 1600, 2900 and 5000mg/kg b.w)

Average Weight of rat 200g = 0.2kg.

$$V = \frac{\text{Dose} \times \text{weight of rat (kg)}}{\text{Stock Concentration}} \qquad V = \frac{5000 \times 0.2}{500} = 0.004\text{ml.}$$

0.004 ml from the stock was prepared and administered.

The same procedure was used in calculation for the dose of aqueous, methanol and hexane extracts and administered to the rats.

APPENDIX IV; Preparation of Extracts for *In Vivo* Studies

Stock concentration = 400mg/ml

Dose = 400mg/kg b.w

$$V = \frac{\text{Dose X weight of rat (kg)}}{\text{Stock Concentration}} \qquad V = \frac{400 \text{ X } 0.2}{400} = 0.2\text{ml.}$$

Dose = 200mg/kg b.w

$$V = \frac{\text{Dose X weight of rat (kg)}}{\text{Stock Concentration}} \qquad V = \frac{200 \text{ X } 0.2}{400} = 0.1\text{ml.}$$

APPENDIX V; Preparation of Standard Drug

Aspirin; 300mg/tablet

Dose = 200mg/kg

Volume = $\frac{300}{200}$ = 1.5ml; One tablet dissolved in 1.5ml of distilled water

Omeprazole; 20mg/tablet

Dose = 10mg/kg

Volume = $\frac{20}{2}$ = 2ml; One capsule dissolved in 2ml of distilled water

APPENDIX VI; Preparation of Standard Albumin Solution (10mg/ml)

Exactly 0.2g of the standard albumin was dissolved in 10ml of distilled water, then made up to 20ml solution. Serial dilutions were made to obtain different concentrations (0.1-1mg/ml) and a standard curve was plotted.

APPENDIX VII; Preparation of Standard Biuret Reagent.

Biuret reagent was prepared by adding 1.5g of copper (II) sulphate pentahydrate and 6g of sodium potassium tartrate to 500 mL water, followed by the addition of 300ml 10% (w/v) NaOH. The resulting solution was then brought to a total volume of 1 litre with water and 1g of potassium iodide was added to inhibit reduction of copper. A series of protein standards (albumin) ranging in concentration from 0.1 to 1mg/ml was prepared and dilutions were made to 1ml solution.

APPENDIX VIII; Preparation of Extracts for *In Vitro* Studies

Stock concentration = 1000µg/ml

Concentrations of Methanol extract and Omeprazole; 10, 30, 50, 100 and 200µg/ml.

10µg/ml; Using the dilution formular; C1V1 = C2V2

C1= Initial concentration (stock concentration)

V1= Initial Volume

C2= Final concentration

V2= Final volume

$$V1 = \frac{C2V2}{C1} = \frac{10 \times 5}{1000} = 0.05ml$$

0.05ml of stock solution was diluted to 5ml to make a solution of 10µg/ml.

The same calculations were applied for preparation of 30, 50, 100 and 200µg/ml of the methanol extract and omeprazole.

APPENDIX IX;

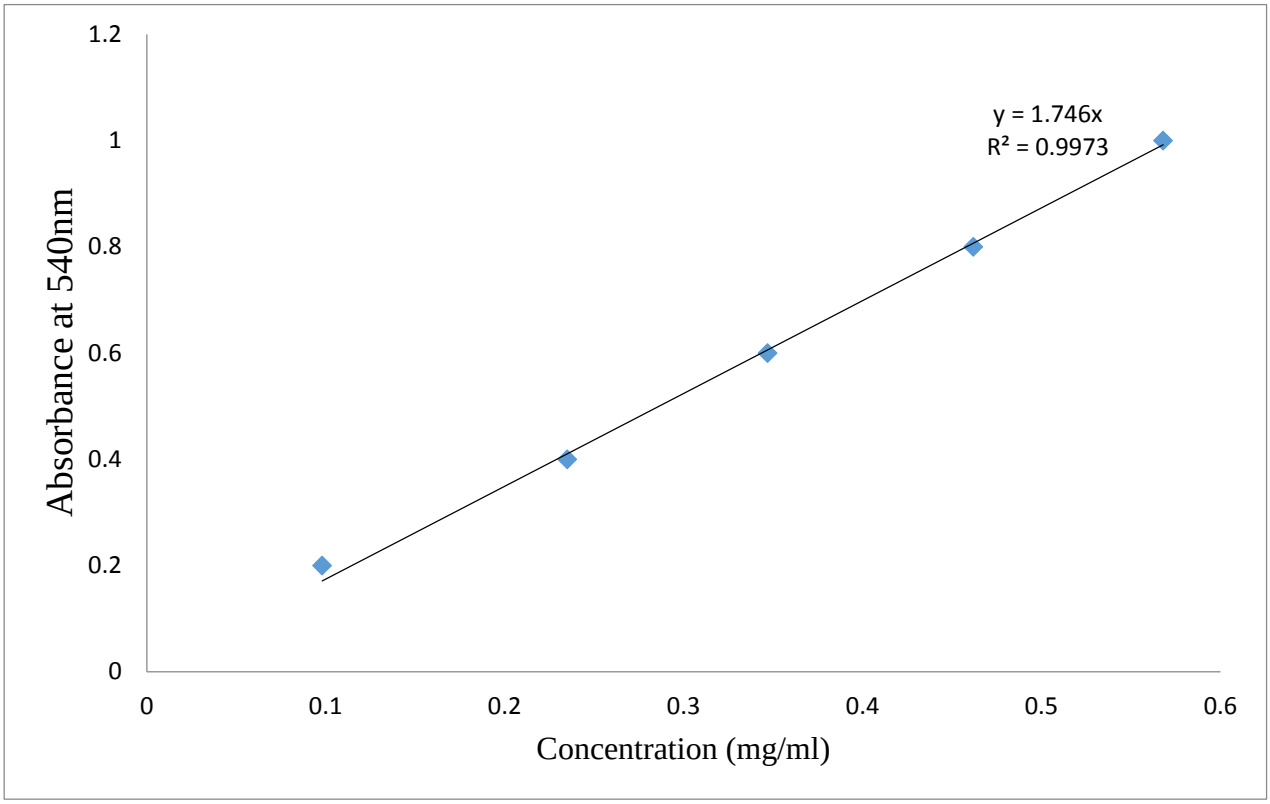

Fig. 1; Standard Curve for Albumin