CHAPTER ONE

1.0 INTRODUCTION

Stomach is a notable organ, because it provides water, electrolytes and nutrients to the body (Soybel, 2005). Stomach has been found to also provide protection from numerous aggressive factors that may be ingested along with food by increasing defensive factors such as gastric mucus, bicarbonate ions, prostaglandins etc. However, an interruption in balance amidst hostile and protective factors results into the formation of gastric diseases like peptic ulcer (Heibashy *et al.,* 2014).

Peptic ulcer is an unwrap sores that develop in the mucosa epithelial coating of the stomach and duodenum. At times it occurs in small and large bowels or in areas of ectopic-gastric tissue as in Zollinger-Ellison syndrome (Sostress *et al*.,2010). Some of the intrinsic hostile factors produced in the stomach are refluxed bile, leukotrienes, abnormal gastric motility, reactive Oxygen species, *Helicobacter pylori* infection, Non–steroidal anti-inflammatory drug used in an unlawful way and environmental substances such as alcohol abuse, stress, smoking and lifestyle constitute what is known as exogenous aggressive factors. The defensive factors involve the regulation of gastric blood flow, endogenous Prostaglandins, mucus secretion, Bicarbonate, Nitric Oxide, metalloproteins, melatonin, protein rich diets and recently discovered peptides that control food intake such as ghrelin, orein-A and leptin (Motalawi *et al*., 2008). Once the innate defending factors of the gastric epithelial cells are overwhelmed via aggressive factors, ulcer is formed. Interestingly, gastric mucosa is being challenged by these aggressive factors and substances continuously.

Rice is among the staple foods in the world (Gibson and Rozelle, 2011). Reports have shown that rice is planted in more than 75% of the African countries with population close to 800 million people (Wilfred and Consultant, 2006). During rice milling process, many by-products are produced, such as rice bran which is gaining significant interests among researchers because of its nutritional values. Rice bran contains significant amount of protein (11-17%), fat (12-22%), dietary fiber (6-14%) like β-glucan, pectin and gum; moisture (10-15%) and ash (8-17%). Also, it has been reported that rice has appreciable amount of vitamins, minerals and more than one hundred antioxidants that are capable of contending against illinesses and maintain good health (Fatemeh *et al.,* 2000).

Studies have shown that a rich- fiber diet reduced the tendency of having ulcers and also help in the healing of already formed ulcers. About 20 to 30 g/day of diet rich in fibers is however recommended to an individual with existing peptic ulcer by World Health Organization. This is because fiber has ability to act as buffers, reduce acid concentrations in the stomach, and reduce abdominal bloating, thereby minimizing distress and pain in the gastrointestinal tract (Marrotta and Floch, 1993).

Civilization has made western diets more fashionable; unfortunately these diets contain little fibers and are rich in refined carbohydrates and low buffering proteins(Oluwole and Bolarinwa, 1986).The Presence of intact bran in rice gives it brown, red or purple color, which is more nutritious than white commonly called commercial rice or polished rice. Interestingly, partially polished rice is rarely available in the market, because it is neither tasty nor appealing to the eyes. However, rice bran is still present in the local rice because it is partially milled. Studies have shown that people are now consuming brown rice, due to increased awareness about its beneficial properties because of high amounts of proteins and minerals than polished rice (Mohan *et al.,* 2012). Nutrients in rice bran reduce the risk of

heart problem by controlling cholesterol and fat absorption, reducing tumor incidence, cancer risk, delaying gastrointestinal emptying and providing gastrointestinal health (Pramod *et al.,*2016). These health benefits of partially milled rice have motivated researchers to study its prowess as an important source of nutrients in food ingredients.

1.1 STATEMENT OF THE PROBLEM

A lot of pharmacologic therapies have been studied for the prevention and management of peptic ulcer. Despite several years of research, controversy still surround the standardization of prophylactic therapy (Mark *et al*., 2014),hence, efficient and less toxic anti-ulcer agents are needed. The use of diet is an alternative approach because studies have shown that glutinous rice extract was protective against different gastric ulcer models (Dong *et al* 2014). Also, Adedeji and Oluwole (2012) reported the gastro-protective properties of two varieties of Nigerian rice. Although literature reviews have kept pace with beneficial properties of local rice and rice bran, none have assessed the possible mechanism(s) by which Nigerian local rice exerts gastro-protective property.

1.2 Aim of the study.

This research work investigated the possible mechanisms of anti-ulcer action of two Nigerian varieties of rice in gastric ulceration induced by indomethacin in male Wistar rats.

1.3 SPECIFIC OBJECTIVES

- To investigate the acclaimed anti-ulcer effect of Nigerian varieties of rice and rice bran using indomethacin-induced gastric ulceration model.
- To evaluate the possible influence of Nigerian varieties of rice and rice bran on the morphology of gastric tissues.

- To investigate possible influence of Nigerian varieties of rice and rice bran on the gastro-protective factors such as gastric blood flow, adherent mucus content, Nitric Oxide and Prostaglandin E_2.
- To investigate the possible influence of Nigerian varieties of rice and rice bran on some biochemical parameters; Lipid Peroxidation,SOD and Sulfhydryl.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 THE GASTROINTESTINAL TRACT

Gastrointestinal tract is like a hollow structure thatstretched from the mouth to the anus. Anatomically, it consists of four layers; the mucosa, lamina propria, muscularis mucosa; and the submucosa (Soybel, 2005). Approximately, sixty tons of food passes through the gastrointestinal tract in a normal lifetime (Johnson, 2001). Although food is essential for living, its passage through the gastrointestinal tract can constitute serious problem to one's health especially when it is contaminated. The gastrointestinal tract provides the body with necessary materials by carring out five prominent roles: motility, secretion, digestion, absorption and storage. The stomach wall is made up of a mucous layer, mucosa, sub-mucosa, muscularisand serosa. The mucus layer provides protection from harmful substances in the lumen that are capable of damaging the epithelial barrier(Duan *et al*., 2006).

The stomach secretes Hydrochloric acid, pepsin, intrinsic factor, mucus and bicarbonate ion majorly. The gastric glands secrete about 2500 mL of gastric juice in a man per day. Gastric juice is made up of Na^{+}, K^{+}, Mg^{2+}, sulphate, gelatinase and enzymes (Shereen-Lehman 2014). Gastric juice is corrosively acidic and its protein-digesting enzymes have potential ability to digest the stomach itself. However, this rarely happens because this organ is secluded via the gastric mucosal barrier. Studies have shown that any subtance that breaks the gel-like mucosal barrier can induce inflammation of the underlying layers of the stomach wall. Conditions such as gastritis and persistent damage to the underlying tissues can produce gastric ulcer. Except in uncommon cases, this organ can oppose the corrosive

effects of noxious substances present in the lumen with a broad range of temperatureand osmolality. The secret behind this engima depends largely on the mucosal lining of the stomach that produces numerous physiological responses in opposition to the potentially harmful luminal agents, as well as the aptitude of the organ to hastely repair the mucosal damage when it occurs. Nevertheless, when the defending mechanisms are overwhelmed by injurious factors, peptic ulcer may develop (Dalia and Mai, 2011).

2.1.1 THE STOMACH

Stomach is the roomiest portion of the GI tract. It serves as a reservoir for ingested food, and its secretory activity provides enzymes and hydrochloric acid required for the initial digestion of protein. Additionally, it provides means of prevention of microorganisms from entering the intestine (Duan *et al.,* 2006). The various roles performed by the stomach depend largely on the quality of the gastric mucosa.Two types of glands are found in the stomach. The gastric gland is about 65–75% of the total glands and is accountable for secretion of mucus, pepsinogen,gastric acid and endogenous factor. The pyloric gland is responsible for gastrin secretion and minute quatity of mucus (Shereen-Lehman, 2014).

The mucus and the HCO_3^- participate in ensuring that the mucosa lining of the stomach is not damaged by excess acidity. Changes in intra-gastric pH are very important signal in the regulation of gastric acid secretion during meal. This is because secretion of acid in the stomach is activated by the presence of food buffers which causes high luminal pH in the stomach (Abdallah *et al.,* 2011).

2.1.3 INNERVATIONS

Two major networks of nerve fibers are intrinsic to the gastrointestinal tract. The nerve supply is from autonomic nervous system. The vagal supply arises via the anterior and posterior trunks, which pass through the diaphragm on either side of the esophagus before giving rise to the hepatic and celiac branches. The hepatic branch supplies other branches to the anterior surface of the body of the stomach and to the pyloric region, while the celiac branch passes to the celiac plexus and the posterior portion of the body of the stomach. The sympathetic nerve supply arises from the spinal cord between T6 and T10 and passes to the sympathetic ganglia. The parasympathetic supply contracts the stomach, relaxes the pylorus and stimulates acid, pepsin and mucus secretion, whereas sympathetic stimulation constricts the blood supply and reduces gastric motor activity and secretion while the pylorus is contracted (Ayman and Martha, 2012).

2.2 PEPTIC ULCER

Peptic ulcer is an erosionof the gastroduodenal mucosa as a result of the sloughing of inflammatory necrotictissue (Ejam *et al.,* 2015). The organs mostly affected are the stomach and the duodenum. Sometimes, peptic lesions are observed in the esophagus and jejunum.Lessions are chronic and single in the majority of cases but can be multiple in about 5% to 20% of cases, simultaneously affecting the stomach and the duodenum or even other segments, as occurs in the Zollinger and Ellison Syndrome (Chinzon *et al*., 2006). The incidence of peptic ulcer varies with the age, gender, geographical location and is associated with severe complications including hemorrhages, perforation, gastrointestinal obstruction and malignancy. This clinical condition represents a worldwide health problem because of its high morbidity,mortality and economic loss (Dimaline and Varro,2007).

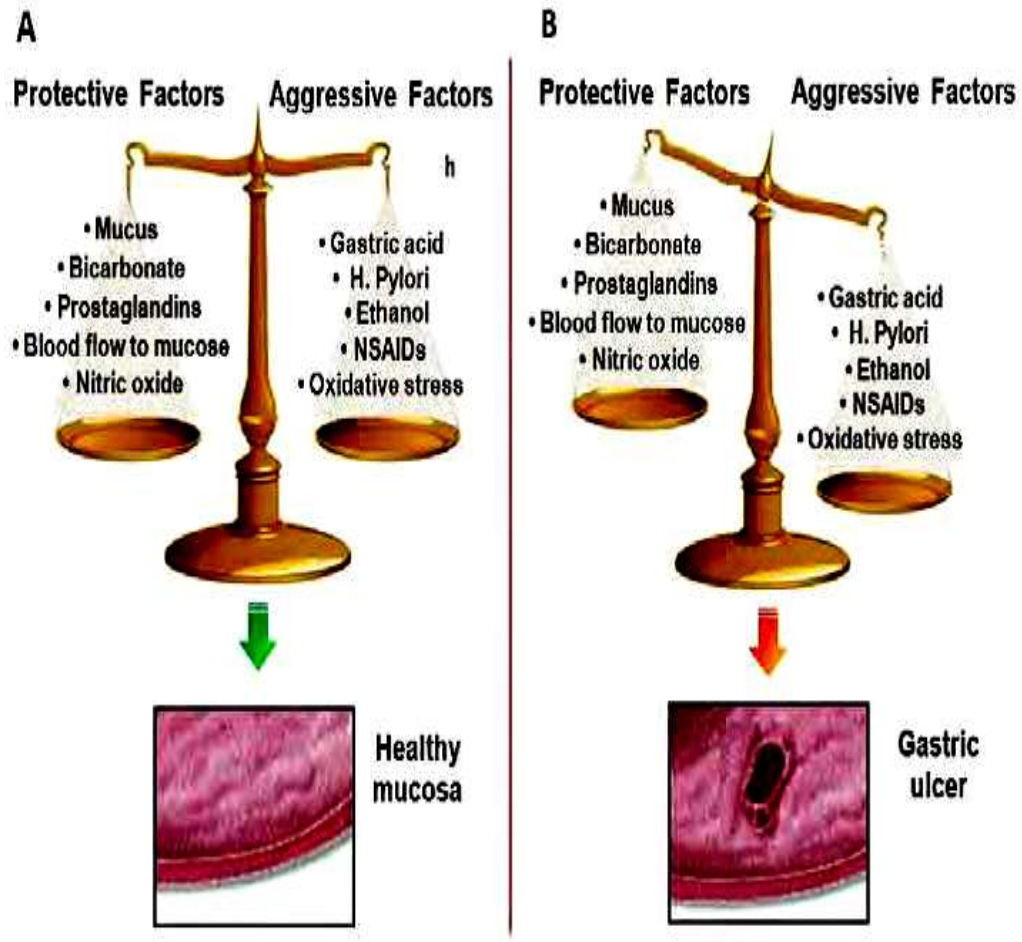


Figure 2.1: (A) Normal gastric mucosa: A balance between gastric damaging and defensive factors. (B) Ulcer development: Animbalance between mucosal destructive and defensive substances (Maria and Francisca, 2011).

Signs of Peptic Ulcers

The use of certain drugs, history of heartburn and gastroesophageal reflux disease can raise the suspicion for peptic ulcer. Drugs associated with peptic ulcer include non-steroidal anti-inflammatory drugs (NSAID) that inhibit cyclooxygenase, an enzyme responsible for the synthesis of prostaglandin. The timing of the symptoms in relation to the meal may differentiate between gastric and duodenal ulcers; a gastric ulcer would give epigastric pain during a meal, as gastric acid is secreted, or after the meal, as the alkaline duodenal contents reflux into the stomach. Symptoms of duodenal ulcer would manifest mostly before meal when acid is passed into the duodenum (Singh and Guha,2012).

Symptoms of peptic ulcers

• Abdominal pain

• Bloating and abdominal fullness

• Loss of appetite and weight loss

• Water brash (rush of saliva after an episode of regurgitation to dilute the acid in esophagus)

• Nausea and copious vomiting.

• Melena (tarry, foul-smelling feces due to oxidized iron from hemoglobin).

• Haemetemesis (vomiting of blood); this can occur due to bleeding directly from a gastric ulcer, or from damage to the esophagus from severe vomiting.

• Rarely, ulcer can lead to a gastric or duodenal perforation, which leads to acute peritonitis. This is extremely painful and requires immediate surgery.

2.2.1 Epidemiology of Peptic Ulcer

Due to multiple causes of peptic ulcers, ulcers are remitting; relapsing often appears with no noticeable precipitating influences and then after a period of weeks to months of active disease heals with or without therapy. Even with healing, the propensity to develop peptic ulcer remains, hence" once a peptic ulcer patient, always a peptic ulcer patient". Thus, it is difficult to obtain accurate data on the prevalence of the active disease (Brown *et al.,* 2012).

In the last few years, imperative changes have been observed in theepidermiology of ulcer disease. The peak incidence was seen among people born toward the end of nineteen century (Cucino and Sonnenbeng, 2002). The prevalence of peptic ulcer disease in Nigeria is not clear, though more than three decades ago Nigeria was listedas an area of high peptic ulcer prevalence. With perforation being the most common signal for surgery, recent studies begin to show comparable re-occurrence for duodenal ulcers and gastric ulcers in Nigeria, and this is attributed to improved diagnostic facilities (Ndubaba and Adeyemi, 2008). Complications of PUD vary in frequency geographically. This observation was supported by diminished rates of peptic ulcers in the United States and European countries in the few years (Sonnenbeng, 2007). Some regional factors that may account for the differences include the rate of NSAID use and the occurence of *H. pylori* disease (Kang *et al.*, 2010). Control of peptic ulcer disease indicates a major triumph for modern pharmacology. Currently, there are two major methods for the treatment of peptic ulcer disease. Reduction of gastric acid secretion is the first one while the second one deals with re-enforcement of gastric mucosal protective factors (Shawon and Gautam, 2012).

2.3 MUCOSAL DEFENSE

Although the stomach secretes substances that can digest the diverse foods ingested, but it rarely destroys itself. The reasons for this engima still remain incompletely understood by scientists (Wallace, 2008). However, early hypothesis stated that continuous passage of alkaline blood through the mucosa neutralizes acid. Homeostasis of gastric mucosa is maintained by several factors that jointly bring about the gastric mucosal defense system and, checkmate hydrogen ions from gaining entrance to the tissue at concentration that can produce cell injury (Wallace and Granger, 1996).

2.3.1 Mucus Secretion

The gastric mucus is made up of a viscous, elastic, adherent and transparent gel secreted by apical expulsion from surface epithelial cells. Mucus is a cohesive mixture of approximately 95% water, 5% mucin glycoprotein molecules, salts, immunoglobulins, cellular and serum macromolecules, and trefoil peptides (Wong *et al.,* 1999). Reports have shown that gastric mucus plays an important role in the gastric ulcer defense mechanism, where it forms a continuous mucus gel-like protective barrier coating the entire gastric mucosa that maintains the mucosal surface at a pH of 6–7 in the acidic environment (pH 1–2). The quality and quantity of gastric mucus is associated with important factor that determines the status of mucosa defense barrier against the injurious effect of acid and pepsin(Zainul *et al*,2013). This mucus acts both as a lubricant, and as a trap for bacteria. Thus mucus can diminish the ability of bacteria to gain access to the epithelium. Unfortunately, this mucus layer in the stomach is the site of colonization by *H. pylori (*Walsh and Peterson,1995).

Glycoprotein mucin is the main component responsible for viscousmucus and elastic gel-like properties. Eleven distinct mucin genes have been discovered (MUC1–4, MUC5AC, MUC5B, MUC6–8, MUC11 and MUC12). In the stomach, MUC5AC is expressed in the surface epithelial cells of cardia, fundus, and antrum; and MUC6 is expressed in the neck cells of the fundus and in antral glands. Alternating layers of MUC5AC and MUC6 have been demonstrated in the mucus layer in human gastric mucosa (Allen and Flemström, 2005). In the small intestine and colon, MUC2 is the dominating mucin product (80% of mucins) secreted from goblet cells. Surface mucus cells show three modes of mucus discharge: single granule exocytosis, apical expulsion or compound exocytosis, and cell exfoliation (Forstner, 1995). Baseline secretion is probably maintained by unstimulated release of single granules by fusion of the peripheral secretory granules with the plasma membrane (Rama and Bradley, 2006). Apical expulsion or compound exocytosis occurs upon mechanical stimulation. This accelerated exocytosis is characterized by the opening of fusion pores in a sequential manner between previously fused and adjacent granules resulting in emptying and cavitations of the apical granule storage area. Apical expulsion is a long process that is due to the fact that loss of cytoplasm and excess granule membrane are involved and may be completed within thirty minutes. However, the intestinal goblet cells recover fairly quickly and are refilled in 60–120 min, with the longer recovery period for goblet cells in the colon and re-annealing when sectioned. Occasionally, cell exfoliation occurs even in the absence of a secretagogue and this is characterized by the migration of the cells into the lumen (Forstner and Forstner, 1994).

Mucus secretion regulation is coupled to neural, hormonal and paracrine effects. Mucus secretion may be increased by NO, Prostaglandin of E series, histamine and substance P (Sababi *et al.,* 1995).

The HCO_3^- is secreted by surface epithelial cells to neutralize acid diffusing into a stable, adherent mucus gel layer and to be quantitatively sufficient to maintain a near neutral pH (~ 7.0) at the mucus-mucosa surface interface (Tulassay and Herszényi, 2010). Little attention has been given to pepsin relatively in contrast to gastric acid as the other endogenous aggressor in the gastric juice (Allen and Flemström, 2005). However, others have provided evidence that it is the carefully regulated secretion of alkali and the trapping of that alkali within the unstirred layer on the surface of the epithelium that is more important to mucosa defense than any impedance of the diffusion of protons by mucus (Baumgartner and Montrose,2004).Moreover, if some oxygen radicals are generated in surface epithelium containing mucus, intracellular mucus could scavenge them, acting as an antioxidant and thus reducing mucosa damage mediated by oxygen free radicals. Even when cells containing mucus are damaged by extracellular oxygen radicals, intracellular mucus is released into the gastric tissue and prevents additional damage by scavenging them (Seno *et al.,* 1995). The efficacy of protective properties of the mucus barrier depends not only on the gel structure but also on the amount or thickness of the layer covering the mucosal surface. The thickness of this layer is the result of a dynamic balance between its secretion and its erosion mechanically by shear forces of the digestive process and by proteolytic degradation, particularly from luminal pepsin in stomach. When mucus layer is overwhelmed or breaks down in different disease conditions, the next series of protective mechanisms come into play, including epithelial repair, and maintenance and distribution of mucosal blood flow (Tulassay and Herszényi, 2010).

2.3.2 The Role of Gastric Sensory Innervations

Gastric mucosal defense is regulated by the central nervous system innervations. Gastric mucosa and submucosa vessels are innervated by primary afferent sensory neurons

and nerves that form a dense plexus at the mucosabase (Appleyard *et al*., 1996).The nerve fibers from this plexus enter the lamina propria and end just beneath the surface epithelial cells. These nerve endings can sense the luminal content or passage of acid into the mucosa through acid-sensing channels. On exposure of gastric mucosa to luminal content or other inrritating chemicals, sensory nerves respond by releasing substance P and calcitonin gene-related peptide, which subsequently cause relaxation of smooth muscle surrounding gastric mucosa arterioles, resulting in an elevation of mucosa blood flow (Morsy and Fouad, 2008).

2.3.3 Prostaglandins

Prostaglandins are groups of hormone-like lipid compounds produced enzymatically from fatty acids and have significant functions in the body. All prostaglandins contain twenty carbon atoms, including a five carbon ring. Prostaglandins are not endocrine hormones,butautocrine or paracrine, which are locally acting messenger molecules. The prostaglandins together with thomboxanes and prostacyclins form the prostanoid class of fatty acid derivatives, a subclass of eicosanoids. Prostaglandins are present all through the gastrointestinal tract and carry out various functions, includingthe control of gastricacid secretion, bicarbonate secretion, mucus production, and mucosa blood flow, and maintenanceof mucosa integrity.

Synthesis of Prostaglandin

Prostaglandins are synthesizedin the cell from poly-unsaturated fatty acids. Cyclooxygenase enzyme (COX) is the rate limiting enzyme in the synthesis of prostaglandins. When an appropriate Poly-unsaturated fatty acid (PUFA) binds to the COX active site, it catalyzes the oxygenation of the arachidonic acid (AAs) to form 5-R,6-R', 2,3-dioxabicyclo heptane through a series of carbon radical intermediates. Both COX-1 and COX-2 primarily, but not

exclusively, oxygenate arachidonic acids into prostaglandin G_2 (PGG_2). PGG_2s are then reduced to prostaglandin H_2 (PGH_2) by peroxides. PGH_2 is subsequently converted into biologically functional molecules-including prostaglandin E_2 (PGE_2), which induces fever and elicits gastric mucus production, bone resorption and uterine contraction; prostaglandin D_2 (PGD_2)mediates vasodilatation, allergic response, and lowers the body temperature; prostacyclin (PGI_2), potentially induce vasodilatation and inhibits platelet activation; and thromboxane (TXA_2),performs acontrast functions to PGI_2 and can also induce vasoconstriction and platelet aggregation (Garavito and Mulichak,2003).

Prostaglandins Signaling

Prostanoids usually act within the tissue, where they are synthesized, via a carrier-mediated process to activate the membrane receptors or in some cases may interact with nuclear receptors(Lim, 1999). These receptors are protermed P receptors and are mainly heterotrimeric G protein coupled receptors (GPCR) (Coleman *et al*., 1994).

Cyclooxygenases

Currently, threeisoforms of COX are known: COX-1, COX-2, and COX-3. COX-1 and COX-2, catalyze the rate limiting step of prostaglandin synthesis (Viola *et al*., 2013). COX-1, encoded by the PTGS1 gene, is constitutively expressed in most mammalian tissues and appears to regulate normal physiological functions, including maintenance of vascular homeostasis, mediation of allergic and immune responses and stimulation of gastric mucosa secretion. COX-2, encoded by PTGS2 shares about 82% homology with COX-1, is usually absent from healthy tissue and is transiently induced by pro-inflammatory stimuli, growth factors, cytokines and tumor promoters to increase the rate of prostaglandin formation after tissue injury (Furstenberger *et al*.,2006).

The biosynthetic activity of COX-I and COX-2 can be inhibited by NSAIDs (Botting, 2010).The traditional NSAIDs such as indomethacin or ibuprofen are dual, nonselective antagonists of both COX-1 and COX-2.Gastric injury was assumed to be correlated withinhibition of gastric mucosaPG production by COX-1.Moreover, inhibition of the COX 1 blocks platelet production of thromboxane, whichincreases bleeding when gastrointestinal active site bleeding is present (Sostres *et al*., 2010). This contention was more supported by the fact that COX-2 selective antagonists, whichdo not affect the mucosa PGs production and do notproduced gross gastric injury in experimental models (Laine *et al*., 2008). Therefore, the development of NSAIDs which selectively inhibit COX-2 (COXIBS), while having little orno effect on COX-1 should resultin effective pain relief with reduced adverse gastrointestinal effects (Rostom *et al*., 2007). In fact, prostaglandins derived from COX-2 emerge to play avital role in ulcer healing by stimulating cell growth, promotion of angiogenesis and restoration of mucosal integrity (Konturek *et al*., 2005). These observations show that, in contrast to the originalperception, COX-2 performs an imperative role in gastric mucosal defense. Moreover, studies haveshown that inhibition of both COX-1 and COX-2 is required for NSAID-induced gastric damage, since inhibition of one did not lead to ulceration (Silverstein *et al.*, 2000).

Studies have shown that indomethacin and related NSAIDs that inhibit both isoforms of the COX enzyme, cause more severe damage in gastric tissue, even gastrointestinal bleeding,than selective COX drugs. Because of this, indomethacin became one of the first choices of drugs to produce an experimental ulcer model (Delaney *et al*., 2007).

Mechanism of Gastric Mucosa Defense

PGE_2 increased mucus and HCO_3^- secretion through EP4 receptors, and inhibited acid secretion or motility through EP3 or EP1 receptors, respectively, Prostaglandins can reduce

the permeability of the epithelium and thus reduce acid back-diffusion. Prostaglandins administered to epithelial cells in culture directly can increase the resistance of these cells to damage induced by exposure of irritants. The inhibitory effect of PGE_2 on acid secretion could be mediated in two ways by EP3 receptors, by inhibiting acid secretion directly at thecells responsible for its secretion (parietal) and indirectly by suppresing histamine release at enterochromaffin-like cells (Kato *et al*., 2005).

Prostaglandins may also enhance the effectivenessof the layer of surface-active phospholipids on the mucosal surface. Result from Kao and Lichtenberger (1993) demostrated that PGE_2analogincrease the volume of certain subcellular organelles within gastric surface epithelial cells that are thought to be storage sites for gastric surfactant.

Prostaglandins of the E and I series are potent vasodilators, producing this effectin the stomachvia EP2/EP4 and IP receptors, respectively (Araki *et al*., 2000), leading to increase in resistance of the gastric mucosa to damage. In addition,a vasodilatory effect of Prostaglandin has been found to facilitate epithelial restitution by contributing to the formation of a relatively high pH microenvironmentwithin the mucoid cap that forms over sites of epithelial injury. The prostaglandins that contribute to basal gastric blood flow are derivedprincipally from COX-1 (Wallace *et al.,*2000), while in conditions in which mucosal integrity is challenged such as during ischemia-reperfusion injury, prostaglandins produced from COX-2- are of increasedimportance for the maintenance of blood flow (Kotani *et al.,*2006). Prostaglandins are also potent inhibitors of leukocyte adherence to the vascular endothelium (Brand *et al.,* 1999). Studies have shown that,leukocyte adherence that occurs within the gastrointestinal microcirculation following administration of anNSAID can be prevented by administration of Prostaglandin (Asako *et al.,*1992), this likely contributes to the defensive roles of Prostaglandins on the gastric mucosa (Wallace,1997).

2.3.4 Nitric Oxide

Nitric oxide (NO) isessential and plays important roles in mammalian life(Moncada *et al.*, 1991). It is a very simple molecule and its synthesis involvs one of the most complicated enzymes in nature, the Nitric Oxide synthase.It is now widely accepted that Nitric Oxide is synthesized from L-arginine, a substance formerly depicted by Furchgott and Zawadzki (1980) as 'endothelium- derived relaxing factor'. NO is produced and released fromvascular endothelium and sensory nerve endings via the enzymatic activity of constitutive NO synthase and inducible NOS(Napoli and Ignarro, 2009).

Macrophages and neutrophils are the major sites for expression of inducible isoform of NOS (iNOS), but with potent signals for induction it may also be found in epithelial cells and neurons (Wallace and Miller, 2000). The constitutive isoforms are primarily regulated by intracellular calcium, via Ca^{2+} activated calmodulin, while the inducible isoform (iNOS, type II) is not regulated by calcium because activated calmodulin is inserted at the time of synthesis. This isoform produces huge amount of NO (micromolar) for longer periods of time compared with the other isoforms and generates a sustained increase in NO (Nathan, 1997).

Several NOS-independent mechanismsof NO formation have been discovered. For example, in hypoxic conditions xanthine oxidoreductase can produce NO by reducing Nitrate (NO^{3-}) and Nitrite (NO^{2-}). Formation of NO from the reaction of hydrogen peroxide with arginine is an example of non-enzymatic NO production (Nagasa *et al.,* 1997).

The action of NO could either be direct or indirect. Activation of soluble guanyl cyclase (GC-S) through formation of second messenger cyclic guanosine monophosphate (cGMP)mediates the direct action of NO. Indirect effect is usually mediated by the

production of reactive oxygen species(Carvajal *et al.*, 2000). When NO is produced in the cells,it is releasedto neighborhood cells and diffuses mainly across the cell membranes of target cells to produce biological responses. Then, NO is attached to the Fe^{2+} of the hem part of soluble guanylyl cyclase (sGC). This cGMP subsequently actby means of protein kinase G leading to relaxation of smooth muscle cell and subsequent increase of vessel diameter and an enhancement of the gastric blood flow (Bian and Murad, 2014).The effect of NO on blood vessels implies that this gaseous molecule contributes to the maintenance of gastric mucosal barrier integrity. This is supported by the observation that the inhibition of NO formation by a nonspecific NG-nitro-L-arginine (L-NNA) not only markedly impaired gastric secretion and motility but also abolished the defensive activity of gastroprotective agents (Kuo *et al*., 2009).

Recent studies revealed that NO is capable of activating potassium channels, mainly calcium dependent potassium channels BKCa and ATP sensitive potassium channel KATP in smooth muscle, neurons, endothelial cells etc., which are cGMP independent processes. After activation of these channels membrane,becomes hyperpolarized with diminished Ca^{2+} ions, which lead to relaxation of vascular or bronchial smooth muscles (Garcia-Calvo *et al.,* 1994).

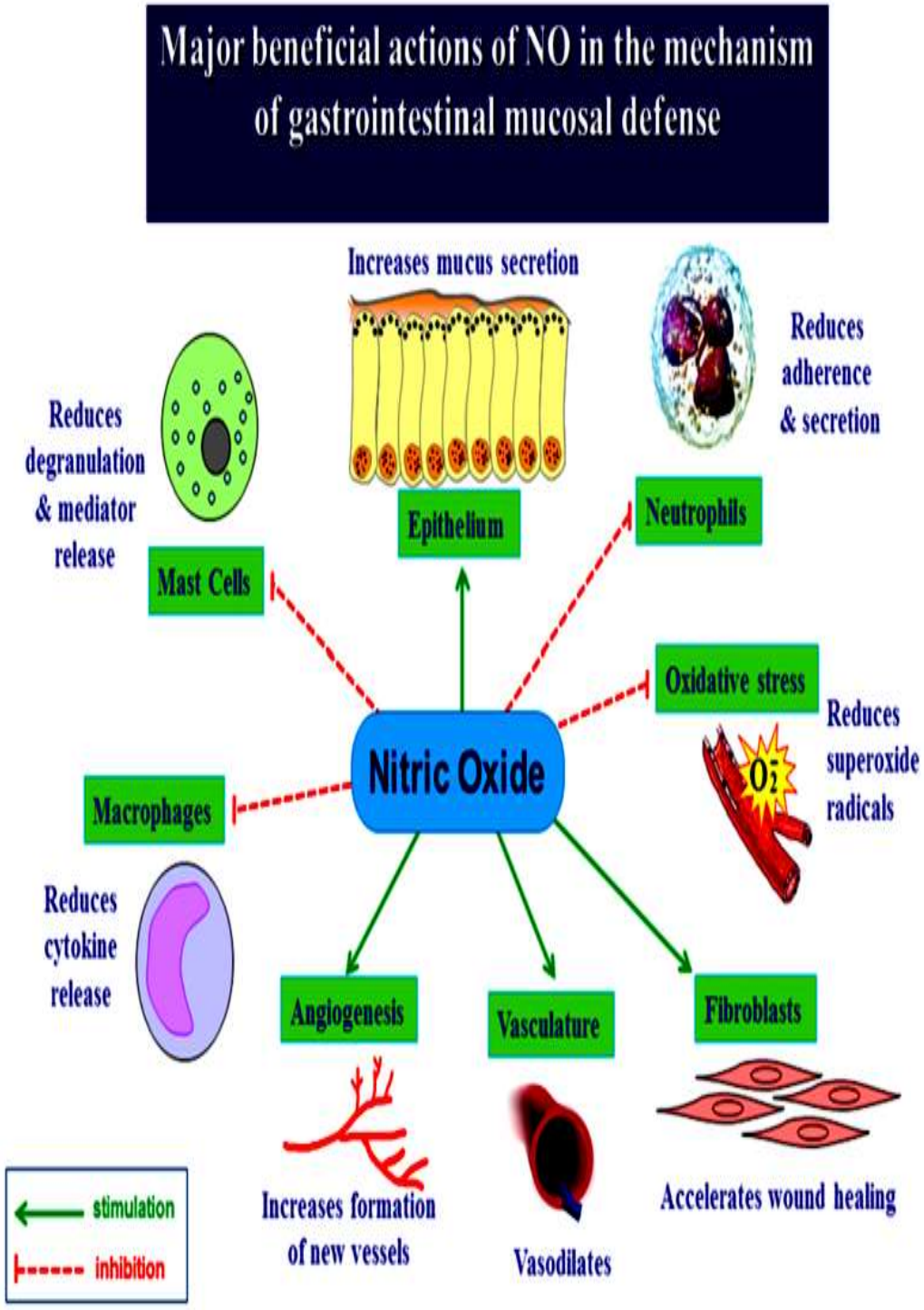


Figure 2.2: Beneficial actions of Nitric oxide in the mechanism of gastrointestinal mucosal protection (Marcin *et al*., 2015).

NO as Two Edges Sword

Just as nitroglycerin has diverse uses that range from very destructive to the assuagement of pain, the effects of NO in the gastrointestinal tract as well as other tissues are wide spread and somewhat mysterious. NO undoubtedly contributes to the modulation of several key physiological functions in the digestive system, but it has also been suggested to be a critical mediator of tissue injury in several intestinal disorders (Motalawi, 2008). This paradox can be explained, at least to some extent, by the ability of different enzymes of NO to produce completely opposite effects in the same tissue. Thus, NO produced by constitutive Nitric oxide synthase (cNos) generally exerts beneficial effects in the gastrointestinal tract, whereas production of NO by inducible Nitric Oxide synthase (iNos) can be detrimental (Ihab, 2010)

The biological action of NO may be mimiced by the exogenous administration of NO donors, such as sodium nitrate, nitroprusside or other organic nitrates, the 3-morpholinosydnonimine (SIN-1), *S*-nitroso-*N*acethyl- D,L-penicylamine (SNAP), gliceryl trinitrate (GTN) or NO-releasing aspirin. Thiols (R-SH), for example, glutathione (GSH) cooperate with NO. Nitric oxide itself or an NO donor could protect the gastric mucosa from damage induced by topically applied ethanol. Moreover, administration of the guanylate cyclase inhibitor, methylene blue, or the nitric oxide scavenger, oxyhaemoglobin, prevented the protective effects of exogenous NO and increased the susceptibility of the gastric mucosa to injury. These findings have now been extended by Kitagawa *et al.,* (1990) who showed protective effects of nitric oxide donors in an acid- induced model of damage in the rat, and that blockade of nitric oxide synthesis greatly increased the susceptibility of the stomach to damage induced by ethanol (Masuda *et al.,* 1995). The administration of NO-donors accelerated healing of gastric mucosa damage and experimental gastric ulcers as it modulate

the activity of mucosal immunocytes and reduce leukocytic endothelial adhesion. In addition, it modulates mucosal blood flow and reduces epithelial permeability, resulting in enhanced mucosal resistance to ulceration. Nitric oxide also prevents adherence of leukocytes to the vascular endothelium. This gaseous mediator has a role also in modulating gastric mucus and bicarbonate secretion. Suppression of nitric oxide synthesis renders the gastric mucosa more susceptible to injury, while administration of nitric oxide donors can protect the stomach from injury. Agents that release nitric oxide in small amounts over a prolonged period have been shown to greatly reduce inflammation and to accelerate ulcerative healing (Kotunrek *et al.,*1993).

2.3.5 Antioxidants

The damaging effects of ROS are neutralized by a broad class of defensive mediatorsnamed antioxidants;which prevent oxidative injury by reacting with the corrosive action of free radicals before any other molecules become a target (Aruoma, 1994). Each living organism is endowed with antioxidant defense system to handle ROS. These antioxidants act at different levels in the body to prevent, scavenge free radical, repair and promote adaptation(Cadenas, 1997). Likewise, some antioxidants promote synthesis of other antioxidants or defense enzymes. Research in the recent past has accumulated enormous evidences revealing that enrichment of body systems with natural antioxidants may correct the vitiated homeostasis and can prevent the onset as well as treat diseases caused and/or fostered due to free-radical mediated oxidative stress. These developments accelerated the research onantioxidant principles that could lead to the discovery of natural resources, isolation of active principles and further modification and refinement of active antioxidant molecules (Aruoma,1994).

2.4 ETIOLOGIES OF GASTRIC ULCER DEVELOPMENT

Despite the robust and multi-faceted nature of gastric mucosa defense mechanisms many factors can directly or indirectly alter the epithelial wall and support the development of mucosal damage. Their actions on the gastric wall signify important mechanisms of the pathogenesis of gastric ulcers, chronic gastritis and other gastric diseases, which are regularly produced via an imbalance between mucosal aggressive and protective factors (Tulassay and Herszényi, 2010).

2.4.1 *Helicobacter Pylori*

Infection with *Helicobacter pylori(H. pylori*) is the most well-defined risk factor for the development of peptic ulcers. The two Australian scientists who identified *H. pylori* as the main cause of stomach ulcers in 1982 were awarded the Nobel Prize in Medicine in 2005 for this discovery. *H. pylori* bacteria are found in about 50% of people with gastric ulcer disease. The success of the pathogen depends on both its virulence and its pathogenicity.*H.pylorus has* cell wall associated lectins which allow them to bind selectively to mucus and epithelial cells. Targets of *H.pylori* lectins exist in the gastric mucus as glycoproteins and glycolipids. *H.pylori* appear to bind to all of these, including sulfated (acid) mucins, L-fucose, D-galactose and sialic acids. *H.pylori* lectins also attach to red blood cells of various animal species thereby damages the mucosal defense system by reducing the thickness of the mucus gel layer (Emody *et al*., 1988). The *H. pylori* cytotoxin-associated gene *CagA* also has an important role: it interferes with gastric epithelial cell-signaling pathways, thereby regulating cellular responses and possibly contributing to apical junction barrier disruption, interleukin-8 secretion and phenotypic changes to gastric epithelial cells. This pathogen multiplies with great efficiency in the hostile environment within the stomach but survives poorly in the gastric lumen. It is mainly found under the

mucous layer and in close proximity, or even attached, to gastric superficial epithelial cells, without substantial invasion of host tissue (Dubois, 1995). The initial response to infection is an interaction of the host epithelial cells with the bacteria; however, the pathogenetic mechanisms of chronic infection with *H. pylori* and gastric ulcer are yet to be full determined (Calvino-Fernández and Parra-Cid, 2010).

A characteristic feature of this pathogen is the synthesis of urease, which was its first virulence factor studied. This enzyme may explain the extraordinary ability of the bacteria to colonize the gastric mucosa and survive in an acidic environment. Because the ecologic niches of these bacteria are rich in urea, it catalyzes urea hydrolysis with the formation of ammonia (NH_3), carbon dioxide and hydroxyl ions. By this mechanism, *H. pylorus neutralizes* the surrounding gastric acid and protectsitself from the strong acidicity of the stomach. On the other hand, although the neutralization of gastric acid benefits the bacteria, metabolites from urease activity are toxic to gastric epithelial cells. The formed ammonia reacts with OCl- produced by activated neutrophils to form highly toxic monochloramine (NH2Cl) in the stomach, a hallmark of *H. pylori* infection. In fact, inhibition of *H. pylori* urease has been showed significantly decrease this toxicity, suggesting that ammonia is at least partially responsible for the cytotoxicity found in association with this bacterium. Moreover, hydroxide ions are also considered toxic to gastric epithelial cells (Smoot, 1991).

H. pylori-infected gastric mucosa showed infiltration of polymorphnuclear leukocytes, lymphocytes, monocytes and plasma cells in the lamina propria, and intraepithelial severe neutrophil infiltration (Fan *et al.,* 1996). The later is well documented to correlate well with mucosal damage due to the effects of various cytokines, free radicals, and monochloramine (Karttunen, 1991). Moreover, *H. pylori*-induced inflammation is implicated in the development of mucosal damage and is characterized by strong

granulocytic and lymphocytic infiltration (Rad *et al.,* 2004). These changes would accelerate apoptosis and proliferation in the mucosal layer (Ohkura *et al.,* 2003). In addition, it had been reported that *H. pylori* infection induced a three-fold increase in the serum gastrin concentration but was without effect on the thickness of the oxyntic mucosa (Zhao *et al.,* 2003). *H. pylori* infection is associated with low acid secretion in gastric cancer patients and with high gastric acid secretion in patients with duodenal ulcers (Calam *et al.,* 1997b). *H. pylori* infection results in the increased secretion of pro-inflammatory cytokines such as IL-1a andIL-6 (Thomson *et al.,* 2003), and IL-8 activity correlates with the histological severity in H. pylori-associated antral gastritis. Thus, the *H. pylori* infection causes chronic inflammations that results in the release of pro-inflammatory cytokines as well as reduced acid secretion, and thereby appear to increase the antisecretory effect of omeprazole (Thomson *et al.,* 2003). This inflammation resolves after eradication of the infection, and presumably the concentrations of the pro-inflammatory and anti-secretory cytokines also fall.

Factors that trigger gastric ulcers in *H. pylori* carriers include genetic factors, which explain the higher incidence of development of ulcers in certain ethnicity. Lifestyle factors as chronic stress, drinking coffee and smoking were long believed to be primary causes of gastric ulcer; it is now thought that they only increase susceptibility to ulcers in some *H.pylori* carriers. Interrupted sleep may be another trigger as people who work at night shifts have a significantly higher incidence of ulcers than day workers. Frequent interruption of sleep is thought to weaken the immune system's ability to protect against harmful bacteria substances. Using certain medications such as non-steroidal anti-inflammatory drugs or corticosteroids may contribute to higher infection rates of *H. pylori*. Patients with prior gastric ulcer, Zollinger-Ellison syndrome, congenital stomach malformations, malignant diseases such as mastocytosis and basophilic leukemia, head trauma, severe traumatic injuries, burns, radiation, or recently had major surgery are also more prone to *H. pylori*

infection. Increased risk of *H. pylori* infection is seen among people who live in crowded places with unsanitary conditions. Some genetic predispositions for *H. pylori* infection cure rate may exist. One example is cytochrome P450-2C19 polymorphism that seems to predict the cure of *H. pylori* infection and predisposition to gastric ulcer. Another example is cytokine genes polymorphism that was significantly associated with persistent infection (Lay and Lin, 2010). Polymorphism of multidrug resistance protein 1 also was reported to influence *H. pylori*-induced gastric inflammation. Therapeutic interventions to eradicate *H. pylori* are needed to prevent ulcer formation and its transformation to gastric cancer, one of the major complications of chronic gastric ulcer. *H. pylori* eradication therapy comprises a combination of two or more drugs including antimicrobials, proton pump inhibitors and gastro-protective agents. Several eradication methods were suggested. Dual eradication therapy using proton pump inhibitor with amoxicillin was tried (Graham *et al.,* 2010). Triple eradication therapy employing two antimicrobials together with proton pump inhibitor also showed some success, but not enough to be considered first-line treatment. Quadruple *H. pylori* eradication was also successfully tried and consisted of 2 antimicrobials, proton pump inhibitor and the gastro-protective agent colloidal bismuth substrate (Zheng *et al.,* 2010). Nowadays, the first line of *H. pylori* eradication therapy is a regimen of 7 or 14 days consisting of a proton pump inhibitor as omeprazole (20 mg 12 hourly), in combination with clarithromycin (500 mg 12 hourly) and metronidazole (400 mg 12 hourly). A second regimen that is equally effective is by using omeprazole as previously mentioned, together with less dose of clarithromycin (250 mg 12 hourly) and substituting metronidazole with amoxicillin (12 hourly). Omeprazole can be replaced with other proton pump inhibitors. Despite that the prevalence of *H. pylori* is decreasing in developed countries, as a result of improvements in living standards and hygiene, *H. pylori* is still a common cause of gastric ulcer in developing countries. Attempts to develop effective vaccination against this bacterium reached phase I

and II clinical trials, and may present effective preventive strategy in preventing gastric ulcer formation and, more importantly, preventing gastric cancer in the future (Majumdar *et al.,* 2011).

2.4.2 Use of Non-Steroidal Anti-Inflammatory Drugs

Administration of Indomethacin is another prominent factor in formation of gastric ulcer. As the prevalence of*H. pylori* infection declined, because of continued efforts to eradicate the organism, the wide spread of NSAID –induced ulcers has risen and is taking on greater clinical importance. It has been shown that NSAIDs are among the most frequently used drugs due to their theraupetic usefulness. Among the most popular NSAIDs is Indomethacin; it was synthesized in 1963 for the treatment of rheumatoid arthritis, ankylosing spondylitis, acute musculo-skeletal disorders and pain associated with primary dysmenorrhea following surgical technique.

Damage to gastric mucosa, exasperation of stress ulcerations and exacerbation of pre-existing gastric ulcerations are the major restriction to their use (Schmassmann *et al*., 1997). This injurious action of conventional NSAID could be attributed to their topical irritating effect, neutrophils activation, reduce microcirculation, enhancement in the motility and the fall in mucosal PGE_2 induced by these drugs. Studies have shown that 20-30% of patients treated with NSAIDs developed gastric ulceration. These ulcerations are usually clinically "not expressed", which often makes perforation the first sign or indication of the disease (Laurence and Bennet, 2013). Binning (2001), reported that almost 80% of deaths resulting from complications of gastric and duodenal ulceration are the cases of NSAID-treated patients.

The discovery that Indomethacin and related drugs exert their anti-inflammatory effects through Prostaglandin inhibition led to the understanding that prostaglandins play an important role in gastric mucosal defense mechanisms.This was further supported by the fact that exogenous Prostaglandins in minute amount defend the mucosa from damage induced by NSAIDs and many other agents (Kotunrek *et al.,* 2005). The beneficial role of Prostaglandins in mucosal defense is underestimated by the evidence that NSAID ability to produce damage in the mucosa is associated with its ability to reduce Prostaglandin synthesis. Moreso,agents that are weak inhibitors of Prostaglandin synthesis, including selective COX-2 inhibitors, are less ulcerogenic and a good correlationexist between decreased gastric Prostaglandin by NSAIDs and their ability to produce gastric ulcers (Wallace *et al.,*2008).

Although the beneficial role of Prostaglandins in mucosa defense has been identified for almost thirty years, a question that remained largely unanswered is why reduction of Prostaglandin production resulted in mucosa injury. This is because to some extent almost all component of mucosa defense are Prostaglandin-dependent, so suppression of Prostaglandin production by NSAIDs raise the susceptibility of gatric mucosa to injury provoke by luminal irritants. Furthermore, alteration in gastric blood flow that seem to be vital in the pathogenesis of ulceration may be due to Prostaglandin production inhibition which can also reduce mucus secretion, epithelial cells growth, immunocytes functions in the lamina propria (Kwiecien *et al.,* 2012).

Two isoforms of COX were recognized in mammalian cells termed COX-1 and COX-2 (Sostres *et al*., 2010). The COX-1 isoforms is constitutively expressed in almost all tissues and is accountable for maintaining gastric mucosal integrity at base line, while COX-2 is responsible for prostaglandin produced in inflammation. Gastric injury was thus considered to be ascribed to gastric mucosal Prostaglandin deficiency by COX-1 inhibition.

Studies have shown that COX-1 dependent PGE_2 depletion causes a decrease in gastric blood flow while COX-2 inhibition promotes leukocyte adherence (Wallace *et al*., 2000). Despite negligible Prostaglandin synthesis, COX-1 or COX-2 deficient mice or mice treated with selective COX-1 or selective COX-2 inhibitors do not develop spontaneous gastric damage. However, they develop severe gastric lesions when both isoenzymes are simultaneously blocked or when given a NSAID (Tanaka *et al*., 2001).Conversely, in impaired gastric mucosa (such as during acid challenge, suppression of NO synthesis or ablation of afferent neurons), isolated inhibition of either COX-1 or COX 2 is ulcerogenic. Inhibition of platelet COX-1 also leads to decreased thromboxane production causing enhanced bleeding tendency and this may be the main factor determining the propensity of an NSAID to cause bleeding complications. Therefore, NSAIDs that selectively inhibit COX-2 were developed and applied in a clinical setting. The result challenges the concept that only COX-1plays a housekeeping role in the stomach. Recent researches suggested that both COX-1 and COX-2 may play a role in Prostaglandin synthesis and maintenance of gastric mucosal integrity, and that COX-2 plays a "back-up" role by alleviating Prostaglandin deficiency which is induced by COX-1 inhibition (Tomisato *et al.,* 2004).

Topical injury initiates the initial mucosal erosions by disrupting the gastric epithelial cell barrier, but Prostaglandin depletion appears essential for the development of clinically significant gastric and duodenal ulcers (Tomisato *et al.,*2004). The ability of an NSAID to cause short-term topical injury depends on its pKa (a measure of its acidity which is also crucial for the inhibition of COX enzymes) and lipid solubility (log P) and can be dissociated from its ability to inhibit COX-1 and deplete gastric mucosal Prostaglandins.

Most NSAIDs are weak organic acids. In gastric juice, they are non-ionized and lipid soluble. These NSAIDs diffuse across gastric mucosal epithelial cell membranes into the

cytoplasm, where pH is neutral. In neutral pH, NSAIDs are converted to an ionized state and cannot diffuse out. Osmotic forces then pull water into the cell, resulting in swelling, sometimes to the point of lyses. Therefore, NSAIDs are trapped and accumulate within cells, leading to the cellular injury (Wallace, 2000). The exart mechanisms by which NSAID induced local injury to the mucosal cell is not known yet, but in-vitro studies revealed that mitochondria are the primary target of NSAIDs. A mitochondrion is a membrane-enclosed organelle with 0.5 to 10 micrometers range. In eukaryotic cells, mitochondria generate most of the cell's chemical energy supply of adenosine triphosphate (ATP). In addition to energy metabolism, the regulation of cell death has recently considered as a second major function of mitochondria (Gretzer *et al.,*2001). Mitochondrial respiratory chain is the major source of reactive oxygen species (ROS), which are mainly generated at Complex I and III of the respiratory chain. More importantly, the mitochondrial respiratory chain is, at the same time, an important target for the damaging effects of ROS. ROS from mitochondria play an important role in the release of cytochrome c and other pro-apoptotic proteins, which can trigger caspase activation and apoptosis. Mitochondria are considered as the target intracellular organelle of absorbed NSAIDs. NSAIDs inhibit, or uncouple, oxidative phosphorylation to dissipate the mitochondrial transmembrane potential (MTP), leading to the liberation of cytochrome c from mitochondrial intermembranous space into cytosol and to the release of ROS such as superoxide (O_2 •−) and hydrogen peroxide (H_2O_2), thereby causing caspase 9 and caspase 3 activation and cellular lipid peroxidation, all resulting in cellular apoptosis (Schmassmann *et al.,*2006). The uncoupling of mitochondria also decreased the intracellular ATP concentration, leakage of Ca^{2+} out of mitochondria, cellular osmotic imbalance and a loss of control over intracellular junctions, resulting in increased permeability and subsequent mucosal damages (To *et al.,*2001).

Topical irritant properties have also been attributed to the ability of NSAIDs to decrease the hydrophobicity of the mucous gel layer in the stomach. Lichtenberger (1995)proposed that this layer is a primary barrier to acid-induced damage in the stomach.

2.4.3 Gastric Acid Secretion

For years, medical practitioners thought that acid secretion was responsible for peptic ulcer disease, since supression of peptic ulcer disease by acid-reduction was substantial and impressive. As a result, acid became the number one target in an effort to enhance understanding and management of ulcer disease, the key medical confrontation as at that time (Fock *et a*l., 2008). Incidence and treatment of peptic ulcer disease has improved in recent time. With the introduction of proton pump blockers, ulcers were effectively managed and cases of surgery became an history (Schubert and Peura, 2008). Because of this, the measurement of gastric acid secretion has become outdated. Although numerous processes are implicated in the progress of gastric lesions, high level of acid secretion still remain a very important condition for ulcer development since their treatment involves elimination of the injurious mediator and acid secretion. Parietal cells produce 160 mmol/L of acid. Gastric acid also acts as first line of defense in the mucosal to prevent penetration of organism thus avoiding bacterial growth and enteric infection that might follow and also aids digestion of proteins and absorption of calcium iron and vitamin B-12. However, severe acid-related clinical conditions occur, when acid levels overwhelm mucosal defense systems, (Schubert and Peura, 2008).

Pharmacological control of gastric acid secretion has long represented a desirable goal in an attempt to protect the gastric mucosa from corrosive action of gastric acid, enhance ulcer healing, and prevent ulcer recurrence (Tuorkey and Karolin, 2009b). Since, gastric acid hypersecretion is one of the major pathogenic factors for the induction of gastric

ulcer disease. Furthermore, luminal acid interferes with the process of restitution, resulting in the conversion of superficial injury to deeper mucosal lesion and inactivates the acid-labile growth factors important for maintenance of mucosal integrity and repair of superficial injury (Wallace and Muscara, 2001). A profound suppression of acid secretion whether by Omeprazole or by a high dose of Famotidine was necessary in order to have a significant impact on the incidence of NSAID-induced ulcers. Furthermore, acid may contribute to NSAID-induced ulcer formation in several ways. First, acid can exacerbate damage to the gastric mucosa induced by other agents, e.g., acid can convert regions of ethanol-induced vascular congestion in the mucosa to actively bleeding erosions (Wallace, 2005). Second, acid will contribute to ulcer formation by interfering with homeostasis, for instance, platelet aggregation is inhibited at a pH of less than four (Green *et al.,* 1978). Third, acid can convert superficial injury to deeper mucosal necrosis by interfering with the process of restitution. Fourth, acid can inactivate several growth factors (e.g., fibroblast growth factor) that are important for the maintenance of mucosal integrity and for the repair of superficial injury, since these growth factors are acid-labile (Szabo *et al.,* 1994). So, the inhibition of their synthesis by NSAIDs can result in an increase in gastric acid secretion. A large amount of studies show that the rate of acid secretion by the human stomach changes little with aging unless there is coexisting disease of the oxyntic mucosa such as atrophic gastritis, infection with *H. pylori* or both. To prevent acid-induced mucosa damage, gastric acid must be precisely regulated through a highly coordinated interaction of neural, hormonal, and paracrine pathways.

2.4.4 Consumption of Alcohol

Alcohol has been used for centuries in social, medical, cultural, and religious settings throughout the world. Currently, it is considered to be one of the most commonly abused

drugs, related to a wide range of physical, mental, and social harms, and responsible for 3.8% of deaths and 4.6% of disability-adjusted life years lost worldwide. The World Health Organization (WHO) has estimated that there are about 2 billion people worldwide who consume alcoholic beverages and 76.3 million with diagnosable alcohol use disorders (Rehm *et al.,* 2009). Among the various organ systems that mediate alcohol's effects on the human body and its health, the gastrointestinal tract plays an important role. The alcohol absorption into the bloodstream occurs throughout the gastrointestinal tract and its direct contact with the mucosa can induce numerous metabolic and functional changes. These alterations can lead to marked mucosal damage, which could result in a broad spectrum of acute and chronic diseases, such as gastrointestinal bleeding and ulcers. Pathogenesis of ethanol-induced gastric lesions is complex. Alcohol may interact directly with the gastric mucosa or it may act through a more general mechanism affecting the release of hormones and the regulation of nerve functions involved in acid secretion (Bode and Bode, 1997). Intragastric application of absolute ethanol has long been used as a reproducible method to induce gastric mucosa lesions in experimental animals (Arafa and Sayed- Ahmed, 2003). The effects of acute administration of absolute ethanol to rats and mice on the gastric mucosa are dose-dependent and the damage appears as early as 30 minutes after ingestion and reaches a peak at about one hour. The ethanol-induced gastric mucosal lesions and erosions are similar to those occurring in gastric ulcer (Stermer, 2002). Thus, alcoholic gastritis leads to the impairment of the integrity of gastric mucosal barrier, contributing to acid reflux into the subluminal layers of the mucosa and submucosa (Oh *et al*., 2005). Chari *et al*. (1993) reported that intravenous, oral, and intragastric administration of alcohol at a concentration of up to 5% increases acid secretion principally by stimulating the secretion of gastrin and to a lesser extent by a direct effect on the parietal cells. On the other hand, an alcohol concentration higher than 5% has no effect on gastric acid secretion. Also, decreased formation of prostaglandins might also

play a role in alcohol-induced mucosal injury, while other studies have indicated that an alcohol-dependent increase in the production of leukotrienes might also contribute to the development of alcohol-induced damage (La Casa *et al*., 2000). It is important to emphasize that changes induced by short-term exposure to alcoholic beverages are rapidly reversible while prolonged alcohol exposure leads to progressive structural mucosal damage. Other mechanisms by which alcohol induced damage to gastric mucosa had been found to be associated with oxidative stress and depletion of anti-oxidants and so they have been widely investigated in a number of studies (Arafa and Sayed-Ahmed, 2003). Ethanol treatment induces intracellular oxidative stress and produces mitochondrial permeability transition and mitochondrial depolarization, which precede cell death in gastric mucosal cells.

2.4.5 Oxidative Stress

Oxygen free radicals are by-products of normal cellular metabolism and play important roles in the modulation of cell survival, cell death, differentiation and cell signaling (Touyz, 2005). Free radicals are said to be molecules or molecular fragments containing one or more unpaired electrons in atomic or molecular orbits (Halliwell and Gutteridge, 1999). This instability makes them very reactive, and attempt to pair up with other molecules, atoms, or even individual electrons to create a stable compound. To achieve this, free radicals can “steal” a hydrogen atom from another molecule or interact in various ways with other free radicals.

Radicals derived from oxygen represent the most important class of radical species generated in living systems (Miller *et al.,* 1990).Several studies have linked oxidative stress to one of the major pathogenic factors that directly impaired cellular functions, promotes organelles damage, including mitochondria, lysosomes, and nucleus (Izyumov*et al*., 2010). Since, ROS could directly disrupt the mitochondrial membrane that subsequently leads to

release of cytochrome C; the later becomes a part of apoptosome complex, or in an additive way leads to membrane rupture of the lysosomes and subsequent release of cathepsins, the later activate caspase and apoptosis cascade, and finally leads to cell death via apoptosis. Additionally, these agents are known to act as second messengers to activate diverse redox-sensitive signaling transduction cascades, including mitogen-activated protein kinases (MAPKs) and downstream transcription factors such as NF-kB and AP-1, which regulate the expression of several pro-inflammatory genes and, thereby, lead to the elaboration of chemical and humoral mediators of tissue inflammation and injury (Ali and Harty, 2009).Under various physiological states, ROS are produced and strongly regulated by various detoxifying enzymes, such as SOD, glutathione peroxidase (GPX), and catalase (CAT), or by different antioxidants, including flavonoids, ascorbic acids, vitamin E, and glutathione (GSH) (Olaleye *et al*., 2007).

Several studies reported that polyunsaturated fatty acids are the most vulnerable to free radical attacks and the initial products of lipid peroxidation are conjugated dienic hydroperoxides. Malondialdehyde (MDA) is an end product resulting from peroxidation of polyunsaturated fatty acids and related esters within cell membranes, and the measurement of this substance represents a suitable index of oxidative tissue damage. On the other hand, sulfhydryl compounds such as GSH are involved in the maintenance of gastric integrity, particularly when reactive oxygen species are implicated in the pathophysiology of tissue injury (Blandizzi *et al.,* 2005). Thus, the appearance of lipid free radicals and MDA in the blood and gastric juice could result from ROS-initiated chain reactions or initiated by indirect mechanisms that suppress the antioxidant capacity in both blood and gastric wall to scavenge ROS (Dotan *et al.*, 2004).Numerous studies have also demonstrated a decrease in GSH level in inflammatory and ulcerated gastric mucosa, as well as the protective effect of

GSH on gastric damage induced by ethanol, nonsteroidal anti-inflammatory drugs, or lipopolysaccharide has been well documented (Silva *et al*., 2009).

The fundamental primary product of ROS is lipid hydroperoxides, which are capable of initiating lipid peroxidation chain reaction and decompose giving rise to secondary oxidation products including: aldehydes, hydrocarbons, acids, ketones and higher polymers. Among these, is MDA that is mutagenic and carcinogenic, and its reaction with thiobarbituric acid is a marker for lipid peroxidation as thiobarbituric acid reactive substances. Identifying lipid peroxidation, as the mediator of acute injury is complicated since MDA, the major component of TBARS undergoes rapid further metabolism. Therefore, it was reported that the use of TBARS is not adequate due to its low sensitivity and interferences with several other substances (Dotan *et al.,* 2004).

2.4. 6 Cytokines

These peptides exercise their biological effects via an interaction with specific membrane receptors leading to transduction of an intracellular message. Abnormal expression of cytokines is correlated with disease morbidity and mortality. Gastric damage is arbitrated by diverse of mechanisms involving numerous inflammatory cytokines are implicated in the development of ulcer. During inflammation, penetration of neutrophils stimulates the secretion of several pro-inflammatory cytokines (Yuan, 2006). The most important pro-inflammatory cytokines are the interlukin-1beta and tumor necrotic factor that are known to perform an imperative role in the generation of acute inflammation following neutrophils infiltration. Neutrophils achieve this via production of superoxide radical anion and ROS. Superoxide radical anion interact with specific receptors on the target cells, resulting in the generation of lipid peroxides that are converted to malondialdehyde (Konturek *et al*., 2000).

2.5 PLANTS AS DIETS

Plant-based natural remedies have been known to offer relief and cure since ancient times, and for many centuries, increasing interest in relationship between health and food, results in increased formulation studies about functional products.

Epidemiological studies and clinical trials have now associated plant-based diet intake (especially diets rich in Polyphenols) with various beneficial biological activities that can ameliorate diseases such as diabetes type 2,cardiovascular disease (CVD), obesity, neuro-degenerative disorders and some types of cancer(Henderson *et al*.,2012). Phytosterols cannot be synthesized by humans, and all plant sterols and stanols in the human body therefore originate from the diet. The class of sterols known as phytosterols is found mainly in plant cell walls and membranes. Their importance stems from their abundance in the diet, their antioxidant properties and ability to regulate various biological/biochemical processes perhaps through a number of cellular signaling pathways and modulation of genes expression. In the last decade, scientific and commercial interest in polyphenols has grown dramatically, and thousands of studies investigating their bioactivities, metabolism and health effects are published every year. However, there is still a lot of information lacking as far as the mechanism of action of these molecules and what happens to them inside the body (Erkkila *et al*., 2005). Dietary antioxidants are referred to as non-enzymatic antioxidants and include polyphenols, carotenoids, flavonoids, phenolic acids, vitamins, minerals and organosulfur compounds. Also enzyme cofactors and low molecular weight molecules are recognized as endogenously produced antioxidants. Nowadays, because of excessive production of reactive oxygen or nitrogen species (RONS), as well as the inadequacy of the body's endogenous defense system to eliminate their production, in the absence of dietary antioxidants, oxidative damage can easily accumulate. Under physiological conditions, RONS are removed by antioxidant enzymes including catalase, superoxide dismutase and

glutathione peroxidase. However, when activities of antioxidants are reduced, often as a consequence of CVD, ageing or excessive production of free radicals, increasing dietary antioxidant consumption is fundamental so that the RONS balance is restored (Wootton-Beard and Ryan, 2011).

The importance of food is not only for the production of energy and body matter of classic metabolism, but also a conditioning environment that modulates the epigenome activity and influences stress adaptive responses, energy metabolism, immune homeostasis, and the physiology of the body. It can be postulated that the pleiotropic effects of plant phytochemicals on body systems can be translated into stable epigenetic patterns of gene expression, and thus diet interventions designed for healthy aging might become a hot topic in nutritional epigenomic research(Maynard and Franklin,2003).

Many bioactive constituents of food have been commercialized in the form of pharmaceutical products in the past few years as pills, capsules, solutions, gels, liquors, powders, granulates that incorporate food extracts or phytochemical-enriched extracts to which a beneficial physiological function has been directly or indirectly attributed. This range of products cannot be truly classified as “food” or “pharmaceutical”, and a new hybrid term has been coined to designate them as nutraceuticals’. This was coined by Stephen DeFelice, founder and chairman of the Foundation for Innovation in Medicine. No official definition exists for the term “nutraceutical”, though it is often used to describe a broad list of products sold under the premise of food components with an expressed intent of treatment or prevention of disease and for enhancing the health and wellbeing of an individual. Nutraceuticals is a unique intersection of the pharmaceutical and food industries. No clear demarcation exists between food and drugs, but the law mandates such distinctions be made. It appears that the nutraceutical industry has found a comfortable middle ground between the food and drug industries. Nutraceuticals are not drugs, which are potential pharmacologically

active substances that potentiate, antagonize, or otherwise modify any physiological or metabolic function. On the other hand a nutraceutical is evidently a food component that not only maintains, supports, and normalizes any physiologic or metabolic function, but one that also potentiates, antagonize, or otherwise modify physiologic or metabolic functions.

Cereals are an important cost effective commodity in the world. Food ingredients from cereals with nutraceutical properties can contribute to health benefits to many people. Cereals like wheat, maize, rice, oats etc are now employed in preparation of foods that are similar in appearance to conventional foods and used in normal diet but have an added advantage of aiding physiological functions along with providing nutrition. In recent years, cereals and its ingredients are accepted as functional foods and nutraceuticals because of providing dietary fiber, proteins, energy, minerals, vitamins and antioxidants required for human health.

2.5.1 Rice Production

Rice (Oryza sativa L) is known as the grain of life is the staple food in many countries of Africa and other parts of the world and second most widely consumed cereal in the world next to wheat (Kent and Evers, 1994). After sugarcane and maize, rice is the 3rd highest agricultural commodity with the worldwide production. Saka and Lawal (2009) classified rice as the most important food depended upon by over 50 percent of the World population for about 80 percent of their food need. It was reported that Nigeria spends over USD$11 billion in the importation of wheat, rice, sugar and fish every year. Rice contributes about USD$3.56 billion to the amount (Akinwumi, 2012). It is reported that the World's stocks of stored rice grain have been falling relative to each year's use, because the consumption has surpassing the production (Roy and Shiina, 2010). The deficiency of food production not only increases the price of food, but also increases the malnourished

population in the World, which might result in different social and economic unrest.Nwite *et al* (2008) indicated that the adoption of technologies and improved management practices should lead to substantial yield increase in rice production. The major rice growing countries are China, India, Indonesia, Bangladesh, Thailand, Burma, Vietnam, Japan and Philippines (Roy *et al.,* 2011).

Rice plant can grow not only in the deep water areas (up to 5 meter) but also on dry land, called semi-aquatic annual grass plants. Rice plant can grow tall up to approximately 1-1.8 m (3.3-5.9 ft), depending on the variety of soil and its fertility. The leaves of rice plant are slender 50-100 cm (12-20 in) long and 2-2.5 cm (0.79-0.98 in) wide. The seed of edible part is a grain, which is 5-12 mm (0.20-0.47 in) long and 2-3 mm (0.079-0.118 in) thick. Nemoto *et al*. (1995) noted that rice can be cultivated in temperate and tropical areas as well as in cool and warm regions. Different environmental condition is considered as important factors to develop rice plant, such as day length, temperature, humidity, planting density and nutrition (Wayne and James, 1994). Global rice production is 645 million tons and this huge amount of production results large amount of rice by products (Al-Okbi *et al*., 2014). The Asian continent accounts for approximately 90 percent of rice production and is also the major consumer. In 1999-2000, land devoted to world rice production was 381 million acres. In 2004–2005, global rice production was forecasted to be 397.8 million tons (milled basis). The US share in global rice production is around 1.5 –2 %. Recently, US domestic market has grown to 60 percent of production. American rice production is concentrated mainly in the south with Arkansas contributing a projected 4.81 million tons of the 10.23 million tons of US rice production forecasted for 2004-05. Louisiana ranks second in the total area and third with respect to total production of rice and is projected to contribute 28.1 million cwt of rice. Per capita consumption of rice in Asia was estimated at 104.32 kg per annum whereas the average global consumption per capita is 65.77kg. American per capita consumption is

12.25 kg/year, but has nearly doubled in the past 20 years. Rice has two major subspecies: the sticky short grained *japonica or sinica* variety and the non-sticky, long grained *indica* variety. *Japonica* varieties are usually cultivated in dry fields, in temperate East Asia, upland areas of South East Asia and high elevations in South Asia, while indica varieties are mainly lowland rice grown mostly submerged, throughout tropical Asia (Molina *et al.,* 2011). Rice endosperm (70%) is obtained as a major product while rice husk (20%), rice bran (8%) and rice germ (2%) are obtained as byproduct of rice milling industry, which is obtained from the seed of the grass species of the *Oryza sativa* (Asian Rice) or *Oryza glaberrima* (African rice) (Hoed *et al*., 2006). Over 2 billion people in Asia alone derive 80% of their energy need from rice, which contains 80% carbohydrates, 7-8% protein, 3% fat and 3% fiber (Juliano,1985).Until recently, rice was considered only a starchy food and a source of carbohydrates and some amount of protein. Rice protein, though small in amount, is of high nutritional value and also a relatively good source of thiamin, riboflavin, niacin, phosphorous, iron and potassium. Non-allergenic and gluten-free characteristics make rice ideal for persons with these special dietary requirements. Rice starch mainly differs in amylose content; amylose molecule determines the grain's gelatinization temperature, pasting behavior and visco-elastic properties (Tavares *et al*., 2010), and has been an important component to be considered in quality breeding of rice (Bhattacharya, 2009). Amylose content in *Indica* rice variety is usually used for manufacturing rice noodles, while *Japonica* rice is partially used to adjust the noodle texture (Huang *et al*., 2007). Amylose content, gelatinization temperature and grain length are used as quality indicators to introduce new rice varieties and the color of rice is an important sensory parameter (Lamberts *et al.,* 2007).

Figure 2.3: A typical structure of *Tapa* / *Alasoosun* rice plant

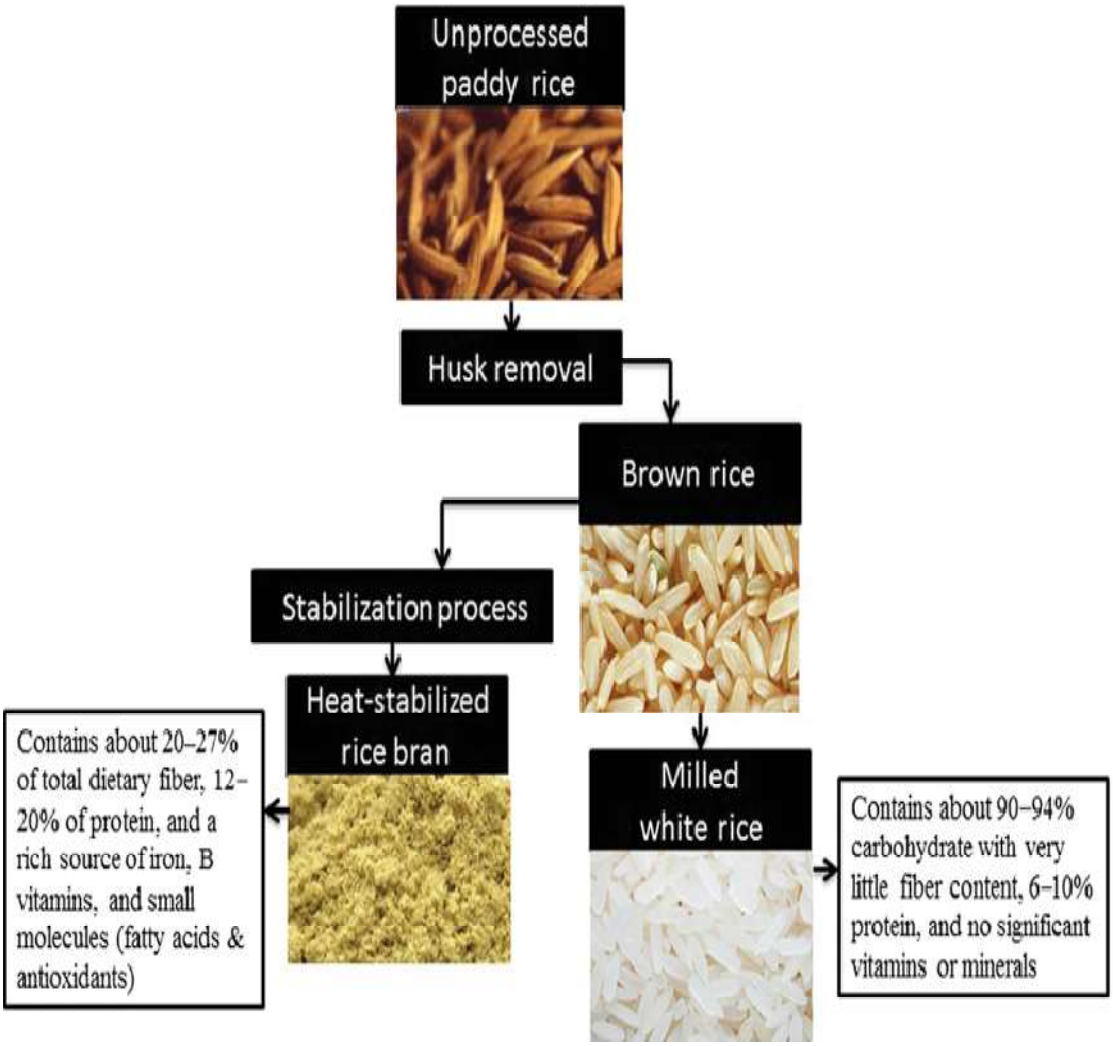


Figure 2.4: Whole grain rice processing into rice and rice bran end products (Juliano, 2007)

2.5.4 Rice Milling

Milling processes is a method of removing bran, hull, and germ with minimum endosperm breakage from rice seed until milled rice (polished white rice) is produced. Harvested rice is in the form of rough rice (paddy) with the edible portion covered with an outer protective layer known as the husk or hull. The outer most layer of the husk contains very little nutrients, but provides protection against insect infestation and fungal infestation (Hu, 1995).

Rice milling step involves pre-cleaning/destoning, husking, paddy separation, whitening (bran removal), polishing, grading, and milling. Each milling step has different function. However, all of steps influent to milled rice quality (Elke and Emanuele, 2013). Removal of the hull from the rice kernel in rice processing results in brown rice, which is called shelling. Removal of thin layer (bran) leads to the production of white rice. White rice is consumed after appropriate polishing to further remove any remaining bran layers and to give a desired degree of whiteness and polish. Moreover, the look of brown rice is not appealing due to its color (Saunders, 1990), as a result brown rice is rarely found in the market. Brown rice is an excellent source of energy, vitamins, and minerals. However, the process of milling and polishing that converts brown rice into white rice destroys virtually all the nutrients (67% of the vitamin B3, 80% of the vitamin B1, 90% of the vitamin B6, half of the manganese, half of the phosphorus, 60% of the iron, and all of the dietary fiber and essential fatty acids) (Ensminger and Ensminger, 1986). In United States by law, fully milled rice must be "fortified" with vitamins B1, B3 and iron. Unfortunately, the form of these nutrients when added back into the processed rice is not the same as in the original unprocessed grain, and at least eleven lost nutrients are not replaced in any form even with rice "enrichment". The difference between brown rice and white rice goes beyond color. Further milling of brown rice to remove the bran coat and most of the germ layer will

produce white rice which is devoid of the bran-based nutrients. Further milling of rice called 'polishing' will result in more whiter rice we buy from the market. During fine polishing the thin aleurone layer containing important nutrients and oil is removed from the grain. The fat layer is removed to extend the shelf life of the grain since this fat is liable to be decomposed by the grain enzyme lipase to produce free fatty acids and leading to the development of rancidity. Thus the final white rice is simply a refined starch that is largely bereft of its original nutrients (Erkkila *et al.,* 2005).

2.5.5 Rice Consumption

Rice is consumed in three categories: it could be used directly as food, processed and in breweries. Comparison study between polished and brown rice, showed high nutritional value in brown than polished rice (Roy *et al.*, 2008). However, advance in technology have transformed the use of rice from direct food to food product and beverages such as rice flour bakery products, cakes, rice breakfast cereals, rice crackers, noodles, germinated brown rice, infant foods, canned and frozen rice, rice snack, vinegar, even alcoholic rice beverages(Elke and Emanuele, 2013).

Since production and milling process of rice extremely influent the nutritional value of the final rice products. By increasing consumption of brown rice, characterized by a higher content of healthy beneficial food components (Roy *et al.*, 2011), compared to the polished (white) rice, will significantly help to avoid malnutrition and other dietary and food-related diseases in the future. Meanwhile, researchers have been encouraged to use rice flour in food industry to substitute wheat flour due to amount of rice production that equivalent to wheat harvest and it is classifieds as gluten-free cereal.

2.6 RICE BRAN

During de-husking and milling of paddy, the brownish portion of rice taken out in form of fine grain (Hernandez *et al*.,2000). Rice bran is the outer covering of rice kernel it consists of pericarp, aleurone, sub-aluerone layer, seed coat, nucellus, part of the germ and small part of starchy endosperm (Friedman, 2013). During milling process, rice bran containing nutrients is completely removed. Approximately sixty million metric tons of rice bran is produced worldwide every year and almost all of it is either thrown away or used as low level animal and poultry feed (Moko *et al.,* 2014). Rice bran constitutes 8% of the weight of the whole grain and contains most of the nutrients (65%), an appreciable amount of protein (11-17%), fat (12-22%), and ash (8-17%). It has also been reported to contain high fiber (99%), low sodium, low sugar (1.1g), but no cholesterol, all these constituents may contribute to the low plasma level of the various parameters of the lipid profile (Neelam *et al.,* 2011). It is also a rich source of vitamins including vitamin E, thiamine, niacin, and minerals like aluminum, calcium, chlorine, iron, magnesium, manganese, phosphorus, potassium, sodium and zinc, dietary fiber and more than 100 antioxidant nutrients that helps to fight against disease and promote good health (Sereewatthanawut *et al*., 2008). Rice bran, on the other hand, can serve as food supplement and as a valuable source of edible oil. Rice bran - both full fat and defatted has been found be rich source of nutrients. Both the bran and oil from rice bran have a range of bioactive phytochemicals with potential for reducing the risk of chronic degenerative diseases (da-Silva *et al*., 2006).There is a need to utilize the full potential of the available rice bran in the country, both as a source of healthy edible oil and as a food supplement for promoting our population's nutrition and health. Many studies reported that rice bran has cholesterol lowering properties, cardiovascular health benefits and anti-tumor activity (Tuncel *et al*., 2014b).

2.6.1 Stabilization of Rice Bran

Rice bran is not properly used despite its beneficial nutritive and biological effects due to lipase enzyme present in it. This enzyme interacts with the lipid in the bran leading to hydrolytic rancidity on the oil content that hydrolyze the ester bonds of triacyglycerol, releasing fatty acids and glycerol and forming of hyperoxides. These problems have now been overcome by destroying the lipolytic activity using an advanced stabilizing technology; the product thus obtained is called "stabilized" rice bran, which has a good taste, readily soluble with a longer shelf life of one year(da-Silva *et al*., 2006).

This process of stabilization is aimed at destroying or inhibiting the activity of lipase enzyme. A variety of methods are available for stabilization of rice bran;.microwave heating, (Ramezanzadeh *et al,.*1999a, b), drier/oven (Issara and Rawdkuen, 2016).Also, Lakkakula *et al.* (2004) reported the stabilization by ohmic heating. It has been reported as most effective method, it has been shown to improve the oil extraction yield. Some chemicals like sodium meta-bisulphate can also be used to stabilize rice bran. Presently, acid with antioxidant properties, such as ascorbic, ascorbyl, palmitate, phosphoric acid and mixture has been reported to be used to stabilize rice bran.

2.6.2 Rice Bran Oil

The beneficial role of rice bran has been attributed to its lipid content which account for 12–22% of rice bran weight (Sharif *et al*., 2014). This is making it to gain attention of several researchers. In 1996, world rice bran oil production was estimated to be 450,000 metric tons (MT), of which 100,000 MT was produced in Japan.Rice bran oil is widely used as edible oil in several countries such as Japan, Korea, China, Taiwan, Thailand and Pakistan (Ruknmani and Raghuram, 1991).

Composition of rice bran oil may vary according to the rice variety, rice bran make up and the procedure employed for bran extraction (Salunkhe *et al.,* 1992).Rice bran contains crude rice bran oil, is rich in unsaturated linoleic and oleic fatty acids and bioactive compounds such as γ-oryzanol, phytosterols, tocopherols, and tocotrienols.In addition to nutritious components and health benefits of rice bran oil, some properties such as good stability, appealing flavor and long fry life, provides the rice bran oil in shortening (Liang *et al*., 2014).

2.6.3 Health Benefits of Rice Bran

As traditional diets shift towards convenience around the world, perceptions regarding healthy foods begin to waver and food decisions are based on cost, ease, and preferences. These are important considerations for incorporating rice bran into meals and snacks, and may influence current beliefs or existing knowledge of whole grain, brown rice preferences. There is continuing growth in the demand for brown rice by health conscious people in developed countries (Dipt*et al*., 2012). Additionally, food scientists and health researchers are aware of the important bioactive compounds in rice bran, yet there continues to be a lack of global consumer awareness regarding the importance of rice bran for chronic disease control and prevention.

Rice bran is used for the enrichment of foods, due to its high dietary fiber content. The role of dietary fiber in health and nutrition has stimulated a wide range of research activities which caught public attention. Accumulating evidence favors the view that increased intake of dietary fiber can have beneficial effects against diseases, such as cardiovascular diseases, gastrointestinal disease, decreasing blood cholesterol, diverticulosis, and diabetes and colon cancer. In view of the therapeutic potential of dietary fiber, more fiber incorporated food products are being developed. Addition of dietary fiber to a wide

range of products will contribute to the development of value-added foods or functional foods that currently are in high demand (Hu, 1995).

Rice bran also plays an important role in decreasing cholesterol and controlling of blood glucose level (Nagendra *et al*., 2011). Stabilized rice bran (SRB), is a powerful source of vitamins, nutrients, proteins and fiber, because it contains an approximate insoluble versus soluble fiber ratio of 5 to 1, which exhibits a high digestive tolerance that occurs along the whole digestive tract with no excessive fermentation in the large intestine. Stabilized rice bran contains a good number of quality synbiotics, tocols, oryzanols, polyphenols, sitosterol, phytosterols with omega-3 and omega-6 fatty acids. Healthy complex carbohydrates found in processed rice bran have "low glycemic index" which means they do not cause spikes in blood glucose (Sayre *et al*., 2007).

Studies have shown the unsaponifiable matters present in the rice bran significantly reduce liver cholesterol level; the low cholesterols influenced coronary heart disease. Also, it has been reported that dietary fiber from cereals grain can reduce the risk of coronary heart disease, blood pressure and improving insulin sensitivity (Truswell, 2002).

Recent studies have shown that rice bran can inhibit microbial growth. In most cases, microorganisms are important factors responsible for diarrheal disease. Minimum inhibitory concentration of rice bran extract has been reported to be effective against *V. cholerae* strain Thus, it seems that rice bran extracts might contribute to the treatment of diarrheal disease (Issara and Rawdkuen,2016). In addition, when rice bran was administered against HIV cell, the result did not only show bacteria inhibition, but that rice bran extracts also promoted against viruses cell (HIV) (Friedman, 2013). Henderson *et al* (2012)found that 10% rice bran consumption modulated mucosal immunity by increasing immunoglobin A concentrations and native gut *Lactobacillus* spp. in a mouse model.Studies have also shown that rice bran

augments phagocytosis and enhances intracellular killing of microbes by human phagocytic cells (Ghoneum *et al*.,2008). Additionally, the human gut microbiome was shown to be altered, with immunological benefits, through consumption of whole grains, including brown rice (Martinez *et al.,* 2013).

It is now recommended that infants should be exclusively breastfed for the first 6 months of life for proper growth and development (Dipt *et al*., 2012). After this period, complementary foods are introduced to an infant's diet; these may include food products based on staples, as well as fruits and vegetables, in liquid to semi-solid forms. Acceptable weaning foods may also include higher amounts of protein and fats to continue healthy development into the toddler years, including animal-based sources such as meat, milk, and eggs. However, in areas that lack food security, these options may not be available to young members of the family. This can lead to inadequate nutrient uptake and underdevelopment of infants' and toddlers immune systems, making them more susceptible to diseases such as viral and bacterial associated pneumonia and diarrhea (Guerrant *et al*., 2008). The protein quality of rice bran is more suitable compared to other cereal bran, because it contains a substantial amount of lysine and the amino acid content meets the requirements of growing children (WHO, 2007).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1. MATERIALS

Dissecting set, oral cannula, dissecting board, cotton wool, stop watch, permanent maker, organ bottles, EDTA bottles, 5 and 10 mLSyringes, nose cover, beakers, conical flask, measuring cylinder, methylated spirit, camera, spectrophotometer, pH meter, centrifuge, desicator, analytical balance.

3.1.1 Drugs, Solvents and Reagents

Alcian blue, sodium acetate, magnesium chloride, sucrose, diethyl ether, sodium chloride, distilled water, hydrochloric acid, sodium hydroxide, griesse reagent, trichloroacetic acid (TCA), thiobarbituric acid (TBA), Enzyme ELISA kits (prostaglandin E), indomethacin, CH_2SO_4 and Acetone.

3. 2 PLANT MATERIALS

Alasoosun and *Tapa* rice werethe Nigerian rice varieties used in this study. They were purchased from Oke-imesi town in Ekiti State and Sapefu village in Lafiagi local government area of Kwara State respectively. Freshly milled rice bran from *Tapa* rice was collected daily from Bodija market to avoid rancidity. The rice and rice bran were ground to fine powder, and was thereafter pelletized.

3.3 PHYTOCHEMICAL SCREENING

A method described by Trease and Evans (1989) was employed to test phytochemicals constituents in the rice extract.

3.3.1 Test for Taninns

Two mililitre (2 mL) of rice extract was heated in 40mL of water and sieved. A drop of 0.2% ferric chloride was added into the filterate. Green or deep blue coloration confirms the presence of tannin.

3.3.2 Test for Phlobatannins

Two mililitre (2 mL) of rice extract was heated with 0.9% Hydrochloric acid; the present of red color was considered as proof for phlobatannins.

3.3.3 Test for Saponin

Twenty mililitre(20mL) of distilled water and 5mL of the rice extract were boiled and sieved. Afterward, 20mL of the filtrate and10mL of water were mixed for a steady persistent froth and 3 drops of olive oil was also added and mixed vigorously, emulsion formation, which verifies the presence of saponin was detected.

3.3.4 Test forFlavonoid

Five mililitre (5 mL) of each of the rice extract was mixed with 3mL of 1% Aluminium chloride solution. Yellow color that confirms the presence of flavonoids was seen. To this mixture, 6mL of ammonia solution and concentrated H_2SO_4 were added. On standing, yellow color disappeared indicating a flavonoids positive test.

3.3.5 Test for Steroids

Four mililitre(4mL) of Acetic anhydride and 4mL of rice extract were added followed by cautious addition of 4mL H_2SO_4. The presence of steroids was confirmed as the color changed to blue.

3.3.6 Test for Terpenoids

Two mililitre(2 mL) of chloroform and 5mL of rice extract were mixed together followed by cautious addition of 3mL H_2SO_4. Reddish brown coloration represents presence of terpenoids

3.3.7 Test for Cardiac Glycosides and Cardenolides

Four mililitre(4 mL) of acetic acid with one drop of ferric chloride solution and 10mL of rice extract were added followed by careful addition of 2mL of sulphuric acid. Brown ring observed shows deoxysugar characteristics of cardenolides which shows a presence of cardenolides. Below the brown ring is a violet-green which indicates the presence of glycoside.

3.3.8 Test for Alkaloids

Ten mililitre(10mL) of 2% hydrochloric acid was stirred with 2mL of the rice extract on a steam bath and sieved immediately. Water was added to the residue and a few drops of Mayer's reagent were added to 1mL of the filtrate. The appearance of a cream color indicates the present of alkaloids.

3.3.9 Test for Anthraquinone

Ten mililitre(10 mL) of Benzene was taken to5mL of the rice extract and sieved, and 5mL of 15% NH_3 was mixed with the filtrate. This mixture was vigorously mixed; the presence of pink in the lower layer represents anthraquinones.

3.3.10. Test for Chalcones

Two mililitre (2 mL) of ammonia was taken to 5mL of the rice extract. The appearance of a reddish color represents chalcones.

3.3.11 Test for Phenol

Ten mililitre(10 mL) of the rice extract was taken into a 60mL test tube, followed by addition of 10mL of water. 10mL of amyl alcohol and 2mL of ammonium hydroxide solution were mixed together and left for thirty minutes to react. The green coloration that was seen confirmed phenol's present.

3.4 PROXIMATE ANALYSIS

Methods decribed by Association of Official Analytical Chemist (2005), were employed to analyze chemical constituents of the rice samples. All the analysis was carried out in duplicate.

3.4.1 Procedure of Crude protein Determination

Semi-micro Kjeldahl procedure or technique was used to determine crude protein in the sample. This comprises three procedures 0.5g of powdered sample was weighed cautiously into the tubes, 1 Kjeldahl catalyst tablet and 15mL of Concentrated H_2SO_4 was added to the tubes and were carefully placed in the proper hole of the Digestion Block

Heaters. After a period of 4 hours, a pure colorless solution was observed. The digest was allowed to cool and cautiously moved into 100mL flask, the digestion tube thoroughly rinsed with distilled water and distilled water was addedto the flask up to mark.

Markham Distillation Apparatus was used for distillation because it permits volatile substances to be steam distilled with whole distillate. Steam producer was taken from the heating mantle to remove the settled water. The steam producer was returned back to the heat source.

Ten mililitre (10 mL)of the digest prepared above and 10mL of 40% (W/V) NaOH were added to the apparatus. This was steam-distilled for 4minutes in a 100mL conical flask containing 20mL of 2% Boric Acid with indicator solution. Changes of color from red to green observed indicate that all the ammonia released has been trapped.

The green color solution was titrated against 0.02N HCL in a 100mL Burette. The green color obtained change to wine color at the end point which confirms that all Nitrogen ensnared as $NH_{42}BO_3$ have been eliminated as NH_4CL.

Nitrogen percentage was estimated by the following formula:

$$\% N = \frac{\text{Titre value x Atomic mass of Nitrogen x Normality of HCL used x 4or}}{\text{Weight of sample digested in milligram x Vol. of digest for steam distillation.}}$$

$$\% N = \frac{\text{Titre value x Normality/Molarity of HCL used x Atomic mass of N x Volume of flask containing the digest x 100}}{\text{Weight of sample digested in milligram x Vol. of digest for steam distillation.}}$$

The crude protein content is determined by multiplying percentage Nitrogen by a constant factor of 6.25 i.e. % crude protein = % N x 6.25.

3.4.2 Determination of Crude fat

One gram (1 gm) of powdered sample was taken into a thimble and blocked calmly with cotton wool. 260 mL soxhlet flask was dried in the oven and allowed to cool and weighed. In to the soxhlet flask petroleum ether (40º – 60ºC) was filed up to $^3/_4$ of its volume. Extractor was put on the heater mantle for 7 hours with regular supply of water and observed closely for ether escape, at the same time the heat mantle was regulated properly. The Ether was left to drain off until it's all disappeared. The thimble was withdrawn and allowed to dry. The flask, extractor and condenser were restored and the distillation was on till the flask was completely dried up. The flask that has oil was disconnected and dried to a stable weight in the oven. If the initial weight of dry soxhlet flask is Wo,

the final weight of oven dried flask + oil/fat is W_1,

percentage fat/oil is obtained by the formula:

$$\frac{W_1 - Wo}{\text{Wt. of Sample}} \times \frac{100}{}$$

3.4.3 Determination of Dry matter and moisture

Four grams of the rice extract was added into a crucible that has been earlier weighed and placed on an oven positioned at 100^0 Cto dried up to a steady weight for one day.After then, the crucible and substance was detached from the oven and moved to dessicator where it was allowed to cool for twenty minutes.

$$\% \text{ Moisture} = \frac{W_1 - W_3}{W_1 - Wo} \times 100$$

3.4.4 Determination of Ash content

Procedures: Four grams of the rice extract was taken into a crucible and placed on heater mantle positioned at 550ºC for 8hours. At this period the color changed to white ash. The percentage of ash was estimated as follows

$$\text{Ash level} = \frac{\text{weight of ash}}{\text{original weight of the substance}} \times 100$$

3.4.5 Determination of Fiber

Procedures: Two grams of the rice sample and 100mL of 0.255N H_2SO_4 were precisely measured into the fiber flask and boiled for 2 hour. When the time was over the hot combination was sieved. The residue was taken back to the fiber flask and 200mL of 0.313N NaOH was taken into the flask and boiled for another 1 hour. The combination was sieved and 10mLof acetone was taken to dissolve any organic constituent. The residue and sieve cloth were clean with 100mL hot water before it was finally taken into the crucible and oven-dried at 105ºC overnight to drain off the water. After that the dried crucible containing the residue was allowed to cool in a desicator and weighed.

3.5 EXPERIMENTAL ANIMALS

Seventy-five male Wistar ratsweighing between 150-170g were used for this study. They were procured from the Central Animal House of the Faculty of Basic Medical Sciences, University of Ibadan. The animals were allowed to adaptto the environment for

two weeks before commencement of the project.They were maintainedunder standard laboratory condition and fed with standard rat's pellet (Ladokun Feeds) and water given ad libtum. After two weeks of acclimatization, the animals were grouped into five main groups of fifteen rats each.

3.5.1 Experimental design and Groupings

This study was divided into three phases:

Phase One: This was carried out to investigate the effect of Nigerian rice varieties on the body weight and ulcer formation.

PhaseTwo- This was carried out to investigate the effect of Nigerian rice varieties on biochemical indices (SOD, Sufhydryl and MDA)

PhaseThree- This was carried out to investigate the effect of Nigerian rice varieties on gastroprotective (NO, gastric blood flow, gastric mucus content and PGE)factors.

Animals Grouping

Group I: (Normal control) animals were fed with standard diet of normal rat pellets (Ladokun feeds) and clean water.

Group II: (Ulcerated control) animals were fed with Standard diet of normal rat pellets (Ladokun feeds) and clean water.

Group III: Animals were fed with *Alasoosun* (local) rice supplemented with 20% of standard feed of normal rat pellets and water.

Group IV:Animals were fed with *Tapa* (local) rice supplemented with 20% of standard feed of normal rat pellets and water.

Group V: Animals were fed with rice bran supplemented with 20% of standard feed of normal rat pellets and water.

3.5.1 Experimental induction of Gastric Ulcer

Indomethacin was used to induce gastric ulcer at a dosage of 40mg/kg body weight in all the groups except for group I (normal control). The rice varieties and rice bran had been appropriately fed to the animals for eight weeks. The animals were fasted for twenty-four hours but were given access to water and were thereafter, anesthetized with 35mg/kg intraperitoneal ketamine. Indomethacin dissolved in 0.5mL of 1.25% Sodium bicarbonate was administered orally four hours prior to sacrifice, and thereafter the stomachs were removed and cut open for scoring (Wallace *et al.,*2000).

Scoring ofUlcer Spots

The ulcer scoring method was according to Alphin and wards (1967). The following criteria were used

Criteria	Score
Normal Stomach	0
Punctuate Ulcer	0.5
Two / More ulcers	1
Ulcers greater than 2mm	2

Index of Ulceration

Paul's Index was used (Martin-Aragon *et al*.,1994).This index is expressed as M x N/100

Where M= mean number of ulcers per rat in the group, N= percentage of rats with ulcer in the group.

Calculation of Percentage Inhibition

$$\%\text{Inhibition} = (I_c - I_t / I_c) \times 100$$

Where I_c is the ulcer index for control group I_t is the ulcer index for the treated group.

3.5.2 Histological Preparation of Slides

Sections were prepared from strips removed from the fundic area of the stomach and stained using Hematoxylin and Eosin stain according to the method of Marks and Drysdale (1995).Stomach was fixed with 10% formalin and embedded in paraffin, sectioned at 5un in an automated microtome. The gastric tissue integrity (mucosa-submucosa) was then assessed for damage.

3.5.3 Estimation of Gastric Mucus Content

Adherent gastric mucus secretion was measured by using the method of Corne *et al* (1974). The excised stomachs were transferred into 0.1% Alcian blue for two hours, in buffer solution containing 0.1 mol/L sucrose and 0.05 mol/L sodium acetate (the pH was adjusted to 5.8 with hydrochloric acid). After washing the stomach twice in 0.25 mol/L sucrose (15min and 45min), the dye complex with mucus was eluted by immersion in 10 mL aliquots of 0.5 mol/L $MgCI_2$ for 2 hours. The resulting blue solution was mixed with equal volumes of diethyl ether and the optical density of the aqueous phase was measured spectrophotometerically at 605nm. The absorbance of each solution was then used to

calculate the various concentration of dye and the weight of dye (expressed in mg) deduced, using a standard curve. The weight of dye was then expressed over the weight of the stomach, to give the weight of mucus secreted, as stated below:

$$\text{Gastric mucus secretion (mg/g tissue)} = \frac{\text{Weight of dye (mg)}}{\text{Weight of stomach (g).}}$$

3.5.4 Gastric Blood Flow (GBF)

Estimation of gastric blood flow was carried out using the T206 dual channel blood meter (Transonic,USA). Unlike conventional Doppler probes that measures velocity; transonic flow probes used in this study measured the volume of blood flow in the target vessel directly and recorded same in mL/min. The miniaturize probes are specifically adapted for the size of mouse and rat vessels thus removing unwanted artifact common in large probes. The rat's celiac trunk was exposed by a midline section just below the sternum and the vessel was hooked to a PS series flow probe to monitor blood flow through the artery which was converted to a digital output on the flow meter (mL/min). Flow was measured at 15minutes interval over a 90minutes period starting from one hour of post-treatment (Kvietys *et al*.,1985).

3.6 Homogenization of Stomach Tissues

The excised stomachs were first blotted on filter papers in order to remove blood and other extraneous tissues that may compromised the assays. The tissues were washed in ice cold 1.15% potassium chloride solution, weighed and chopped into bits before homogenizing in four volumes of the homogenizing buffer (50mM Tris HCL,1.15% KCL, P^H 7.4) using a potter-elvegin homogenizer. The resulting homogenate was centrifuged at 10,000g and at 4^0 C for 10 minutes. The supernatant was collected and then used for biochemical analysis.

3.6.1 Lipid peroxidation assay

Lipid peroxidation (LPO) was assayed for by measuring the thiobarbituric acid reactive products (TBARS) present in the test sample. This was based on the method of Vashney and Kale (1990) and expressed as micromolar of malondialdehyde (MDA/g tissue). It is based on the principle that the ratio of chromogenic reagent (2-thiobarbituric acid) to MDA (an end product of LPO) under acidic condition to yield a stable pink chromophore read with a spectrophotometer at a maximum absorption of 532nm wavelength.

MDA level (units/mg protein) was calculated according to the method of Adam-Vizi and Sergei (1982) with a molar extinction coefficient of 1.56×10^5 M^{-1} cm^{-1}

$$\text{MDA level (units/mg protein)} = \frac{\text{Absorbance X Volume of mixture}}{E_{532nm} \text{ X Sample volume X mg protein}}$$

3.6.2 Superoxide Dismutase (SOD) assay

The level of SOD activity was determined by the method of Misra and Fridovich (1972).This assay method involves the inhibition of autooxidation of adrenaline to adrenochrome by SODat pH 10.2. Superoxide anion generated by the xanthine oxidase reaction causes the oxidation of epinephrine to adrenochrome. The yield of adrenochrome produced per superoxide anion introduced increases in increasing concentration of epinephrine. The result led to the proposal that autoxidation of epinephrine proceeds by at least two distinct pathways only one of which is a free radical chain reaction involving superoxide radicals and hence inhibitable by SOD.

0.05M Carbonate buffer (pH 10.2)

Sodium carbonate dehydrates, $Na_2C0_3.10H_20$ (1.97 g), and 0.525g of Sodium hydrogen carbonate, Na_2HCO_3, was dissolved in 100 mL of distilled water. The pH was then adjusted to 10.2 and then made up to 125 mL with distilled water.

0.3mM Epinephrine

Adrenaline (epinephrine) (0.01 g) was dissolved in 200 mL of distilled water which was prepared fresh as needed.

Assay Protocol: To make a 1 in 5 dilution, 0.2 ml of sample was diluted in 0.8 mL of distilled water. An aliquot of 0.2 mL of the diluted sample was added to 2.5 mL of 0.05M carbonate buffer (pH 10.2) in the cuvette and allowed to equilibrate in the spectrophotometer. The reaction is started by addition of 0.3 mL of freshly prepared 0.3mM epinephrine to the mixture which was quickly substrate (epinephrine) and 0.2 mL water. The increase in absorbance at 480nm was monitored every 30seconds for 150seconds.

Calculation

$$\text{Increase in absorbance per minute} = \frac{A3-A0}{2.5}$$

Where A_0=absorbance at 30 seconds

A_3= absorbance at 150seconds

$$\% \text{ inhibition} = 100-\left(100X \frac{\text{increase in absorbance for substrate}}{\text{Increase in absorbance for blank}}\right)$$

1 unit SOD activity was given as the amount of SOD necessary to cause 50% inhibition of the oxidation of epinephrine.

3.6.3 Determination of Sulfhydryl Concentration

Sulfhydryl concentration was measured according to Sedlak and Lindsay (1968). Briefly, 0.2M phosphate buffer: 8.40g of NaH_2PO_4 and 9.9., 4 of Na^2HPO_4 were dissolved in distilled water and made up to 1000 mL mark in volumetric flask. The buffer was adjusted to pH 8. To 150 µL of serum, 1.5 mL of 10% TCA was added and centrifuged at 1500g for 5 minutes. The supernatant (1mL) was treated with 0.5mL of Ellman‟s reagent (19.8 mg of 5, 5‟- dithiobis nitro benzoic acid (DNTB) in 100mL of 0.1% sodium nitrates), and 3mL of phosphate buffer (0.2 M, pH 8). The absorbance was read at 412nm.

3.6.4 Determination of Total Protein

Protein concentration of the homogenate was determined by means of the Biuret reaction as described by Gornal *et al* (1949) with some modification.

Estimation of protein in test samples

One mililitre (mL) of the diluted sample (1 in 10 dilutions) was taken and added to 3mL of biuret reagent in triplicate. The mixture was incubated at room temperature for 30 minutes after which the absorbance was read at 540nm using distilled water as blank. The protein content of the samples were derived from the standard curve and multiplied by 10 to get the exact amount in the fraction. A standard curve was prepared from serial dilution of stock BSA solution containing 2-10mg protein/mL and the optical densities plotted against concentration.

3.6. 5 Determination of Nitric Oxide (NO)

Nitric oxide determination was done using the method described by Ignarro *et al* (1987). The gastric mucosa was allowed to cool in ice-cold distilled water, homogenized and centrifuged at 21.000 g for 20 min at 4^0C. Aliquots of the supernatant were taken to

determine nitric oxide level. The amount of nitrite was measured in the gastric mucosa by performing the Griess reaction. 100mL of sample was incubated with 100mL of Griess reagent at room temperature for 20 min. Nitrite level was determined by measuring the absorbance at 550nm using a spectrophotometer.

3.6.6 Determination of Prostaglandin E level

Prostaglanding E_2 (PGE_2) has been widely studied due to its role in inflammation, atherosclerosis, cancer, sepsis and auto immune disease. Oxidation of arachidonic acid which is done by prostaglanding synthases cyclooxygenase 1 and 2 produces prostaglanding H_2 (PGH_2), which is then metabolized to PGE_2. PGE_2 is of greatinterest as atherapeutic target either because its synthesis can be modulated by the COX inhibitors (NSAIDS) or by modulation of its receptors by down-regulation or binding antagonists. PGE_2 production in a variety of tissues has been shown to modulate numerous physiological processes including smooth muscle elasticity in the blood vessels, natriuresis in the kidney, and the inflammatory response to damaged tissues by the cell lines such as the monocytes and macrophages.

PGE_2 immunoassay kit was procured from Elabscience, China. Based on the principles of specific antigen –antibody interaction. The standard method of immobilizing reactants to the bottom of 96 well plate and then conjugated to an antibody that is linked to an enzyme. Detection is accomplished by estimating the amount of end-product resulting from the conjugated enzyme activity.

Principle

The Elisa Kit uses a Competitive –Elisa as the method. The micro ELISA plate wells provided in the kit were pre-coated with an antibody specific for PGE_2. One hundred

microliters of the supernatant derived from each centrifuged gastric homogenate sample was added to the micro ELISA plate wells successively and reacted with the specific PGE_2 antibody inside the wells. The homogenate samples were added to the bottom of the wells, avoiding contact with the inside walls to prevent the coated antibody from being grazed. 100µL of a reference sample (20ng/mL) was also added to a blank well, which was positioned adjacent to the well containing the last homogenate sample. The plate was sealed and left to incubate for ninety minutes at thirty seven degree Celsius (37^0C). The liquid in each well was removed and without washing the wells, 100µL of Biotinylated Detection Antibody specific for PGE_2 was then added successively into each well, in the same order that the sample and standard were added. The wells were gently tapped at the bottom of the microplates to ensure thorough mixing; the plate was sealed again and incubated for one hour at thirty –seven degree celsius. After this, each well was drained of its content and washed three times by decanting with approximately three hundred and fifty microliters (50µL) of the Wash Bufer prepared from reagents in the ELISA kit. After washing, the plate was patted against thick clean absorbent paper. 100µL of Avidin-Horseradish Peroxidase (HRP) conjugate which was also prepared from reagents in the kit was added successively into each well; the plate was sealed again and incubated for thirty minutes at37^0C. The wash process was repeated five times again, after which ninety microliters of Substrate Reagent Solution was added to each well in the same order. The plate was sealed and incubated for fifteen minutes at 37^0C, protecting the plate from contact with light. After incubation, there was a color change in the liquid in each of the well to blue, indicating a reaction and the presence of PGE_2 in the samples. Fifty microliters (50µL) of Stop Solution provided in the ELISA kit was then added to each of the wells in the same order and there was an immediate color change to yellow. Immediately, the microplate was placed in the microplate reader (Biobase Biodustry Co. Ltd, Shandong, China), set at four hundred and fifty nano-meters (450nm)

and the optical density of the liquid in each well was determined and recorded in nano-gram per milliliter (ng/mL) as the concentration of PGE_2 receptors in that well.

3.7 Statistical Analysis

Data were expressed as Mean ± SEM. Statistical comparisons between variables were calculated using one way analysis of variance(ANOVA) and Tukey's post-hoc test. Graph pad prism version 6 statistical packages. Values of $P < 0.05$ were considered significant

CHAPTER FOUR

4.0 RESULTS

4.1 PHYTOCHEMICAL SCREENING OF ALASOOSUN, TAPA RICE VARIETIES AND RICE BRAN

This result shows the presence of alkaloids, tanin,saponin and phenol in an appreciable amount in all the samples. However, little flavonoids and anthraquinones were observed in all the samples.terpenes, chacones and cardenolides were absent in the samples (Table 4.1).

Table 4.1: Phytochemical screening of some Nigerian rice varieties and rice bran.

Phytochemicals	Alasoosun	Tapa	Rice Bran
Alkaloids	+++	+++	+++
Tannin	++	+++	+++
Phlobatannin	+	+	++
Saponin	+++	+++	+++
Flavonoids	+	+	+
Anthraquinones	+	+	++
Steroids	-	+	+
Terpenes	-	_	+
Cardenolides	-	_	_
Phenol	+++	+++	+++
Chalcones	-	_	_
Cardiac Glycosides	++	+++	+++

Remarks key:

+++ present in an appreciable amount

++ present in a moderate amount

+ present in a minute amount

- Completely absent

4.2 ANALYSIS OF CHEMICAL CONSTITUENTS

This result shows the presence of protein, fat, carbohydrate, ash, moisture and fiber in Alasoosun, Tapa and rice bran. Fiber content was higher in rice bran than the two local rice (Table 4.2).

Table 4.2: Chemical constituents on proximate determination.

Sample	%Pr	%FAT	%FIBR	%ASH	%M	%CHO
Alasoosun	4.87	3.34	1.67	3.69	8.58	77.85
Tapa	5.49	4.28	1.96	3.88	6.97	77.42
Rice bran	8.18	7.79	38.47	11.97	9.89	23.7

All values are expressed in percentage.

Remarks Key :

Pr = protein

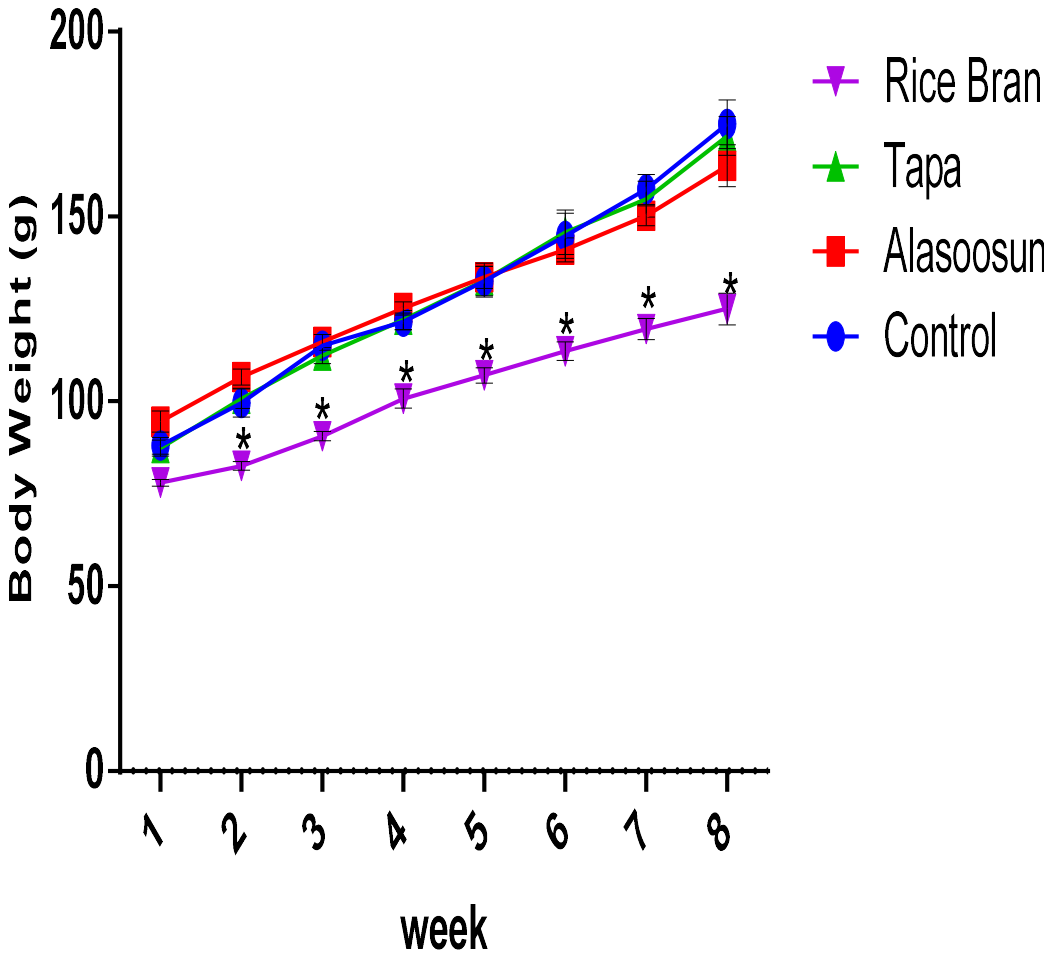
M = moisture

FIBR = fiber

CHO = carbonhydrates

4.3 EFFECT OF NIGERIAN RICE VARIETIES AND RICE BRAN ON BODY WEIGHT AFTER EIGHT WEEKS OF PRE-TREATMENT.

This result shows significant decrease in the weight of the group pre-fed with the rice bran in relation with control. However, no significant difference was observed in the groups fed with local rice(Figure4.1).



Figur 4.1: Effect of Nigerian varieties of rice and Rice bran on the rat body weight. Expressed as Mean±SEM, n =5. * $p < 0.05$ when compared with control.

4.4 EFFECT OF NIGERIAN VARIETIES OF RICE AND RICE BRAN ON MEAN ULCER SCORE, ULCER INDEX AND PREVENTIVE INDICES.

There was significant increase in ulcer score in ulcerated control group (7.58±0.71) comparedwith the groups fed with varieties of Nigerian rice, $p<0.05$ as presented in Table 4.1.

The ulcer score was significantly reduced in the group fed with the rice- bran (1.250 ± 0.72) compared with the other groups (*alasoosun*;2.00 ± 0.29 and *tapa*:3.83± 0.17). The ulcer index in the group fed with rice bran (0.05) was significantly decrease compared to ulceratedcontrol (0.20) and the other groupsas shown in Table 4.3.

The preventive indices show that Rice bran inhibited ulcer formation by 75.0% as compared with control, *Alasoosun* rice inhibited ulcer information by 73.0 %, while *Tap*a rice variety inhibited ulcer by 42.0 % ascompared to control. The result is shown in Table 4.4.

Table 4.3: Effect of Nigerian varieties of rice and rice bran on ulcer score and ulcer index

Group	**Mean Ulcer Score (Mean ±SEM)**	**Ulcer Index**
Ulcerated Control	7.583±0.7120	0.20
***Alasoosun*-fed rats**	2.000±0.2887*	0.05
***Tapa*-fed rats**	3.833±0.1667*	0.12
Rice Bran-fed rats	1.250±0.7217*	0.05

Expressed as Mean±SEM;n =5.* $p < 0.05$ when compared with ulcerated control.

Table 4.4: Effect of Nigerian rice and rice bran on the percentage inhibition of ulcer score

TREATMENT	% INHIBITION OF ULCER
Ulcerated Control	
Alasoosun	73
Tapa	42
Rice Bran	75

4.5 EFFECT OF NIGERIAN RICE VARIETIES AND RICE BRAN PRE-TREATMENT ON THE MICROSCOPIC ASSESSMENT IN INDOMETHACIN-INDUCED GASTRIC ULCERATION IN RATS.

Morphological findings of the gastric mucosa obtained in this study shows ulcer combined with distorted gastric glands, a damage mucosal epithelium, necrosis of the mucosa (black arrow), mild infiltration of inflammatory cells into the gastric gland, lamina propia and the submucosal layer (blue arrow) in ulcerated control group (Plate 1A). However, Nigerian rice varieties and rice bran pre-treated groups show protection against these morphological changes and these resulted in the maintenance of glandular organisation and the structure of the muscularis mucosa (Plate 1B-D).

4.3 Effect of Nigerian rice varieties and rice bran pre-treatment on the microscopic assessment of Indomethacin-induced gastric ulceration in rats.

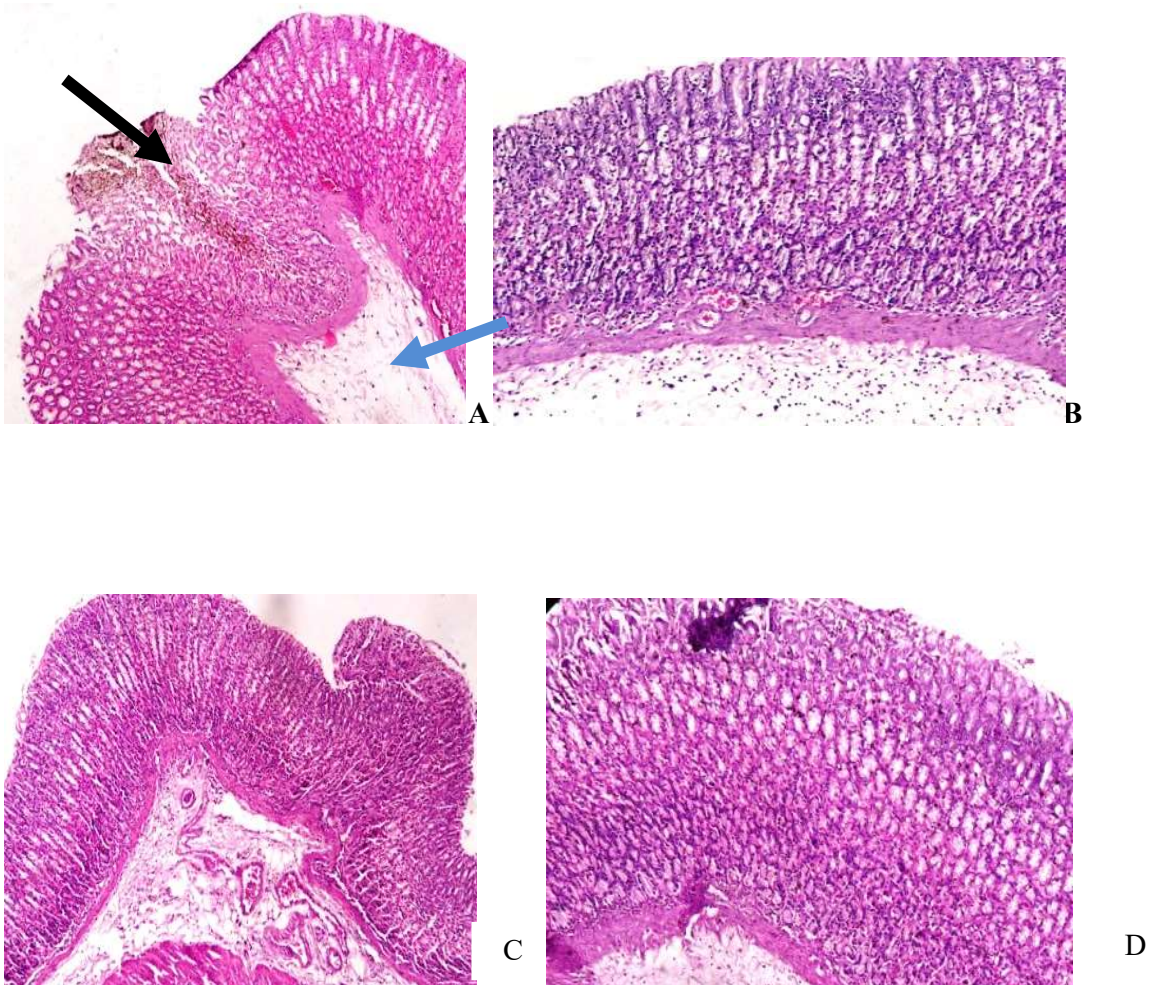


Fig. 4.2 :Arrows point to lymphocyte infiltration in the mucosa, edema, erosion and ulcer are seen in ulcerated control group following indomethacin exposure when compared with the groups pre-fed with local rice and rice bran(magnification.×100).

A – ulcerated control

B- *Alasoosun* rice fed group

C – *Tapa* rice fed group

D – Rice bran fed group

4.6. EFFECT OF NIGERIAN VARIETIES OF RICE DIETS AND RICE BRAN ON THE GASTRIC MUCUS CONTENT.

The gastric mucus content was significantly increased in rice bran pre-fed rats (12.88 ± 0.39ug alcian blue/g wet tissue) compared to ulcerated control (1.94 ± 0.26ug alcian blue/g wet tissue) and normal control (1.26 ± 0.09 ug alcian blue/g wet tissue). The local rice groups showed no significant difference (1.82 ± 0.26and 1.70 ± 0.14 ug alcian blue/g wet tissue) for *Alasoosun* and *Tapa* rice respectively as compared to the ulcerated control (1.94 ± 0.26 ug alcian blue/g wet tissue). (Figure 4.3.)

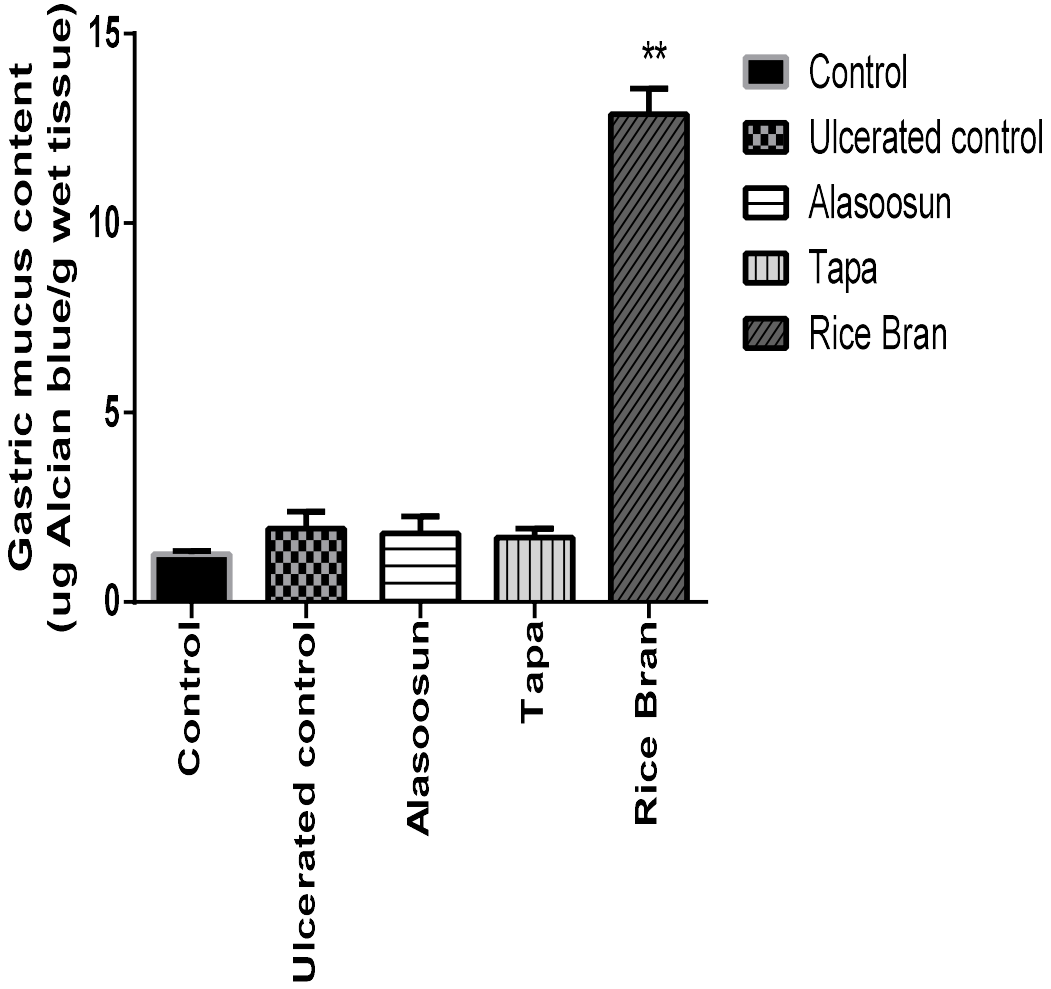


Figure 4.3: Effect of Nigerian varieties of rice and Rice Bran on Gastric Mucus content. Data expressed as Mean±SEM;n =5. * p< 0.05when compared with ulcerated control.

4.7 EFFECT OF NIGERIAN VARIETIES OF RICE DIETS AND RICE BRAN ON GASTRIC BLOOD FLOW

The gastric blood flow in all the pre-treated groups; 4.23 ± 0.11 mL/min, 3.30± 0.15mL/min and 3.60 ±.12mL/min, for *Alasoosun* ,*Tapa*and rice bran respectively was significantly increased as compared with the ulcerated control (02.20 ± 0.12mL/min). However, no significant difference was observed in normal control (2.04 ± 0.11 mL/min) as compared to ulcerated control. (Figure 4.4)

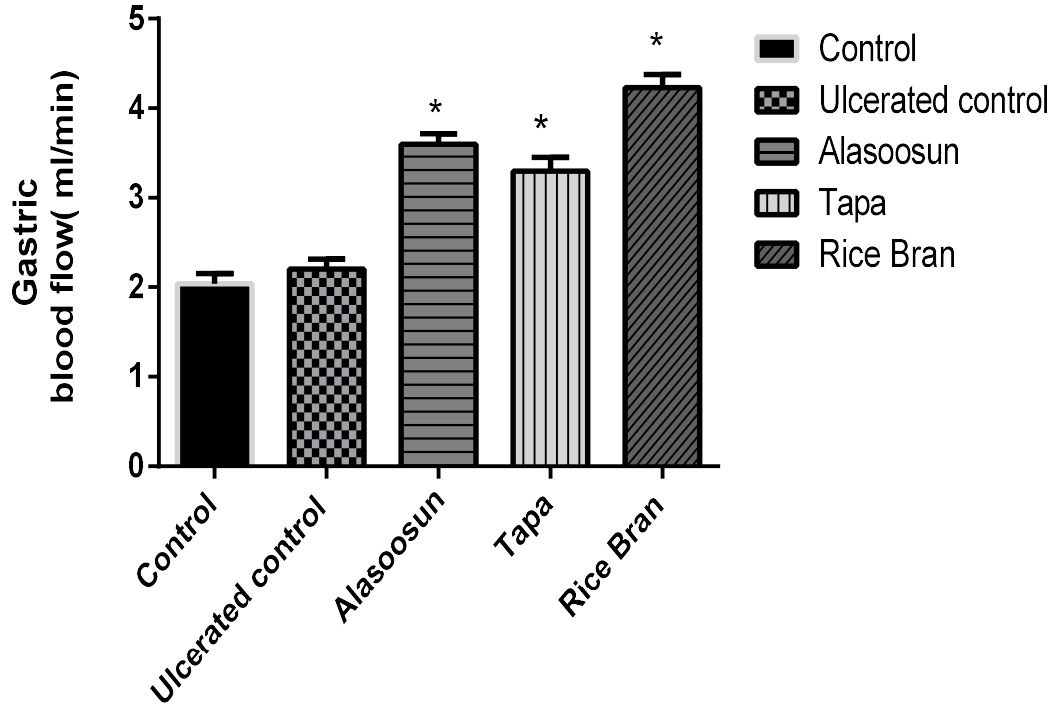


Figure 4.4: Effect of Nigerian varieties of rice and rice bran on gastric blood flow.Data expressed as Mean±SEM;n =5, * p< 0.05 compared with ulcerated control

4.8 EFFECT OF VARIETIES OF NIGERIA RICE DIETS ON BIOCHEMICAL PARAMETERS

4.8.1 Effect of varieties of Nigeria rice diets on Malondialdehyde (MDA) levels

In all the Nigerian rice varieties and rice bran pre-treated groups, there was a significant decrease ($P < 0.05$) in the mean values of MDA (*Alasoosun*(0.52 ± 0.04nmol/mg protein/),*Tapa* (0.77 ± 0.03nmol/mg protein) and Rice bran (0.51 ± 0.12nmol/mg protein) when compared with the ulcerated control (1.73 ± 0.03nmol/mg protein). Significant decrease was observed in normal control (0.79 ± 0.07) when compared with ulcerated control (Figure 4.5).

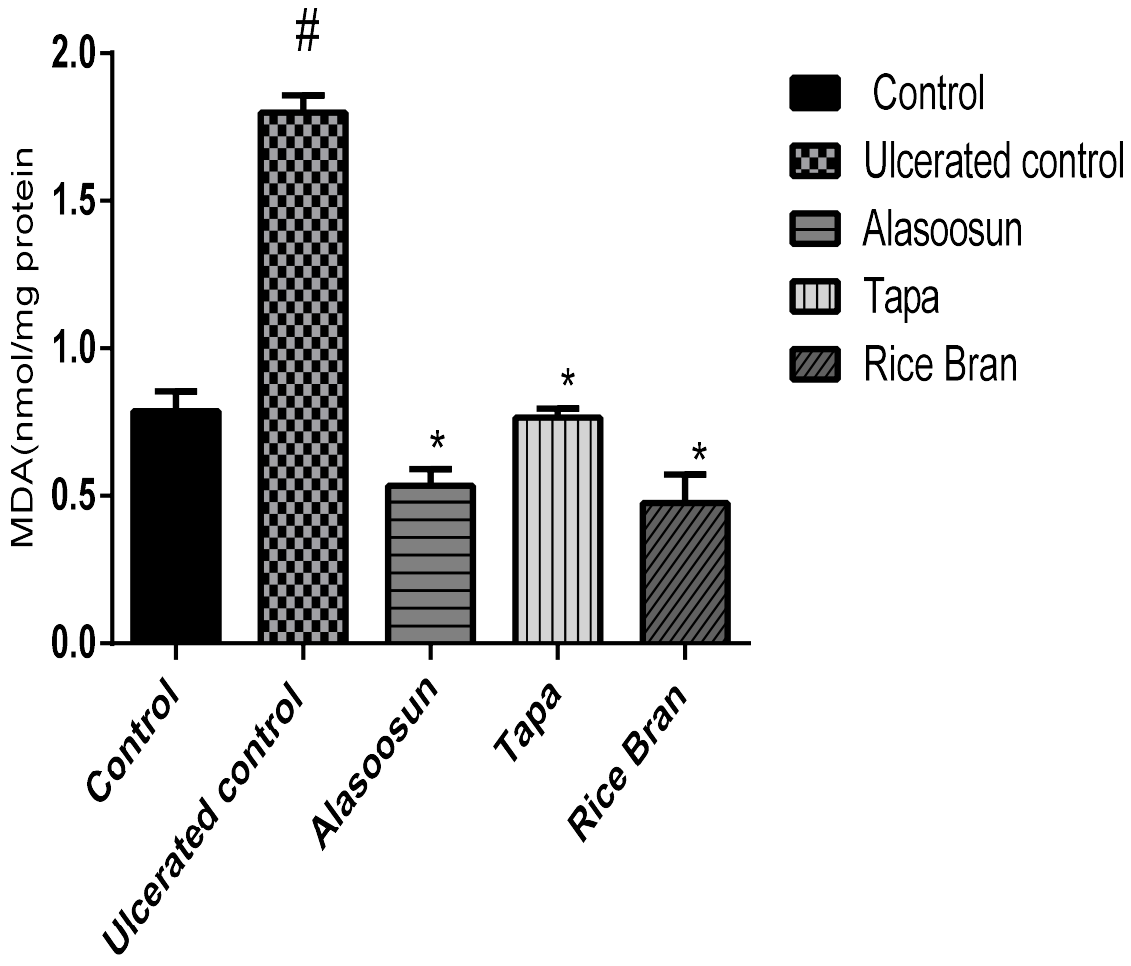


Figure 4.5: Effect of Nigerian varieties of rice and rice bran on Malondialdehyde level in Indomethacin induced gastric ulcerated rats.

Data expressed as Mean ±SEM, n =5, * p< 0.05 compared with ulcerated control.

4.8.2 Effect of Nigerian varieties of rice and rice bran on superoxide dismutase in indomethacin induced ulceration

In all the Nigerian rice varieties and rice bran pre-treated groups, there was a significant increase ($P < 0.05$) in the mean values of SOD(*Alasoosun* (353.6 ± 7.59µg/ml),*Tapa* (350.9 ± 6.53µg/ml) and Rice bran (350.3 ± 5.77µg/ml) when compared with the ulcerated control (298.1 ± 10.66µg/ml). However, no significant difference was observed in the normal control group *(*260.20 ± *5.98*µmol/100g wet tissue) when compared with ulcerated control. Figure 4.6.

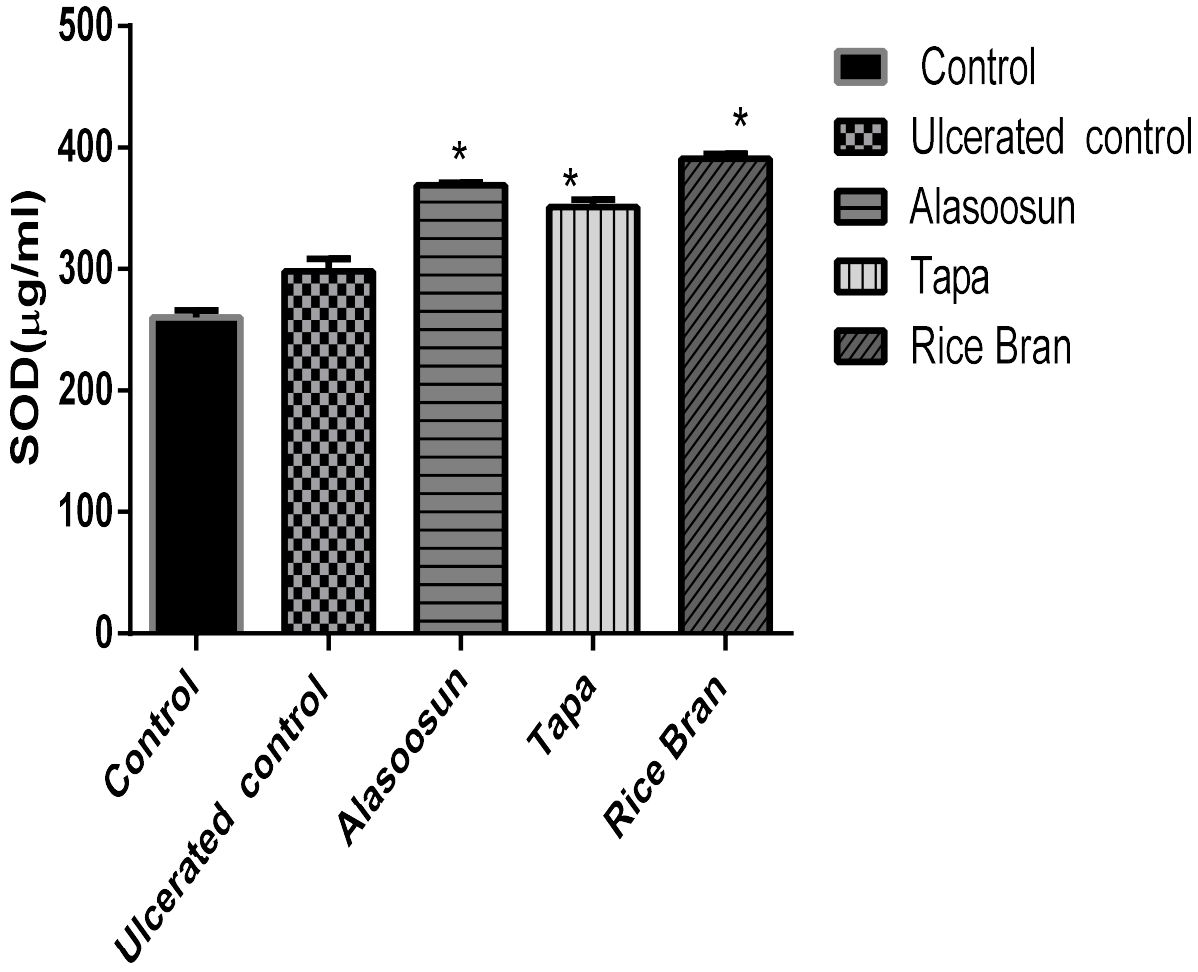


Figure 4.6: Effect of Nigerian varieties of Rice and Rice Bran on Superoxide Dismutate (SOD) level.

Data expressed as Mean±SEM;n =5. * p< 0.05 compared with ulcerated control.

4.8.3 Effect of Nigerian varieties of rice and rice bran on Sulfhydryl concentration in indomethacin induced ulceration.

Sulfhydryl concentrations in the normal control (3.67 ± 0.0663µmol/100g wet tissue), *Tapa* rice (3.58 ± 1.63µmol/100g wet tissue) variety and rice bran(2.68 ± 1.60µmol/100g wet tissue) pre-treated groups were significantly increased as compared to ulcerated control (1.86 ± 1.82µmol/100gwet tissue) ($P<0.0$). However, no significant difference was observed in *Alasoosun (*2.10 ± 1.33µmol/100g wet tissue) when compared with ulcerated control. Figure 4.7.

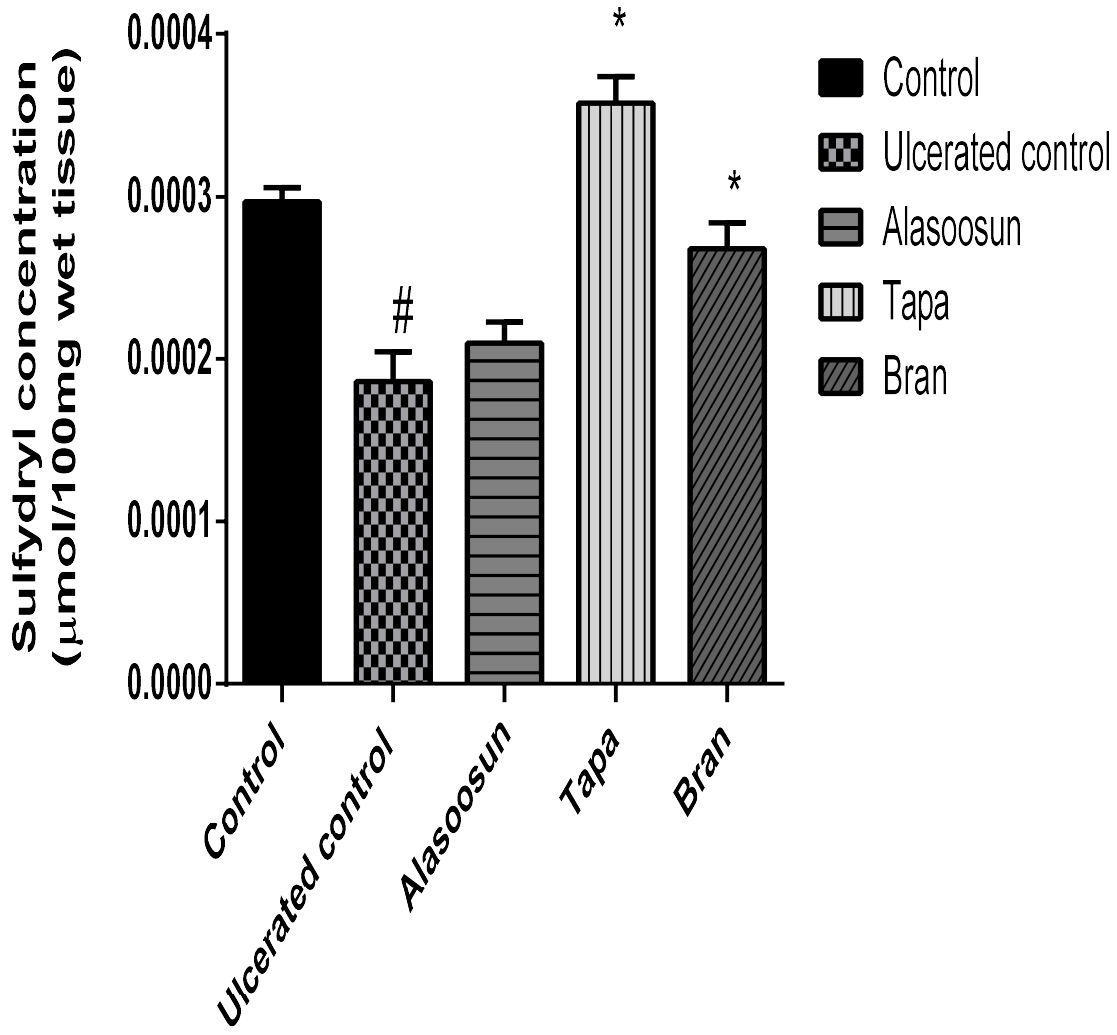


Figure 4.7: Effect of Nigerian Rice varietiesand Rice Bran on Sulfhydryl level.

Data expressed as Mean ± SEM; n =5. #, * p< 0.05 compared with ulcerated control

4.8.4 Effect of Nigerian varieties of rice and rice bran on tissue protein level in indomethacin induced ulceration.

Pre-treatment with Nigerian rice varieties and rice bran shows no significant difference in the total gastric protein concentrations. (Alasoosun (0.24 ± 0.01mg/ml), Tapa (0.29 ± 0.01mg/ml), rice bran (0.24 ± 0.00mg/ml) and control (0.27 ± 0.02mg/ml) compared with ulcerated control(0.25 ± 0.00mg/ml). Figure 4.8

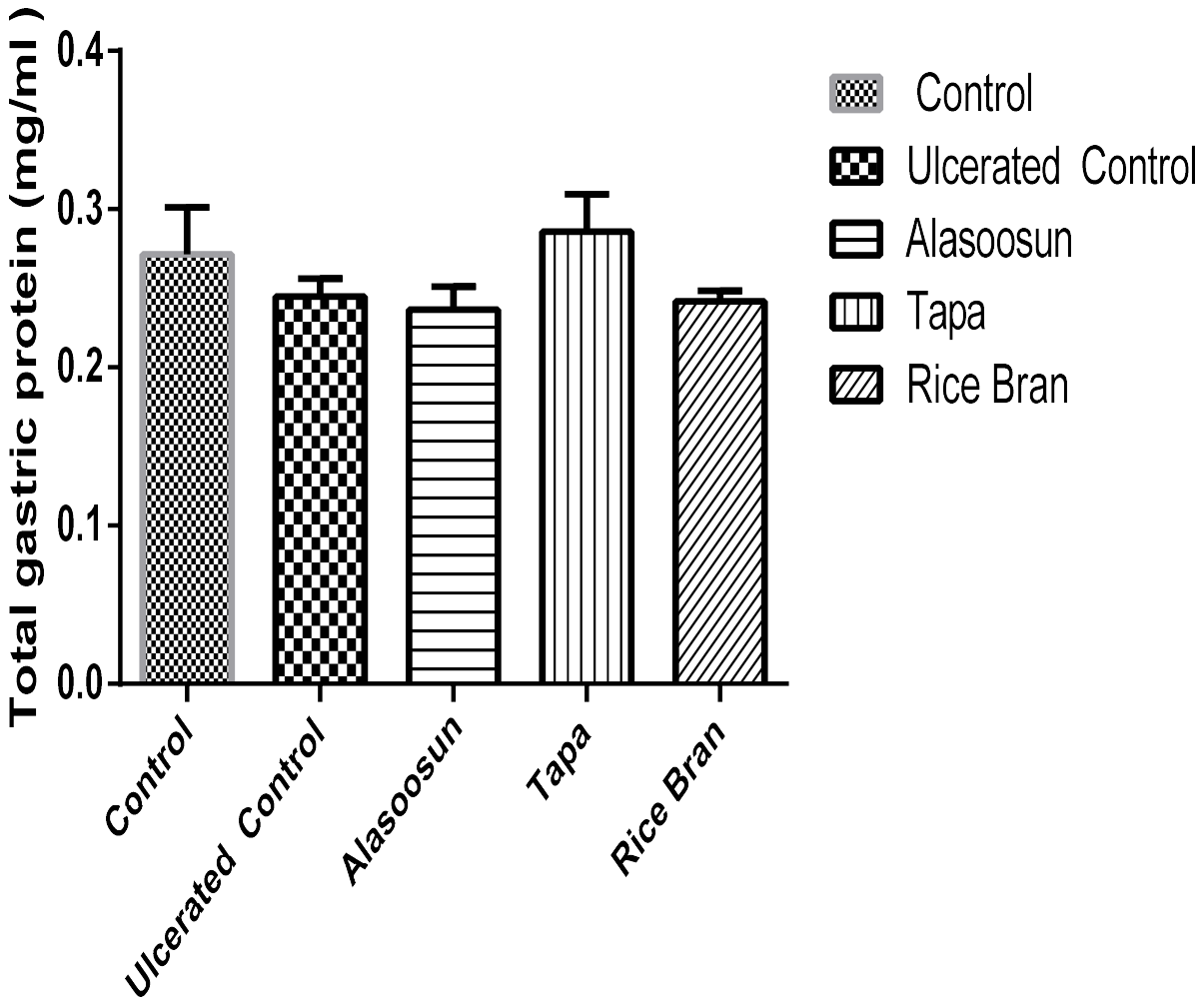


Figure 4.8: Effect of Nigerian varieties of rice and rice bran on tissue Protein level

Data expressed as Mean±SEM;n =5.

4.8. 5 Effect of Nigerian varieties of rice and rice bran on gastric Nitric Oxide level.

There was significant increase in Nitric oxide level in normal control (56.96 ±1.45nmol/g wet tissue) *Tapa* rice (64.66 ± 12.07 nmol/g wet tissue) and rice bran(110.07 ± 4.17 nmol/g wet tissue) pre-treated group when compared with ulcerated control (9.57 ± 1.77 nmol/g wet tissue), $p<0.05$.No significant difference was observed in*Alasoosun* group (18.84 ± 1.63 nmol/g wet tissue) when compared to ulcerated control. Figure 4.9

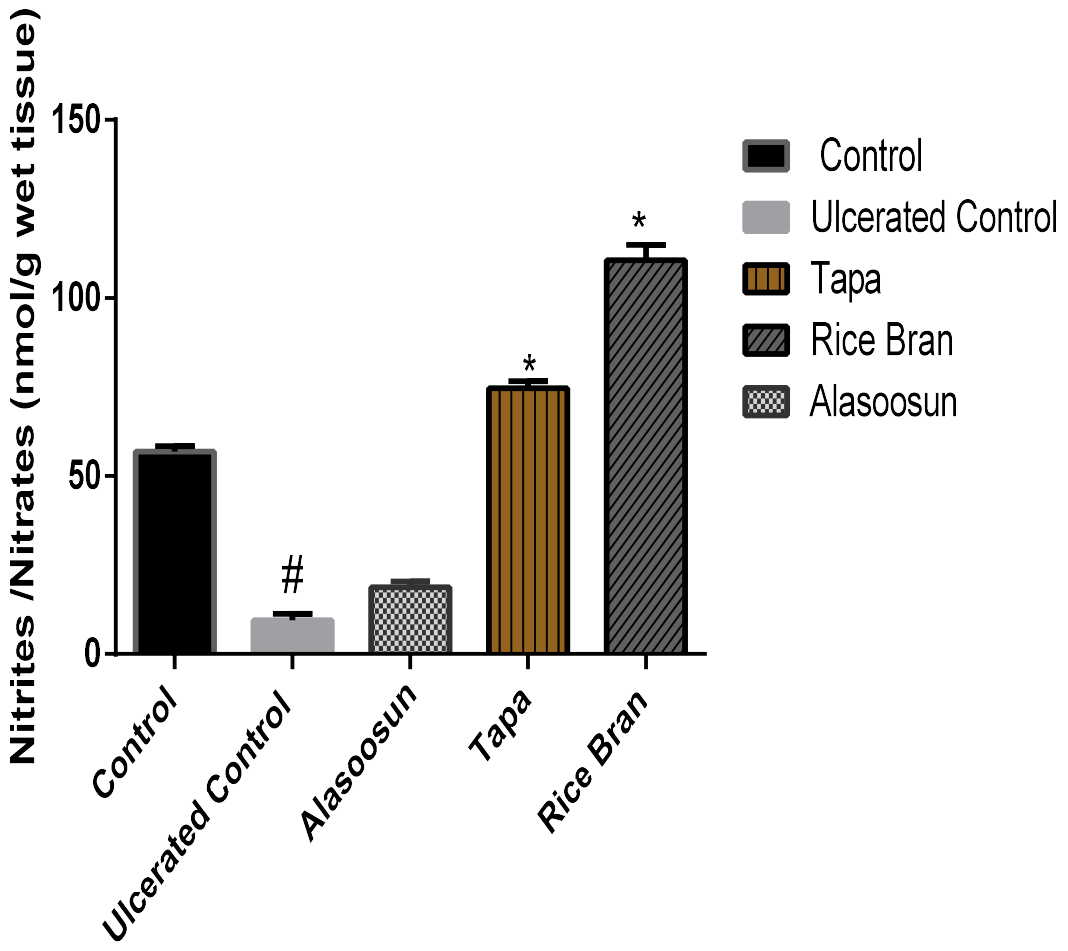


Figure 4.9: Effect of Nigerian varieties of rice and rice bran on gastric Nitric Oxide level.

Data expressed as Mean±SEM;n =5. #, * p< 0.05 compared with ulcerated control

4.9 Effect of Nigerian varieties of rice and rice bran on gastric prostaglandin E (PGE) concentration.

The gastric mucosal synthesis of PGE_2 in the *Alasoosun* rice (919.00 ± 11.27pg/mol) and rice bran (798.2 ± 44.86 pg/mol) pretreated rats increase significantly ($P< 0.05$) compared to ulcerated control group (433.05 ± 31.38 pg/mol). However, in the *Tapa* rice pre-treated group PGE_2 mean value (1345.00 ± 64.43pg/mol) was more enhanced compared to *Alasoosun*, rice bran and control (263.6 ± 5.657 pg/mol), ($p<0.05$). Figure 4.10

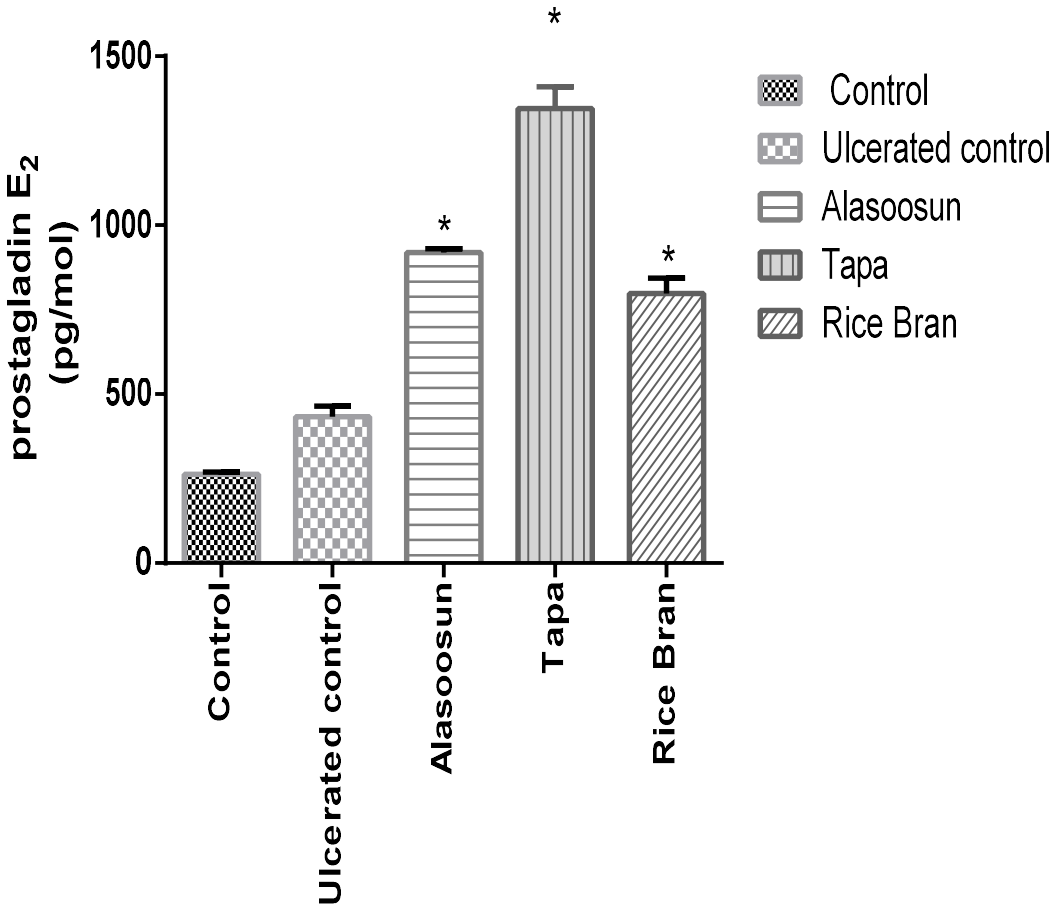


Figure 4.10: Effect of Nigerian varieties of rice and rice bran on gastric PGE_2.

Data expressed as Mean±SEM;n =5. * $p< 0.05$ compared with ulcerated control.

CHAPTER FIVE

5.0 DISCUSSION

In this study, pre-treatment with different varieties of Nigerian rice diet shows no significant difference in the total body weights of animal compared with control, except rice bran pre-fed group that revealed a significant decrease when compared with the other groups. The significant decrease observed in the rice bran pre-fed groupcould be attributed to the present of dietary fiber in the rice bran. Several epidemiological studies have alsoshown that dietary fiber intake causes weight loss (Slavin, 2005). Drewnowski (1998),stated that fiber content has a great impact on the palatability of food and possibly reduces energy intake. This could be attributed to the fact that dietary fiber stimulates increase level of satiation and satiety to help promote energy balance and weight stability (Chambers *et al*., 2015).

All the Nigerian varieties of rice pre-fed groups showed significant reduction in ulcer index compared to the ulcerated control group with the rice bran pre-fed group having the highest percentage inhibition. This is in agreement with work of Adedeji and Oluwole (2012), where it was reported that Tapa rice was protective and rice bran conferred a high degree of protection against gastric ulceration and this is in agreement with the report of this study. These results could be attributed to the fact that prostaglandins normally protect the gastrointestinal mucosa from damage by maintaining blood flow and increasing mucosal secretion of mucous and bicarbonate (Voultilainen *et al.,* 2001). Indomethacin causes mucosal damage by interfering with prostaglandin synthesis, increasing acid secretion and block diffusion of H^+(Roa *et al*., 1999). Studies have shown that blockade of cyclooxygenase-1 (Cox-1) and (Cox-II) by indomethacin results in reduction of prostaglandin synthesis. The interruption of prostaglandin synthesis results in impairment of mucosal damage repair, thus facilitating mucosal injury (Burke *et al*., 2006). Indomethacin

and related non-steroidal anti-inflammatory drugs can aggravate or interfere with the healing of peptic ulcers.

In the present study, flattenned mucosal folds were observed which suggests that gastroprotective effect of Nigerian rice varieties and rice bran might be due to a decrease in gastric motility. Studies have shown that changes in the gastric motility might play a role in the development and prevention of experimental gastric lesions. Relaxation of circular muscles may protect the gastric mucosa through flattening of the folds. This will increase the mucosal area exposed to necrotizing agents and reduce the volume of the gastric irritants on rugal crest. Indomethacin produces a marked contraction of the circular muscles of rat fundic strip. Such a contraction can lead to mucosal compression at the site of the greatest mechanical stress, at the crests of mucosal folds leading to necrosis and ulceration (Mahmood *et al*., 2011).

Gastric mucus is an important protective factor in the gastric mucosa. A strong correlation between the protection afforded against experimental ulcers and mucus secretion has been reported (Tulassay and Herszényi, 2010). Furthermore, evidence obtained with several cell culture systems indicates that stimulation of NO production through inducible Nitric oxide synthase (iNOS) leads to the inhibition of proteoglycan synthesis and the loss of extracellular matrix proteins (Kim*, 2001*). Mucus is released predominantly by the process of exocytosis, consists of a viscous, elastic, adherent and transparent gel formed by 95% water and 5% glycoproteins that cover the entire gastrointestinal mucosa to forms a protective barrier between mucosal surface and luminal content (Praveen *et al*., 2014).

Moreover, mucus is capable of acting as an antioxidant, and thus can reduce mucosal damage mediated by oxygen free radicals (Repetto and Liesuy, 2002). The protective properties of the mucus barrier depend not only on the gel structure but also on the amount or

thickness of the layer covering the mucosal surface (Penissi and Piezzi, 1999). In this study, the reduced mucus secretion in the ulcer control group indicated reduced ability of the mucosal membrane to protect the mucosa from damage induced by indomethacin and this was effectively reversed by pretreatment with rice bran. This suggests that rice bran might protect against gastric ulcer by increasing gastric mucus secretion from gastric mucosal cells. These stimulatory effects of rice bran on gastric mucus secretion may be similar to that Prostaglandin analogue such as sucralfate and misoprostol (Poonam *et al*, 2003). An increase in mucus production usually assists the healing process by protecting the ulcer crater against irritant stomach secretions (HCl and pepsin) thereby enhancing the rate of the local healing process (Inas *et al.,*2011).

Gastric blood flow has been shown to be reduced in animals by indomethacin treatment (Abdallah, 2011). In the current study, gastric blood flow was increased in Rice Bran and the two Nigerian rice varieties (*Alasoosu*n and *Tapa*) pre-fed group as compared with control. The increase in gastric blood flow observed in Rice Bran and *Tapa*groups could be as result of nitric oxide which helps to improve blood flow to the site of inflamed tissue. The vasodilatory effect of NO contributes to gastroprotection and ulcer healing by an increase of both, blood flow and mucus secretion as well as the inhibitory effect on platelet aggregation and leukocyte recruitment resulting in the protection of gastric mucosa against damaging agents (Yamaguchi *et al.,* 2005). The apparent reduction in the activity of cNOS following the application of a non-specific NOS inhibitor increases the susceptibility of gastric mucosa to damage in the presence of corrosive and irritating agents (Modliun and Sachs, 2005).

The ulceration induced by indomethacin may also be attributed to various processes which include inhibition of prostaglandin synthesis, generation of ROS and initiation of lipid

peroxidation. Abdallah *et al*, (2011) reported that stomach ulceration induced by indomethacin was accompanied with a severe oxidative stress in gastric tissue causing damage to key biomolecules such as lipids and stimulation of lipid oxidation causing increased accumulation of MDA as well as reduction in the gastric activity of antioxidant levels. In this study, gastric MDA were increased in ulcer control group and decreased by pre-treatment with Nigerian rice and rice bran, another indicator of a possible antioxidant activity of these diets. Brown (unmilled) rice and rice bran have been shown to contain antioxidants (Liu *et al*., 2013) and it is likely that gastroprotective activity exerted by these diets could be attributed to their antioxidant properties.

Free radicals and reactive oxygen species (ROS) that are continuously produced in human body are the cause of cell damage. Therefore, there is need for tissue to be protected from oxidative injury through intracellular as well as extracellular antioxidants. Superoxide dismutase (SOD) is considered as the first line of defense against deleterious effects of oxygen radicals within the cells. SOD-mediated catalysis of superoxide radical anion into less noxious hydrogen peroxide (H_2O_2) (Johansen *et al*., 2005).In this study, SOD activities were significantly reduced after indomethacin administration in ulcerated control group, and this reduction was prevented by pretreatment with Nigerian rice and rice bran. Reduced activities of SOD in gastric tissue homogenate in ulcerated control group that have been observed in our study may be due to increased production of reactive oxygen radicals that can themselves reduce the activity of these enzymes (Yasar *et al*., 2003). The reduction of these enzymes in gastric tissue homogenate may lead to a number of deleterious effects. Ichikawa *et al*. (2001) reported that colored rice is efficient, and two fold stronger with respect to antioxidant activities of blue berries due to the presence of anthocyanin, an important antioxidant. Nigerian rice varieties and rice bran may antagonize indomethacin-induced gastric ulcer directly or indirectly: directly through capturing of superoxide anions,

which will consequently prevent its interaction with generated nitric oxide to form peroxynitrite thus preserving the beneficial functions of nitric oxide. Indirectly through up-regulation of cNOS-derived nitric oxide which improves tissue perfusion leading to attenuation of neutrophil infiltration and free radicals production.

Sulfhydryls have been reported to be involved in protecting gastric mucosa against various noxious substances (Alqasoumi*et al.,* 2011). Sulfhydryl reacts with reactive oxygen species to transform them to inert products and also keep sulfhydryl groups of proteins in reduced form.It helps in recycling endogenous antioxidant vitamins, such as vitamin C and E thereby preventing lipid peroxidation. In addition, sulfhydryl scavenges superoxide anions, thus preventing their interaction with nitric oxide to form peroxynitrite. Nitric oxide thus preserved can improve tissue perfusion and attenuate neutrophil infiltration and free radical generation. The facts that concentration of sulfhydryl in the gastric mucosa is relatively high and treatment of animals with sulfhydryl blockers results in induction of gastric mucosal injury suggest the importance of this compound in gastro-protection and maintenance of gastric mucosal integrity. Decreased levels of endogenous sulfhydryls have been associated with tissue damage by ethanol, indomethacin and various chemical agents (Saleh, 2012). In this study, groups pre-fed with Rice Bran and Tapa rice caused increase sulfhydryl level when compared with control. This is in accordance with the work of Saleh (2012) that diets that cause an increase in endogenous sulfhydrl group like cinnamon suspension offered a profound effect in replenishing the NP-SH (Non protein sulfhydryl) concentration and preventing the decreased level of gastric wall mucus contents induced by ethanol. Thus, sulfhydryl seemed to be involved in gastro-protection mechanism by enhancing mucus secretion. The enhanced gastric NP-SH and mucus levels may have contributed to the Nigerian rice varieties and rice bran anti-ulcer activity and this agrees with the reports of (Dong *et al*., 2014).

. Gastric mucosal NO was increased in all pre-fed groups with rice bran more profound when compared to ulcerated control group. Probably, the gastro-protective effect of rice bran and Nigerian rice varieties could be explained on the basis that these diets stimulate cNos in the gastric tissue.In the digestive system, nitric oxide produced by cNOS is cytoprotective whereas nitric oxide produced by iNOS is cytotoxic. Nitric oxide at low concentration (from cNOS) plays a role in protecting the integrity of epithelial tissues by improving the mucosal blood flow in the gastric mucosa. This protective effect of nitric oxide could be attributed to inhibition of activation, adhesion and migration of leucocytes to the inflammatory area (Motawi *et al.,* 2007). Indomethacin was reported to decrease tissue cNOS-derived nitric oxide and increase iNOS-derived nitric oxide (Motawi et al., 2008). This could explain the decrease in nitrite ⁄ nitrate levels (as an indicator of nitric oxide production) in gastric mucosa of indomethacin- treated rats which may be due to decreased production of cNOS in gastric tissue and up-regulation of iNOS in macrophages and neutrophils (nitric oxide utilized to form peroxynitrite).This result is in agreement with report of Ihab (2010) reporting that indomethacin-induced gastropathy is associated with significant decrease in gastric mucosal nitric oxide levels

Prostaglandin E (PGE) plays an important role in the regulation of gastric mucus secretion. PGE has protective effects against various gastric injury models (Abdel-Salem *et al*., 1997). Indomethacin has been shown to inhibit cyclooxygenase enzymes (a rate limiting step in the synthesis of PGE). PGE is the most abundant gastrointestinal prostaglandin and it regulates functions of the gut, including motility and secretion. PGE has also been shown to exert a protective action on the stomach through the activation of EP receptors (Takeuchi *et al.,* 2002). The role of PGE in mediating the gastroprotective effect of Nigerian rice and rice bran were investigated. The results of the present study suggest that the gastroprotective effects of these diets are mediated partially by PGE as direct measurement of its mucosal

level confirmed that its biosynthesis was significantly enhanced by these diets. It has been shown that prostaglandins influence virtually every component of the mucosal defense: stimulating mucus and bicarbonate secretion, maintaining mucosal blood flow, enhancing the resistance of epithelial cells to injury induced by aggressive agents, and inhibiting leukocyte recruitment (Kato *et al.,* 2005).

5.1. SUMMARY OF THE STUDY

Nigerian rice varieties and rice bran could significantly protect the gastric mucosa against indomethacin-induced injury. Such protection was ascertained grossly by reduction of ulcer areas in the gastric wall as well as by the reduction or inhibition of edema and leukocytes infiltration of the submucosal layers as shown by the histological studies. Significant increase in the gastric mucus content with increased theSOD, sulfhydryl and decreased the level of lipid peroxidation (MDA) in the treated group compared to the ulcerated control group contributes to the antiulcer properties of rice bran and varieties of Nigeria rice diets . It is suggested that this could be due to the present of nutrients such as protein, minerals (Ca, P, Fe, and Zn) and dietary fiber contents that are higher in colored rice compared to polished rice. In addition measurements of gastric blood flow, NO and PGE showed that these diets exert gastro-protective properties in pre-fed groupswhen compared with ulcerated control group.

5.2 CONCLUSION

Findings in this study therefore revealed that the mechanisms by which*Alasoosun* rice, *Tapa* rice and rice bran pre-treatments exert gastro-protective activities may be via vasodilatation, increasedgastric blood flow,gastric mucus content,prostagladin E_2level and anti-oxidative activities.

5.3 RECOMMENDATIONS

It is recommended that the incidence of peptic ulcer could be reduced significantly if patient with pre-existing ulcer or people predisposed to factors that can lead to formation of peptic ulcer could consume the Nigeria indigenous rice diets especially the rice bran which is known to contain nutrients that are beneficial to the body.

5.4 CONTRIBUTIONS TO KNOWLEDGE

Pre-treatment of rats with Nigerian rice varieties and rice bran showed gastro-protective effect in indomethacin –induced gastric ulceration.It significantly decrease lipid peroxidation, increases level of endogenous antioxidant, gastric mucus content, gastric blood flow, NO and prostaglandin E_2 levels.

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