THE EFFECT OF BEVI-MIX (ARTIFICIAL SWEETENER) AND ASPARTAME ON THE INTEGRITY OF GASTRIC MUCOSA AND SOME BIOCHEMICAL PARAMETERS IN MALE WISTAR RATS

BY

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DECLARATION

I declare that the work in this Dissertation entitled effect of bevi-mix (artificial sweetener) and its major constituent aspartame on the integrity of gastric mucosa in male wistar rats has been performed by me in the Department of human physiology. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this project dissertation was previously presented for another degree or diploma at this or any other institution.

Zainab Abdullahi MIKO ---------- ----------

Name of student Signature Date

CERTIFICATION

The effect of Bevi-mix (artificial sweetener) and aspartame on the integrity of gastric mucosa and some biochemical parameters in male Wistar rats by Zainab Abdullahi MIKO meets the regulations governing the award of the degree of M.Sc. in Human Physiology of the Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

This work is dedicated to my beloved parents Alhaji Miko Abdullahi and Hajiya Hauwa Abdurrahman Shuwari. They are the most giving, caring people I know, my motivation for greatness, my passion for life and goal for future happiness.

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ABSTRACT

Bevi mix is a powdered drink consisting of mixture of artificial sweetener including aspartame. The present study was designed to investigate the effect of Bevi-mix (artificial sweetener) and its major constituent aspartame on the integrity of gastric mucosa in male Wistar rats. Fourty two male Wistar rats were divided into 6 groups of 7 animals each (n = 7). Group 1: Normal control group, Group 2: Bevi mix 330 mg/kg, Group 3: Bevi mix 660 mg/kg, Group 4: Aspartame 20 mg/kg, Group 5: Aspartame 40 mg/kg and Group 6: Aspartame 80 mg/kg). After ligature, gastric juices were collected for evaluation/assessment of gastric secreation and mucus component, then the stomachs were harvested for mucus secration. Blood sample were collected for biochemical analysis. The kidney and the liver were homogenized for determination of kidney electrolytes and liver enzymes. From the result, Administration of Bevi mix 660 mg/kg and aspartame 80 mg/kg showed a significant ($P < 0.05$) increase in volume of gastric juice when compared with normal control and bevi mix 330 mg/kg, Aspartame 80 mg/kg significantly ($p< 0.05$) increases volume of gastric juice when compared with aspartame 20 or 40 mg/kg. Administration of Bevi mix 660 mg/kg and aspartame (20, 40 or 80 mg/kg) showed a significant ($P < 0.05$) increase in titratable acidity and total acid output when compared with the control. Administration of bevi 660 mg/kg and aspartame (40 mg/kg or 80 mg/kg) showed a significant ($P < 0.05$) decrease in mucus secretion when compared to the normal control. Administration of 80 mg/kg of aspartame showed a significant ($P < 0.05$) decrease in mucus secretion when compared to bevi mix 330 mg/kg. Bevi mix 660 mg/kg and aspartame 80 mg/kg showed a significant ($P < 0.05$) increase in free sialic acid and bound sialic acid when compared with normal control and bevi mix 330 mg/kg. Administration of bevi mix (660mg/kg) and aspartame (80 mg/kg) caused a significant ($P <0.05$) increased in malondialdehyde (MDA) concentrations when compared with normal control and bevi mix (330mg/kg). Administration of bevi mix or aspartame showed a significant ($P \leq 0.05$) decrease in serum activities of superoxide dismutase (SOD) and reduce glutathione (GSH) levels when compared with the control group. Group administered with Bevi mix 330 mg/kg showed a significant ($P \leq 0.05$) increase in GSH when compared with the aspartame groups. Bevi mix 330 mg/kg and the entire aspartame groups showed a significant ($P < 0.05$) increase in alanine aminotransferase (ALT), when compared with the control and bevi mix 660 mg/kg. Aspartame (20, 40 or 80 mg/kg) showed a significant ($P< 0.05$) increase in the enzymatic activities of aspartate aminotransferase (AST) when compared with control, bevi mix 330 and 660 mg/kg. Additionally all the administered groups showed a significant ($P < 0.05$) in alkaline phosphatase when compared with the control. Aspartame and Bevi mix significantly at ($P < 0.05$) increased sodium ion and bicarbonate ion concentrations in all the administered groups when compared with the control. While aspartame alone groups significantly increase potassium ion concentration at ($P < 0.05$) when compared with the control and Bevi mix groups. In conclusion, Aspartame and bevi mix caused changes in gastric secretion and oxidative stress in the rats by increasing MDA concentrations level and decreasing serum antioxidant enzymes. The result also revealed that the observed decreased in mucus secretion and its components (Sialic acid), might be due to the administration of bevi mix and its component aspartame.

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CHAPTER ONE

1.0 INTRODUCTION

1.1 ARTIFICIAL SWEETENER

Sweeteners are food additives that are used to improve the taste of foods (Gafar *et al.,* 2016). There are two types of sweeteners found in food and beverages. Sugars (natural sweeteners) are sweeteners extracted from natural products without any chemical modifications which add some energy value to food (Gafar *et al.,* 2016), and artificial sweeteners (AS) which are substances used to substitute sugar in foods and beverages, which may be derived from naturally occurring substances, including herbs, or sugar (Azeez and Alkass, 2018**).** Artificial sweetener can be divided into two: Nutritive sweeteners, which add some energy value to food; and non-nutritive sweeteners, which are also called high-intensity sweeteners because they are used in extremely minute quantities, adding no energy value to food (Polyák*et al.,* 2010).

Bevi mix is a powdered drink consisting of mixture of artificial sweetener which was introduced by Foster Clark in 2016. Bevi Mix was designed to add 'A Splash of Fun' to the consumers' drinks, and it is available in six flavours – Cola, Mango, Pineapple, Berries, Pineapple Coconut and Orange. Bevi Mix has been launched in Nigeria, Niger, Democratic Republic of Congo, Liberia, Ethiopia, Cameroon, Mali, Mauritania and Senegal (Foster, 2016). By the end of 2016, it is planned that Bevi Mix will be available in all the leading markets across the Middle East, Africa and Asia. Bevi mix is composed of Artificial sweetener like Aspartame E951 (0.36g/L), Sodium Cyclamate E952 (0.29g/L), Sodium Saccharine E954 (0.08g/L), Sugar, Maltodextrine, Acidity Regulator (E330), Artificial Berries Flavouring, Colours (E129, E133), Vitamins and Anti- Caking Agent (E551) (Foster, 2016).

Aspartame (N-Alpha-Aspartyl-L-phenylalanine) is a white crystalline powder belonging to the non-nutritive type of artificial sweetener that is approximately 200 times sweeter than sucrose (Celik *et al.*, 2014). It is widely used in the industrial formulation of food and beverages, especially for the low caloric intake and low cost (Nallely *et al.*, 2018). The global production of aspartame is assumed to be more than 16000 tons per year (Ardalan *et al.*, 2017). Aspartame is hydrolyzed into its componentsin the gastrointestinal tract, soon after absorption into the circulation (Serkan and Aslı, 2014). Aspartame consumption has been associated with a risk of developing gastrointestinal disturbances, seizures, headache etc due to the formation of its metabolites, especially methanol (10%), aspartic acid (40%), and phenylalanine (50%), (Ashok and Sheeladevi, 2015).

The most important structural feature of the stomach is the gastric mucosa which plays an important role in the physiological function of the stomach (Motilva *et al.*, 2008). Powdered drink like bevi mix has also been associated with oxidative stress due to the present of aspartame in it which is a risk factor for gastric mucosal induced tissue damage (Mourad and Noor, 2011). The mucosa acts as gastric barrier, which protects deeper located cells against the detrimental action of the gastric secretory components (Kwieclen *et al.*, 2012). Damage of the gastric mucosa occurs when aggressive factors (endogenous, exogenous and/or infectious agent) overcome mucosal defense mechanisms in the stomach. Endogenous aggressive factors include gastric acid, pepsin, refluxed bile, leukotrienes, abnormal motility, while *Helicobacter pylori* infection, indiscriminate use of non-steroidal anti-inflammatory drugs (NSAIDs), alcohol abuse, stress, smoking and lifestyle factors constitute part of the exogenous aggressive factors (Ajeigbe *et al.*, 2014). Mucosal defense mechanisms which protect the integrity of the stomach wall, on

the other hand, are mucus-bicarbonate barrier, prostaglandin from the mucosal cells mucosal blood flow, cell renewal and migration, and some growth factors (de Souza *et al.*, 2017)**.**

The pathogenesis of gastric mucosal damage involves reactive oxygen species (ROS) due to the presence of uncoupled electron within their molecules and / or shortage of antioxidant system. Therefore, they cause tissue damage, mainly due to enhance lipid peroxidation (Kwieclen *et al.,* 2014). The antioxidant enzymes and molecules with free radical scavenger properties in gastric tissue have an important role in the protection of gastric mucosal integrity in addition to the mucus, bicarbonate and prostaglandins secreted from surface epithelial cells of gastric tissue (Yildirim *et al.,* 1986).

Although the renal excretion is the major process for the removal of toxic substances, the renal participation in the assimilation of many substances including product with aspartame has not been considered. In the last decades, growing concern about health and life quality has encouraged people to exercise, eat healthy food and decrease the consumption of food rich in sugar, salt and fat (Prokić *et al.,* 2014). Consequently, the use of products with aspartame has increased concurrently with an increasing incidence of liver and kidney damage (Choudhary, 2017).

Methanol as an aspartame metabolite is very toxic and are recognized as a substance that damages the liver cells, when it is oxidized to formaldehyde and then to formate (Gehan *et al.,* 2015)**.** However, methanol was reported to produce altered oxidant/ antioxidant balance and surface charge density in the liver (Chourdary *et al.,* 2016). Administration of aspartame even at a dose lower than the recommended daily intake is accompanied with a significant increase in

methanol levels. Therefore, the long-term health risk associated with the ingestion of bevi mix needs to be evaluated.

1.2 STATEMENT OF RESEARCH PROBLEM

Studies have demonstrated the association of aspartame consumption with metabolic diseases such as diabetes, cardiac oxidative stress,and gastrointestinal tract disorders (Fernanda *et al.,* 2013; Shastry *et al.,* 2012; Choudhary *et al.,* 2016; Hanaa *et al.,* 2017**).** When aspartame is consumed through soft drinks and other products it comes in contact with the stomach which could lead to digestive symptoms including stomach cramps (Nallely *et al.,* 2018)**.**

1.3 JUSTIFICATION

There is increase consumption of Bevi Mix due to its sweet taste and low cost, that may have concurrently increased the intake of aspartame exceeding the threshold of the recommended maximum daily intake of 40 mg/kg bodyweight.

Many consumers report headaches, dizziness and gastrointestinal disorders after ingesting artificial sweeteners (Nallely *et al.,* 2018)**.** These side effects on the body could build up over time and cause serious long-term diseases with regular consumption of these artificial sweeteners (Gehan *et al.,* 2015).

The health consequences of long-term consumption of Bevi mix are not known, although previous studies on aspartame – the major constituent of bevi-mix have indicated altered microbial metabolic pathways (Suez *et al.,* 2015) and increases in the rate of lymphomas,

leukemias, and mammary tumors (Soffritti *et al.,* 2007). However little or no information concerning the effect of aspartame on gastric secretion and mucosal membrane damage have been addressed hence, the need for this study.

1.4 AIMS OF THE STUDY

The aim of the study is to evaluate the effect of Bevi-mix (artificial sweetener) and aspartame on the integrity of gastric mucosa and some biochemical parameters in male Wistar rats.

1.5 SPECIFIC OBJECTIVES

Objectives of the study are to determine the effect of bevi mix and its constituent aspartame on:

(1) Gastric secreation by determining: volume of the gastric juice (VG), titratable acidity (TA), total acid output (TO) and pepsin content (PC).

(2) Gastric mucus secreation by determining: Gastric mucus secretion and mucus components (sialic acid and galactose).

(3) Oxidative stress biomarkers: Serum malondialdehyde (MDA), superoxide dismutase (SOD) and reduce glutathione (GSH)

(4) Liver enzymes activity (Alanine aminotransferase (ALT), Aspartate aminotransferase (AST) and Alkaline phosphatase (ALP) and electrolyte composition of the kidney (sodium, potassium andbicarbonate).

1.7 NULL HYPOTHESIS

Bevi mix and aspartame does not alter the integrity of gastric mucosa and biochemical parameters in male Wistar rats

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Sugar-Sweetened Beverages

In the past few years, the health effects of sugar-sweetened beverages (SSBs) have been a matter of scientific and public interest. Sugar-sweetened beverages include any beverage with added sugar or other sweetener, such as carbonated and noncarbonated soft drinks, fruit punch, fruit juice concentrates, powdered drink mixes, and energy drinks (WHO, 2019). Sugar-sweetened beverages typically contain 140 to 150 kcal and 35 to 37.5 g of sugar per 12-oz serving, and they are the largest source of added sugars in the diet. Substantial effort has been devoted to discourage the consumption of SSBs, including policies for taxation and restrictions on marketing to children (WHO, 2019). The global flavored powdered drinks is to grow at a CAGR of 8.16% during the period 2017-2021 based on Research and markets (Wood, 2017).

2.2. Chemical and Physical Properties of Aspartame

Aspartame has the molecular formula $C_{14}H_{18}N_2O_5$ along with the molar mass 294.31 g/mol. The density of Aspartame is 1.347g/cm^3 with a high melting point, between 246- 247°C (Shinggu and Joytide, 2018). Under strong alkaline and acidic conditions aspartame hydrolyzed. In aqueous solution the relationship between pH and stability of aspartame is a bell-shaped curve with the maximum stability at pH 4.3. The solubility of aspartame changes in respect of changes in pH and temperature. Aspartame is more soluble in higher temperature acidic solutions. Solubility of aspartame in water is about 0.03gm/ml on acidic pH 3 at room temperature. Being a dipeptide, aspartame has some limitations (Zafar *et al.,* 2017). Aspartame becomes unstable during heating and become inappropriate for use in cooking and baking (Choudhary *et al.,* 2016).

2.3. Aspartame

Aspartame (ASP) has been drastically consumed among the population worldwide, most commonly among the diabetic and diet conscious individual (Zafar *et al.,* 2017) Consumers use aspartame irrespective of their clinical conditions and age. Presently aspartame is consumed widely by the population via cold drinks, powdered drink, diet soda, low calorie sweet products, sugar free sweet products and medications (Zafar *et al.,* 2017).

Aspartame was discovered by a chemist named James Schlatter working in 1965 in Gideone Danie Searle Laboratories discovered during one intermediate reaction of gastrin inhibitor preparation. Mistakenly some of the solution spilled on his hand, irrespective of all safety measures he licked his finger to pick up the piece of paper and came to know about the intense sweet taste of the chemical (Zafar *et al.*, 2017). In 1970 Cloninger and Baldwin published report in Science to propose its use as an artificial sweetener (Nallely *et al.,* 2018)

Aspartame is widely used in more than 6000 products worldwide with a massive commercial outcome under numerous brand names (Zafar *et al.,* 2017). The Food and drug administration in (2014) established acceptable daily intake (ADI) levels for the approved high-intensity sweeteners. Acceptable daily intake as 40mg/kg BW/d) (Hanaa *et al.,* 2017).

Aspartame is composed of amino acids such as aspartic acid and phenylalanine as well as methanol. The metabolism of aspartame provides just about 4 kcal/g of energy which is insignificant that little is needed to be added to foods to attain sweetness (Mourad and Noor,

2011). The derivatives of those breakdown substances are more toxic than the original substance itself (Ashok and Sheeladevi, 2015).

2.3.1. Metabolites of Aspartame

Aspartic acid and Phenylalanine are both amino acids which are found in natural proteins and under normal circumstances are beneficial, and essential, for health (Mourad and Noor, 2011).

Aspartic acid in ASP is a well-documented excitotoxin. Excitotoxins are usually amino acids, such as glutamate and aspartate. They are substances that react with specialized receptors in the brain that lead to destruction of certain types of neurons (Saleh, 2015). Aspartate a component of aspartame is an excitatory neurotransmitter which is found in high levels in the brain where it stimulates N-Methyl-D-aspartate (NMDA) receptors (Prokić *et al.,* 2014). N-methyl D-aspartate (NMDA) receptors are distributed throughout the central nervous system including the hypothalamus, amygdala and hippocampus, regulating vital metabolic and autonomic functions including energy homeostasis, glucose sensing and non-insulin mediated hepatic glucose uptake (Choudhary, 2017).

Phenylalanine is a large neutral amino acid that competes with other important large neutral amino acids for binding on the large neutral amino acid transporter. However, excess phenylalanine concentrations are associated with decreased concentrations of catecholamine, serotonin, and dopamine (Choudhary and Pretorius, 20187). Phenylalanine can be converted into tyrosine in the body, this acts as a precursor for several important neurotransmitters like dopamine, noradrenalin (norepinephrine) and adrenalin (epinephrine) and for such substances as

thyroid hormone and melanin (Shinggu and Joytide, 2018). Phenylalanine and its derivatives after ingestion of aspartame accumulate in the blood, tissues and urine; this is unsafe to individuals who have the disorder called, phenylketonuria (PKU); an inborn disorder caused by lack of Phenylalanine Hydroxylase Enzyme, where phenylalanine cannot be metabolized to tyrosine (Mourad and Noor, 2011). Consequently, such accumulation result in mental retardation, growth retardation (El Attug *et al.,* 2015).

Methanol as a metabolites of aspartame is a toxicant that causes systemic toxicity. The primary metabolic fate of methanol is the direct oxidation to formaldehyde and then into formate. The severity of clinical findings in methanol intoxication correlated better with formate levels (Choudhary *et al.,* 2016). Methanol is more readily generated by the body (thus becoming even more dangerous) when it is heated above 30°C before being ingested. This occurs when soft drinks are left out in the sun or foods containing aspartame are heated. Formaldehyde is an embalming fluid, as a preservative in vaccines and a deadly neurotoxin. Formic acid causes cells to become too acidic, thereby producing metabolic acidosis. Acidosis damages cellular health by causing enzymes to stop functioning (Choudhary and Rathinasamy, 2014).

Although methanol production is not very high during aspartame metabolism yet it contributes to the toxicities because of its metabolites (Zafar *et al.,* 2017). It is increasingly recognized as a substance that damages the liver cells, when it gets oxidized. These processes are accompanied by rise in NADH level and the formation of superoxide anion that may be involved in lipid peroxidation. Methanol intoxication is associated with mitochondrial damage and increased microsomal proliferation, which results in the overproduction of oxygen radicals. These factors,

together with the excess of formaldehyde formed during acute methanol intoxication, cause an increase in lipid peroxidaion (Ashok and Sheeladevi, 2015).

Free radicals or reactive species through oxidative stress have been evidently implicated in the incidence and progression of several health conditions (Ighodaro and Akinloye, 2017). Oxidative stress is cellular phenomenon or condition which occurs as a result of cumulative damage in the body by free radicals, incompetently neutralized by antioxidants. Stressor is a stimulus by either internal or external, which activates the hypothalamic pituitary adrenal axis and the sympathetic nervous system resulting in a physiological change (Melekh *et al.*, 2017).

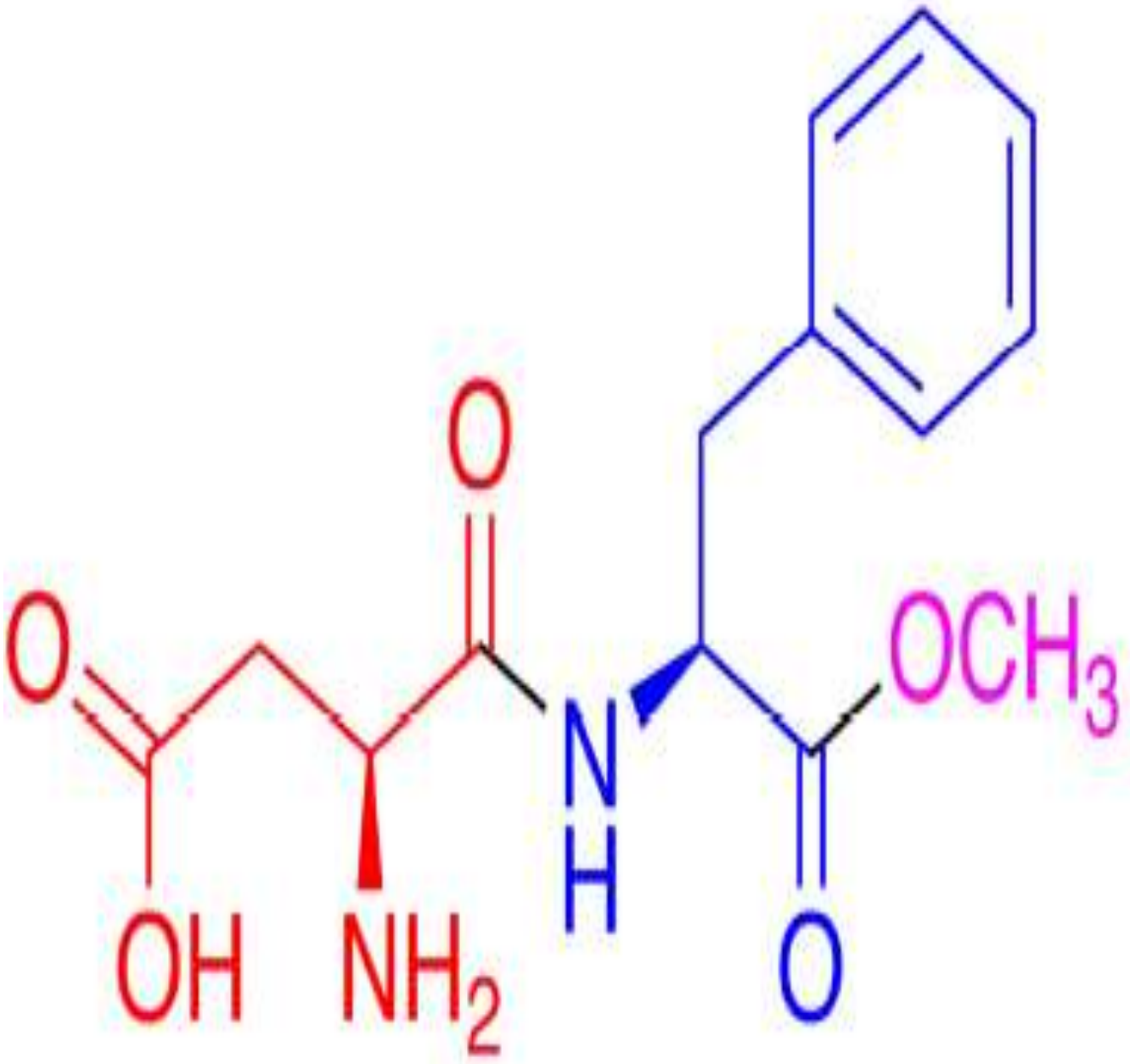

Figure 2:1: Chemical structure of Aspartame (El Attug *et al.*, 2015).

2.3 PHYSIOLOGIC ANATOMY OF THE STOMACH

The stomach otherwise known as the *gastros* is the most dilated part of the digestive tube roughly J-shaped, even though it sizes and position vary considerably, depending on the position of the body, the phase of respiration and whether it is full or empty and is situated between the end of the oesophagus and the beginning of the small intestine (Ellis, 2011). The stomach lies in the epigastric, umbilical, and left hypochondrial regions of the abdomen occupying a recess bound by the upper abdominal viscera, the anterior abdominal wall and the diaphragm (Daniels and Allum, 2017) which also lined by simple columnar epithelial cells (Adjene and Igbigbi 2010). Topographically, the stomach has five regions; the cardia and gastroesophageal (GE) junction, fundus, corpus, antrum and the pylorus (Soybel, 2005). The left part of the stomach is the cardiac region which is a thin band near the opening of the esophagus, which contains cardiac glands. The glands are branched simple tubular glands and are composed almost entirely of mucus secreting cells with few odd entero-endocrine cells present and few secretory cell characteristics for the fundi regions may be present (Stephen, 1977). The stomach have a very marked fundus (*fundus ventriculi*), which formed a distinct craniodorsal blind ventricular sac (*saccuscecus ventriculi*) on the left side, near the cardiac part. The fundic glands are simple tubular structure with a base, neck, and isthmus that are continuous with the glandular gastric pit. The fundic gland has the following cells

(1) Mucous neck cell that are situated between the parietal cells in the neck region of the gland**,** which produces mucus under vagal stimulation (Ettarh *et al.*, 2003).

(2) Parietal cell or oxyntic cells are present mostly in the neck of the gland that interspersed among the mucous neck cells, which are situated deeper, between and below

chief cells in the lower part of the gland with a cellular product consisting of hydrochloric acid (HCl) and intrinsic factor (Ettarh *et al.,* 2003). The HCl secreted is stimulated mainly by the hormone called gastrin; which activate pespinogen and effectively sterilize the stomach. The intrinsic factor is a glycoprotein that binds vitamin B12, they are necessary for reabsorption of vitamin B12 which is essential for maturation of red blood cells (Fawett, 1994)

(3) Chief or zymogen cell they are the most abundant of all the cell types and are located primarily in the body of the glands and are protein secreting cells. They secrete pepsinogen which is a precursor of the proteolytic enzyme pepsin to which it is being converted upon contact with gastric acid, the optimum pH of pepsin is 2, this enzyme is able, to break collagen (Ojo *et al.,* 2010).

(4) The Argentaffin cells that produce a variety of endocrine and paracrine hormones. In the pyloric antrum, glands are lined by mucus containing pyloric glandular cells (Ojo *et al.,* 2010).

The right part of the stomach is the pyloric region (*parspylorica*). The stomach of the rat has two surfaces. The cranial, parietal surface (*facies parietalis*) is in contact with the diaphragm and left abdominal wall. The part of the parietal surface is covered by left lobe of the liver. The caudal, visceral surface (*facies visceralis*) is attached to the intestine (Vdoviaková *et al.,* 2016).

The stomach has two curvatures: the lesser curvature is concave that forms the right border of the stomach; a sharp angular notch at the lesser curvature which indicates the junction of the body

and the pyloric part of the stomach. The greater curvature is convex and forms the left border of the stomach; it's about five times longer than the lesser curvature (Daniels and Allum, 2017)). These two surfaces are fused in greater and lesser curvature (*curvature major* and *minor*). The greater curvature is directed caudoventrally. The esophagus entered in the middle of the lesser curvature, which is directed craniodorsally (Vdoviaková *et al.*, 2016).

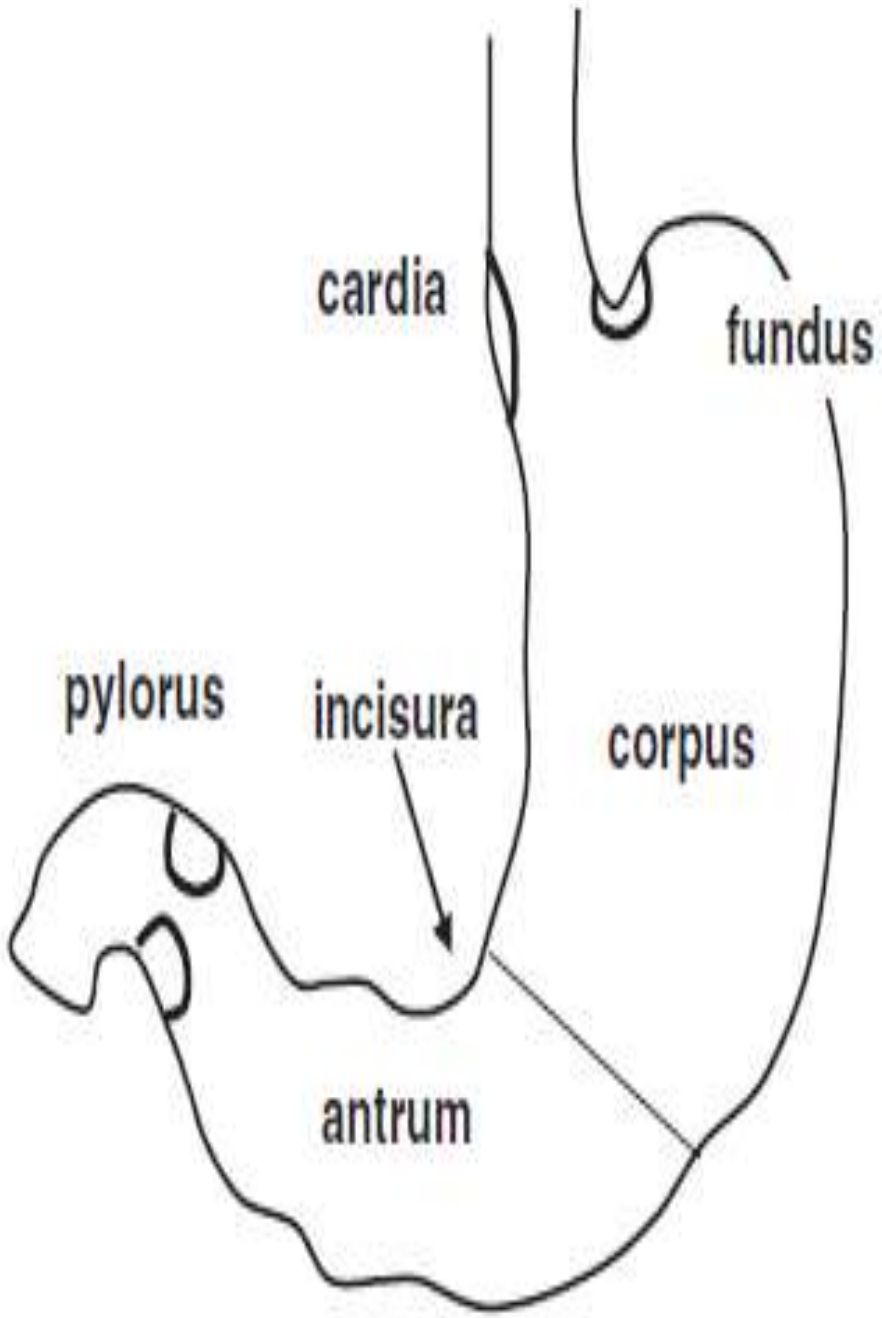

Figure 2:2: Regions of the stomach (Soybel, 2005).

2.3.1 Layers of the Stomach

I. Gastric Mucosa: The surface of the stomach is lined by simple columnar epithelium whose Cells are made up of surface mucous cells. There are numerous invaginations of the surface epithelium that extends into the lamina propria (Ojo *et al.,* 2010). Gastric mucus is secreted to form a gel layer on the gastric mucosa, which acts as a lubricant for ingested food and protects against injury from digestive juice, alcohol, hypertonic or hypotonic foods, spices, drugs, *etc*. However, even in the absence of food in the stomach, the gastric mucosa should be coated with mucus against unexpected invasions. Therefore, it is thought that gastric mucus secretion is controlled by many factors in different ways (Tani *et al.,* 2002). Mucosal surface epithelium is a subject of attack by physical, chemical or microbiological agents acting from the gastric lumen, which are involved in multiple pathologies, such as gastritis, peptic ulcer or gastric cancer (Zayachkivska *et al.,* 2005). The mucosa is thrown into longitudinal folds also called gastric folds or ruggae, which disappear when the stomach is fully distended (Ojo *et al.,* 2010).

II. Submucosa: This is made up of smooth muscle which supports the mucosa consisting of dense connective tissue which contains large blood vessels, lymph vessels and nerves plexus of Meissner (Stephen, 1977).

III. Muscularis Externa or Muscularis Propria: This consists of three fairly indistinct layers; an inner oblique middle circular and outer longitudinal layer (Reitel and Travill, 1978). The layers are somewhat randomly oriented and some are absent or poorly developed in some areas. The muscularis mixes the chyme and expels it into the small intestine, the muscularis contains Meissner's plexus, but they are located between the circular and longitudinal fibers of the

muscularis externa and concentrated in the submucosa, myentericplexus, superior mesenteric plexus and Auerbach's plexus (Ojo *et al.,* 2010).

IV. Adventitia or Serosa: The stomach is covered by the serosa which is continuous with the peritoneum of the body via the omentum, it consists of blood vessels, nerves and adipose tissues (Ojo *et al.,* 2010).

2.4 Gastric Mucosal Defense.

Mucosal defense is a term used to describe the various factors and components that permit the mucosa to remain intact despite its frequent exposure to substances with a wide range of temperature, pH, and osmolarity, as well as to substances with detergent or cytotoxic actions, and bacterial products capable of causing local and systemic inflammatory reactions (Wallace, 2008). The defense mechanisms of the gastric mucosa are crucial for the maintenance of an effective barrier and for preventing the stomach mucosa from digesting itself. The defense is arranged at different levels, which work in concert for effective protection (Phillipson, 2004).

2.4.1 The pre-epithelial layer

This first line of defense consists of the mucus layer and bicarbonate secreted into the mucus, creating a pH gradient within the mucus.

2.4.1.1 Mucus Layer.

Epithelia that are frequently exposed to hostile environments often protect themselves by the secretion of mucus (Dekker *et al.,* 1988). Gastric mucus is present in the mucus granules of the

mucus-producing cells, the insoluble mucus gel layer adhering to the mucosal surface and the gastric lumen in a solubilized condition (Takafumi and Kazuhiko 2016). This mucus forms a continuous layer of mucus gel, secreted by the surface epithelial cells and the mucous neck cells, covers the gastric mucosa (Phillipson, 2004). The mucus gel layer is an integral structural component of the stomach, acting as a medium for protection, lubrication, and transport between the luminal contents and the epithelial lining. Mucus secretion is stimulated by agents such as prostaglandins and nitric oxide, whereas the mucus layer is degraded by pepsin. The adherent mucus barrier is continuously being turned over as part of the protective functions of the mucosa surface. Therefore, degradation of mucus is a normal feature of balance between mucus synthesis, secretion of preformed mucus and breakdown of existing adherent mucus (Yusuf *et al.,* 2005). The viscoelastic, polymer-like properties of mucus are derived from the major gel-forming glycoprotein components called mucins (5%). Other components include water (95%) with electrolytes, sloughed epithelial off cells, enzymes and various other materials, including bacteria and bacterial products depending on the source and location of the mucus. The thickness of the mucus layers depends on the secretion of mucins and the degree of erosion and proteolytic degradation of the layers. Mucus contains as its major components large molecular weight glycoproteins known as mucins secreted by mucous neck cells in the stomach. Mucins consist of a peptide backbone containing alternating glycosylated and nonglycosylated domains, with *O*-linked glycosylated regions comprising 70–80% of the polymer (Bart and Gaskins 2001). In the stomach, three mucins have been identified MUC1, MUC5AC, and MUC6. MUC1 is a non gel forming membrane bound mucin, whereas the latter two form the mucus gel in the stomach (Namulema *et al.,* 2018). They have a very distinct expression pattern; MUC5AC is present in the surface epithelial cells and MUC6 is present in the mucus neck cell (Flemström and Jon

2001). On the molecular level, gel formation by mucins depends mainly on two features of the molecules. One mucin subunit contains hundreds of oligosaccharides comprising up to 80 % of the weight of the molecule, which are 0-linked at serine and threonine residues. In a mucus gel the mucin molecules interact by formation of hydrophilic non-covalent bonds between the oligosaccharides. Therefore, the number and structure of the oligosaccharides partly determine the rheological properties of the mucin gel (Dekker *et al.*, 1991). The second feature of gastric mucin molecules, and one that is essential for gel formation, is their disulphide bound oligomeric structure. Chemical reduction of the disulphide bonds of oligomeric gastric mucins results in complete loss of gel-forming properties. Sialic acid, *N*-Acetylglucosamine, *N*-acetylgalactosamine, fucose, and galactose are the 5 primary mucin oligosaccharides (Sadiq *et al.*, 2008).

2.4.1.1.1. Sialic Acid as a Component of Mucus

Sialic acids are a diverse family of carbon derivatives of neuraminic acid. There are more than 30 natural derivatives of neuraminic acid, the most abundant of which is Neu5Ac (Oto *et al.*, 2016). Sialic acids are components of glycoproteins, glycolipids such as gangliosides, or polysaccharides, and usually occupy the terminal position of the oligosaccharide chain linked to other sugars such as galactose or N-acetyl galactosamine by glycosidic bonds (Cecile *et al.*, 1993). These terminal residues have a significant influence on the mucus charge, mucus rheology and mucus degradation. In this way they contribute to the high viscosity of the mucus lining and protect the endothelial layers of the stomach (Sadiq *et al.*, 2008). While the usual location of sialic acids is at the terminal position of oligosaccharides, they may also be located in the side chain of oligosaccharide located in the side chain of oligosaccharide units in

gangliosides and some glycoproteins (Cecile *et al.,* 1993). In the structure of sialic acid the amino group is replace with an acetyl group that form N-acetylneuraminic acid. Alternatively, the amino group could also be substituted by a glycolyl residue. The hydroxyl groups could either be methylated or esterified with acetyl, lactyl, phosphate, or sulfate groups (Narayanan, 1994).

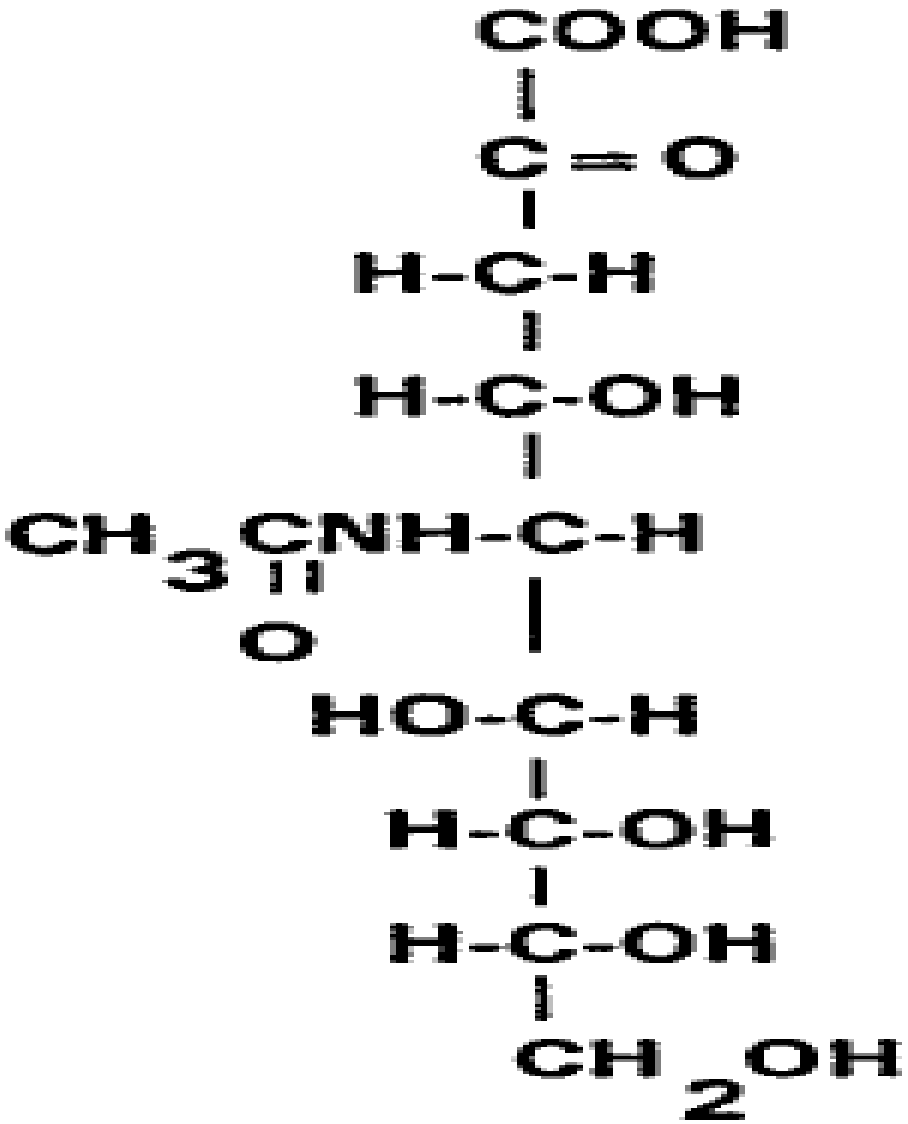

Figure 2:3: Structure of Sialic Acid (Narayanan, 1994).

2.4.1.1.1.1. Biosynthesis of Sialic Acid

The sialic acids (N-acetylneuraminic acid) have in common a 9-carbon carboxylic acid, neuraminic acid, which is regarded as a condensation product of a 3 carbon and a 6-carbon unit. Neuraminicacid itself is not found naturally, but several N- and O-substituted derivatives are widely distributed in nature. Chemical and enzymic degradations of N-acetylneuraminic acid shows that is composed of pyruvic acid and an N-acetylhexosamine (Schauer, 2000).

Heimer and Meyer were the first to explained an enzyme called neuraminic acid aldolase, which made the important observation that the N-acetylhexosamine component of N-acetylneuraminic acid is N-acetyl-D-mannosamine. This compound was a product of the reversible N-acetylneuraminic acid aldolase-catalyzed reaction (Warren and Felsenfeld, 1961). Sialic acid is synthesized by glucosamine 6 phosphate and acetyl-CoA through a transferase, resulting in N-acetylglucosamine-6-P. This becomes N-acetylmannosamine-6-P through epimerization, which reacts with phosphoenolpyruvate producing N-acetylneuraminic-9-P (sialic acid). For it to become active to enter in the oligosaccharide biosynthesis process of the cell, a monophosphate nucleoside is added, which comes from a cytidine triphosphate, turning sialic acid into cytidine monophosphate-sialic acid (CMP-sialic acid). This compound is synthesized in the nucleus of the animal cell (Fulcher, 2009).

2.4.1.2 Bicarbonate

Bicarbonate, secreted from the surface epithelial cells into the mucous gel, neutralizes back-diffused acid and that endogenous acid traverses the mucus only in distinct “channels” leading from the gland openings toward the lumen (Phillipson *et al.*, 2001). Bicarbonate production in the surface epithelial cells is stimulated by vagal stimulation, prostaglandins, gastric distension,

and acid in the gastric lumen all increase gastric bicarbonate secretion. Furthermore, Teorell demonstrated that for each proton secreted from the parietal cell, one bicarbonate ion is released from the basolateral membrane of the parietal cell to the capillaries leading to the surface epithelium (Phillipson, 2004). This blood borne transport of bicarbonate during acid secretion is of importance because a maintained pH gradient is formed, with pH at the epithelial cell surface considerably higher than that in acid-containing luminal bulk solutions, and diffusion of macromolecules, including pepsin, from the lumen to the epithelial surface is prevented or restricted (Flemström, 1980).

The proton concentration in the gastric lumen is million-fold higher than in the blood. The pH at the surface epithelial cells (Juxtamucosal pH) is neutral. pH in the mucosal was measured using sensitive microelectrodes and the gradient succeed in keeping the pHjm neutral while the lumen pH is 2 in both acid secreting and non-secreting mucosae (Ross *et al.,* 1981, Schade *et al.,* 1994). Bicarbonate secreted from epithelial cells is responsible for causing this gradient. However, the mucus layer is essential for the establishment of the gradient as it creates an unstirred layer in which neutralization of back-diffused acid can occur (Lamont, 2000). In addition, it has been proposed that the gastric epithelium is covered by a hydrophobic phospholipid layer, which likewise might impede the diffusion of luminal H1 inside the mucosa (Nylander-Koski *et al.,* 2001).

2.4.2. The epithelial layer

It consists of intercellular tight junctions and proton and bicarbonate transport systems.

2.4.2.1. Intercellular tight junctions

Under normal conditions the mucosa is relatively impermeable to transport of luminal contents due to a very tight epithelium. The apical membrane of the gastric surface cells is highly impermeable to proton (Phillipson, 2004). Furthermore, it has been shown that the apical membranes of the parietal and chief cells in the gastric gland are very resistant to H^+, NH_3^+, NH_4^+, CO_2 and HCO_3^-, whereas the basolateral membranes show normal permeability properties (Boron *et al.*, 1994). Between the cells tight junction give rise to a highly impermeable paracellular route (Hirst, 1989).

2.4.3. The Post Epithelial layer

It consists mainly of an effective blood flow and the gastrointestinal autonomic nervous system, the enteric nervous system (ENS).

2.4.3.1. Blood flow and vascular response of the gastrointestinal mucosa

Underlying the surface epithelium of the stomach is a dense network of capillaries. In addition to supplying of nutrient and oxygen to the epithelium, the microcirculation also removes, dilutes, and neutralizes toxic substances that have diffused into the mucosa from the lumen. When the epithelium is damaging the microcirculation plays a critical role in creating a microenviroment over the site of injury conducive for repair (Wallace and Granger 1996). The microcirculatory response of the mucosa is possibly the most important component of mucosal defense. It is modulated by the extrinsic and intrinsic nervous systems (Martin and Wallace 2006). Mucosal blood flow can be modulated by many endogenous substances. Among the more important of these are calcitonin gene-related peptide (CGRP), prostaglandins and nitric oxide (Wallace and Granger, 1996). For example, gastric hyperemia caused by mucosal acidification by luminal acid

and barrier-breaking substances (e.g., ethanol or sodium taurocholate) there is a profound and rapid increase in mucosal blood flow and haemorrhagic lesions are formed. This activate the sensory afferent nerves, leading to release of calcitonin gene-related peptide (CGRP) in the vicinity of the submucosal arterioles and generation of nitric oxide (NO) (Li *et al.*, 2002). This increase in blood flow acts to dilute and neutralize the toxin, as well as to prevent the toxin from accumulating within the mucosa to cytotoxic concentrations (Martin and Wallace, 2006). Nitric oxide is produced and released from the vascular endothelium, epithelial cells and sensory nerve endings via the activity of NO synthase (NOS). A substrate for this enzyme is amino acid L-arginine and NO-synthase (NOS) puts molecules of oxygen (O2) into molecule of L-arginine, capable of producing NO (Kwiecien *et al.,* 2014). Nitric oxide diffuses to the adjacent vascular smooth muscle cells where, through the stimulation of soluble guanylate cyclase (increasing intracellular cGMP level) it causes them to relax which is accompanied by an increase of blood flow through this vessel (Wallace and Granger, 1996). The importance of this vascular response is evident from experiments in which ablation of capsaicin sensitive primary afferent nerves or pretreatment with CGRP/substance P antagonists abolished the reactive hyperemic response, rendering the mucosa more susceptible to injury (Schmidt *et al.*, 2003).

2.5 Factors That Modulate Mucosal Defense

Since the gastric mucus is secreted to form a gel layer on the gastric mucosa. It is therefore thought that gastric mucus secretion is controlled by many factors in different ways. The various components of mucosal defense can be modulated by a number of endogenous substances, including prostaglandins (Wallace, 2008). Prostaglandins are a group of fatty acids that were first isolated from seminal fluid by Von Euler. Indeed, they are so named because they were believed

(incorrectly) to be a prostatic secretion. A crucial discovery, in terms of understanding the role of prostaglandins in the stomach, was the finding by Vane in 1971 that aspirin and other NSAIDs inhibited the synthesis of prostaglandins (Wallace, 2008).

2.5.1. Prostaglandins (PGs)

Prostaglandins (PGs), biosynthesized from arachidonic acid by cyclo-oxygenase (COX) and various PG synthesizing enzymes, these enzymes are present throughout the gastrointestinal (GI) tract and bring about various actions, including the control of bicarbonate secretion, mucus production, and mucosal blood flow, and maintenance of mucosal integrity. Indeed, PGs protects the GI mucosa against necrotizing agents, stress, and nonsteroidal anti-inflammatory drugs (NSAIDs) (Takeuchi and Amagase, 2017). Prostaglandins (PGs) prevent damage of deeper structures due to an increase of mucus secretion, intensification of bicarbonate anions production (HCO3-), which neutralise acidic gastric content and the stimulation of mucosal phospholipids (Tache, 2012). Prostaglandins (PGs) PGs were shown to evoke increment of gastric blood flow thus enhancing oxygen and nutrients delivery to the gastric mucosa (Kwiecien *et al.*, 2014). Two isoforms of COX were proposed: the constitutive isoform COX-1 which delivers PG for the physiological functionsand the inducible isoform, COX-2 stimulated by inflammatory conditions, lipopolysaccharide, growth factors and cytokines. Classic approach to their functions has established that COX-1plays a gastro-protective role, because PGs generated by this pathway were shown to exert gastroprotective effects. On the other hand, high levels of PGs, produced by COX-2 activation, contribute to detrimental effects, such as inflammation, increase of vessels permeability, transmission of pain sensation and fever. In addition to PG, several other mediators modulate significantly to mucosa defence and repair. For example, nitric oxide (NO) modulates

many of the same elements of mucosal defence (e.g. blood flow, mucus and bicarbonate secretion) as do PGs (Brown *et al.,* 1993), and can promote the healing of ulcers (Elliott *et al.,* 1995).

2.5.2 Hydrogen sulphide (H_2S)

Like NO, hydrogen sulphide (H_2S) is a vasodilator and neuromodulator with physiological and pathophysiological roles in many organs (Wang, 2002) and contributes to the resistance of the gastric mucosa to injury. H_2S is produced by the healthy gastric mucosa, and both of the key enzymes for mammalian H_2S synthesis, CSE and CBS, are constitutively expressed in the mucosa. Cystathionine- β- synthase (CBS) and cystathionine -λ- lyase (CSE) are the principal enzymes involved in the generation of H_2S from cysteine, cystine, and homocysteine.

Induction of gastric ulceration was associated with a profound increase in the expression of these two enzymes, and of H_2S synthesis. This is a defensive response, aimed at reducing gastric mucosa injury and accelerating repair. Enhanced ulcer healing was observed following administration of three chemically distinct hydrogen sulfide donors. Moreover, administration of l-cysteine, a precursor of endogenous H_2S synthesis, resulted in enhanced healing (Wallace *et al.,* 2007) This beneficial effect of l-cysteine was prevented by co-administration of an inhibitor of CSE, strongly suggesting that the effect of l-cysteine was mediated via H2S production. Taken together, these results therefore suggest an important role for H_2S in the process of healing of chronic gastric mucosa damage (Wallace *et al.,* 2007).

2.5.3. Pepsin and Increase Acidity

Gastric acid and pepsin secretions result from the interplay of neurohormonal factors with stimulatory and inhibitory actions on oxyntic glands. At the turn of XIX century, the notion of nervism or entire neural control of digestive functions, developed by Pavlov prevailed. However, in the second part of XX century, hormonal control has been thought to play a major role in the mechanism of gastric secretion, especially gastrin, which was isolated and synthesized in 1964 by Gregory (Konturek, 2003).

Gastric juice is a digestive fluid formed in the stomach and it is composed of high proportion of hydrochloric acid, potassium chloride and sodium chloride, in small proportion (Ikwebe *et al.,* 2017). Gastric acid secretion is influenced by the interplay of stimulatory and inhibitory factors arising from both the central nervous system and within the gastrointestinal system (Ajeigbe *et al.,* 2012). Addition to these regulatory processes are factors arising from daily assaults on the gastrointestinal tract such as food and drugs. Hydrochloric acid is produced by the parietal cells in the stomach. In the resting state the parietal cell is filled with vesicles. During acid secretion morphological changes occur to parietal cells, including the movement of the tubovesicles to the apical membrane with formation of secretory canaliculi (Ajeigbe *et al.,* 2012). Activation of the parietal cell increases cytoplasmic calcium, followed by activation of a cAMP-dependent kinase cascade resulting in movement of the proton pump to the apical surface. The proton pump, H+/K-ATPase, actively pumps H+ ions in exchange for K+ (Davies, 1951)). Parietal cell mitochondria provide ATP for ATP hydrolysis generating the necessary energy for the membrane embedded proton pump. The different receptor pathways converge to stimulate the gastric H+/K-ATPase, the pump responsible for acid secretion by the stomach. This enzyme is an

alpha, beta heterodimer, present in cytoplasmic membrane vesicles of the resting cell and in the canaliculus of the stimulated cell. It has been shown that acid secretion by the pump depends on provision of K+Cl- efflux pathway becoming associated with the pump. As secretion occurs only in the canaliculus, this K+Cl- pathway is activated only when the pump inserts into the canalicular membrane (Hirschowitz et al., 1995. Transport by the enzyme involves reciprocal conformational changes in the cytoplasmic and extracytoplasmic domain. These result in changes in sidedness and affinity for H3O+ and K+, enabling active H+ for K+ exchange (Hirschowitz *et al.,* 1995).

For every hydrogen ion secreted by the oxyntic cells there is an uptake of one molecule of carbon dioxide, and one bicarbonate ion is formed and exchanged for a chloride ion from the sodium chloride of the blood in vivo, or of the saline solution in vitro (Davies, 1951) The consequent increase in the sodium bicarbonate content of the blood leads to the 'alkaline tide' of the urine following acid secretion, and the hydration of carbon dioxide to form these bicarbonate ions is so rapid that carbonic anhydrase activity is required within oxyntic cells(Hirschowitz et al., 1995). The amount of this enzyme found in oxyntic cells is easily able to produce the required extra uptake of carbon dioxide. Virtually complete inhibition of carbonic anhydrase in isolated gastric mucosa leads to inhibition of acid secretion (Davies, 1951).

2.5.4. Oxidative Stress

Oxidative stress (OS) can be defined as an imbalance between the levels of ROS produced and the ability of the biological system to neutralize them, creating a state of possible cellular damage (Danboyi *et al.,* 2019).The mucosa acts as gastric barrier that protects deeper located

cells against the detrimental action of the gastric secretory components, such as acid and pepsin. Integrity of this gastric mucosa depends upon a variety of factors, such as maintenance of microcirculation, mucus-alkaline secretion and activity of the antioxidizing factors (Kwiecien *et al.,* 2014). The pathogenesis of gastric mucosal damage includes reactive oxygen species (ROS), because of their high chemical reactivity, due to the presence of uncoupled electron within their molecules. Therefore, they cause tissue damage, mainly due to enhanced lipid peroxidation. Lipid peroxides are metabolized to malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE). The local increase of MDA and 4-HNE concentration indicates ROS-dependent tissue damage. Free radicals created during gastric mucosal damage attack the protein in gastric mucosa which leads to a reduction in the protein level (Choudhary *et al.,* 2016). Administration of HCl and ethanol has been shown to produce ulcerative lesions and increased lipid peroxidation in the gastric mucosa with depletion of endogenous antioxidants. In gastroprotection, the first line of antioxidative enzyme is SOD which catalyses the dismutationof superoxide radical anion (O2) into less noxious hydrogen peroxide (H_2O_2) (Olaleye *et al.,* 2007).Hydrogen peroxide is then inactivated by the degradation into water by catalase or glutathione peroxidase. Depletion of these enzymes, therefore predisposes the stomach to a greater impact of the free radicals produced *via* increased lipid peroxidation, hence increased ulcer formation (Ighodaro and Akinloye 2017).

2. 6 Mechanism of Stimulation of Gastric Acid Secretion

The first 25 years of the twentieth century defined the major mechanisms of stimulation of acid secretion. Gastrin, released from G cells of the gastric antrum (hormonal), and acetylcholine, released from the vagus and histamine released from ECL cells (paracrine) (Sachsa *et al.,* 1994).

These agents interact with receptors coupled to two major signal transduction pathways: adenylate cyclase in the case of histamine and an increase intracellular calcium in the case of gastrin and Ach (Takeuchi *et al.,* 2016).

Histamine in the gastric mucosa is found within enterochromaffin-like (ECL) cells and mast cells, the relative proportion of the two cell types being species and site dependent. Histamine is formed by the decarboxylation of histamine by histidine decarboxylase (HDC). After release histamine is enzymatically deactivated by two pathways. The majority is methylated onto one of the nitrogen atoms in the imidazole ring byimidazole-*N*-methyltransferase, and asmaller proportion is degraded by oxidativedeamination to imidazole aceticacid (Beales, 2000). Histamine binds to H2 receptors on the parietal cell that activate adenylatecyclase (AC) via G protein and generate cAMP. Gastrin, released from G cells, binds to CCK2 receptors that activate phospholipase C to induce release of cytosolic calcium (Ca2+). Gastrin stimulates the parietal cell directly and, more importantly, indirectly by releasing histamine from ECL cells. Ach, released from intramural neurons, bind to muscarinic acetylcholine receptors (mAChRs)that are coupled to activation of phospholipase C with generation of inositoltriphosphate and release of intracellular calcium (Mitchell and David, 2008).

The muscarinic acetylcholine receptors consist of five subtypes (M1–M5) and are widely expressed in many peripheral organs as well as central nervous system to mediate diverse autonomic functions, including acid, pepsin and mucus as well as bicarbonate secretions (Tobin *et al.,* 2009). These receptors are G protein-coupled receptors; M1, M3, and M5 receptors are coupled to Gq protein, while M2 and M4 receptors are coupled to Gi protein. It is generally

accepted that the acid stimulatory action of carbachol (CCh), a muscarinic agonist, is mediated by the activation of M3 receptors Muscarinic receptors on parietal cells are of the M3 subtype. Like CCK2 receptors (Takeuchi *et al.*, 2016).

The intracellular cAMP- and calcium-dependent signaling systemsactivate downstream protein kinases ultimately leading to fusion and activation of H+K+-ATPase, the proton pump. The main inhibitor of acid secretion is somatostatin, released fromoxyntic and pyloric D cells (paracrine). Each of these agents acts directly on the parietal cellas well as indirectly by modulating the secretion of neuroendocrine cells (Takeuchi *et al.*, 2016).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Materials

3.1.1 Drugs and Chemicals

Alcian blue, Methylated spirit, Normal saline (0.9% w/v NaCl), Distilled water, sucrose, sodium acetate, magnesium chloride, diethylether, periodic acid reagent, pipette, thiobarbituric acid solution, acid butanol, hydrochloric acid,

3.1.2 Experimental animals

Forty two (42) apparently healthy male Wistar rats between the age of 7 – 8 weeks, were purchased from the Department of Human Physiology, Faculty of Basic Medical Sciences, College of medical Sciences, Ahmadu Bello University, Zaria, Nigeria, where they were housed in cages and were provided with growers feed and water *ad libitum.*

3.2. Methodology

3.2.1. Experimental Animal grouping

The forty two (42) male Wistar rats were divided into six (6) groups containing seven Wistar rats each, (n=7).

Group 1: Rats received 1 ml/kg distilled water orally, once daily for four weeks.

Group 2: Rats received 330 mg/kg of bevi mix orally, once daily for four weeks.

Group 3: Rats received 660 mg/kg bevi mix orally, once daily for four weeks.

Group 4: Rats received 20 mg/kg of aspartame orally, once daily for four weeks (Gabr *et al.*, 2011).

Group 5: Rats received 40 mg/kg of aspartame orally, once daily for four weeks (Azeez and Alkass, 2018).

Group 6: Rats received 80 mg/kg of aspartame orally, once daily for four weeks (Azeez and Alkass, 2018).

3.2.2. Method of gastric juice

Gastric juice was collected according to the method of Shay *et al.* (1954). The animals were fasted 48 hours to ensure complete emptying of the stomach and water was permitted ad libitum. The animals were weighed before fasting and at the end of the 48 hours fast. Under light ketamine anaesthesia, the abdomens of the rats were shaved and a midline incision was made extending 2 cm downwards from the xyphoid. The junction between the pylorus and the duodenum was picked up gently with a curved probe; while the stomach itself was not disturbed. A pyloric ligature was made using silk thread, care being taken to avoid damage of blood vessels or traction on the stomach. The abdominal wound was cleaned thoroughly with physiological saline, dried and covered with a solution of flexible collodion. The animals were allowed to recover from the anesthesia and four hours later, the animals were again anaesthetized with high dose of ketamin, the abdomen was opened and the cardiac junction was ligated. Blood sample were collected via cardiac puncture using 5ml syringes into plain bottles and were centrifuge at 3000 × g for 10 minutes to obtain serum which were used for biochemical assays (Eno *et al.*, 2004). The stomach was removed and washed in physiological saline and dried. An opening was made near the pyloric ligature and gastric juice was drained. After centrifugation (6000 g for 5 min) the volume of the supernatant and solids was recorded.

3.2.3. Experimental Procedures for Gastric Secretion

3.2.3.1 Determination of gastric juice.

0.2 ml of centrifuged gastric juice was titrated using phenol red as an indicator with end point at 7.0 pH against 0.01 NaOH (Grossman, 1963).). It was calculated as milliequivalent per liter (mEq/L)

Titratable acid output was calculated as micro (μ-) equivalents, by multiplying the volume in ml by the acid concentration in m-equivalent per liter (Brodie and Hooke, 1971). The results were then divided by four to give output per hour.

3.2.3.2 Determination of pepsin content

One ml from various concentrations of bovine pepsin, ranging from 0.1-1.0 mg/100 ml in 0.1N HCl, was transferred to a test tube and incubated for 30 min with 3.9 ml of the substrate in a water bath at 37^{o}C. Then, 10 ml of trichloracetic acid (TCA) were added and the tubes allowed to stand for 10 min and filtered using Whatman filter paper No. 1. Blanks were made for each concentration by adding 10 ml of TCA before the addition of the enzyme. Duplicate determinations were performed for each enzyme concentration. The optical density of the filtrate was measured at 280 nm wavelength. A standard curve was constructed from which pepsin content of gastric secretion were determined by extrapolation (Hawk *et al.,* 1960).

To 0.2 ml of centrifuged gastric juice, 3 ml of casein 3 % for each rat test and blank was added. Then 10 ml of 6 % trichloracetic acid added to blank to stop enzyme activity. Both blank and test tubes incubated in water bath with temperature 37^{0}C for 30 minutes. At the end of incubation period the tubes were taken out from the water bath and enzymatic reaction was stopped in the

test by adding 10 ml of trichloracetic acid to test tubes, it was shaken well. The solution in the test tubes was allowed to stand for 10minutes and was filtered using No.1 whattman filter paper. Proteolytic activity was determined from the absorbance readings of the filtrate which was measured at 280 (mp) wave length of ultraviolet light using spectrophotometer (Hawk *et al.*, 1960).

3.2.3.3. Thiobarbituric acid assay of sialic acids

To 0.2 ml of gastric juice, 0.25 ml of periodic acid was added. The tubes were shaken and incubated at 37^0C for 30 min. Of a solution of sodium arsenite 0.1 ml were added to destroy the excess periodate and the tubes shaken until the yellow-brown colour disappeared. Thiobarbituric acid solution (1 ml) was added, the tubes shaken, capped with a glass bead and then heated in a vigorously boiling water bath for 15 min. The solution cooled on ice to room temperature. The chromophore was extracted with 2.5 ml of HCl/ butanol (95:5 v/v) mixture by vigorous shaking. After 3 min of centrifugation at 3000 g, the clear organic phase was transferred to a 10-mm cuvette and the absorbance was read at 549 nm against the butanol phase of a blank in which the gastric juice sample was substituted with water. A standard curve was prepared with standard sialic acid (N-acetylneuraminic acid, Type IV-S). The amount of sialic acid in the sample was extrapolated from the standard curve. As the procedure outlined above measures only free sialic acids, gastric juice samples were heated at 80^0C for 1 h in 0.1 N HCl (0.1 ml) to release bound sialic acids without degradation. Subsequently, the total sialic acid content was estimated quantitatively by the procedure outlined above. From the difference between total and free sialic acid, the bound sialic acid was calculated. Secretory rates were given as mg ml $^{-1}$ bound sialic acid produced per period (Sadiq *et al.*, 2008).

3.2.4. Gastric mucus secretion

Procedure for measurement of gastric mucus secretion was that described by Corney *et al.,* (1974). The inverted glandular portion of the stomach was inversed in 0.1% Alcian blue and 0.16M sucrose and 0.05M sodium acetate, adjusted to pH 5.8 with hydrochloric acid for two hours. The uncomplexed dye was removed by two successive washes at 15 and 45 minutes in 0.25M sucrose after then inverse the mucosa in 10ml of 0.5M Magnesium Chloride for 2 hours. The resulting blue solution was shaken briefly with equal volume of diethyl ether and absorbance of the aqueous phase was measured at 605nm using spectrophotometer. The absorbance of each solution was then used to calculate the various concentrations of dye. The weight of dye (expressed in mg) was deduced using a standard curve. The weight of dye was then expressed over the weight of the stomach, to give the weight of mucus secret (Onwuchekwa and Oluwole, 2015).

3.2.5 Assay of oxidative stress biomarkers

3.2.5.1 MDA Concentration.

Lipid peroxidation was determined as thiobarbituric acid reactive substance according to Okhawwa *et al.,* (1979), with slight modification by Atawodi *et al.* (2011), using 15% trichloroacetic acid (TCA) and 0. 67% thiobarbituric acid (TBA). Lipid peroxidation generates peroxide intermediate which upon cleavage release MDA, a product which react with TBA. The product of the reaction is a coloured complex which absorb light at 532nm and hence was measured.

3.2.5.2 Assay of Superoxide Dismutase (SOD) Activity

The enzyme was assayed by the method described by Fridovich (1980)

Principle of the assay: the principle works on the basis of superoxide dismutase to inhibit auto oxidation of adrenaline at pH 10.2.

Reagents:

0.05M carbonate buffer: 14.3g of Na_2CO_3 and 4.2g of $NaHCO_3$ were dissolved in distilled water and made up to 1000 ml in volumetric flask. The buffer was be adjusted to pH 10.2

0.3Mm Adrenaline: 0.01g of adrenaline was dissolved in 17ml of distilled water. The solution solution was prepared fresh.

Procedure:

To 0.1ml of the serum was diluted in 0.9 ml of distilled water to make 1:10 dilution of serum. A aliquant mixture of 0.2 ml of the diluted serum was added to 2.5 ml of 0.05M carbonate buffer. The reaction started with the addition of 0.3 mm of 0.3mM adrenaline. The reference mixture contains 2.5ml of 0.05M carbonate buffer, 0.3 ml of 0.3mM adrenaline and 0.2 ml of distilled water. Absorbance was measured over 30s up to 150s at 450nm.

Calculation

Increased in absorbance per minute = (A5- A1)/ 2.5

% inhibition = 100- increase in absorbance for substrate/increase in absorbance of blank×100.

50% inhibition of adrenaline oxidation → IU

% Inhibition of adrenaline oxidation → ×U

1 unit (U) of SOD activity is the quantity of SOD necessary to elicit 50% of the oxidation of adrenaline to the serum in 1 minute.

3.2.5.3 Reduce Glutathione Assay:

Reduce Glutathione (GSH) was assayed according to the method of Ellman (1959), as described by Rajagopalan *et al.* (2004).

Principle of the assay:

The principle of the assay is based on the reaction of 5,5' -dithiobis nitro benzoic acid (DNTB) and reduced glutathione (GSH).

Reagents

To 0.2M phosphate buffer **(**8.40g of NaH_2PO_4) was dissolved in distilled water and made up to 100 ml mark in a volumetric flask. The buffer was adjusted to pH 8.0. 10 % Trichloroacetic acid (TCA): 10 g of TCA was dissolved in distilled water and made up to 1000 ml mark in a volumetric flask.

Ellman's Reagent : (19.8g of 5,5' -dithiobis (nitro benzoic acid) (DNTB) in 100 ml of 0.1% sodium).

Procedure:

To 150µL of serum (in phosphate – saline pH 7.4), 1.5ml of 10% TCA was added and centrifuged at 1500g for 5minutes. 1ml of the supernatant was treated with 0.5ml of Ellman's reagent and 3ml of phosphate buffer (0.2M, Ph 8.0). The absorbance was be read at 412nm. The quantity of GSH was obtained from the graph of the GSH standard curve.

3.2.6 Effect of Bevi Mix and Aspartame in the Liver Tissues of Wistar Rats

3.2.6.1 Effects of Bevi Mix and Aspartame on Aspartate aminotransaminase activity (ALT) in Male Wistar rats.

Using Reitman and Frankel (1957) methods the activity of ALT was determined. The liver supernatant of 100 µl of the test sample and 500 µl of buffer (100 mM phosphate buffer at pH 7.4, 2 mM α-oxoglutarate, and 200 mM L-alanine) were produced, subsequently, the mixture was incubated at 37°C for 30 minutes. 500 µl of 2 mM 2,4 dinitrophenylhydrazine was added into the mixture and the samples were left at room temperature (25°C) for 20 minutes. 500 µl of 0.4 mMNaOH waslateradded and vigorously mixed together, then, the absorbance were measured at 546 nm for 5 minutes against a reagent blank and the activity of ALT was determined.

3.2.6.2 Effects of Bevi mix and aspartame on Alanine aminotransferase (AST) activity in male Wistar rats.

This was achieved by using the Reitman and Frankel (1957). The liver supernatant of 100 µl of the test sample and 500 µl of buffer (100 mM phosphate buffer pH 7.4, 2 mM α-oxoglutarate, and 100 mM L-aspartate) were generated. This mixture was subsequently incubated at 37°C for 30 minutes, afterward 500 µl of 2 mM 2,4dinitrophenylhydrazine was added to the reaction mixture and incubated at 25°C (room temperature) for 20 minutes. 500 µl of 0.4 mMNaOH was added and vigorously mixed together, then the absorbance were measured after 5 minutes at 546 nm against a reagent blank and the AST activity will be evaluated.

3.2.6.3 Effects of Bevi mix and Aspartame on Alkaline phosphatase (ALP) activity in Male Wistar rats.

This was achieved by Deutsche Gesellschaft fur KlinischeChemie, DGKC (1972). The mixture of 20 µl of the liver supernatant and 1 ml of reacting mixture (1 M Diethanolamine buffer pH 9.8, 10 mM*p*-nitrophenyl phosphate, and 0.5 mM MgCl2) were produced. The absorbance wasmeasured between 1-minute intervals for 3 minutes at 405 nm and the activity of ALP was evaluated.

3.2.8. Determination of Kidney Electrolyte

3.2.8.1. Sodium Determination.

Step 1: Precipitation

Standard	**Test**
Sodium R1 (precipitating Reagent) 1000 µL	1000 µL
Sodium standard 10 µL	_
Tissue _	10 µL

For the precipitation of test sample, exactly 10 µL of kidney tissue homogenate was added to the 1000 µL of precipitating reagent (Sodium R1) in a test tube. While for precipitation of standard sample, sodium standard (10 µL) was added to 1000 µL of precipitating reagent (Sodium R1) in another test tube. The two test tubes containing standard and test samples were incubatedin room temperature for 5 min. Then centrifuged at 2000-3000 RPM for 2 minutes and a cleared

supernatants were obtained. The absorbance of the supernatant of the testwas measured against the standard.

Step 2: Sodium estimation s

	Blank	**Standard**	**Test**
Sodium R2 (colour reagent)	1000µL	1000µL	1000µL
Supernatant from step 1	-	20µL	20µL
Sodium R1	20µL	-	-

It was allowed to stand at room temperature for 5 minutes. The absorbanceof standard were taken and sample against reagent blank.

CALCULATION

Sodium concentration (mmol/L) =

Abs of Blank-Abs of test ÷ Abs of Blank-Abs of standard × Standard Concentration (Trinder, 1951)

3.2.8.2. Potassium Determination

Procedure

	Reagent Blank	**Standard**	**Sample**
Reagent R	1ml	1ml	1ml
Standard	20µL		
Sample		-	20µL

The reagent was mix and incubated for 3 minutes at 37^{o}c it was mix again thoroughly absorbance of sample (A_{sample}) and standard ($A_{standard}$) against blank was taken

Calculation

Serum potassium conc (mmol/L) = $(A_{sample}) \div (A_{standard}) \times 5$ (Henry, 1974).

3.2.8.3. Bicarbonate Determination.

10mul of kidney homogenate was used to determined Co_2 as bicarbonate. phosphoenolpyruvate carboxylase catalyzes the reaction of HCo_3^- with phosphoenolpyruvate to give oxalacetate, The resulting NADH, in the presence of malate dehydrogenase, was oxidized to NAD^+, and the decreased in absorbance at 34onm was directly proportional to the amount of Co_2 present in the sample. Reaction was completed in 3 to 6min under assay conditions, and was linearly related to Co_2 concentrations between 8 and 65 mmol/litre (Forrester *et al.*, 1976).

3.3 STATISTICAL- ANALYSIS.

Data from the study were analyzed using one-way analysis of variance (ANOVA) followed by *Turkey's post hoc* test to compare the effects between control and test groups. The results are expressed as mean ± Standard Error of (SEM). Values of $p<0.05$ were considered statistically significant.

CHAPTER FOUR

4.0 RESULTS

4.1. Effect of Bevi Mix and Aspartame on Volume of Gastric juice in Male Wistar rats.

The volume of gastric juice was significantly ($p < 0.05$) increased with administration of bevi mix at 660 mg/kg (2.66 ± 0.05) when compared with the control group (1.7 ± 0.04) and bevi mix 330 mg/kg (1.64 ± 0.17) in Table. 4.1. Aspartame administration at doses of 80 mg/kg (3.2 ± 0.15) shows a significant ($p < 0.05$) increase in volume of gastric juice when compared with control (1.7 ± 0.04), bevi mix 330 mg/kg (1.64 ± 0.17), aspartame 20 mg/kg (2.02 ± 0.10) and aspartame 40 mg/kg (2.22 ± 0.25).

4.1.2. Effect of Bevi Mix and Aspartame on Titratable Acidity in Male Wistar rats.

The titratable acidity was significantly ($p < 0.05$) increased with administration of bevi mix 660 mg/kg (92 ± 1.26) when compared with the control (18.4 ± 0.24) and 330 mg/kg (21.6 ± 0.68) of bevi mix Table 4.1. There was a significant ($p < 0.05$) increase in titratable acidity with aspartame administration at 20 mg/kg (120 ± 1.3) when compared with control (18.4 ± 0.24), 330 mg/kg (21.6 ± 0.68) and 660 mg/kg (92 ± 1.26) of bevi mix. However, administration of 40 mg/kg (131 ± 1.18) or 80 mg/kg (149 ± 1.58) caused a significant ($p < 0.05$) increase in titratable acidity when compared with control (18.4 ± 0.24), 330 mg/kg (21.6 ± 0.68), 660 mg/kg (92 ± 1.26) of bevi mix, and aspartame 20 mg/kg (120 ± 1.3). Aspartame administration at 80 mg/kg (149 ± 1.58) alone significantly ($p < 0.05$) increase the titratable acidity when compared to aspartame 40 mg/kg (131 ± 1.18).

4.1.3 Effect of Bevi Mix and Aspartame on Total Acid Output in Male Wistar rats.

Total acid output was significant ($p < 0.05$) increased with administration of bevi mix 660 mg/kg (4.89 ± 0.41), aspartame 20 mg/kg (4.88 ± 0.43), and 40 mg/kg (5.81 ± 0.72) when compared with control (0.62 ± 0.02) and bevi mix 330 mg/kg (0.76 ± 0.08) shown in Table 4.1. Administration of aspartame 80 mg/kg (9.57 ± 0.66) caused a significant ($p < 0.05$) increase in total acid output when compared with control (0.62 ± 0.02), bevi mix 330 mg/kg (0.76 ± 0.08), bevi mix 660 mg/kg (4.89 ± 0.41), aspartame 20 mg/kg (4.88 ± 0.43), and 40 mg/kg (5.81 ± 0.72) of aspartame as shown in Table 4.1.

4.1.4 Effect of Bevi Mix and Aspartame on Pepsin Concentration in Male Wistar rats.

There was a significant ($p <0.05$) increase in pepsin concentration with administration of bevi mix 330 mg/kg (5.5760 ± 0.10) when compared with control (2.1480 ± 0.16) and a significant ($p <0.05$) decrease and increase was seen with administration of bevi mix 660 mg/kg (4.6600 ± 0.04) when compared with control (2.1480 ± 0.16) and bevi mix 330 mg/kg (5.5760 ± 0.10) respectively. Administration of aspartame 20 mg/kg (3.7660 ± 0.23) significantly ($p < 0.05$) increase in pepsin concentration when compared with control (2.1480 ± 0.16) but decrease the pepsin concentration when compared with bevi mix 330 mg/kg (5.5760 ± 0.10) and 660 mg/kg (4.6600 ± 0.04). Administration of aspartame 40 mg/kg (7.3340 ± 0.15) or 80 mg/kg (10.0580 ± 0.17) caused a significant ($p < 0.05$) increase in pepsin concentration when compared with the control (2.1480 ± 0.16), bevi mix 330 mg/kg (5.5760 ± 0.10), and bevi mix 660 mg/kg (4.6600 ± 0.04) as shown in Table 4.1. Administration of aspartame 40 mg/kg (7.3340 ± 0.15) or 80 mg/kg (10.0580 ± 0.17) caused a significant ($p < 0.05$) increase in pepsin concentration when compared with 20 mg/kg (5.5760 ± 0.10) of aspartame but administration of aspartame 80 mg/kg (10.0580

± 0.17) alone showed a significant ($p < 0.05$) increase when compared to aspartame 40 mg/kg (7.3340 ± 0.15).

Table 4.1. Effect of Bevi Mix and Aspartame on Gastric Secretion in Male Wistar rats.

GROUPS	VG (ml)	TA (m- equil/L)	TO (mEq/L)	PC (**µg/ml)**
NC	1.7±0.04	18.4±0.24	0.62±0.02	2.1480±0.161
BM330mg/kg	1.64±0.17	21.6±0.68	0.76±0.08	5.5760±0.10[a]
BM 660mg/kg	2.66±0.05[ab]	92±1.26[ab]	4.89±0.41[ab]	4.6600±0.04[ab]
ASP 20mg/kg	2.02±0.10	120±1.30[abc]	4.88±0.43[ab]	3.7660±0.23[abc]
ASP 40mg/kg	2.22±0.25	131±1.18[abcd]	5.81±0.72[ab]	7.3340±0.15[abcd]
ASP 80mg/kg	3.2±0.15[abde]	149±1.58[abcde]	9.57±0.66[abcde]	10.0580±0.17[abcde]

All values are means ± SEM, n=7. Superscript a significantly different compared to normal control, b = significantly different compared to bevi mix 330mg/kg, c = significantly different compared to bevi mix 660mg/kg, d = significantly different compared to aspartame 20mg/kg, e = significantly different compared to aspartame 40mg/kg, VG=Volume of gastric juice. TA= Titratable acidity, TO= Total acid output and PC= Pepsin concentration.

4.2. Effect of Bevi Mix and Aspartame on Mucus Secretion in Male Wistar rats

Mucus secretion was significantly ($p<0.05$) decreased with administration of bevi mix 660mg/kg (0.03±0.01µg/ml) when compared with control but shows no significant ($p >0.05$) effect when bevi mix 330mg/kg was compared with control at (0.03±0.01µg/ml) as shown in Fig 4.1. Administration of aspartame 40 mg/kg or 80 mg/kg caused a significant ($p < 0.05$) decrease in mucus secretion when compared to control but no significant ($p > 0.05$) effect seen with 20 mg/kg of aspartame with the control. There was no significant ($p > 0.05$) effect in mucus secretion between the groups administered with low or high dose of bevi mix when compared with aspartame 20 and 40 mg/kg, although group administered 80 mg/kg showed a significant ($p < 0.05$) decrease when compared to low dose of bevi mix as shown in Fig 4.1.

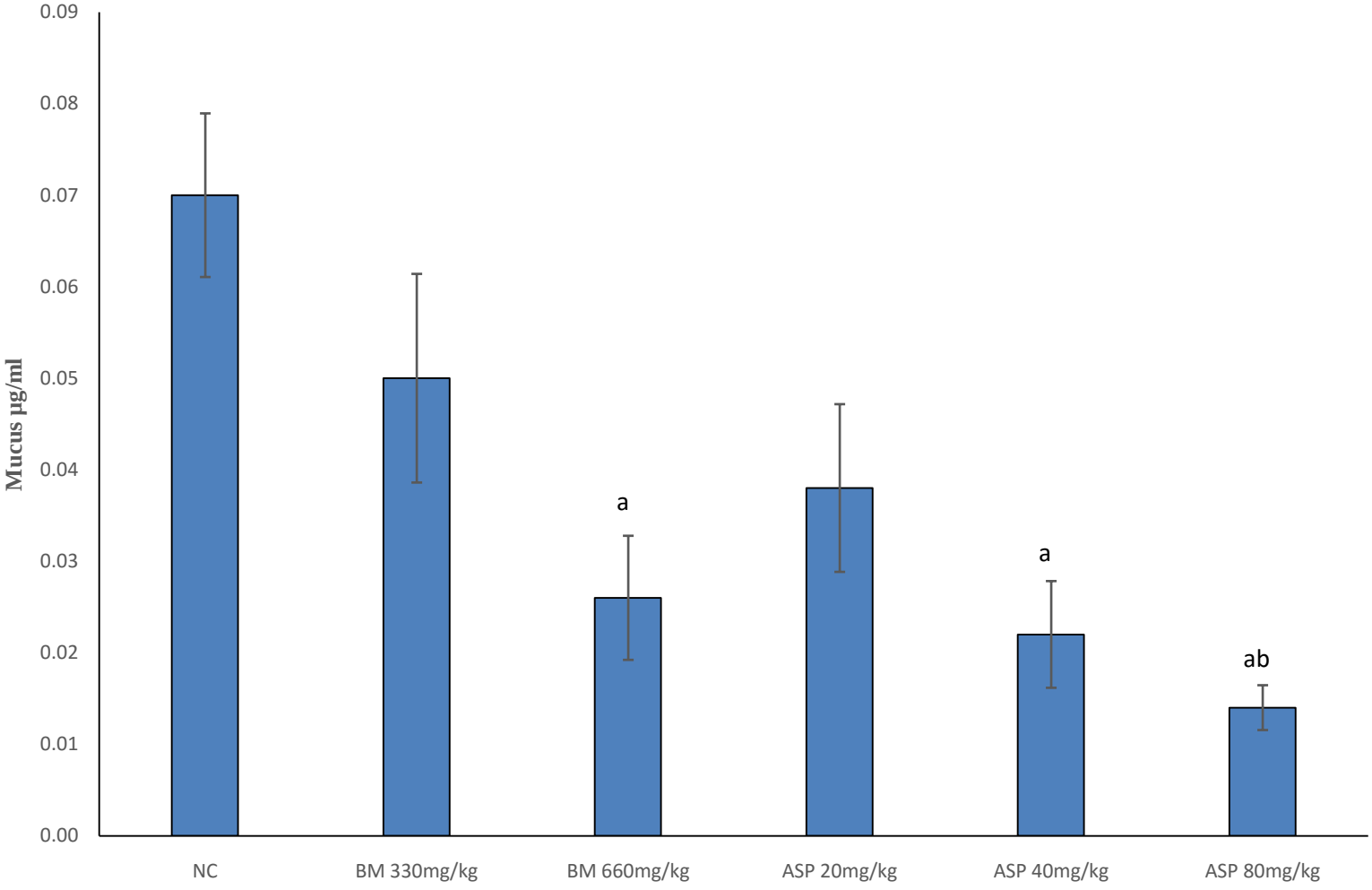

Figure 4.1: Effect of Oral Administration of Bevi mix and Aspartame on Mucus Secretion in male Wistar rats. Letters with different superscript [a,b] significantly different compared to normal control and experimental groups

4.2.1 Effect of Bevi Mix and Aspartame on Mucus Component (sialic acid) in Male Wistar rats.

Table 4.2 shows administration of 660 mg/kg (42.52 ± 0.66) caused a significant ($p < 0.05$) increase in free sialic acid and bound sialic acid when compared with the control (29.88 ± 1.35) and bevi mix 330 mg/kg (30.92 ± 1.23). Administration of aspartame at 20 mg/kg caused a significant ($p < 0.05$) decrease on free (32.02 ± 0.73) and bound sialic acid (2.69 ± 0.67) when compared with bevi mix 660 mg/kg (42.52 ± 0.66) and (15.60 ± 1.28) respectively. Administration of aspartame at 40 mg/kg (28.42 ±1.37) caused a significant ($p < 0.05$) decrease on free sialic acid when compared with bevi mix 660 mg/kg (42.52 ± 0.66. There was a significant ($p < 0.05$) decrease with administration of 40 mg/kg (14.03 ± 1.36) on total sialic acid when compared with control (28.58 ± 1.60), bevi mix 330 mg/kg (28.97 ± 1.06) , bevi mix 660 mg/kg (26.92 ± 1.48) and aspartame 20 mg/kg (29.33 ± 0.86). Administration of aspartame 40 mg/kg (14.39 ± 2.32) or 80 mg/kg (16.79 ± 1.20) showed a significant ($p < 0.05$) increase on bound sialic acid when compared with control (1.30 ± 2.65), bevi mix 330 mg/kg (1.95 ± 2.13) and aspartame 20 mg/kg (2.69 ± 0.67) respectively. Administration of aspartame 80 mg/kg (16.79 ± 1.20) showed a significant ($p < 0.05$) increase in bound sialic acid when compared with control (1.30 ± 2.65), bevi mix 330 mg/kg (1.95 ± 2.13), and aspartame 20 mg/kg (2.69 ± 0.67). Administration of aspartame 80 mg/kg in both free sialic acid (42.00 ± 0.74) and total sialic acid (25.21 ± 1.10) showed a significant ($p < 0.05$) increase in free sialic acid (28.42 ±1.37) and total sialic acid (14.03 ± 1.36) when compared with aspartame 40 mg/kg respectively.

Table 4.2. Effect of Bevi mixand Aspartame on Free, total and bound sialic acid in male Wistar rats.

GROUPS	FSIA **(µg/ml)**	TSIA **(µg/ml)**	BSIA **(µg/ml)**
NC	29.88±1.35	28.58±1.60	1.30±2.65
BM 330mg/kg	30.92±1.23	28.97±1.06	1.95±2.13
BM 660mg/kg	42.52±0.66^{ab}	26.92±1.48	15.60±1.28^{ab}
ASP 20mg/kg	32.02±0.73^{c}	29.33±0.86	2.69±0.67^{c}
ASP 40mg/kg	28.42±1.37^{c}	14.03±1.36^{abcd}	14.39±2.32^{abd}
ASP 80mg/kg	42.00±0.74^{abde}	25.21±1.10^{e}	16.79±1.20^{abd}

All values are means ± SEM, n=7. Superscript a significantly different compared to normal control, c = significantly different compared to bevi mix 660mg/kg, d = significantly different compared to aspartame 20mg/kg, e = significantly different compared to aspartame 40mg/kg. FSIA= Free sialic acid, TSIA= Total sialic acid and BSIA= Bound sialic aci, Aspartame (ASP), Bevi mix (BM).

4.3. Effect of Bevi Mix and Aspartame on Malondialdehyde concentrations (MDA) in male Wistar rats.

Table 4.3 shows the administration of bevi mix 660 mg/kg (26.08 ± 1.48) caused a significant ($p < 0.05$) increase in MDA concentrations when compared with control (14.26 ± 1.22) and bevi mix 330 mg/kg (18.70 ± 1.49). Administration of aspartame 20 mg/kg (18.74 ± 1.38) caused a significant ($p < 0.05$) decrease in MDA concentration when compared with bevi mix 660 mg/kg (26.08 ± 1.48). Administration of aspartame 40 mg/kg (24.56 ± 1.37) showed a significant ($p < 0.05$) increase in MDA concentration when compared with control (14.26 ± 1.22). Administration of aspartame 80 mg/kg (32.22 ± 1.6) caused a significant ($p < 0.05$) increase in MDA concentration when compared with control (14.26 ± 1.22), bevi mix 330 mg/kg (18.70 ± 1.49), bevi mix 660 mg/kg (26.08 ± 1.48), aspartame 20 mg/kg (18.74 ± 1.38), and 40 mg/kg (24.56 ± 1.37) of aspartame

4.3.1 Effects of Bevi Mix and Aspartame on Serum Superoxide Dismutase Level in Male Wistar rats.

Administration of bevi mix 330 mg/kg (66.35 ± 0.45) or 660 mg/kg (56.60 ± 1.14) caused a significant ($p < 0.05$) decrease in SOD level compared to control (79.02 ± 0.52), however, administration of bevi mix 660 mg/kg (56.60 ± 1.14) showed a significant ($p < 0.05$) decrease in SOD when compared with bevi mix 330 mg/kg (66.35 ± 0.45). Aspartame administration at a dose of 20 mg/kg (73.63 ± 1.52) caused a significant ($p < 0.05$) decrease when compared with the control (79.02 ± 0.52) but showed a significant ($p < 0.05$) increase when compared with bevi mix 330 mg/kg (66.35 ± 0.45) and bevi mix 660 mg/kg (56.60 ± 1.14). Administration of aspartame 40 mg/kg (63.16 ± 1.29) or 80 mg/kg (45.37 ± 0.54) caused a significant ($p < 0.05$)

decrease in SOD level when compared with control (79.02 ± 0.52) and 20 mg/kg (73.63 ± 1.52) of aspartame. In addition, administration of aspartame 40 mg/kg (63.16 ± 1.29) caused a significant ($p < 0.05$) increase in SOD level when compared with bevi mix 660 mg/kg. Meanwhile, aspartame 80 mg/kg showed a significant ($p < 0.05$) decrease in SOD level when compared with the entire administered group.

4.3.2 Effects of Bevi Mix and Aspartame on Serum Reduced glutathione (GSH) Level in male Wistar rats.

Administration of bevi mix 330 mg/kg (121.13 ± 1.10) or 660 mg/kg (58.16 ± 1.76) caused a significant ($p < 0.05$) decreased in GSH level compared to control (168.25 ± 0.90), it also showed a significant decreased in GSH level with administration of 660 mg/kg (58.16 ± 1.76) when compared to bevi mix 330 mg/kg (121.13 ± 1.10) in Table 4.3. Aspartame administration of 20 mg/kg (23.26 ± 1.08), 40 mg/kg (19.43 ± 1.53) or 80 mg/kg (42.14 ± 1.25) significantly ($p < 0.05$) decrease the GSH level when compared with control (168.25 ± 0.90), bevi mix 330 mg/kg (121.13 ± 1.10), bevi mix 660 mg/kg (58.16 ± 1.76), and aspartame 20 mg/kg (23.26 ± 1.08). Also, the administration of aspartame 80 mg/kg (42.14 ± 1.25) caused a significant decrease in GSH level compared with aspartame 20 mg/kg (23.26 ± 1.08) and 40 mg/kg (19.43 ± 1.53).

Table 4.3. Effects of Bevi Mix and Aspartame on Serum Oxidative Stress Biomarkers in male Wistar rats.

GROUPS	MDA(µmol/mgprotein)	SOD(µg/ml)	GSH (µg/ml)
NC	14.26±1.22	79.02±0.52	168.25±0.90
BM 330mg/kg	18.70±1.49	66.35±0.45[a]	121.13±1.10[a]
BM 660mg/kg	26.08±1.48[ab]	56.60±1.14[ab]	58.16±1.76[ab]
ASP 20mg/kg	18.74±1.38[c]	73.63±1.52[abc]	23.26±1.08[abc]
ASP 40mg/kg	24.56±1.37[a]	63.16±1.29[acd]	19.43±1.53[abc]
ASP 80mg/kg	32.22±1.61[abcde]	45.37±0.54[abcde]	42.14±1.25[abcde]

All values are means ± SEM, n=7. Superscript a significantly different compared to normal control, b = significantly different compared to bevi mix 330mg/kg, c = significantly different compared to bevimix 660mg/kg, d = significantly different compared to aspartame 20mg/kg, e = significantly different compared to aspartame 40mg/kg, Aspartame (ASP), Bevi mix (BM), MDA (Malondiaaldeyde), SOD (Superoxide dismutase), GSH (Reduced glutathione).

4.4. Effects of bevi mix and aspartame on Alanine aminotransferase (ALT) activity in male Wistar rats.

Group administered with bevi mix 330 mg/kg (100.6 ± 1.21) showed a significant ($p < 0.05$) increase in ALT activity compared with control (84.6 ± 1.72) and a significant ($p < 0.05$) decrease was seen with bevi mix 660 mg/kg (86.6 ± 1.47) when compared with bevi mix 330 mg/kg (100.6 ± 1.21). There was a significant increase ($p < 0.05$) on the activity of ALT in the groups administered with aspartame 20 mg/kg (98.0 ± 1.38), 40 mg/kg (105.2 ± 1.98) or 80 mg/kg (97.6 ± 1.29) when compared to the control (84.6 ± 1.72) and bevi mix 660 mg/kg (86.6 ± 1.47) as shown in Table 4.4. The group administered 40 mg/kg (105.2 ± 1.98) of aspartame showed a significant increase ($p < 0.05$) in ALT activity compared to aspartame 20 mg/kg (98.0 ± 1.38). However, there was a significant ($p < 0.05$) reduction with 80 mg/kg (97.6 ± 1.29) when compared with administration of bevi mix 330 mg/kg (100.6 ± 1.21) and 40 mg/kg (105.2 ± 1.98) of aspartame.

4.4.1. Effects of Bevi Mix and Aspartame on Aspartate aminotransaminase activity (AST) in Male Wistar rats.

The activity of AST was significantly ($p < 0.05$) increase with administration of bevi mix 660 mg/kg (8.0 ± 0.32) when compared with bevi mix 330 mg/kg (3.4 ± 0.24) as shown in Table 4.4. Aspartame adiministration with 20 mg/kg (49.0 ± 1.58), 40 mg/kg (32.4 ± 0.51) or 80 mg/kg (31.8 ± 0.86) showed a significant ($p < 0.05$) increase on the activity of AST when compared with control (4.6 ± 0.6), bevi mix 330 mg/kg (3.4 ± 0.24) and 660 mg/kg (8.0 ± 0.32). The activity of Aspartate aminotransaminase in 40 mg/kg (32.4 ± 0.51) or 80 mg/kg (31.8 ± 0.86) of

aspartame group were significantly ($p< 0.05$) decrease when compared with 20 mg/kg (49.0 ± 1.58).

4.4.2 Effects of Bevi mix and Aspartame on Alkaline phosphatase (ALP) activity in Male Wistar rats.

Admistration of bevi mix 330 mg/kg (23.85 ± 1.09), 660 mg/kg (24.48 ± 1.28) or 20 mg/kg (27.34 ± 0.87) caused a significant ($p < 0.05$) increase in the activity of ALP level when compared with the control (15.37 ± 1.27) as shown in Table 4.4. Administration with 40 mg/kg (32.84 ± 1.16) with aspartame caused a significant ($p < 0.05$) increase in ALP activity when compared with control, bevi mix 330 mg/kg (23.85 ± 1.09), 660 mg/kg (24.48 ± 1.28). Groups administered with aspartame 80 mg/kg (34.45 ± 2.43) showed a significant ($p < 0.05$) increase in ALP activity when compared with control (15.37 ± 1.27), bevi mix at 330 mg/kg (23.85 ± 1.09) and 660 mg/kg (24.48 ± 1.28) and 20 mg/kg (27.34 ± 0.87) of aspartame

Table 4.4 Effect of Bevi Mix and Aspartame on Liver Enzymes (ALT, AST and ALP) in Male Wistar rats.

GROUPS	**ALT(IU/mg of protein)**	**AST(IU/mg of protein)**	**ALP(IU/mg of protein)**
NC	84.6±1.72	4.6±0.60	15.37±1.27
BM330mg/kg	100.6±1.21[a]	3.4±0.24	23.85±1.09[a]
BM660mg/kg	86.6±1.47[b]	8.0±0.32[b]	24.48±1.28[a]
ASP20mg/kg	98.0±1.38[ac]	49.0±1.58[abc]	27.34±0.87[a]
ASP40mg/kg	105.2±1.98[acd]	32.4±0.51[abcd]	32.84±1.16[abc]
ASP80mg/kg	97.6±1.29[ace]	31.8±0.86[abcd]	34.45±2.43[abcd]

All values are means ± SEM, n=7. Superscript a significantly different compared to normal control, b = significantly different compared to bevimix 330mg/kg, c = significantly different compared to bevimix 660mg/kg, d = significantly different compared to aspartame 20mg/kg, e = significantly different compared to aspartame 40mg. ALT= Alanine aminotransferase, AST = Aspartate aminotransaminase and ALP = Alkaline phosphatase, Aspartame (ASP), Bevi mix (BM).

4.5. Effect of Bevi Mix and Aspartame on Sodium ion level in Male Wistar rats.

Administration of bevi mix 330 mg/kg (176.03 ± 1.52) or 660 mg/kg (179.21 ± 1.57) caused a significant ($p < 0.05$) increase in the kidney sodium ion level when compared with control (142.67 ± 1.02) as shown in Table 4.5. Aspartame administration of 20 mg/kg (163.74 ± 1.21) caused a significant ($p < 0.05$) increase on kidney sodium ion level when compared with control (142.67 ± 1.02), but showed a significant ($p < 0.05$) decrease on the kidney sodium ion level when compared with bevi mix 330 mg/kg (176.03 ± 1.52) and 660 mg/kg (179.21 ± 1.57) Administration of aspartame 40 mg/kg (173.55 ± 1.18) showed a significant ($p < 0.05$) increase when compared with control (142.67 ± 1.02) and aspartame 20 mg/kg (163.74 ± 1.21), but showed a significant ($p < 0.05$) decrease when compared with 660 mg/kg (179.21 ± 1.57). Administration of 80 mg/kg (163.89 ± 1.26) showed a significant ($p < 0.05$) increase when compared with control (142.67 ± 1.02) but showed a significant decrease when compared with 330 mg/kg (176.03 ± 1.52), 660 mg/kg (179.21 ± 1.57) and aspartame 40 mg/kg (173.55 ± 1.18).

4.5.1 Effect of Bevi Mix and Aspartame on potassium ion level in Male Wistar rats.

Administration of aspartame 20 mg/kg (11.6 ± 0.8) caused a significant ($p < 0.05$) increase on the kidney potassium ion level when compared with bevi mix 330 mg/kg (8.16 ± 0.59) or 660 mg/kg (8.03 ± 0.45) as shown in Table 4. 5. Administration with aspartame 40 mg/kg (12.23 ± 0.35) caused a significant ($p < 0.05$) increase on kidney potassium ion level when compared with the control (9.36 ± 0.52), bevi mix 330 mg/kg (8.16 ± 0.59) and 660 mg/kg (8.03 ± 0.45). There was a significant ($p < 0.05$) increase with administration of 80 mg/kg (13.52 ± 0.9) on kidney potassium ion level when compared with the control (9.36 ± 0.52), bevi mix 330 mg/kg (8.16 ± 0.59) and 660 mg/kg (8.03 ± 0.45)

4.5.2 Effect of Bevi Mix and Aspartame on bicarbonate ion level in Male Wistar rats.

Administration of bevi mix at 330 mg/kg (90.6 ± 1.83) or 660 mg/kg (90 ± 1.45) caused a significant ($p < 0.05$) increase on kidney bicarbonate ion level when compared with control (82.2 ± 1.02) as shown Table 4.5. Aspartame 20 mg/kg (100 ± 1.26), 40 mg/kg (112.4 ± 0.98) or 80 mg/kg (100.2 ± 1.46) caused a significant ($p < 0.05$) increase on kidney bicarbonate ion level when compared with control (82.2 ± 1.02), bevi mix 330 mg/kg (90.6 ± 1.83) and 660 mg/kg (90 ± 1.45). A significant ($p < 0.05$) increase was also observed with administration of 40 mg/kg (112.4 ± 0.98) when compared with aspartame 20 mg/kg (100 ± 1.26. At 80 mg/kg (100.2 ± 1.46) of aspartame there was a significant decrease when compared with 40 mg/kg (112.4 ± 0.98)

Table4.5: Effect of aspartame and bevi mix on kidney electrolytes (sodium, potassium and bicarbonate) Level in male Wistar rats

GROUPS	Na(mmol/mgprotein)	K(mmol/mgprotein)	HCO_3(mmol/mgprotein)
NC	142.67±1.02	9.36±0.52	82.2±1.02
BM330mg/kg	176.03±1.52[a]	8.16±0.59	90.6±1.83[a]
BM660mg/kg	179.21±1.57[a]	8.03±0.45	90±1.45[a]
ASP20mg/kg	163.74±1.21[abc]	11.6±0.80[bc]	100±1.26[abc]
ASP40mg/kg	173.55±1.18[acd]	12.23±0.35[abc]	112.4±0.98[abcd]
ASP80mg/kg	163.89±1.26[abce]	13.52±0.9[abc]	100.2±1.46[abce]

All values are means ± SEM, n=7. Superscript a significantly different compared to normal control, b = significantly different compared to bevimix 330mg/kg, c = significantly different compared to bevimix 660mg/kg, d = significantly different compared to aspartame 20mg/kg, e = significantly different compared to aspartame 40mg/kg. Na= Sodium, K= Potassium and HCO_3= Bicarbonate, Aspartame (ASP). Bevi mix (BM).

CHAPTER FIVE

5.0 DISCUSSION

The present study was aimed at investigating the effect of bevi mix and its major constituent aspartame on the integrity of gastric mucosa in male Wistar rats. Bevi mix is a powdered drink consisting of mixture of artificial sweetener including aspartame which among others is the most used artificial sweetener in the world. Artificial sweetener has been widely approved as a sweetener for powdered drinks and liquid carbonated beverages around the world. It has had a wide acceptance as an additive in many dry food applications. However, its consumption is associated with many complaints including gastrointestinal disturbances, pancreatitis, episodes of high blood pressure and depression (Iroghama *et al.,* 2017).

The result of our present-day study showed a significant increase in the volume of gastric juice. The aspartic acid present in aspartame stimulate the NMDARs which are also located in Meissner's plexus in the stomach causes membrane depolarization. The membrane depolarization together with the NMDA effect causes NMDAR opening. This results in the additional stimulation of neurons and additional ACh release from the presynaptic membrane. In turn, ACh-activated m3AChRs stimulate parietal cells providing additional HCl release (Golovynska *et al.,* 2018). There was a significant decrease in gastric mucus secretion with administration of bevi mix and aspartame in the study. Mucosal barriers are the most significant factors for gastric protection. The more the secretion of mucous, the lesser the degree of damage to the mucosa lining (Shakeerabanu *et al.*, 2011). The decrease in mucus secretion with bevi mix and Aspartame could have probably been attributed to disruption and/or degeneration of gastric mucosa might have cause decrease in gastric defensive factors or increase of offensive factors,

such as gastric acid back-diffusion and generation of oxy-radicals. Acid back-diffusion is a critical factor of acid-induced gastric ulcer. The back-diffused free acid may in the long run damage gastric mucosal cells by increasing oxyradicals and the release of histamine.

The observed increase in free sialic acid in the present study may have indicated an increased break down of the mucus or the production of defect mucous molecules. Oral administration of Asp resulted in decreased total sialic acid and an increase in bound sialic acid in the gastric juice. These findings suggest that Bevi mix and ASP possibly decreased mucus production due to exhaustion of the mucus cells. The increase free sialic acid in this study might be an expression of increased mucus cell components destruction (Menguy and Masters 1965).

The increase in MDA concentrations which is an index of lipid peroxidation and determined the integrity of cell membrane observed in this study is as a result of bevi mix and aspartame is an evidence of oxidative stress. This may be ascribed to aspartame metabolite. Methanol undergoes oxidative processes with the formation of superoxide anion and hydrogen peroxide which probably stimulate lipid peroxidation that resulted in cell damege (Saleh, 2015). As demonstrated by Parthasarathy *et al*. (2006), that methanol administration significantly increased MDA concentrations level in the lymphoid organs. The result of the study showed a significant decrease in the activities of antioxidant enzymes (Superoxide dismutase) in bevi mix aspartame groups. Superoxide dismutase constitutes an important link in the biological defense mechanism through dismutation of endogenous cytotoxic superoxide radicals to H_2O_2 and O_2 which are deleterious to polyunsaturated fatty acids and proteins (Mourad and Noor, 2011). The decrease in the SOD level with bevi mix 330 and 660 mg/kg which corresponds to 20 and 40 mg/kg of

aspartame might probably be due to the aspartame metabolite and high aspartame has been implicated as a one of the higher constituents of bevi mix.

The decrease in serum SOD level could have been as a result of inactivation of endogenous antioxidant enzymes (SOD) in the administered groups that received bevi mix and aspartame which could probably be due to rapid consumption, or exhaustion of storage of this enzyme in fighting free radicals generated in the administered.

Glutathione plays a critical role in protecting cells from oxidative stress. Reduction of cellular glutathione content recorded in this study may be one of the reasons for the increase in cell vulnerability to oxidative stress (Ibrahim and Hussein, 2014). The significant decrease in GSH level with bevi mix and aspartame could have been due to its rapid reaction with formaldehyde formed during methanol metabolism, to produce lipid peroxidation products and nucleophilic adducts. Hence, the decrease in GSH content may be owing to the excessive exploitation of this antioxidant to scavenge the free radicals produced during aspartame metabolism. Depletion in the activities of this enzymatic and non-enzymatic antioxidant could be due to methanol metabolite of aspartame. This clearly indicates the generation of free radicals by aspartame administration.

The increase level of liver enzymes in this study could be attributed to bevi mix consumption which increases the level of methanol that causes damage to the hepatocytes which in the long run causes elevation in the liver enzymes as a result of oxidation of methanol to formaldehyde.

This result is in consonant with the findings of Prokić *et al*. (2014), who demonstrated an increase in serum liver enzymes with administration of aspartame.

The kidney as an excretory organ is central to the normal functioning of the body. Its role in maintenance of the body homeostasis, excretion of waste products of metabolism, drugs and chemical are vital to maintenance of health. Among the waste products of metabolism excreted by the kidney are urea and creatinine while in the tubule's electrolytes are reabsorbed in maintenance of body's homeostasis (Nwankpa *et al.,* 2018). Electrolyte concentrations assay is one of the process that gives a better measure of kidney functions and a guide for choosing the treatment option (Uroko *et al.,* 2019). The significant increase in Na^+ concentration seen in the kidney homogenate in the study could be from excessive loss of Na^+ pool in the body fluids. The study is in harmony with the study of Saleh, (2014), who studied synergistic effect of N-acetyl cysteine and folic acid against aspartame induced nephrotoxicity in rats.

Potassium is the principal cation of the intracellular fluid. It is also an important constituent of the extracellular fluid due to its influence on muscle activity. Its intracellular function parallels that of its extracellular function, namely influencing acid-base balance and osmotic pressure, including water retention. The excessive potassium concentration on the kidney as seen in group 40 and 80 mg/kg that received aspartame may have probably occurred because of renal malfunction associated with the inability of the kidney to excrete potassium ions and the increased amount of potassium released from the damaged cells in other word it may be due to increased sensitivity of the nephron to aldosterone and other mineral corticoids responsible for re-absorption and retention of electrolytes respectively. Bicarbonate is produced by the pancreas

and liver to neutralize the acidic pH produced by the acid in the gastrointestinal tract. Bicarbonate ions also maintain the acid-base buffering system of the blood (Zuberu *et al.,* 2018). Increase in bicarbonate level in the all the administered groups may imply that either of the bevi mix or aspartame overwhelmed the pH regulatory mechanism of the blood. The pH of the mammalian system depends on the ratio of (HCO_3^-) to the partial pressure of exhaled CO2 (*p*CO2). While bicarbonate concentration is controlled by the kidney, *p*CO2 is rapidly controlled by the lung.

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATION

6.1 Conclusion

Based on our findings, it could be concluded that bevi mix intake alters the integrity of gastric mucosa at 660 mg/kg bevi mix and 40 and 80 mg/kg of aspartame with decrease in gastric mucus secretion, increase volume of gastric juice, increase in titratable acidity, increase in total acid output and increase in pepsin concentration which may probably predispose the animals to gastric ulceration. It induces oxidative stress by increasing MDA concentration and decreases SOD and GSH.

6.2 Recommendations

(i) Further studies should focus on other components of bevi mix other than aspartame.

(ii) Extensive toxicity studies should be carried out on aspartame present in other sweeteners.

(iii) These studies suggest that aspartame, even at 20 mg/kg, might not be safe, so further research should be carried out.

6.3 Contribution to Knowledge

(i) The study established that oral administration of bevi mix at 660 mg/kg significantly increase, titratable acidity (92±1.26m- equil/L), total acid output (4.89±0.41m- equil/L) and pepsin concentration (4.66±0.04µg/ml)when compared to the control (18.4±0.24, 0.62±0.02 and 2.14±0.16).

(ii) Bevi mix at 660 mg/kg showed a significant decrease in mucus secreation (0.03±0.01µg/ml) when compared to the control (0.07±0.01)

(iii)Bevi mix 660 mg/kg showed a significant increase in free sialic acid (42.52±0.66µg/ml) and bound sialic acid (15.60±1.28µg/ml)when compared to the normal control (29.88±1.35 and 1.30±2.65).

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