PROTECTIVE AND ANTIOXIDANT PROPERTIES OF "AQUEOUS ANTI-ULCER DRUG "AQAUD", AGAINST ASPIRIN-INDUCED GASTRIC ULCERS IN RATS

BY

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CERTIFICATION

I certify that this thesis **"Protective and antioxidant properties of "Aqueous Anti-Ulcer Drug "AQAUD", against aspirin-induced gastric ulcers in albino rats."** was carried out by Mba, Blessing Amarachi in the Department of Biochemistry, Federal University of Technology Owerri, Imo State, under my supervision.

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DEDICATION

This research work is dedicated to God almighty for his immense help, wisdom and guidance in the course of this research work. He deserves all the glory and praise.

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ABSTRACT

The protective and antioxidant properties of Aqueous Anti-ulcer Drug (AQAUD) against Aspirin-induced gastric ulcers in albino rats were investigated. In this study, thirty male albino rats were divided into five groups. Rats in group I served as normal control and received food and water. Animals in group II received food and water in addition to aspirin 400mg/kg.b.wt orally on the 14th day. Rats in groups III, IV and V received "AQAUD" 250mg/kg.b.wt, 500mg/kg.b.wt and Omeprazole 20mg/kg.b.wt respectively for 14 days and aspirin 400mg/kg.b.wt orally on the 14th day. Antioxidant property of "AQAUD" was determined by its ability to scavenge nitric oxide and hydroxyl radicals *in vitro*. The ulcer protective effect of "AQAUD" was assessed by determining the free and total acidity, ulcer index, % protection, and total protein and nitrite concentrations in the stomach homogenates. The antioxidant potential in animals was evaluated by determining the concentrations of malondialdehyde (MDA) and reduced glutathione (GSH). Superoxide dismutase (SOD) and Catalase (CAT) activities were assayed in the stomach homogenates to further assess antioxidant potential. Acute toxicity testing was done to ascertain the safety of "AQAUD". Total phenolics and flavonoid compounds were quantified to know the antioxidant content. Histopathological assessment of the gastric mucosa was used to assess the protective potentials of "AQAUD". The results revealed that free and total acidity, ulcer indexes were significantly ($p<0.05$) reduced by "AQAUD". There were significant changes in SOD and CAT activities of the stomach homogenates as well as in nitrite, MDA and GSH concentrations when compared to the control. Result of acute toxicity testing showed that "AQAUD" is generally safe. Our results revealed that treatment with aspirin caused loss of gland architecture with erosion of epithelial layer, but AQAUD treatment ameliorated the effect of aspirin administration. The study revealed that "AQAUD" has considerable antioxidant potentials and can effectively protect against gastric ulcers.

KEYWORDS: "AQAUD", Antioxidants, Omeprazole, Gastric ulcers, Aspirin.

CHAPTER ONE

1.0 INTRODUCTION

Medicinal plants have been identified and used throughout human history. Plants have the ability to synthesize a wide variety of chemical compounds that are used to perform important biological functions and to defend against attacks from predators such as insects, fungi and herbivorous mammals. At least twelve thousand (12000) of such compounds have been isolated so far; a number estimated to be less than 10% of the total (Tapsell *et al.,* 2006).

The use of plants as medicines predates human history. In 2001, researchers identified one hundred and twenty two compounds used in modern medicine which were derived from plants sources, 80% of which had an ethno-medical use identical or related to the current use of the active element of the plant (Fabricant and Farnsworth, 2001). Many of the pharmaceuticals currently available to physicians have a long history of use as herbal remedies, including aspirin, quinine and opium (Swain and Tony, 1968).

The World Health Organization (WHO) estimates that 80% of some Asian and African countries presently use herbal medicine for some aspect of primary healthcare. The traditional system of medicine is based on the experiences in the use of plant products in amelioration of common diseases. A vast majority of our population particularly those living in the villages depend largely on herbal medicine (Gupta, 1994). The importance of traditional system of medicine and of certain traditional medical practices has now been recognized all over the world (Satyabati, 2006).
The World Health Organization notes however that inappropriate use of traditional medicine and practices can have negative and dangerous effects and that further research is needed to ascertain the efficacy and safety of several of the practices and medicinal plants used by traditional medicine system.

The term “Peptic ulcer” refers to an ulcer in the lower oesophagus, stomach or duodenum. Ulcer in the stomach (gastric ulcer) may be acute or chronic. Quincke was probably the first to use the term ‘Peptic ulcer (Quincke, 1963)’. Because of its frequency and worldwide distribution, peptic ulcer continues to be a subject of numerous investigations, both experimentally and in clinical practices. In this respect peptic ulcer occupies a place secondary to carcinoma in the field of gastroenterology.

There are medicines to treat peptic ulcer (Tierney *et al.,* 2001). These drugs have brought about remarkable changes in peptic ulcer therapy but the efficacy of these drugs is still debatable. Reports on clinical evaluation of these drugs show that there are incidences of relapses, adverse effects and danger of drug interactions during ulcer therapy (Barrowman and Pfeiffer, 1982). Hence, the search for an ideal anti – ulcer drug continues and has also been extended to medicinal plants in search for new and novel molecules, which afford better protection and decrease the incidence of relapse.

Numerous medicinal plants showed anti gastric ulcer activity. Sanyal *et al.,*(1963) found that vegetable banana is efficacious not only for experimentally induced gastric ulcers in albino rats, guinea pigs etc. but also for human beings suffering from gastric ulcers. Akah and Nwafor (1999) demonstrated anti gastric ulcer activity of the herb *Cassampelos mucronata*. Likewise Shetty *et al.,*(2000), Sairam *et al.,* (2001), Maity *et al.,* (1995) and Dharmani and Palit (2006) confirmed anti gastric ulcer activities of *Ginkgo biloba*, *Convolvulus pluricaulis Chois,* tea root extract and *Vernonia lasiopus* respectively.

Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) represent one of the most widely used classes of drugs to alleviate the symptoms (pain and swelling) of osteoarthritis, rheumatoid arthritis and other inflammatory disorders. However the use of NSAIDs is limited by their ability to induce the formation of erosions and ulcers in the gastro intestinal tract (Kearney *et al.,* 2006).

Reactive oxygen species which include superoxide anions and hydroxyl radicals have been implicated in several degenerative diseases including digestive system disorders such as hyper-secretion and gastric mucosal damage (Gates *et al.,* 2008).

An antioxidant can be broadly defined as any substance that delays or inhibits oxidative damage to a target molecule (Yamagishi and Matsui, 2011). The characteristic feature of an antioxidant is its ability to scavenge the free radicals due to their redox hydrogen donators and singlet oxygen quenchers (Anokwuru *et al.,* 2011). The free radicals can be scavenged by the natural (plant) and synthetic (butylated hydroxyl toluene, butylated hydroxyl anisol and quercetin) antioxidants (Mbaebe *et al.,* 2012). But the use of these synthetic antioxidants is now minimized because the natural antioxidants could be considered safer without any side effects. In recent times, many researchers are interested in medicinal plants for identification of antioxidant phytochemicals, such as phenols, flavonoids and tannins which have received more attention for their role in prevention of human diseases (Upadhyay *et al.,* 2010).

Various classes of synthetic anti-ulcer drugs like antacids, proton pump inhibitors, H_2-receptor antagonist and cyto-protective agents are being used in clinical practices, but these drugs have been associated with undesirable side effects and drug to drug interactions. Therefore, search for an ideal anti-ulcer drug continues and has been extended to herbal drugs for their easy availability, better protection, low cost and lesser toxicity.

Antioxidants are considered as possible protective agents, reducing oxidative damage to human body (Sies, 1997). In recent years there has been growing interest in the use of natural antioxidants, especially those derived from edible materials such as fruits, spices, herbs and vegetables.

The herbal formulation Aqueous Anti-Ulcer Drug (AQUAD) is one of the widely consumed medicinal formulations in the eastern parts of Nigeria. It is used locally in treating peptic ulcers. The medicament consists of juice from *Citrus aurantifolia* (lime), gbogbonise, aqueous root extract of *Strophantus hispidus* (osisikagiri) and *Hippocratea welwitschii* (Ovuru mgbede).

1.1 Justification of the Study

Various synthetic anti-ulcer drugs have been associated with high adverse drug reactions and drug to drug interactions and search for an ideal anti-ulcer drug has been extended to herbal drugs.

This study sought to discover if "AQAUD" herbal formulation can be used as an alternative anti-ulcer drug with minimal drug to drug interactions and adverse effects.

1.2 Aim of the Study

The aim of this study is to evaluate the protective and antioxidant properties of Aqueous Anti-Ulcer Drug "AQAUD" against aspirin-induced gastric ulcers in albino rats.

1.3 Specific Objectives

- To determine the antioxidant potential of the "AQAUD".
- To ascertain the protective effect of the "AQAUD" against aspirin-induced gastric ulcers in albino rats.
- To assess the safety of animals taking the drug and assess toxicity through the determination of the median lethal dose (LD_{50}) of the "AQAUD".
- To determine the phytochemical content of the "AQAUD".
- To conduct histopathological study of the stomachs taking of rats the "AQAUD".

CHAPTER TWO

LITERATURE REVIEW

2.1 Ulcers

An ulcer is a discontinuity or break in bodily membrane that impedes the organ of which the membrane is a part from continuing its normal function.

Common forms of ulcer recognized, include:

Skin ulcer: a discontinuity of the skin or break in the skin.

Genital ulcer: an ulcer located on the genital organ.

Ulcerative dermatitis: a skin disorder associated with bacterial growth often initiated by self-trauma.

Corneal ulcer: an inflammatory or infective condition of the cornea.

Mouth ulcer: an open sore inside the mouth.

Peptic ulcer: a discontinuity of the gastro-intestinal mucosa.

Venous ulcer: a wound thought to occur due to improper functioning of valves in the veins (Kumar *et al.,* 2004).

2.1.1 Peptic Ulcers

A peptic ulcer is a sore or break in the protective lining of the stomach, small intestine or esophagus. A peptic ulcer in the stomach is called gastric ulcer.

A duodenal ulcer is a peptic ulcer that develops in the first part of the small intestine (duodenum). An esophageal ulcer occurs in the lower part of esophagus. Gastric or duodenal ulcers are as a result of caustic effects of acid and pepsin in the lumen.

Historically, peptic ulcer is identified as necrosis of mucosa which produces lesions equal to or greater than 0.5cm. It is the most common ulcer of an area of the gastro- intestinal tract that is usually acidic and thus extremely painful (Kumar *et al.,* 2004).

Peptic ulcers can be minor (they only go through the first or second layer of the stomach), or can be considered a medical emergency (when they go through every layer of the stomach or duodenum lining, causing major internal bleeding).

2.1.2 Signs and Symptoms of Peptic Ulcers

- Abdominal Pain: classically epigastric, strongly correlates to meal times. In the case of duodenal ulcer, the pain appears about three hours after taking a meal.
- Bloating and abdominal fullness.
- Water brash (rush of saliva after an episode of regurgitation to dilute the acid in the esophagus).
- Nausea and copious vomiting.
- Loss of appetite and weight lost.
- Hematemesis (vomiting of blood): this can occur due to bleeding directly from a gastric ulcer or from damage to the esophagus from severe or continuous vomiting.
- Melena (tarry, foul-smelling faeces due to the presence of oxidized iron from hemoglobin).
- Rarely an ulcer can lead to gastric or duodenal perforation, which leads to acute peritonitis: extreme stabbing pain (Bhat, 2013) and requires immediate surgery.

2.1.3 Complications of Peptic Ulcer

- Gastrointestinal bleeding is the most common complication. Sudden large bleeding can be life-threatening (Cullen *et al.,* 1997). It occurs when the ulcer erodes one of the blood vessels such as gastro-duodenal artery.

- Perforation (a hole in the wall of the gastro-intestinal tract) often leads to catastrophic consequences if left untreated. Erosion of the gastro-intestinal wall by the ulcer leads to spillage of the stomach or intestinal content into the abdominal cavity.

- Perforation and penetration are when the ulcer continuous into adjacent organs such as the liver and pancreas.

- Gastric outlet obstruction is the narrowing of pyloric canal by scarring and swelling of gastric antrum and duodenum due to peptic ulcer. Patients often present with severe vomiting without bile.

2.1.4 Causes of Peptic Ulcers

A major causative factor (60% of gastric and up to 90% of duodenal ulcer) is the chronic inflammation due to *Helicobacter pylori* that colonizes the antrial mucosa. The immune system is unable to clear the infection despite the appearance of antibodies. Thus, the bacterium can cause a chronic active gastritis. Gastrin stimulates the production of gastric acid by parietal cells. In *H. pylori* colonization response to increase in gastrin, the increase in acid can contribute to the erosion of the mucosa and therefore ulcer formation.

Another major cause of peptic ulcer is the use of Non-Steroidal Anti-Inflammatory Drugs (NSAIDS). The gastric mucosa protects itself from gastric acid with a layer of mucus, the secretion of which is stimulated by certain prostaglandins. NSAIDS irreversibly block the function of cyclo oxygenase 1 (Cox-1), which is essential for the production of prostaglandins. Although, some studies have found correlations between smoking and ulcer formation(Kato *et al.,* 1992), others have been more specific in exploring the risks involved and have found that smoking by itself may not be much of a risk factor unless associated with *H. pylori* infection (Salih *et al.,* 2007).

Some suggested risk factor such as diet and spice consumption were hypothesized as ulcerogens (helping to cause ulcers) until late in the 20th century, but have been shown to be relatively of minor importance in the development of peptic ulcer. (Ryan-Harshman and Aldoori, 2004). Similarly, while studies have found that alcohol consumption increases risk of ulcer when associated with *H. pylori* infection, it does not seem to independently increase the risk, and even when coupled with *H. pylori* infection, the increase is modest in comparison to the primary rick factor (Salih *et al.,* 2007).

Also Gastrinomas (Zollinger Ellison Syndrome), rare gastrin-secreting tumors, also cause multiple and difficult-to-heal ulcers.

2.1.5 Diagnosis of Peptic Ulcers

Peptic ulcers can be diagnosed through the following:

An Esophago-gastroduo-denoscopy (EGD), a form of endoscopy, also known as gastroscopy is carried out on patient in whom a peptic ulcer is suspected. By direct visual identification, the location and severity of an ulcer can be described. Moreover, if no ulcer is present, EGD can often provide an alternative diagnosis.

Endoscopies or barium contrast X-rays which are typically ordered if the symptoms do not resolve after a few weeks of treatment.

Blood tests are not reliable for accurate peptic ulcer diagnosis on their own because of their inability to differentiate between past exposure to the bacterium and current infection. Additionally, a false negative result is possible with blood test if the patient has recently been taking certain drugs such as antibiotics or proton pump inhibitors (Sonnenberg *et al.,* 1981).

The diagnosis of *Helicobacter pylori* can be made by:

- Urea breath test (non-invasive and does not require EGD),
- Direct culture from an EDG biopsy specific.
- Direct detection of urease activity in a biopsy specimen by rapid urease test.
- Measurements of antibody level in the blood.
- Stool antigen test.
- Histological examination and staining of an EDG biopsy.

2.1.6 Treatment of Peptic Ulcers

Patients with ulcer-like symptoms are often treated with antacids or H_2 antagonists like Ranitidine and famotidine, to provide relief of peptic ulcer, heart burn, indigestion and excess stomach acid. They work by decreasing the amount of acid the stomach produces, allowing healing of ulcer.

Patients who are taking Non-Steroidal Anti-Inflammatory Drugs (NSAIDS) may also be prescribed a prostaglandin analogue (misoprostol) in order to help prevent peptic ulcers, which are side effects of NSAIDs.

When *H. pylori* infection is present, the most effective treatments are combination of two antibiotics (e.g. Clarithromycin and Amoxicillin, tetracycline and metronidazole) and one proton pump inhibitor (PPI), sometimes with a bismuth compound. In the absence of *H. pylori*, long term higher dose of proton pump inhibitors are often used.

Perforated peptic ulcer is a surgical emergency and requires surgical repair of the perforation.

2.2 Helicobacter pylori

Helicobacter pylori, previously named *Campylobacter pylori* is a gram-negative micro-aerophilic bacterium found in the stomach and may be present in other parts of the body, such as the eyes (Giusti, 2004). It was identified in 1982 by Australian scientists Barry Marshal and Robin Warren. With further research, it was found to be present in patients with chronic gastritis and gastric ulcer (Blaser, 2006). *H. pylori* contains a hydrogenase which can be used to obtain energy by oxidizing molecular hydrogen (H_2) produced by intestinal bacteria (Olson and Maler, 2002). It produces oxidase, catalase and urease. It is capable of forming bio-films (Stark *et al.,* 1999) and can convert from spiral to possibly viable but non culturable coccoid form (Chan *et al.,* 1994), both likely to favour its survival. These are factors in the epidemiology of the bacterium.

H. pylori harm the stomach and duodenal linings by several mechanisms. The ammonia produced to regulate pH is toxic to epithelial cells, as are bio-chemicals produced by *H. pylori* such as proteases, vacuolating cytotoxin A (which damages pithelial cells, disrupts tight junctions and causes apoptosis), and certain phospolipases. Also cytotoxin associated gene Cag A can also cause inflammation and potentially a Carcinogen (Smoot, 1997).

2.3 Non-Steroidal Anti-Inflammatory Drugs (NSAIDs)

Non-Steroidal Anti-Inflammatory Drugs are a class of drugs that provide analgesic, anti-pyretic effects, and in higher doses, anti-inflammatory effects (Green, 2001). The most prominent members of this group of drugs are Aspirin, Ibuprofen and Naproxen. Paracetamol (acetaminophen) is not considered as one of the NSAIDs because it has only little anti-inflammatory activity and treats pain mainly by blocking cyclooxygenase-2 (Cox-2) mostly in the central nervous system, but not much in the rest of the body (Hinz *et al.,* 2008).

NSAIDs inhibit the activity of both cyclooxygenase-1 (Cox-1) and cyclooxygenase-2 (Cox-2), and thereby the synthesis of prostaglandins and thromboxanes. It was thought that inhibiting Cox-2 leads to anti-inflammatory, analgesic and anti-pyretic effects and those NSAIDs also inhibiting Cox-1 , particularly, aspirin, may cause gastro-intestinal bleeding and ulcer (Kearney *et al.,* 2006).

Aspirin, the only NSAIDs able to irreversibly inhibit Cox-1 is also indicated for inhibition of platelet aggregation. This is useful in the management of arterial thrombosis. It

inhibits the action of thromboxane synthase, an enzyme that synthesizes thromboxane A_2 (Green, 2001).

2.4 Strophanthus hispidus

Figure 2.1 *Strophanthus hispidus* plant

Strophanthus hispidus is a medicinal plant widely used in traditional African medicine in the treatment of rheumatic afflictions, ulcers, conjunctivitis, leprosy and skin diseases (Ishola *et al.,* 2013). *Strophanthus hispidus* belongs to the family Apocynaceae and is called "osisikagiri" in Igbo language. It is found all over Africa and in the eastern, western and southern parts of Nigeria (Ishola *et al.,* 2013). The plant contains an amorphous glycoside (pseudo-strophantin) with heavy oil, two alkaloids (trigonelline and choline), resin, mucilage, and a rhamnose sugar (Irvine, 1961). Also methanolic root extract of *S. hispidus* indicated the presence of starch, tannin, glycosides, flavonoids, steroids and alkaloids (Wagner and Bladt, 1996).

2.5 Citrus aurantifolia

Figure 2.2 *Citrus aurantifolia* plant.

Citrus aurantifolia (Lime) is a citrus fruit, which is typically round, green in colour, about 3-6cm in diameter and containing acidic juice vesicles (Taylor and Francis, 1986).

Limes are an excellent source of vitamin C, and are often used to accent the flavours of foods and beverages. They are grown year-round in tropical climates and are usually smaller and less sour than Lemon, although varieties may differ in sugar and acid content. In cooking, lime is valued both for the acidity of its juice and the floral aroma of its zest.

Lime juice is highly rich in vitamins, which is an essential nutrient that is needed for the growth and repair of tissues, as well as the repair and maintenance of our bones (schagen *et al.,* 2012). It also helps the body to produce collagen, a protein that plays a vital role in the production of blood vessels, skin, tendons, ligaments and cartilages (schagen *et al.,* 2012).

C. aurantifolia juice contains diverse phytochemicals like phenols, terpenes and flavonoids, which have anti-carcinogenic properties that prevent the invasion of cancer cells (Hatherill, 2014). Limes contain unique active compounds called flavonol glycosides, which not only offer anti-cancer antioxidant benefits, but also have antibiotic properties (Bowe and Logan, 2011). The antibiotic properties of these compounds have been shown to protect against the

contraction of cholera, a diseases caused by a bacteria called *Vibrio cholera* (Bowe and Logan, 2011).

Also limes contain eight different liminoids, which are compounds that promote the activity of an enzyme in the liver called glutathione-s-trasnferase or GST, according to Hatherill, (2014). This liver enzyme detoxifies a variety of cancer-causing chemicals, turning them into harmless chemicals that are then removed from the body through the urine.

2.6 *Hippocratea welwitschii*

Figure 2.3: *Hippocratea welwitschii* plant

Hippocratea welwitshcii is a medicinal plant used in Igbo (Nigeria) ethnomedicine. It is locally known as ovuru mgbede in Igbo language and its root is used locally in the treatment of gastro-intestinal problems. It is a climber and belongs to the family of celastrales. Phytochemical screening on the root extract indicated the presence of triterpenes, glycosides and saponins (Maurice, *et al.,* 2008).

2.7 Oxidative Stress

Oxidative stress is essentially an imbalance between the production of free radicals and the ability of the body to counteract or detoxify their harmful effects through neutralization by antioxidants. In humans, oxidative stress is thought to be involved in the development of cancer, atherosclerosis, parkinson's disease, sickle cell disease etc. (Halliwell, 2007).

Disturbances in the normal redox state of cells can cause toxic effects through the production of oxygen species and other free radicals that damage the components of the cell, including proteins, lipids and DNA. However, reactive oxygen species can be beneficial, as they are used by the immune system as a way to attack and kill pathogens (Segal, 2005).

Chemically, oxidative stress is associated with increased production of oxidizing species or a significant decrease in effectiveness of antioxidant defenses such as glutathione (Schafter and Buettner, 2001). The effects of oxidative stress depend upon the size of these changes, with a cell being able to overcome small perturbations and regain its original state. However, more severe oxidative stress can cause cell death and even moderate oxidation can trigger apoptosis, while more intense stress can cause necrosis (Lennon *et al.,* 1991).

2.8 Free Radicals

A free radical is any atom (example oxygen, nitrogen) with at least one unpaired electron in the outermost shell, and is capable of independent existence (Karlsson, 1997). Free radicals are highly reactive due to the presence of unpaired electron(s). When free radicals steal an electron from the surrounding compound or molecule to be stable, a new free radical is formed in its place. In turn the newly formed radical then looks to return to its ground state by stealing electron with anti-parallel spins from cellular structures or molecules. Thus, the chain reaction continues and can be thousands of events long (Goldfarb, 1999). The cumulative production of free radicals (Reactive Oxygen Species / Reactive Nitrogen Species) through either endogenous or exogenous means results to oxidative stress.

The most important oxygen-containing free radical in many disease states are hydroxyl radical (OH^{-}), Superoxide anion radical (O_2^{-}), hydrogen peroxide (H_2O_2), oxygen singlet (O^{-}), hypochlorate (HClO), nitric acid radical (NO^{-}) and peroxynitrite radical (NOO^{-}).

2.8.1 Production of free radicals in the human body

Free radicals and other reactive oxygen species (ROS) are derived either from the normal essential metabolic processes in the human body or from external sources. The production of free radicals in the body is continuous and in escapable. The basic causes include the following:

- The immune system: The immune system cells deliberately create free radicals and reactive oxygen species as weapons to fight infectious organisms.

- Energy production: The energy producing process in every cell generates oxy radicals and oxygen species as toxic waste continuously and abundantly (Sharma and Clark, 1998). Oxygen is used to burn glucose molecules that act as the body's fuel. In this energy freeing operation, oxy radicals are thrown off as destructive by-products. Given the insatiable hunger for oxygen, there is no way to have it suffusing the body's energy-producing processes without the constant creation of oxy radicals and reactive oxygen species.

- Stress: The pressure common in industrial societies can trigger the body's stress response. In turn, the stress response creates free radicals in abundance. The stress response races the body's energy creating apparatus, increasing the number of free radicals as a toxic by-product (Liu *et al.,* 1999). Moreover, the hormones that mediate the stress reaction in the body: Cortisol and catecholamines will themselves degenerate into particularly destructive free radicals. Researchers now know one way in which stress may cause diseases. A stressful life mass-produces free radicals.

- Pollution and other external substances: The pollutants produced by modern technologies often generate free radicals in the body. The food most of us buy contains farm chemicals, including fertilizers and pesticides that produce free radicals when we ingest them. Prescription drugs often have the same effect; their harmful side effects may be caused by the free radicals they generate (Sharma and Clark, 1998). Processed foods frequently contain high levels of lipid peroxide, which produce free radicals that damage the cardiovascular system. Cigarette smoke generates high free radical concentration. Most of the lung damage associated with smoking is caused by free radicals (Bagchi and Puri, 1998). Air pollution has similar effects. Alcohol is a potent generator of free radicals (although red wine contains antioxidants that counteract this effect) (Sharma and Clark, 1998).

  In addition, free radicals can result from all types of electromagnetic radiations including sun light. Exposure to sun light generates free radicals that age the skin, causing roughness and wrinkles. If the exposure is prolonged, skin cancer may result (Bagchi and Puri, 1998).

2.8.2 Benefits of free radicals

Many chemical reactions that occur in the body inevitably produce free radicals. The body can however, keep these free radicals under control.

Moreover, despite the long list of problems they cause, free radicals are not all bad. The body tries to harness the destructive power of the most dangerous free radical (the oxygen and the ROS) for use in the immune system and in inflammatory reactions. Certain cells in this system engulf bacteria or viruses, take up oxygen molecules from the blood stream, remove an electron to create a flood of oxy-radicals and ROS, and bombard the invader with the resulting toxic shower. The aggressive use of toxic oxygen species is remarkably effective in protecting the body against infectious organisms.

Unfortunately, the process may go out of control, creating a chain reaction that leads to the over production of free radicals. These reactions are no less damaging to the body than other formation of free radicals.

2.9 Antioxidants

An antioxidant is a molecule that inhibits the oxidation of other molecules. Oxidative reactions can produce free radicals which can start chain reactions. Antioxidants terminate these chain reactions by removing free radicals intermediates, and inhibit other oxidation reactions. They do this by being oxidized themselves, so antioxidants are often reducing agents such as thiols, ascorbic acid, or polyphenols (Sies, 1997).

Early researches on the role of antioxidants in biology focused on their use in preventing the oxidation of unsaturated fats, which is the cause of rancidity (German, 1999). The possible mechanism of action of antioxidants were first explored when it was recognized that a substance with antioxidant activity is likely to be one that is itself readily oxidized (Wolf, 2005).

Research on how vitamin E prevents the process of lipid peroxidation led to the identification of antioxidants as reducing agents that prevent oxidation reaction often by scavenging reactive oxygen species before they cause damage to cell (Wolf, 2005).

Insufficient levels of antioxidants or inhibition of antioxidant enzymes can cause oxidative stress and may damage or kill cells. In general, antioxidant systems either prevent these reactive species from being formed or remove them before they can damage vital cellular components such as DNA, proteins and lipids (Davies, 1995). However, reactive oxygen species also have useful cellular functions, such as redox signaling. Thus, the function of antioxidant system is not to remove oxidants entirely, but instead to keep them at an optimum level (Rhee, 2006).

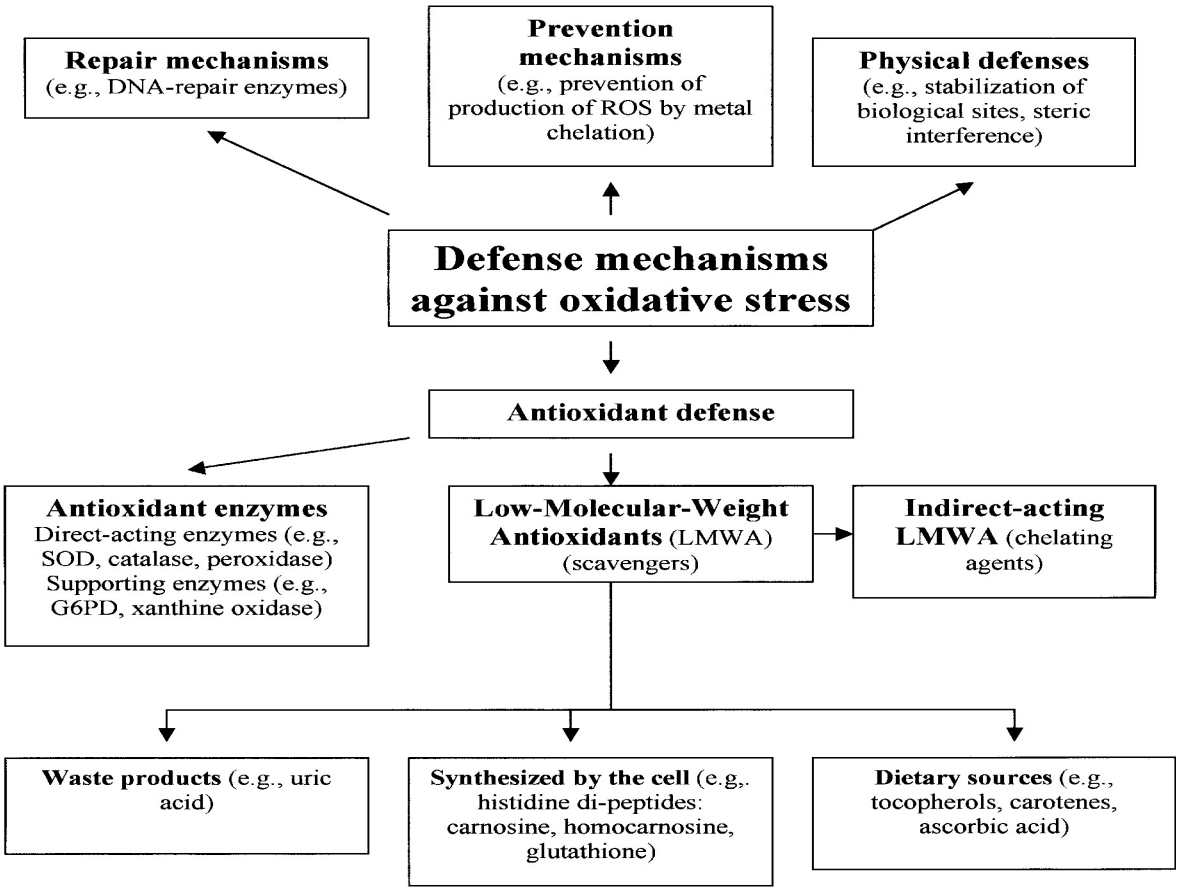


Figure 2.4: Classification of antioxidant cellular-defense mechanisms (Brigelius-Flohē, 1999).

The following are some of the antioxidants we have:

- ASCORBIC ACID (VITAMIN C)

  Ascorbic acid is a monosaccharide redox catalyst found in both plants and animals. As one of the enzymes needed to make ascorbic acid is not found in human, they must obtain it from the diet; it is therefore a vitamin (Smirnoff, 2001).

  Ascorbic acid is required for conversion of the procollagen to collagen by oxidizing proline residues to hydroxyproline. It is also a redox catalyst which can reduce and thereby neutralize reactive oxygen species such as hydrogen peroxide (Linster and Van, 2007).

- MELATONIN

  Melatonin is a powerful antioxidant that easily crosses cell membrane and blood-brain barrier (Reiter *et al.,* 2009). Unlike other antioxidants, melatonin does not undergo

redox cycle, which is the ability of a molecule to undergo repeated reduction and oxidation, thereby promoting free radical formation. Hence, it has been referred to as a terminal or suicidal antioxidant (Tan *et al.,* 2000).

- TOCOPHEROLS AND TOCOTRIENOLS (VITAMIN E)

  Vitamin E is the collective name for a set of eight related tocopherols and tocotrienols, which are fat-soluble vitamins with antioxidants properties (Herrera and Barbas, 2001). Of these, α –tocopherols is the most important which protects the membrane from oxidation by reacting with lipid radicals produced in the lipid peroxidation chain reaction (Wang and Quinn, 1999), thus, removing free radicals.

- GLUTATHIONE

  Glutathione is a cysteine-containing peptide found in most forms of aerobic life. It is not required in diet and is synthesized in cells from its constituent amino acids (Meister and Anderson, 1983). In cells, glutathione is maintained in the reduced form by the enzyme glutathione reductase and in turn reduces other metabolites and enzyme systems, such as ascorbase in the glutathione-ascorbate cycle and glutathione peroxidases (Meister, 1994). Glutathione has antioxidant properties since the thiol group in its cysteine moiety is a reducing agent and can be reversibly oxidized or reduced.

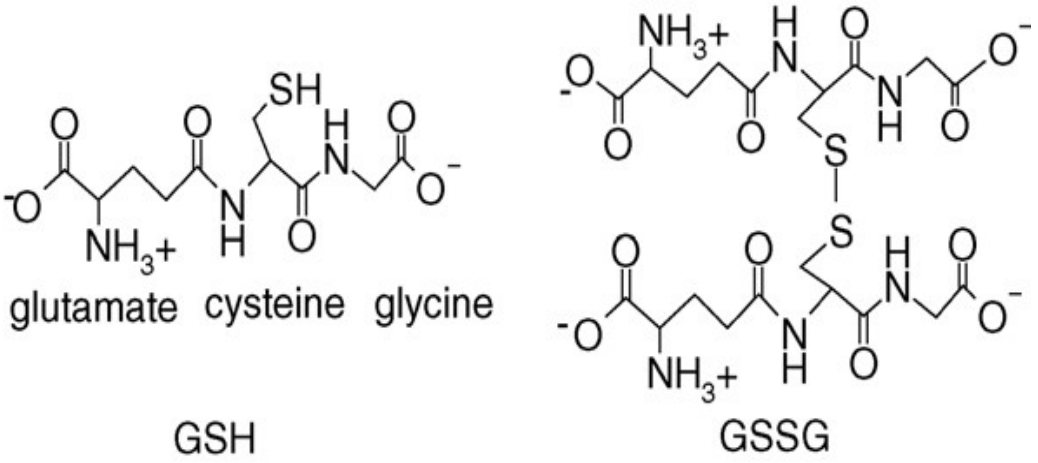


Figure 2.5: Structures of reduced (GSH) and oxidised (GSSG) glutathione

The major thiol antioxidant is the tripeptide, glutathione. Glutathione (GSH) is a multifunctional intracellular non-enzymatic antioxidant. It is considered to be the major thiol-disulphide redox buffer of the cell (Ji *et al.,*1999). Glutathione is highly abundant in the cytosol (1–11 mM), nuclei (3–15 mM), and mitochondria (5–11 mM) and is the major soluble antioxidant in these cell compartments (Ji *et al.,*1999). The reduced form of glutathione is GSH, glutathione, and the oxidised form is GSSG, glutathione disulphide. GSH in the nucleus maintains the redox state of critical protein sulphydryls that are necessary for DNA repair and expression. An oxidative environment leads to rapid modification of protein sulphydryls (protein-SH): two electron oxidation yields sulphenic acids (protein-SOH) and one-electron oxidation yields thiyl radicals (protein- S•) (Ji *et al.,*1999). These partially oxidised products react with GSH and form *S*-glutathiolated protein (protein-SSG), which is reduced further by the glutathione cycle through glutathione reductase and small proteins such as glutaredoxin and thioredoxin, to restore protein sulphydryls (protein-SH). However, if the process of oxidation of protein sulphydryls is not trapped by GSH, further oxidation leads to the formation of irreversibly oxidized forms such as sulphinic (protein-SO2H) and sulphonic (protein-SO3H) acids.

Generally, the antioxidant capacity of thiol compounds is due to the sulphur atom which can easily accommodate the loss of a single electron (Karoui *et al.,* 1996). In addition the lifetime of sulphur radical species thus generated, i.e. a thiyl radical (GS•), may be significantly longer than many other radicals generated during the stress. The reaction of glutathione with the radical R• can be described:

GSH + R• → GS• + RH

Thioyl radicals generated may dimerise to form the nonradical product, oxidised glutathione (GSSG):

GS• + GS• → GSSG

Oxidised glutathione GSSG is accumulated inside the cells and the ratio of GSH/GSSG is a good measure of oxidative stress of an organism (Hwang *et al.,*1992). Too high a concentration of oxidised glutathione GSSG may damage many enzymes oxidatively. GSSG can react with protein sulphydryl groups to produce protein–glutathione mixed disulphides:

GSSG + protein-SH ↔ protein-SSG + GSH

The mixed disulphides (protein-SSG) have a longer half-life than GSSG, most probably due to protein folding. The main protective roles of glutathione against oxidative stress are (Masella *et al.,* 2005) that (i) glutathione is a cofactor of several detoxifying enzymes against oxidative stress, e.g. glutathione peroxidase (GPx), glutathione transferase and others; (ii) GSH participates in amino acid transport through the plasma membrane; (iii) GSH scavenges hydroxyl radical and singlet oxygen directly, detoxifying hydrogen peroxide and lipid peroxides by the catalytic action of glutathione peroxidase; (iv) glutathione is able to regenerate the most important antioxidants, vitamins C and E back to their active forms; glutathione can reduce the tocopherol radical of Vitamin E directly, or indirectly, via reduction of semi dehydroascorbate to ascorbate.

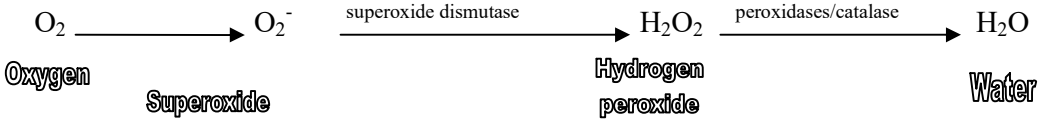
The capacity of glutathione to regenerate the most important antioxidants is linked with the redox state of the glutathione disulphide–glutathione couple GSSG/2GSH (Jones *et al.,*2000). This, in turn, has a high impact on the overall redox environment of the cell. The values of the half-cell reduction potential for the GSSG/2GSH couple depend on the redox environment in which the couple is functioning.

The intracellular content of glutathione is responsive to environmental factors and is a function of the balance between use and synthesis. Exposure to ROS (involving H_2O_2)/RNS or to compounds that can generate ROS including 4-hydroxy-2-nonenal (HNE) can increase the content of GSH by increasing the rate of GSH synthesis. HNE is removed from many cells by reactions with GSH, catalyzed by glutathione *S*-transferases (GSTs) that have relative specificity for alkenals.

2.9.1 Antioxidant Phytochemicals

Phytochemicals are antioxidants that are naturally used by plants to protect themselves against free radicals. Studies show that humans who eat sources of phytochemicals also benefit from the antioxidant properties of the plant. Examples of these antioxidant phytochemicals are Carotenoids, Flavonoids, Ally sulfides and Polyphenols. Sources of these antioxidants are whole grains, fruits and vegetables.

2.9.2 The Enzyme Systems



Enzymatic pathway for detoxification of reactive oxygen

i. Superoxide Dismutases (SODs):

These are class of closely related enzymes that catalyze the breakdown of superoxide anion into oxygen and hydrogen peroxide (Johnson and Gluliri, 2005). SOD enzymes are present in almost all aerobic cells and in extracellular fluids (Johnson and Giuhri, 2005). Superoxide dismutase enzymes contain metal ion cofactors that, depending on the isozyme, can be copper, zinc, manganese or iron. In humans, the copper/zinc SOD is present in cytosol, the manganese SOD is present in the mitochondrion, while the extracellular fluid SOD contains copper and zinc (Zelko *et al.,* 2002).

The mitochondrial isozyme seems to be the most biologically important of these three since mice lacking the enzyme die soon after birth (Melor *et al.,* 1998). In contrast, the mice lacking copper/zinc SOD (SOD) are viable but have numerous pathologies and reduced life span, while mice without the extracellular SOD have minimal defects (sensitive to hyperoxia) (Beaume *et al.,* 1996).

In plants, SOD enzymes are present in the cytosol and mitochondria, with an iron SOD found in the chloroplasts that is absent in vertebrates and yeast (Van *et al.,* 1997).

ii. Catalases:

These are enzymes that catalyze the conversion of hydrogen peroxide to water and oxygen, using either iron or manganese cofactor (Chelikani *et al.,* 2004). This protein is localized to peroxisomes in most eukaryotic cells. Catalase is an unusual enzyme since, although hydrogen peroxide is its only substrate, it follows a ping-pong mechanism. Here, its cofactor is oxidized by one molecule of hydrogen peroxide and then regenerated by transferring the bound oxygen to the second molecule of the substrate (Hiner *et al.,* 2002).

Despite its apparent importance in hydrogen peroxide removal, humans with genetic deficiency of catalase- acatalasemia or mice genetically engineered to lack catalase completely, suffer few ill effects (Ogata, 1991).

iii. The Glutathione System:

The glutathione system includes glutathione, glutathione reductase, glutathione peroxidases and glutathione-s-transferases. This system is found in animals, plants and microorganisms (Meister and Anderson, 1983). Glutathione peroxidase is an enzyme containing four selenium cofactors that catalyses the breakdown of hydrogen peroxides and organic hydroperoxides.

There are at least four different glutathione peroxidase isozymes in animals. Glutathione peroxidase 1 is the most abundant and is a very efficient scavenger of hydrogen peroxide, while glutathione peroxidase 4 is most active with lipid hydro-peroxide (Brigelius-Flohē, 1999).

Also, glutathione-s-transferases show high activity with lipid hydro-peroxides. These enzymes are at particularly high level in the liver and also serve in the detoxification metabolism (Hayes *et al.,* 2005).

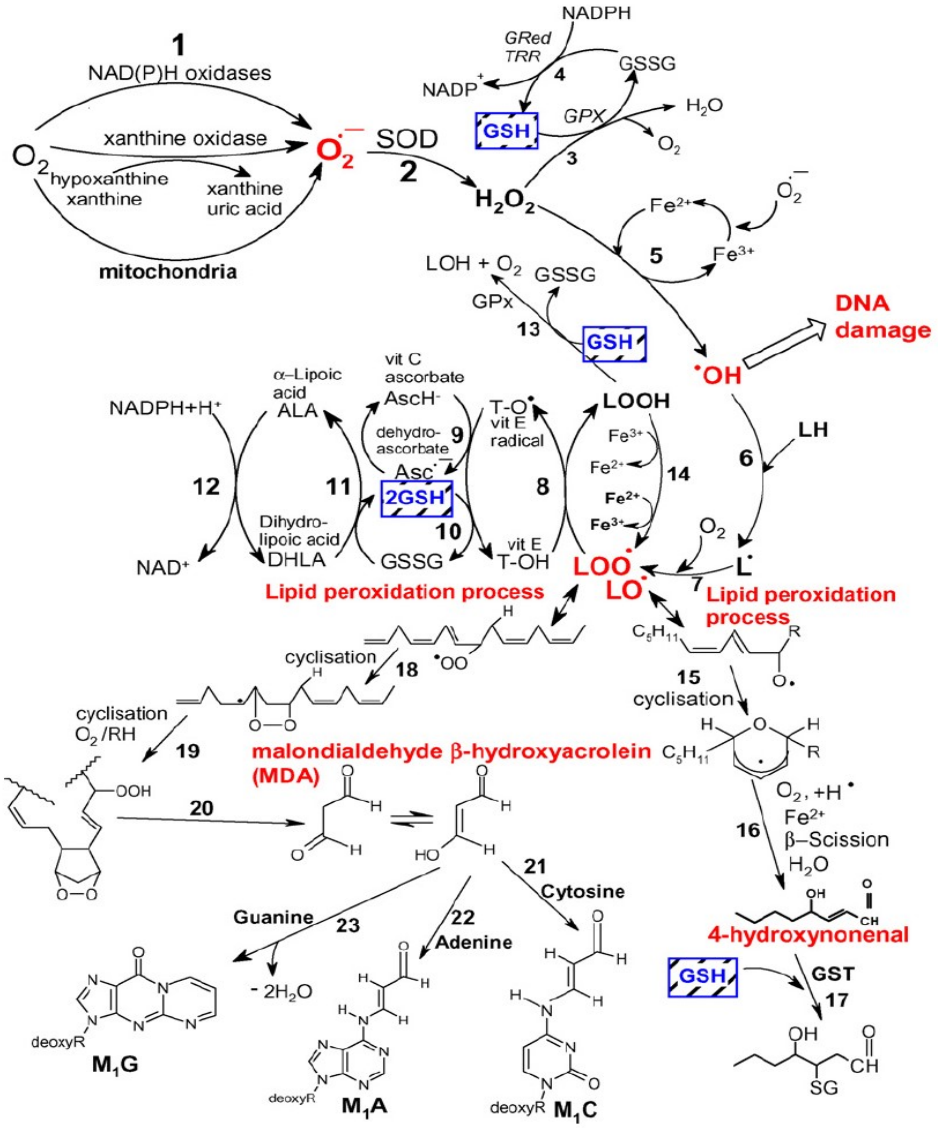


Figure 2.6: Pathways of ROS formation, the lipid peroxidation process and the role of glutathione (GSH) and other antioxidants in the management of oxidative stress. (Valko *et al.,*2007).

2.10 Pathways of ROS formation

Reaction 1: The superoxide anion radical is formed by the process of reduction of molecular oxygen mediated by NAD(P)H oxidases and xanthine oxidase or non-enzymatically by redox-reactive compounds such as the semi-ubiquinone compound of the mitochondrial electron transfer chain.

Reaction 2: Superoxide radical is dismutated by the superoxide dismutase (SOD) to hydrogen peroxide.

Reaction 3: Hydrogen peroxide is most efficiently scavenged by the enzyme glutathione peroxidase (GPx) which requires GSH as the electron donor.

Reaction 4: The oxidised glutathione (GSSG) is reduced back to GSH by the enzyme glutathione reductase (Gred) which uses NADPH as the electron donor.

Reaction 5: Some transition metals (e.g. Fe2+, Cu+ and others) can breakdown hydrogen peroxide to the reactive hydroxyl radical (Fenton reaction).

Reaction 6: The hydroxyl radical can abstract an electron from polyunsaturated fatty acid (LH) to give rise to a carbon-centred lipid radical (L•).

Reaction 7: The lipid radical (L•) can further interact with molecular oxygen to give a lipid peroxyl radical (LOO•). If the resulting lipid peroxyl radical LOO• is not reduced by antioxidants, the lipid peroxidation process occurs (reactions 18–23 and 15–17).

Reaction 8: The lipid peroxyl radical (LOO•) is reduced within the membrane by the reduced form of Vitamin E (T-OH) resulting in the formation of a lipid hydroperoxide and a radical of Vitamin E (T-O•).

Reaction 9: The regeneration of Vitamin E by Vitamin C: the Vitamin E radical (T-O•) is reduced back to Vitamin E (T-OH) by ascorbic acid (the physiological form of ascorbate is ascorbatemonoanion, AscH−) leaving behind the ascorbyl radical (Asc•−).

Reaction 10: The regeneration of Vitamin E by GSH: the oxidised Vitamin E radical (T-O•) is reduced by GSH.

Reaction 11: The oxidised glutathione (GSSG) and the ascorbyl radical (Asc•−) are reduced back to GSH and ascorbatemonoanion, AscH−, respectively, by the dihydrolipoic acid (DHLA) which is itself converted to _-lipoic acid (ALA).

Reaction 12: The regeneration of DHLA from ALA using NADPH.

Reaction 13: Lipid hydroperoxides are reduced to alcohols and dioxygen by GPx using GSH as the electron donor. Lipid peroxidation process:

Reaction 14: Lipid hydroperoxides can react fast with Fe^{2+} to form lipid alkoxyl radicals (LO•), or much slower with Fe^{3+} to form lipid peroxyl radicals (LOO•).

Reaction 15: Lipid alkoxyl radical (LO•) derived for example from arachidonic acid undergoes cyclisation reaction to form a six-membered ring hydroperoxide.

Reaction 16: Six-membered ring hydroperoxideudergoes further reactions (involving β-scission) to form 4-hydroxy-nonenal.

Reaction 17: 4-hydroxynonenal is rendered into an innocuous glutathiyl adduct (GST, glutathione S-transferase).

Reaction 18: A peroxyl radical located in the internal position of the fatty acid can react by cyclisation to produce a cyclic peroxide adjacent to a carbon-centred radical.

Reaction 19: This radical can then either be reduced to form a hydroperoxide (reaction not shown) or it can undergo a second cyclisation to form a bicyclic peroxide which after coupling to dioxygen and reduction yields a molecule structurally analogous to the endoperoxide.

Reaction 20: Formed compound is an intermediate product for the production of malondialdehyde.

Reactions 21, 22, 23: Malondialdehyde can react with DNA bases Cytosine, Adenine, and Guanine to form adducts M1C, M1A and M1G, respectively.

Production of O2•− by activated phagocytic cells in response to inflammation is one of the most studied free radical-producing systems (Gutteridge and Mitchell, 1999). If oxygen attracts two hydrogen molecules, hydrogen peroxide (H_2O_2) is formed. H_2O_2, though not

technically considered an oxygen free radical, is a member of the ROS family and may selectively participate in free radical generation (Kerr *et al.,*1996). The majority of the H_2O_2 is broken down to oxygen and water by the cellular enzyme catalase.

In addition to catalase, the enzyme glutathione peroxidase is responsible for the breakdown of H_2O_2 and any peroxides that form on lipids within the body (Gutteridge and Mitchell, 1999). The hydroxyl radical (•OH) is the most reactive of the free radical molecules (Droge, 2002). The hydroxyl radical damages cell membranes and lipoproteins by a process called lipid peroxidation. Lipid peroxidative damage to lipids in low density lipoprotein (LDL) plays an important role in atherosclerosis (Kerr *et al.,*1996).

2.11 Oxidative challenge in Biology

A paradox in metabolism is that while the vast of majority of complex life on earth require oxygen for their existence. Oxygen is a highly reactive molecule that damages living organisms by producing oxygen species (Davies, 1995). The reactive oxygen species produced in cells include hydrogen peroxide, (H_2O_2), hypchlorous acid (HClO) and free radicals such as hydroxyl radical (OH^-) and the superoxide anion (O_2^-) (valko *et al.,* 2007).

The hydroxyl radical is particularly unstable and will react rapidly and non-specifically with biological molecules. This species is produced from hydrogen peroxide in metal catalyzed redox reactions such as the fenton reaction (Stohs and Bageli, 1995). These oxidants can damage cells by starting chemical chain reactions such as lipid peroxidation or by oxidizing DNA or proteins (Sies, 1997). Damage to DNA can cause mutations and possibly cancer, if not reversed by DNA repair mechanism (valko *et al.,* 2004), while damage to proteins causes enzymes inhibition, denaturation and protein degradation (Stadtman, 1992).

2.12 Gastric Juice

Gastric juice or acid is a digestive fluid produced in the stomach by the parietal cells of the stomach. It is composed of hydrochloric acid (HCl) (around 0.5%) as high as 0.5M of potassium chloride (KCl) and sodium chloride (NaCl) (Marieb and Hoehn, 2010). The acid plays a key role in digestion of proteins by activating digestive enzymes (pepsin and rennin), making ingested proteins unravel or denature so that digestive enzymes can break down the long chains of amino acids and inhibiting the growth of many microorganisms, which is helpful in preventing infection.

Other cells in the stomach produce bicarbonate, a base, to buffer the fluid, ensuring that it does not become too acidic. These cells also produce mucus, which forms a viscous physical barrier to prevent gastric juice from damaging the stomach.

The pH of gastric acid is 1.5 to 3.5 in the human stomach lumen and the acidity is being maintained by the proton pump H^+/K^+ ATPase (Marieb and Hoehn, 2010).

2.13 Malondialdehyde (MDA)

Malondialdehyde (MDA) is an organic compound with the formula $CH_2(CHO)_2$. This reactive specie that occurs naturally is a marker for oxidative stress and results to lipid peroxidation of poly unsaturated fatty acids (Davey *et al.,* 2005). The degree of lipid preoxidation can be estimated by the amount of malondialdehyde in the tissue. Malondialdehyde reacts with deoxyadenosine and deoxyguanosine in DNA, forming DNA adducts and other protein adducts, generally referred to as advanced lipoxidation end-products (ALE), resulting in biomolecular damage, because they are mutagenic (Marnett, 1999).

2.14 Omeprazole

Omeprazole (under the brand name of miraprazole, Prilosec and Losec among others) capsule is a substituted benzimidazole, that is (5-methoxy-2-[(4-methoxy-3,5-dimethylpyridin-2-yl)sulfinyl]benzimidazole). It is a compound that inhibits gastric acid secretion. It is used to treat Gastroesophageal Reflux Diseases (GERDs), peptic ulcer disease and Zolinger-Ellison syndrome (Wilde and Mctavish, 1994).

Omeprazole is a proton pump inhibitor (PPI), and as such blocks the release of stomach acid (Wilde and Mctavish, 1994). It is a selective and irreversible proton pump inhibitor. It suppresses stomach acid secretion by specific inhibition of the H^+/K^+-ATPase system found at the secretory surface of gastric parietal cells. Because this enzyme system is regarded as the acid (proton or H^+) pump within the gastric mucosa, omeprazole inhibits the final step of acid production. Omeprazole also inhibits both basal and stimulated acid secretion irrespective of the stimulus (Tajima *et al.,* 2008).

The absorption of omeprazole takes place in the small intestine and is usually completed within 3 to 6 hours. Omeprazole as well as other PPIs, are only effective on active H^+/K^+ ATPase pumps. These pumps are stimulated in the presence of food to aid in digestion. Thus, omeprazole is better taken on an empty stomach about 30-60 minutes before a meal to allow the drug to reach peak levels once food is ingested (Kirchheiner *et al.,* 2009).

The most frequent significant adverse effects of omeprazole include headache, dizziness, upper respiratory tract infection, abdominal pain, diarrhea, nausea, flatulence, acid

regurgitation constipation, muscle weakness and pains, osteoporosis-related fractures, hypomagnesaemia, vitamin B_{12} and iron mal-absorption (Mccoll and Kenneth, 2009), (Neal and Logan, 2001).

Omeprazole is completely metabolized by the cytochrome P_{450} system, mainly in the liver. Identified metabolites are the sulfone, the sulfide and hydroxyl-omeprazole, which exert no significant effect on acid secretion. About 80% of an orally taken dose is excreted as metabolites in the urine, and the remainder is found in the feaces, primarily originating from bile secretion (Shi and Klotz, 2008).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Equipment and Apparatus

Digital PH meter (Labtech, india), water bath (Grant, England), automatic micro pipettes (Teco, diagnostics ,USA), tissue homogenizer BLK 397 (kenwood Ltd, japan), magnifying lens, refridgerator.Digital Spectrophotometer model 390 (VIS Spectrophotometer 21D,Life Assistance Scientific Instrument Company China),Weighing balance- Mettler PT 320 (Mettler-Wagen,Switzerland), Bench centrifuge (Clay Adams, USA), Intubator, Tissue homogenizer(Janke& Kunkel, Gmbh), Deep freezer (Fresh point FDF-196).

3.1.2 Chemicals/Reagents

Quercetindihydrate (Sigma-Aldrich Mo USA), 2-Thiobarbituric acid (Sigma-Aldrich Mo USA), Sodium dodecyl Sulphate (Fluka-Chemie, Switzerland), Acetic acid, Trichloroacetic acid, Sodium dihydrogen orthophosphate, Disodium hydrogen orthophosphate, Potassium Chloride, Glutathione ((Fluka-Chemie, Switzerland), Protein test kit, Sodium nitroprusside, Nitroblue tetrazolium, Naphthyl ethylene diamine dihydrochloride, Deoxyribose, Aluminium chloride, Folin Dennis, All other chemicals and reagents used were of analytical grade.

3.1.3 Animals

Thirty male albino (wistar) rats weighing 200 – 250g and fifteen swiss albino mice, weighing 20 – 30g were purchased from an animal breeding station (Animal friend) at No. 92 Royce road Owerri, Imo state. The animals were maintained under standard laboratory condition in a stainless steel cages with free access to standard animal food (Pelletised, Vital finisher) and portable drinking water. Albino mice were used for the determination of the acute toxicity of the drug and the albino rats were used for the ulcero-protective studies. The animals were acclimatized in the laboratory for two weeks before the commencement of the study.

3.1.4 Aqueous Anti-Ulcer Drug (AQAUD)

The Aqueous Anti-Ulcer Drug (AQAUD) was obtained as prepared by a traditional medicine practitioner in Owerri, Imo state. Briefly, the herbal medicament was prepared by mixing clean and sorted portion of the plants' roots (100g of *Ipomoea mauritana* + 50g of S*trophantus hispidus*) in a pot. These were boiled with portable water (1.0litre) for 5 hours. The aqueous extract obtained was cooled to room temperature (25 ±2 °C), Thereafter, the C*itrus aurantifolia* fruit juice (20ml) and Gbogbonise (10g) were added to the extract, thoroughly mixed and volume made up to 1litre to obtain the AQAUD. The drug was collected in plastic containers and preserved in the freezer for the studies.

3.1.5 Aspirin and Omeprazole

Aspirin and omeprazole (the standard anti-ulcer drug) were purchased from Bright Way Pharmacy at No 25 Mbaise road, Owerri, Imo state. They were kept hygienically for the studies.

3.2 METHODOLOGY

3.2.1 Grouping of Animals

The animals were divided into groups to serve for the ulcer-protective and acute toxicity studies. Thirty albino rats were divided into five groups to serve for the ulcer-protective studies and fifteen albino mice were divided into five groups for the acute toxicity studies.

3.2.1.1 Grouping of Animals for Ulcer-protective study

A total of thirty healthy albino rats weighing 200g-250g were divided into five groups with six animals in each group according to their weight.

Group A or normal control group received normal rat diet and water orally for 14 days.

Group B or intoxicated control group received aspirin 400mg/kg body weight orally as a single dose on the 14th day in addition to normal rat diet and water for 14 days.

Group C or intoxicated test group 1 received 250mg/kg body weight of the AQAUD for 14 days and aspirin 400mg/kg body weight orally as a single dose on the 14th day in addition to normal rat diet and water.

Group D or intoxicated test group 2 received 500mg/kg body weight of the AQAUD for 14 days and aspirin 400mg/kg body weight orally as a single dose on the 14th day in addition to normal rat diet and water.

Group E or intoxicated standard group received omeprazole 20mg/kg body weight orally for 14days and aspirin 400mg/kg body weight on the 14th day in addition to normal rat diet and water.

After administration for 14 days, all the animals were fasted for 24 hours and sacrificed under light anaesthesia with dichloromethane to bring out the stomachs which were used to check for the ulcer parameters. They include ulcer index, free and total acidity of the stomach content. Nitrite, glutathione, malondialdehyde and total protein concentration of the stomach homogenate was also determined. Also superoxide dismutase and catalase activities in the stomach homogenate were assayed.

3.2.1.2 Grouping of Animals for Acute toxicity study

A total of 15 albino mice weighing 20-30g were grouped into groups according to their weight (Lorke, 1983).

Phase 1: This phase required twelve animals divided into three groups with three animals in each group. Each group of animals were administered different doses (10, 100 and 1000mg/kg.b.wt.) of the AQUAD and then observed for 24 hours to monitor their behavior as well as if mortality will occur.

Group 1 or control group received normal rat diet and water orally.

Group 2 received 10mg/kg.b.wt. of the AQUAD orally once in addition to normal rat diet and water.

Group 3 received 100mg/kg.b.wt. of the AQAUD orally once, in addition to normal rat diet and water.

Group 4 received 1000mg/kg.b.wt. of the AQAUD orally once, in addition to normal rat diet and water.

Phase 2: This phase required three animals divided into three groups with one animal in each group. Each group of animals was administered different doses (1600, 2900 and

5000mg/kg.b.wt.) of the AQUAD and then observed for 24 hours to monitor their behavior as well as if mortality will occur.

Group 1 received 1600mg/kg.b.wt. of the AQUAD orally once, in addition to normal rat diet and water.

Group 2 received 2900mg/kg.b.wt. of the AQUAD orally once in addition to normal rat diet and water.

Group 3 received 5000mg/kg.b.wt. of the AQAUD orally once, in addition to normal rat diet and water.

Then the LD_{50} is calculated thus: $LD_{50} = \sqrt{(D_0 \times D_{100})}$

D_0 = Highest dose that gave mortality.

D_{100} = Lowest dose that produced mortality.

3.2.2 Preparation of Stomach Homogenate

The stomach samples were homogenized in phosphate buffer (pH 7.4) in 4 parts of homogenizing buffer i.e 1:4 ratio, and centrifuged at 12,000rpm for 30 mins. The supernatant was collected and kept in the freezer at -4°C and used to assay for catalase, superoxide dismutase activities. Malondialdehyde, glutathione, nitrite, and protein concentrations were also estimated in the homogenate.

3.3 Qualitative Phytochemical Screening

Qualitative phytochemical screening was done on the AQAUD using standard procedures to identify its bioactive components.

3.3.1 Test for the presence of Tannins

An aspect for the estimation of tannins by AOAC (1984) was adopted. 1ml of the AQAUD was mixed with 0.5ml of Folin-Denis reagent and 1.0ml of 17% Na_2CO_3. The mixture was allowed to stand for three minutes at room temperature for colour development. The sample tested positive for tannins when it developed blue colour (intensity varying with the concentration of tannins in the test sample). Also 1.0ml was treated with 0.5ml of 15% ferric chloride solution. A blue colour indicated the presence of hydrolysable tannins (Odebiyi and Sofowora, 1978).

3.3.2 Test for the presence of Glycosides

To 2ml of the sample, 15ml of 2% of sulfuric acid was added. The mixture was heated on a water bath for 5 minutes. To 5ml of the solution obtained, 0.2ml of Fehling solution A was added and then Fehling solution B, until it turned alkaline (tested with litmus paper). The mixture was heated on a water bath for 2 minutes. A brick red coloration indicated the presence of glycosides.

3.3.3 Test for the presence of Saponins

The method of Harbone (1990) was used for the analysis.

Frothing test: 1.0ml of the sample was diluted with 4ml of distilled water, shaken vigorously and observed on standing for stable froth.

Emulsion test: To 1.0ml of the sample, 2 drops of olive oil were added. The solution was shaken and observed for the formation of emulsion.

3.3.4 Test for the presence of Flavonoids

To 1.0ml of the sample, 10ml of ethyl acetate was added and the solution heated for 3 minutes.

a. To 4ml of the solution obtained, 1% aluminum chloride solution was added and shaken. It was observed for a light yellow coloration on ethyl acetate layer.
b. 4ml of the solution obtained was shaken with 1ml of 2% ammonia. The layers were allowed to separate and the ammonia layer was observed for yellow coloration.

3.3.5 Test for the presence of Steroids (Salkowski's test)

To 1ml of the sample, 2.0ml of chloroform was added. Concentrated sulfuric acid was added carefully along the tube to form a layer. A reddish brown colour at the interface was indicative of the presence of steroidal ring (Sofowora, 1982).

3.3.6 Test for the presence of Phenolic compounds

To 4ml of sample, 0.2ml of 2N NH_4OH was added. 0.1ml of 2% aminoantipyrene and 0.1ml of potassium ferricynide were added. The mixture was shaken vigorously and observed for colour change. A pink to red colouration indicates the presence of phenol.

3.4 Quantitative Phytochemical analysis

3.4.1 Total Phenolic Content

Principle: A colorimetric assay using the Folin-Dennis reagent for the determination of phenolic compounds is based on the reaction between the Folin-Dennis reagent and phenolic compounds, which results in the formation of a blue colour complex that absorbs radiation and allows quantification.

Procedure:

The total phenolic content was determined using the method of Swian and Hillis as described by Wattashinghe and Shaidi (2000). 50mg of aqueous extract was diluted with 100ml of distilled water to obtain a concentration of 0.5mg/ml (Solution A). Folin-Denis reagent (0.5ml) was added to centrifuge tube containing 0.5ml of Solution A. Tubes were shaken and 1ml of saturated Sodium Carbonate solution was added into each tube. The volume for each tube was then adjusted to 10ml by the addition of 8ml of deionised water and the content was mixed vigorously. Tubes were allowed to stand at ambient temperature for 25mins and then centrifuged for 5mins at 4000xg. Absorbance of the supernatants was measured at 725nm. Content of total phenolics in the extract was determined using a standard curve prepared for (+) Tannic acid ($y=0.0039x$)$R^2 = 0.989$. Total extracted phenolic compound was expressed as mg (+) Tannic acid equivalent/g extract.

3.4.2 Total Flavonoid content

Principle: This method uses aluminum chloride and is based on the formation of a complex between the aluminum ion (Al^{3+}), and the carbonyl and hydroxyl groups of flavones and flavonols that produce a pink colour, that is absorbance is determined colorimetrically (Popova *et al.,*2004).

Procedure:

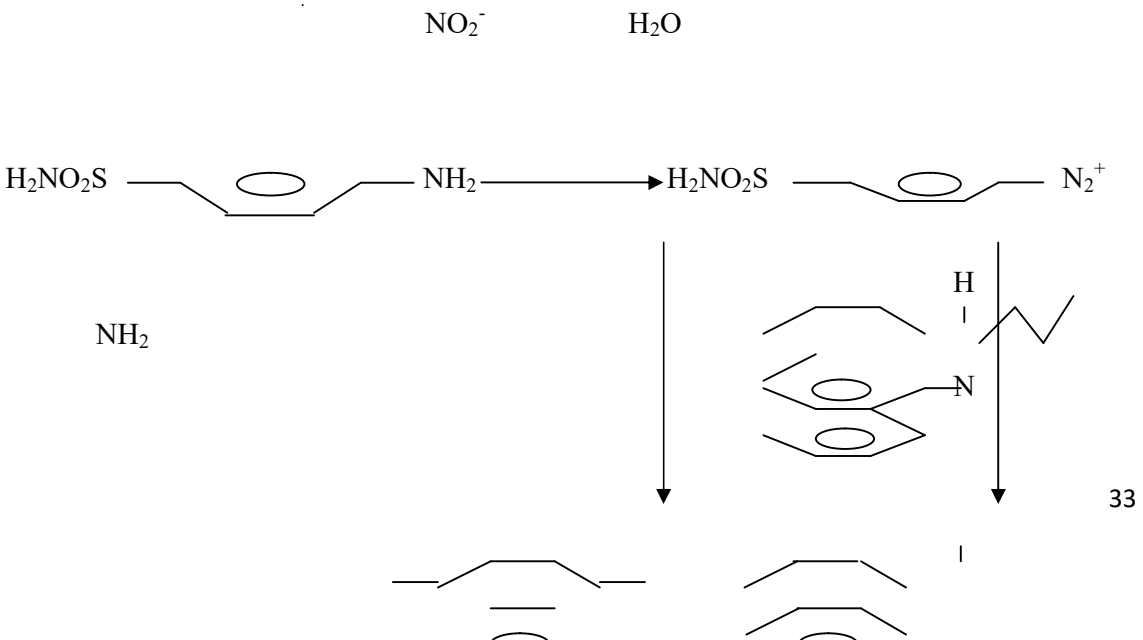
The total flavonoid content of the aqueous plant extract was determined colorimetrically as described by Zuo *et al.,* (2004). In brief, 0.5ml of Solution A above was mixed with 2ml of distilled water and subsequently 0.15ml of 5% of $NaNO_3$ solution. After 6mins of incubation, 0.15ml of 10% of $AlCl_3$ solution was added and then allowed to stand for 6mins, followed by addition of 2ml of 4% NaOH solution to the mixture. Immediately, water was added to the sample to bring the final volume to 5ml. The mixture was thoroughly mixed and allowed to stand for another 15mins. The absorbance was read at 510nm. The total flavonoid content was expressed in milligrams of Quercetin equivalent per gram of the plant extract. Content of total flavonoid in each extract was determined using standard curve prepared for (+) Quercetin ($y = 0.0024x$) R^2=0.9816

Total extracted flavonoid was expressed as mg (+) Quercentin equivalent /g extract.

3.5 Determination of Radical Scavenging Ability

3.5.1 Determination of Nitric Oxide Radical Scavenging Ability of the Extract

Principle: The compound Sodium Nitro Prusside (SNP) is known to decompose in aqueous solution at physiological pH (7.2) producing NO^-. Under aerobic condition, NO^- reacts with oxygen to produce stable products: nitrate and nitrite, the quantities which can be determine using Griess reagent (Marcocci *et al.,* 1994). The Griess reagent system is based on the chemical reaction shown below which uses sulfanilamide and N-1-napthyethylenediaminedihydrochloride (NED) under acidic (phosphoric acid) conditions.



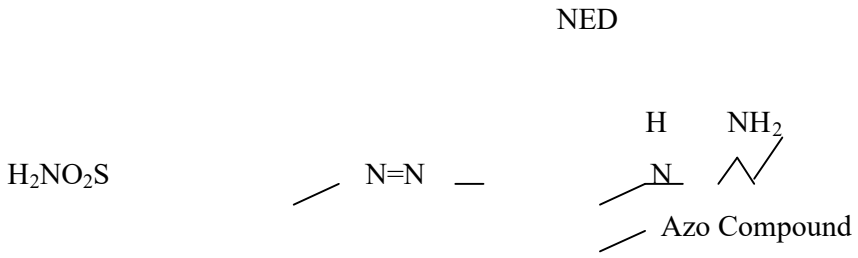


Figure 3.1 Griess reagent system.

Procedure: The scavenging effect of the extract on nitric acid was measured according to the method of Marcocci *et al.,*1994 with little modification (Alisi and Onyeze, 2008). 4ml of extract solution at different concentrations were added to 1ml of sodium nitro prusside(SNP) solution(10mM) and the tubes were incubated at 29^{oc} for 2 hours. An aliquot (2ml) of incubated solution was removed and diluted with 1.2ml of Griess reagent (1% sulfanilamide in 5% H_3PO_4 and 0.1% Naphthyl ethylenediamine dihydrochloride). The absorbance of the chromophore that formed during diazotization of nitrite with sulfanilamide and subsequent coupling with Naphthylethylenediamine dihydrochloride was immediately read at 550nm and compared with the absorbance of standard solution of sodium nitrate salt treated in the same way as Griess reagent. Inhibition of nitrate formation by the extract or the standard antioxidant (Quercetin) was calculated relative to the control.

$$\%\ Inhibition = \frac{Absorbance\ control - Absorbance\ test}{Absorbance\ control} \times 100$$

3.5.2 Determination of Hydroxyl Radical Scavenging Ability of the Extract

Principle: Free radical dependent 2-deoxyribose degradation was studied using fenton oxidant reaction mixture of F^{3+}/ ascorbic acid and H_2O_2 as described by Halliwell, *et al.,* (1987).

Fenton oxidant reaction mixture releases hydroxyl radicals which attack and degrade 2-deoxyribose into fragments. These fragments formed from 2-deoxyribose degradation react with Thiobarbituric acid on heating at low PH to form a pink product (Thiobarbituric Acid Reacting Substances {TBARs}), whose optical density was read at 532nm.

Procedure: Hydroxyl radical scavenging ability of extract was measured by studying the competition between deoxyribose and the test extract for hydroxyl radicals generated from the Fe^{3+}/ ascrbate/ EDTA/H_2O_2 system. The reaction mixture contained: Deoxyribose (2.8mM), $FeCl_3$ (0.1mM), EDTA (0.1mM), H_2O_2 (1mM), Ascorbic acid (0.1mM), NaH_2PO_4-NaOH buffer (20Mm, pH 7.4) and the extract (0-200µg/ml) in a final volume of 1.0ml. After incubation for 1hr at 37^{oc}, the deoxyribose degradation was measured as TBARs by the method of Ohkawa, *et al.,* (1979), as modified by Lui, *et al.,* (1990). Briefly, 1.5ml of 20% acetic acid (pH 3.5), 1.5ml of 0.8% thiobarbituric acid (TBA), 0.2ml of 8.1% sodium dodecyl sulphate (SDS) and the incubated mixtures were heated at 100^{oc} for 1hr, cooled and 2ml of trichloroacetic acid added. The mixture was vortexed vigorously and centrifuged at 3000g for 10mins and the absorbance was read at 523nm. Concentration of thiobarbituric acid reactive substances (TBARs) was determined using the molar extinction coefficient of Malondialdehyde. Inhibition of deoxyribose degradation which gives an indication of hydroxyl radical scavenging action was calculated by:

$$\%\ \text{OH Radical Scavenging} = \frac{Absorbance\ MDA\ control - Absorbance\ MDA\ test}{Absorbance\ MDA\ control} \times 100$$

3.6 Determination of total Protein concentration

Principle: Cupric ions, in an alkaline medium, interact with protein peptide bonds resulting in the formation of a coloured complex. (Gornall *et al*.,1949).

Procedure: The reagents were brought to room temperature then pipetted into labelled test tubes as follows:

Reagents	Reagent Blank	Standard	Sample
Distilled water	0.02ml	-	-
Standard	-	0.02ml	-
Sample	-	-	0.02ml
RL.(Biuret Reagent) Sodium hydroxide100Mm/L, Na-K-tartrate 16Mm/L, Potassium iodide 15Mm/L, Cupric sulphate 6Mm/L.	1.0ml	1.0ml	1.0ml

The test tubes content were mixed thoroughly and incubated for 30mins at room temperature. The absorbance (A) of samples or standard was read against the reagent blank (Sodium hydroxide 100Mm/L and Na-K-tartrate 16Mm/L) at 546nm in a spectrophotometer.

Calculations: The total protein concentration in the sample was calculated as follows

$$C_{sample} = \frac{A_{Sample}}{A_{Standard}} * C_{Standard}$$

Where C_{sample} is the concentration of protein in stomach homogenate

$C_{standard}$ is the concentration of protein in standard (g/l)

A_{sample} is the absorbance of test sample

$A_{standard}$ is the absorbance of standard sample

3.7 Determination of Catalase (CAT) activity

Principle: The catalase enzyme activity in stomach was assayed following the procedure of Sinha (1972). The principle of the assay is based on the rate of peroxide removal by catalase. As soon as the enzyme mixture comes in contact with the dichromate solution, its activity is destroyed. Any hydrogen peroxide which hasn't been split by the catalase will react with the dichromate to give a blue precipitate of perchromic acid. This unstable precipitate is then decomposed by heating to give the green solution that its absorbance is read with spectrophotometer.

Procedure: Homogenate (0.1 ml) was incubated with H_2O_2 (0.2 M, 0.5 mI), in the presence of 2.0ml of 0.01 M phosphate buffer (pH 7.4). Reaction was incubated for 15 minutes in the dark and stopped by adding 1.0ml of 5% dichromate solution. Samples were incubated for 15 min and stopped by boiling. Phosphate buffer (2.0 ml, 0.01M, P^H 7.4) was added and shaken vigorously. The upper layer of the mixture was taken and the absorbance read at 570 nm.

3.8 Superoxide Dismutase (SOD) assay

Principle: The principle of the assay is based on the reduction of Nitro-Blue Tetrazoleum (NBT). The ability of superoxide radical to interact with NBT, reducing the yellow tetrazoleum to a blue precipitate is measured colorimetrically. (Vijayalakshmia and Kumar, 2013).

Procedure: The SOD was assayed by autoxidation of hydroxylamine at PH 10.2(1.2ml phosphate buffer), accompanied by reduction of Nitro-Blue tetrazoleum (NBT). Nitrite production in the presence of 0.2ml EDTA (40mg/l) was detected colorimetrically. One enzymatic unit of SOD corresponds to the amount of proteins present in 100 µl of 10% stomach homogenate required to inhibit the reduction of 0.6 mM NBT (0.3ml) by 50% and is expressed as units per mg of proteins.

3.9 Assessment of Lipid Peroxidation in stomach homogenate

Principles: Lipid peroxidation in the supernatant fractions was determined spectrophotometrically by assessing the concentration of Thiobarbituric Acid Reactive Substances (TBARs) as described by Liu *et al.,*1990. The results were expressed in

malondiadehyde (MDA) formed relative to an extinction coefficient of 1.56 x 10^6mol/cm. Small quantities of MDA are produced during lipid peroxidation. These react with Thiobarbituric Acid (TBA) to generate a pink coloured complex which in acid solution absorb light at 532nm and flourescences at 532nm and is readily extractable into organic solvents such as butan-1-ol.

Procedure: Lipid peroxidation was estimated by the method of Liu *et al.,* 1990. Acetic acid 1.5ml (20%; P^H 3.5), 1.5mL of thiobarbituric acid (0.8%) and 0.2ml of sodium dodecyl sulphate (8.1%) was added to 0.1mLof supernatant and heated at 100 ◦C for 60 min. After centrifugation at 1200×*g* for 10 min, the organic layer was separated and absorbance measured at 532 nm using a spectrophotometer. Malondialdehyde (MDA) is an end product of lipid peroxidation, which reacts with thiobarbituric acid to form pink chromogen–thiobarbituric acid reactive substance. It was calculated using a molar extinction coefficient of $1.56\times10^5 M^{-1}$ $cm^{-1}$and expressed as nanomoles of MDA/gTissue.

3.10 Determination of Glutathione concentration in stomach homogenate

Principles: Glutathione (reduced) was measured according to the method of Ellman (1959) as described by Raja *et al.,*2007. Reduced glutathione (GSH) forms the bulk of non-protein sulfhydryl groups. This method is based on the formation of relatively stable yellow colour when Ellman's reagent is added to a sulfhydryl compound. 2-nitro-5-thiobenzoic acid, the chromophoric product resulting from the reaction of Ellman's reagent with reduced glutathione.

Procedure: Equal quantity of homogenate was mixed with 10% trichloroacetic acid and centrifuged at 4000g for 15 minutes to separate the proteins.

To 0.5ml of the supernatant, 4.5ml of Ellman's reagent were added. Mixture was vortexed and the absorbance of mixture read at 412 nm within 15 min.

Calculations: concentration of glutathione was calculated by using standard glutathione of known concentration when subjected to the same experimental conditions.

3.11 Estimation of Nitrite/Nitrate

The estimation of nitrite in the supernatant was determined using a colorimetric assay with the Griess reagent as described by Green *et al.,* 1982. Equal volumes of supernatant and the Griess reagent (0.1% NED, 1% sulfanilamide and 2.5% phosphoric acid) were mixed. Then,

the mixture was incubated for 10 min at room temperature in the dark, and the absorbance was measured at 540 nm (Green *et al.,*1982; Kumar and Kumar, 2008).

3.12 Determination of Ulcer Index

Antiulcer activity was assessed using Aspirin-induced gastric ulcer. Ulcers were induced by administering Aspirin 400mg/kg body weight to the test groups (T1 and T2), experimental group and the standard group as a single dose to each rat on the 14^{th} day. After 24hours, all the rats were sacrificed by cervical dislocation, stomach was cut open along the greater curvature and gently rinsed under tap water and gastric content was collected. The stomachs were stretched on a board and the ulcer index was obtained according to scoring method of Suzuki as follows: Score 1: maximal diameter of 1mm; Score 2: maximal diameter of 1-2mm Score 3: maximal diameter of 2-3mm; Score 4: maximal diameter of 3-4mm; Score 5: maximal diameter of 4-5mm; Score 10: an ulcer over 5mm in diameter; Score 25: a perforated ulcer (Suzuki *et al.,* 1976).

3.13 Determination of Free and total acidity:

Free acidity and total acidity were determined as described by Kulkarni, 1999. After centrifuging the gastric contents with 5ml of water, 4 ml of the supernatant was taken in a conical flask and 2 drops of methyl orange indicator was added to it. 0.01N NaOH was taken in a burette and allowed to titrate into the conical flask until the solution in the conical flask changed colour to colourless, at end point. Then 2 drops of phenolphthalein was added and titration was continued till pink colour as end point was reached.

The amount of 0.01N NaOH required to titrate to the methyl orange end point is the measure of the free acid present. The amount of 0.01N NaOH required to titrate from the beginning to the phenolphthalein end point is a measure of the total acid present in the sample. The acidity was calculated by the following formula and expressed in mEq/l (Kulkarni, 1999).

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

3.15 Statistical Analysis

Data were analyzed using appropriate software (Microsoft Excel, 2007). Results were presented as mean ± S.D. for body weights while data for biochemical values were expressed as mean ± S.D. of six observations and statistically analyzed using one-way analysis of variance on statistical program for social sciences (SPSS version 21). The degree of statistical difference was accepted at $P< 0.05$.

CHAPTER FOUR

RESULTS AND DISCUSSIONS

4.0 RESULTS

4.1 Qualitative Phytochemical composition of AQAUD

The "AQAUD" was found to contain tannins, glycosides, saponins, flavonoids, phenols and alkaloids. The "AQAUD" however did not show a positive reaction for steroids and cyanogenic glycosides (Table 4.1).

Table 4.1: Qualitative phytochemical result of Aqueous Anti-Ulcer Drug (AQAUD)

Phytochemicals	Presence/Absence
Tannins	+
Glycosides	+
Saponins	+
Flavonoids	+
Phenols	+
Alkaloids	+
Cyanogenic Glycosides	-
Steroids	-

(+) represents presence of the phytochemical (-) represents absence of the phytochemical

4.2 Total Phenolics and Flavonoid content of "AQAUD"

Table 4.2 shows the total phenolics and flavonoid content of "AQAUD". Total phenolics content of "AQAUD" is 1.900 ± 0.190(mg Tannic acid equivalent/g extract) and the total flavonoid content of "AQAUD" is 1.040 ± 0.140(mg Quercetin equivalent/g extract).

Table 4.2

Quantitative phytochemical content of AQAUD

Total Phenolic Content (mg Tannic acid equivalent /g extract)	**Flavonoid Content (mg Quercetin equivalent /g extract)**
1.90 ± 0.190	1.04 ± 0.14

4.3 Threshold Inhibitory Concentrations of "AQAUD" and Quercetin against nitric oxide radicals.

Table 4.3 shows the Threshold Inhibitory Concentrations (IC) of "AQAUD" and Quercetin against nitric oxide radicals. The IC_{20} for "AQAUD" and Quercetin were 34.92±1.75μg/ml and 12.56±0.63 μg/ml respectively and the IC_{40} for both were 80.91±4.05μg/ml and 27.64±1.38μg/ml respectively.

Table 4.3: Threshold inhibitory concentrations of AQAUD and Quercetin against nitric oxide radicals.

Threshold Inhibitory Concentrations (IC)	**% Nitric Oxide radical scavenging**						
	5	10	20	40	50	70	80
AQAUD (μg/ml)	9.22 ± 0.46	17.46 ± 0.87	34.92 ± 1.75	80.91 ± 4.05	114.66±5.73	238.90±11.95	383.73±19.19
Quercetin (μg/ml)	3.62±0.18	6.57±0.33	12.56±0.63	27.64±1.38	38.44±1.92	77.78±3.89	124.31±6.22

4.4 Threshold Inhibitory Concentrations of "AQAUD" and Quercetin against hydroxyl radicals.

Table 4.4 shows the Threshold Inhibitory Concentrations (IC) of "AQAUD" and Quercetin against hydroxyl radicals. The IC_{10} for "AQAUD" and Quercetin were 75.92±3.80μg/ml and 99.23±11.82μg/ml respectively and the IC_{20} for both were 146.28±7.31μg/ml and 236.36±11.82μg/ml respectively.

Table 4.4 : Threshold inhibitory concentrations of AQAUD and Quercetin against hydroxyl radicals.

	Threshold Inhibitory Concentrations (IC)					
	5.00	10.00	20.0	50.00	70.00	80.00
AQAUD (*ug*/ml)	74.92±3.75	75.92±3.80	146.28±7.31	ND	ND	ND
Quercetin (*ug*/ml)	49.57±2.48	99.23±4.96	236.36±11.82	1017.62±50.881	2218.37±110.92	3525.66±176.28

ND= Non determinable

4.5 Nitric oxide radical scavenging effect of graded concentrations of Quercetin and Aqueous Anti-Ulcer Drug (AQAUD).

Nitric oxide radical scavenging result (figure 4.1) showed that "AQAUD" dose dependently scavenged nitric oxide ie nitrite concentration diminished with increase in "AQAUD" concentration. The threshold inhibitory concentrations of AQAUD were IC_{50}=114.660±5.73µg/ml, IC_{70} =238.900±11.95µg/ml and IC_{80} =383.730±19.19µg/ml.

Quercetin dose dependently scavenged nitric oxide ie nitrite concentration diminished with increased concentration of quercetin. The threshold inhibitory concentrations of quercetin were IC_{50}=38.440±1.92µg/ml, IC_{70} =77.780±3.89µg/ml and IC_{80} =124.310±6.22µg/ml (Table 4.3).

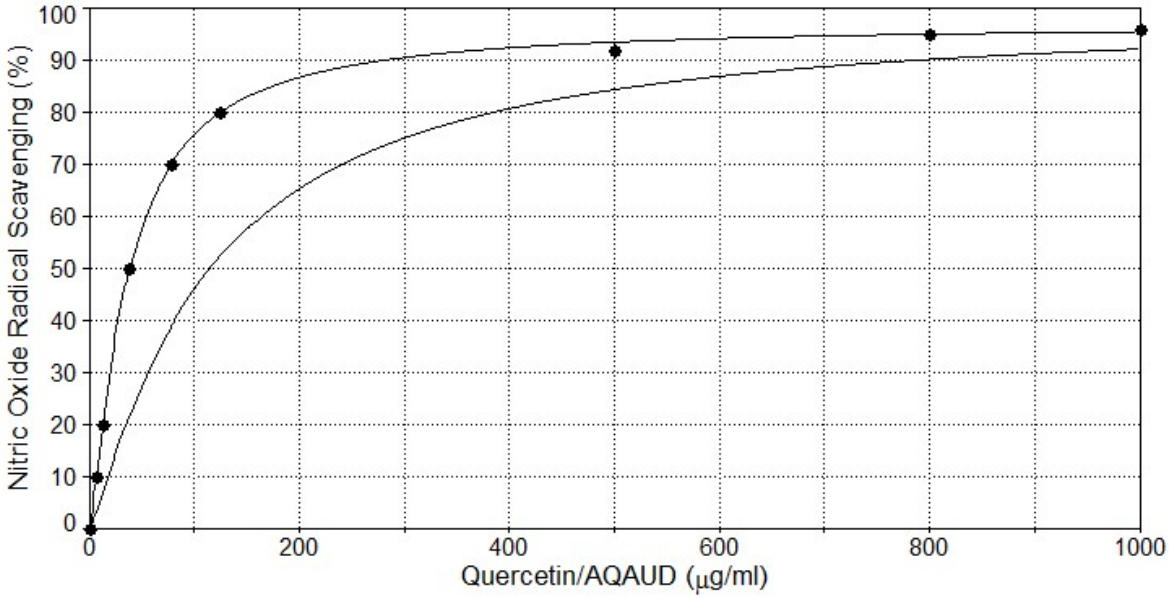


Figure 4.1: Nitric oxide radical Scavenging effect of graded concentrations of quercetin •—• and Aqueous Anti-Ulcer Drug ——(AQAUD).

4.6 Hydroxyl radical scavenging effect of graded concentrations of quercetin and Aqueous Anti-Ulcer Drug (AQAUD).

Hydroxyl radical scavenging result (fig 4.2) showed that the "AQAUD" scavenged hydroxyl radicals. With increasing concentration of the "AQAUD", hydroxyl radicals generated decreased. The threshold inhibitory concentrations were IC_5 =74.920±3.75µg/ml and IC_{20} =146.280±7.31µg/ml (Table 4.4). Thus, 146.280±7.31µg/ml of "AQAUD" was able to scavenge the hydroxyl radicals generated by 20%.

Quercetin also scavenged hydroxyl radicals with increased concentration of quercetin. The threshold inhibitory concentrations were IC_5=49.570±2.48µg/ml, IC_{20} =236.360±11.82µg/ml, and IC_{80}=3525.660±176.28µg/ml.

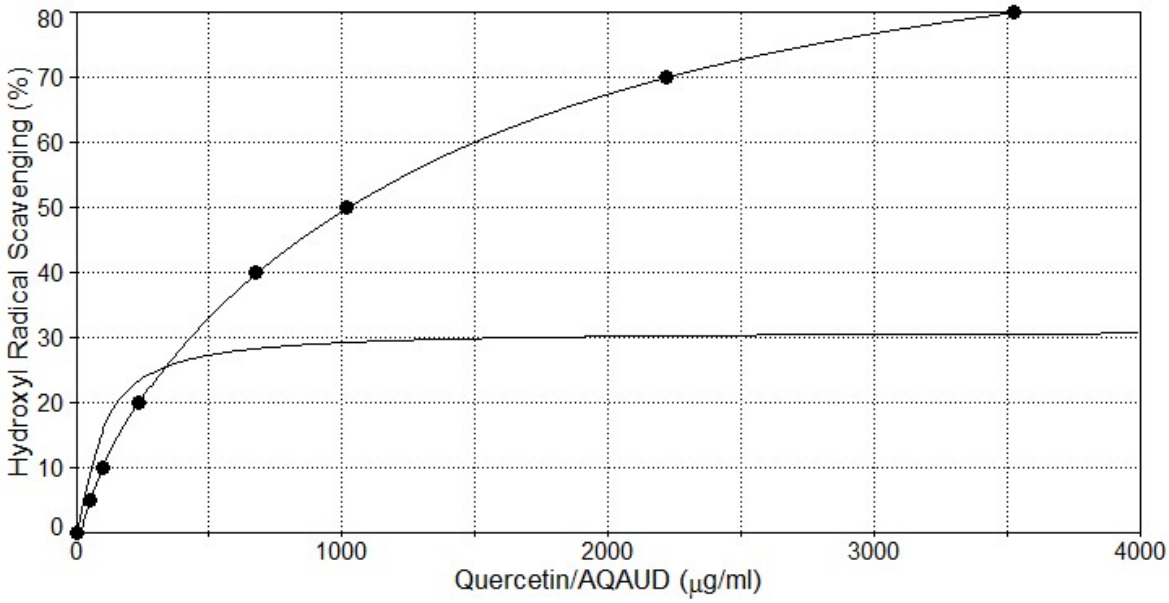


Figure 4.2: Hydroxyl radical Scavenging effect of graded concentrations of quercetin •——•

and Aqueous Anti-Ulcer Drug —— (AQAUD).

4.7 Effect of "AQAUD" administration on free acidity of the gastric content in aspirin induced gastric ulcers in albino rats.

Figure 4.3 shows the effect of "AQAUD" on free acidity of gastric content during aspirin induced ulcers in albino rats. Aspirin significantly increased the free acidity of the gastric content ($p<0.05$) when compared to the control. Free acidity in control group was 8.800±2.646. For aspirin group, the value was 13.600±2.160."AQAUD" 250mg/kg.b.wt., "AQAUD" 500mg/kg.b.wt. and omeprazole had the values 12.000±2.082, 8.750±1.732 and 11.00±1.414 respectively.

"AQAUD" 500mg/kg.b.wt. significantly decreased the free acidity ($p<0.05$) of the gastric content when compared to the aspirin while "AQAUD" 250mg/kg.b.wt. and omeprazole did not show a significant ($p>0.05$) difference when compared to the control.

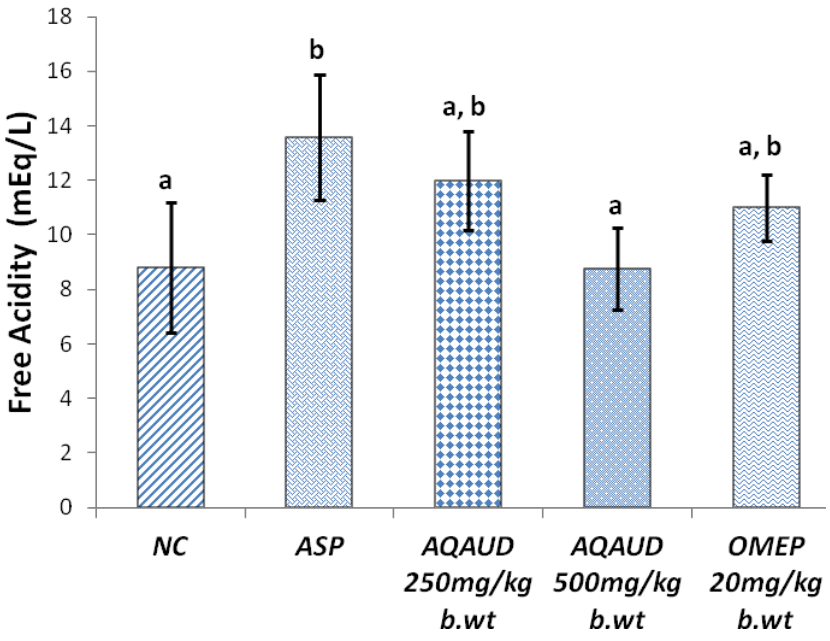


Figure 4.3: Effect of *Aqueous* Anti-Ulcer Drug (AQAUD) administration on the free acidity of gastric content in aspirin induced gastric ulcers in male albino rats. Results are in mean ± Standard deviation of 5 determinations and statistical significance among groups was at ($P<0.05$). Values with different superscripts are significantly different at $P<0.05$.

4.8 Effect of "AQAUD" administration on the total acidity of the gastric content in aspirin induced gastric ulcers in albino rats.

Figure 4.4 shows the effect of "AQAUD" on total acidity of the gastric content during aspirin induced gastric ulcers in albino rats.

Aspirin significantly increased the total acidity of the gastric content ($p \leq 0.05$) when compared to the control. Total acidity in control group was 18.800±4.899. For aspirin group, the value was 27.600±2.645. "AQAUD" 250mg/kg.b.wt., "AQAUD" 500mg/kg.b.wt. and omeprazole decreased the raised total acidity of the gastric content (23.500±3.000, 21.500±2.000 and 21.600±5.315 respectively) but the decrease was not significant ($p \geq 0.05$) when compared to the control.

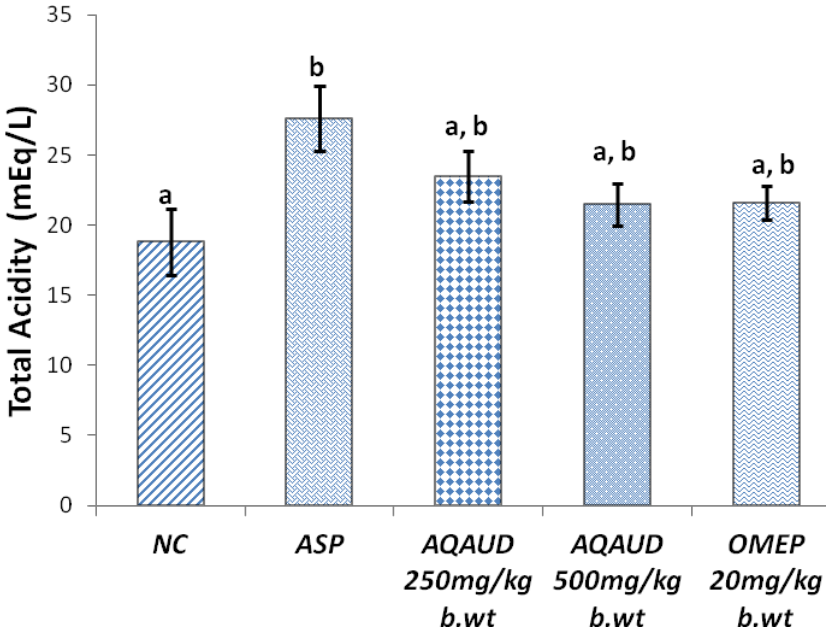


Figure 4.4: Effect of *Aqueous* Anti-Ulcer Drug (AQAUD) administration on the total acidity of gastric content in aspirin induced gastric ulcers in male albino rats. Results are in mean ± Standard deviation of 5 determinations and statistical significance among groups was at ($P<0.05$). Values with different superscripts are significantly different at $P<0.05$.

4.9 Effect of "AQAUD" administration on the ulcer index of the gastric mucosa in aspirin induced gastric ulcers in albino rats.

Figure 4.5 shows the effect of "AQAUD" on ulcer index of the gastric mucosa during aspirin induced gastric ulcers in albino rats. The ulcer index values for the control, aspirin, "AQAUD" 250mg/kg.b.wt, "AQAUD" 500mg/kg.b.wt and omeprazole groups were: 0.000, 8.500±0.425, 0.833±0.042, 2.500±0.125 and 1.333±0.067 respectively. Aspirin significantly ($p<0.05$) increased the ulcer index when compared to the control. "AQAUD" 250mg/kg.b.wt significantly reduced the elevated ulcer index when compared to the control. "AQAUD" 500mg/kg.b.wt and omeprazole significantly ($p<0.05$) reduced the ulcer index and were maintained near normal

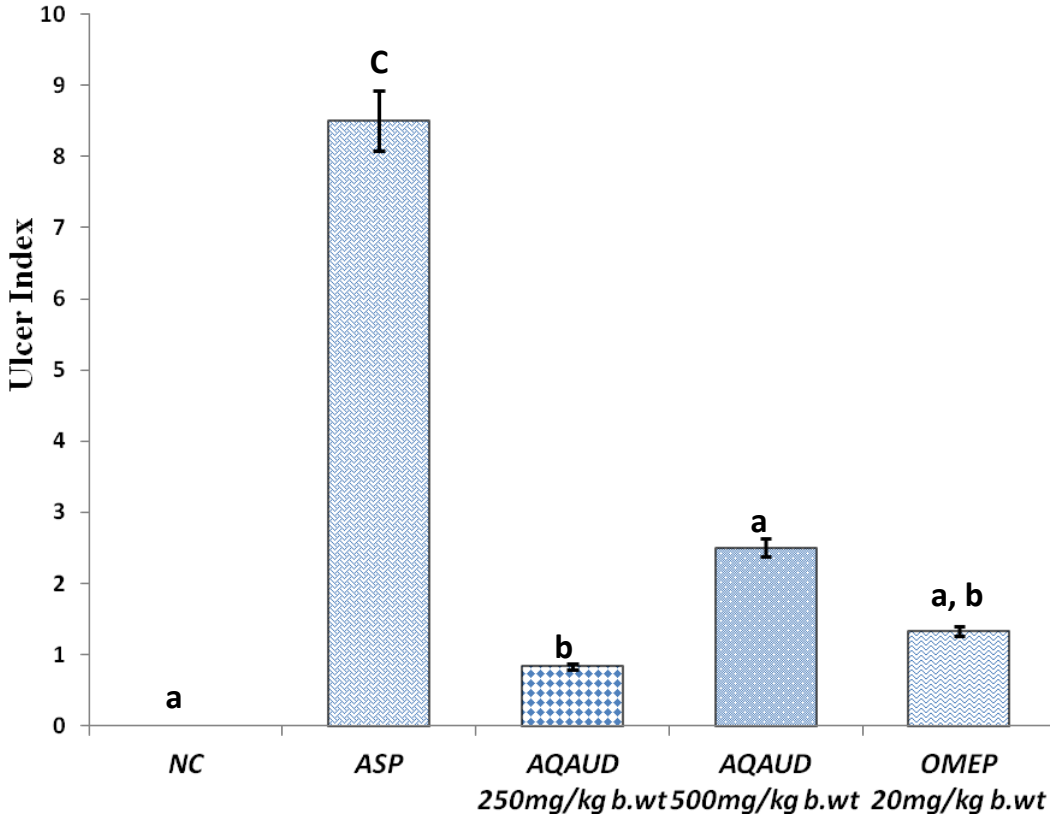


Figure 4.5: Effect of *Aqueous* Anti-Ulcer Drug (AQAUD) administration on the ulcer index of gastric mucosa in aspirin induced gastric ulcers in male albino rats. Results are in mean ± Standard deviation of 5 determinations and statistical significance among groups was at ($P<0.05$). Values with different superscripts are significantly different at $P<0.05$.

4.10 Effect of "AQAUD" administration on the % protection of the gastric mucosa against aspirin induced gastric ulcers in albino rats.

Figure 4.6 shows the effect of "AQAUD" on the % protection of the gastric mucosa against aspirin induced gastric ulcers in albino rats. "AQAUD" 250mg/kg.b.wt, 500mg/kg.b.wt and omeprazole offered the % protection of 90.196%, 70.588%and 84.314% respectively.

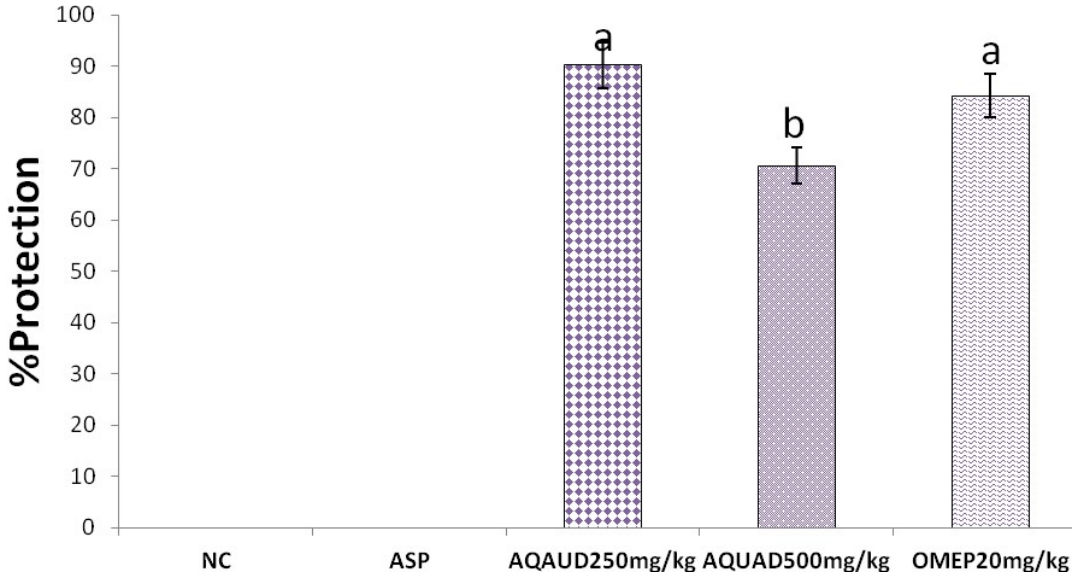


Figure 4.6: Effect of *Aqueous* Anti-Ulcer Drug (AQAUD) administration on the %protection of gastric mucosa against aspirin induced gastric ulcers in male albino rats. Results are in mean ± Standard deviation of 5 determinations and statistical significance among groups was at ($P<0.05$).Values with different superscripts are significantly different at $P<0.05$.

4.11 Effect of "AQAUD" administration on the total protein concentration of the stomach homogenates in aspirin induced gastric ulcers in albino rats

Protein concentration values for the control, aspirin, "AQAUD" 250mg/kg.b.wt, "AQAUD" 500mg/kg.b.wt and omeprazole groups were: 7.200±0.910, 5.290±0.830, 5.770±0.290, 6.100±0.170 and 7.130±0.690 respectively.

"AQAUD" 500mg/kg.b.wt significantly increased the protein concentration of the stomach homogenate ($p \leq 0.05$) and was maintained near normal. The same was observed for omeprazole.Aspirin significantly decreased the total protein concentration of the stomach homogenate ($p \leq 0.05$) when compared to the control. "AQAUD" 250mg/kg.b.wt elevated the reduced protein concentration but not significantly ($p \geq 0.05$) when compared to the aspirin group (Fig 4.7).

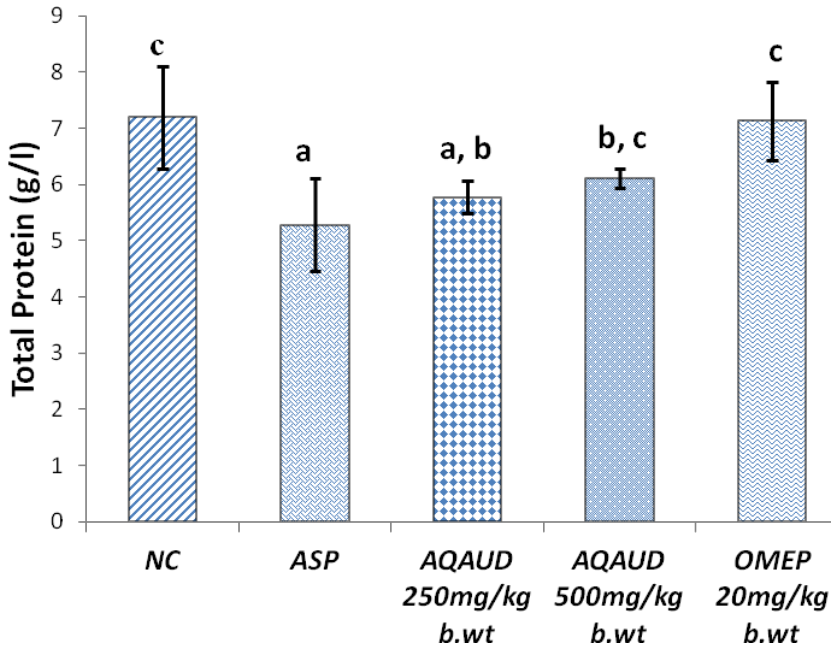


Figure 4.7: Effect of *Aqueous* Anti-Ulcer Drug (AQAUD) administration on the total protein concentration of the stomach homogenate in aspirin induced gastric ulcers in male albino rats. Results are in mean ± Standard deviation of 5 determinations and statistical significance among groups was at ($P<0.05$). Values with different superscripts are significantly different at $P<0.05$.

4.12 Effect of "AQAUD" administration on nitrite concentration of the stomach homogenates in aspirin induced gastric ulcers in albino rats.

Nitrite concentration values for the control, aspirin, "AQAUD" 250mg/kg.b.wt, "AQAUD" 500mg/kg.b.wt and omeprazole groups were: 0.076±0.009, 0.164±0.009, 0.120±0.011, 0.091±0.009 and 0.111±0.013 respectively (Fig. 4.8).

Nitrite concentration of the stomach homogenates was increased significantly ($p \leq 0.05$) by aspirin when compared to the control. "AQAUD" 250mg/kg.b.wt and "AQAUD" 500mg/kg.b.wt significantly ($p \leq 0.05$) decreased the elevated nitric oxide radicals when compared to the aspirin group. Omeprazole also decreased significantly the aspirin-raised nitrite concentration when compared to the control (Fig 4.8).

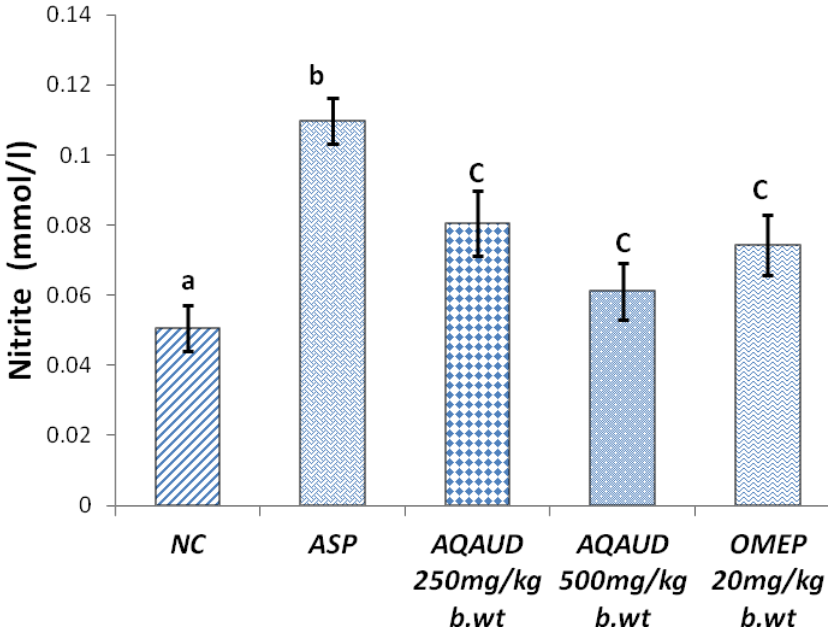


Figure 4.8: Effect of *Aqueous* Anti-Ulcer Drug (AQAUD) administration on nitrite concentration of the stomach homogenate in aspirin induced gastric ulcers in male albino rats. Results are in mean ± Standard deviation of 5 determinations and statistical significance among groups was at ($P<0.05$). Values with different superscripts are significantly different at $P<0.05$.

4.13 Effect of "AQAUD" administration on Superoxide Dismutase (SOD) activity of the stomach in aspirin induced gastric ulcers in albino rats.

SOD activity values for the control, aspirin, "AQAUD" 250mg/kg.b.wt, "AQAUD" 500mg/kg.b.wt and omeprazole groups were: 37.240±5.387, 23.638±2.906, 32.311±2.338, 34.880±5.895 and 30.403±4.564 respectively (Fig. 4.9).

Aspirin significantly ($p < 0.05$) decreased the SOD activity of the stomach homogenate when compared to the control. "AQAUD" 250mg/kg.b.wt and "AQAUD" 500mg/kg.b.wt significantly increased the SOD activity and restored enzyme activity to normal.Omeprazole increased the SOD activity but not significantly ($p > 0.05$) when compared to the aspirin group. (Fig. 4.9).

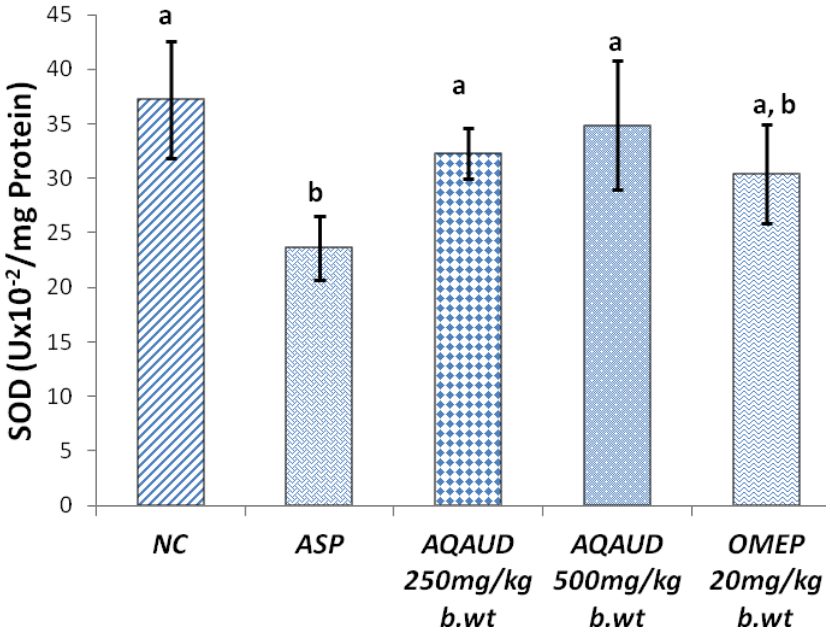


Figure 4.9: Effect of *Aqueous* Anti-Ulcer Drug (AQAUD) administration on Superoxide dismutase activity of the stomach homogenate in aspirin induced gastric ulcers in male albino rats. Results are in mean ± Standard deviation of 5 determinations and statistical significance among groups was at ($P<0.05$). Values with different superscripts are significantly different at $P<0.05$.

4.14 Effect of" AQAUD" administration on Catalase (CAT) activity of the stomach homogenate in aspirin induced gastric ulcers in albino rats.

CAT activity values for the control, aspirin, "AQAUD" 250mg/kg.b.wt, "AQAUD" 500mg/kg.b.wt and omeprazole groups were: 63.383±1.425, 35.632±1.898, 43.560±6.467, 63.255±5.879 and 61.604±5.582 respectively.

Aspirin significantly ($p<0.05$) decreased the CAT activity when compared to the control. "AQAUD" 250mg/kg.b.wt could not significantly ($p>0.05$) increased the CAT activity when compared to the aspirin group. "AQAUD" 500mg/kg.b.wt and omeprazole significantly increased the CAT activity when compared to the aspirin group and were able to restore Catalase activity to its normal control activity.

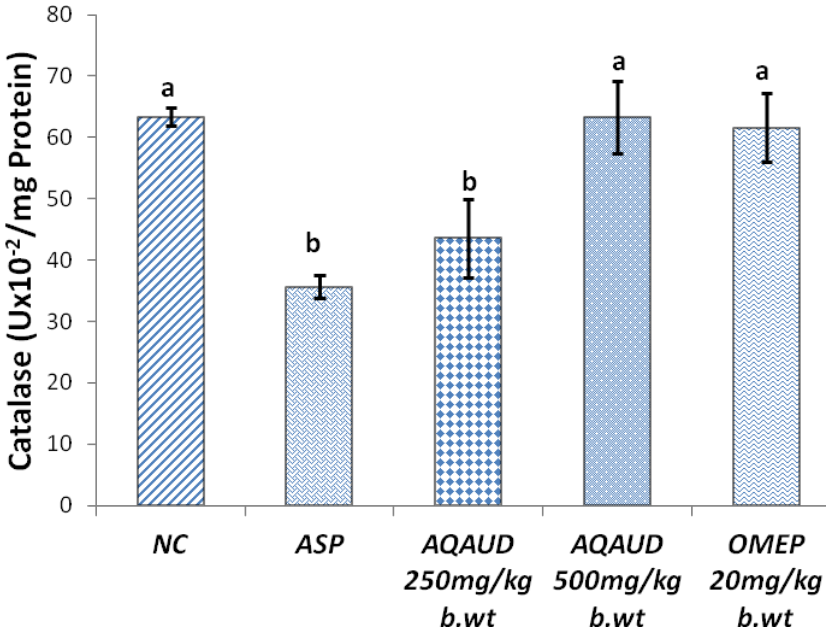


Figure 4.10: Effect of *Aqueous* Anti-Ulcer Drug (AQAUD) administration on Catalase activity of the stomach homogenate in aspirin induced gastric ulcers in male albino rats. Results are in mean ± Standard deviation of 5 determinations and statistical significance among groups was at ($P<0.05$). Values with different superscripts are significantly different at $P<0.05$.

4.15 Effect of "AQAUD" administration on lipid peroxidation of the stomach homogenate in aspirin induced gastric ulcers in albino rats.

Result of our study showed that lipid peroxidation measured as MDA concentration for the control, aspirin, "AQAUD" 250mg/kg.b.wt, "AQAUD" 500mg/kg.b.wt and omeprazole groups were: 7.530E-04±3.370E-05, 1.010E-03±4.900E-05, 9.410E-04±5.800E-05, 8.730E-04±3.250E-05 and 6.920E-04±1.830E-04 respectively.

Aspirin significantly ($p \leq 0.05$) increased MDA concentration when compared to the normal control. "AQAUD" 500mg/kg.b.wt significantly reduced the aspirin- elevated MDA concentration when compared to the aspirin group. There was a significant difference ($p \leq 0.05$) between omeprazole and aspirin group (Fig. 4.11). "AQAUD" 500mg/kg.b.wt and omeprazole conferred significant protection against peroxidation more than "AQAUD" 250mg/kg.b.wt when compared to aspirin group.

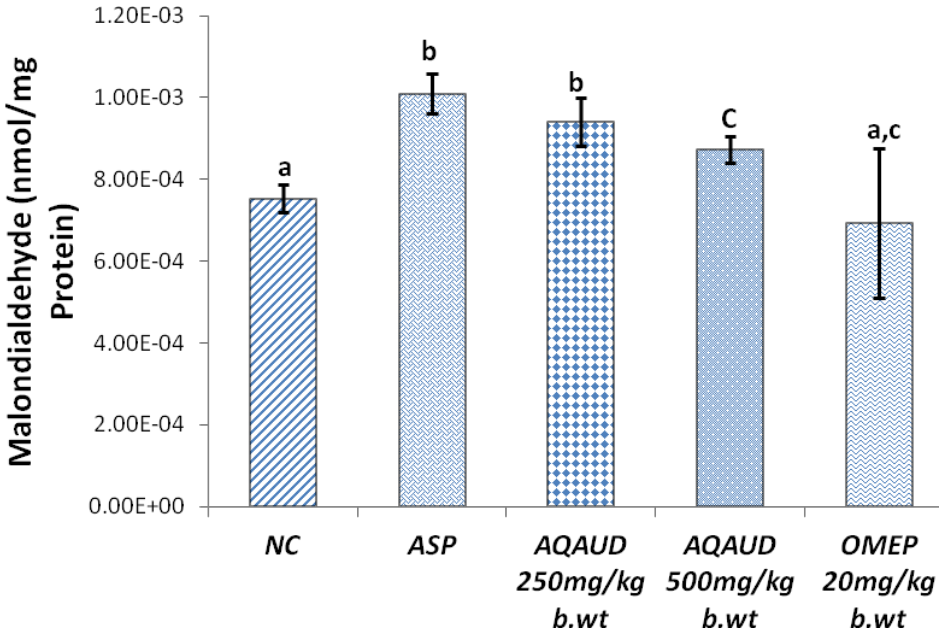


Figure 4.11: Effect of *Aqueous* Anti-Ulcer Drug (AQAUD) administration on Malondialdehyde concentration of the stomach homogenate in aspirin induced gastric ulcers in male albino rats. Results are in mean ± Standard deviation of 5 determinations and statistical significance among groups was at ($P<0.05$). Values with different superscripts are significantly different at $P<0.05$.

4.16 Effect of "AQAUD" administration on reduced glutathione (GSH) concentration of the stomach homogenate in aspirin induced gastric ulcers in albino rats.

GSH concentration values for the control, aspirin, "AQAUD" 250mg/kg.b.wt, "AQAUD" 500mg/kg.b.wt and Omeprazole groups were: 7.369±1.193, 6.922±1.752, 12.303±2.940, 10.455±1.609 and 6.737±0.941 respectively.

From the result, aspirin non-significantly ($p>0.05$) decreased the GSH concentration when compared to the control."AQAUD" 250mg/kg.b.wt significantly ($p<0.05$) increased the GSH concentration when compared to the control while the difference between "AQAUD" 500mg/kg.b.wt and omeprazone is not significant ($p>0.05$) when compared to the control and aspirin groups (Fig. 4.12).

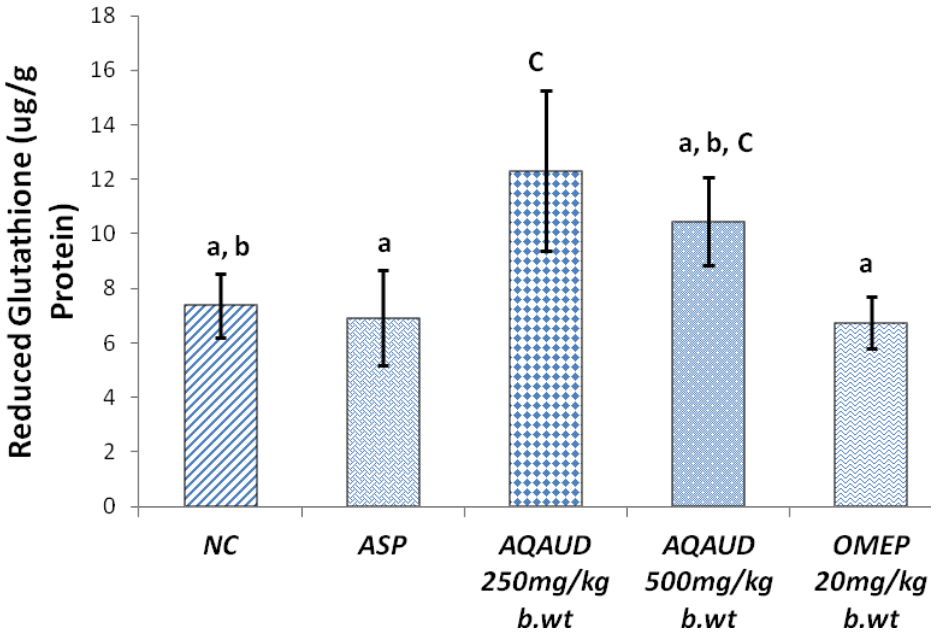


Figure 4.12: Effect of *Aqueous* Anti-Ulcer Drug (AQAUD) administration on reduced glutathione concentration of the stomach homogenate in aspirin induced gastric ulcers in male albino rats. Results are in mean ± Standard deviation of 5 determinations and statistical significance among groups was at ($P<0.05$). Values with different superscripts are significantly different at $P<0.05$.

4.17 Determination of the LD_{50} of the Aqueous Anti-Ulcer Drug (AQAUD).

Acute toxicity studies revealed the non-toxic nature of the "AQAUD". No toxic reaction was found in any of the selected doses (10mg, 100mg, 1000mg, and 5000mg) till the end of the study. All mice were healthy and active during the experimental period. Not a single rat died during the study. This shows that "AQAUD" is generally safe (LD_{50} > 5000mg/kg.b.wt).

Table 4.5: Determination of the LD_{50} of the Aqueous Anti-Ulcer Drug (AQAUD).

Group of rats	Dose of AQAUD (mg/kg b.wt.)	No. of death recorded
Phase1		
1	10	0/3
2	100	0/3
3	1000	0/3
Phase 2		
4	1600	0/2
5	2900	0/2
6	5000	0/2

4.18 Histology studies of Rat stomachs administered with Aspirin, "AQAUD" 250mg/kgb.wt., 500mg/kgb.wt. Omeprazole 20mg/kgb.wt.

Histology observation indicated ulceration of the surface epithelium, Lamina proporia and muscularis mucosa of the gastric mucosa in aspirin intoxicated rats (plate 2). It also showed regeneration of cells of the gastric mucosa in rats treated with "AQAUD" 250mg/kgb.wt. (Plate 3), with little ulceration evident in "AQAUD" 500mg/kgb.wt. and Omeprazole 20mg/kgb.wt. (Plate 4 and 5)

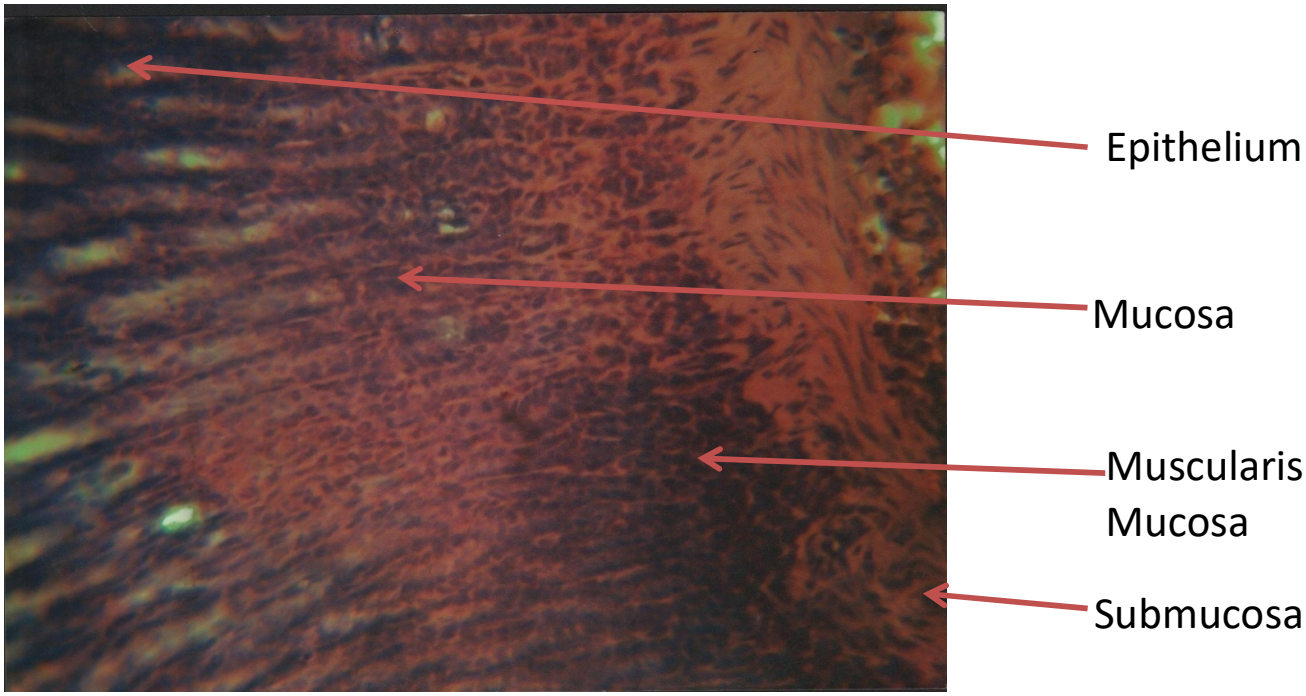


Plate 1 (Normal control):The gastric mucosa consists of a surface epithelium that invaginates to various extents into the lamina propria, forming gastric pits. Emptying into the gastric pits are branched, tubular glands. The lamina propria of the stomach is composed of loose connective tissue. Separating the mucosa from the underlying submucosa is a layer of smooth muscle, the muscularis mucosa. The morphological features of this section are in line with that of a normal stomach (x400), stain: Hematoxylin (H) and Eosin (E).

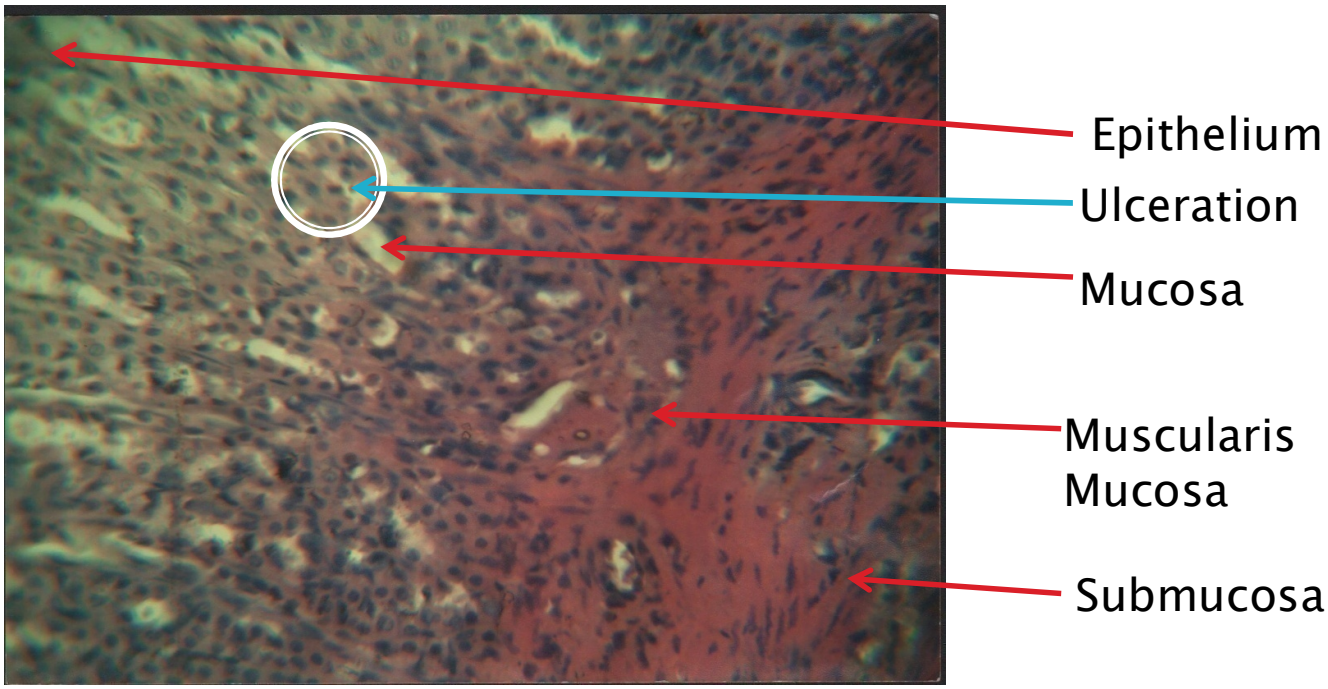


Plate 2 (Stomach of rat intoxicated with Aspirin 400mg/kg.b.wt to induce ulcer): There is ulceration of the surface epithelium, lamina propria and the muscularis mucosa. (x400), stain: H and E.

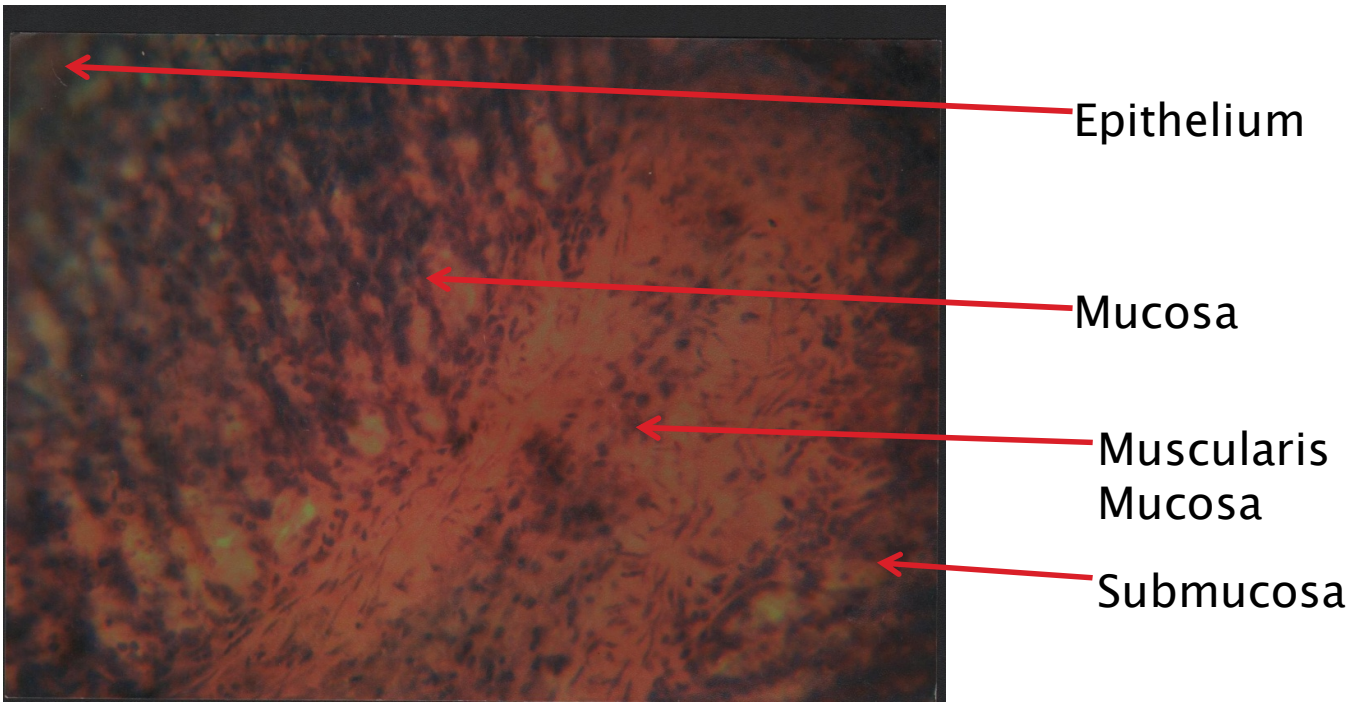


Plate 3 (Stomach of rat intoxicated with Aspirin 400mg/kg.b.wt to induce ulcer + treatment with AQAUD 250mg/kg.b.wt): There is regeneration of the gastric mucosa, submucosa and muscularis mucosa. (x400), stain: H and E.

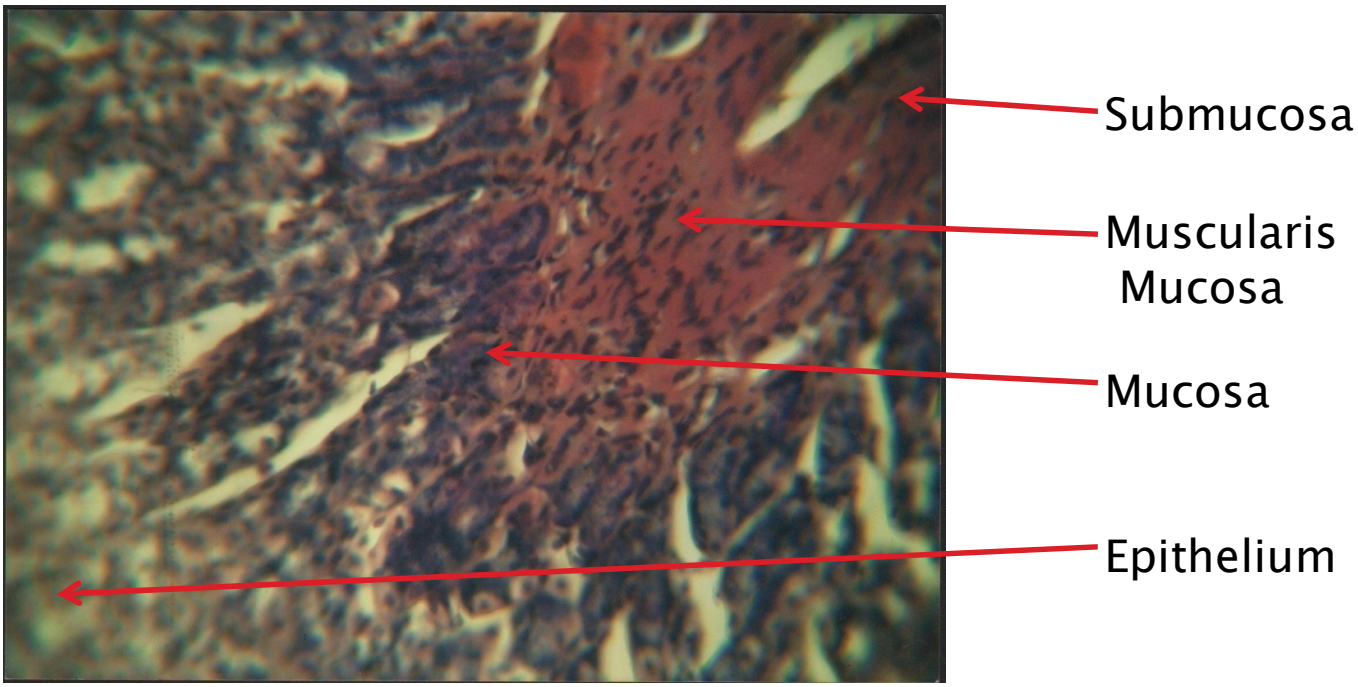


Plate 4 (Stomach of rat intoxicated with Aspirin 400mg/kg.b.wt to induce ulcer + treatment with AQAUD 500mg/kg.b.wt.):

There is ulceration of the gastric mucosa, submucosa and muscularis mucosa. (x400), stain: H and E.

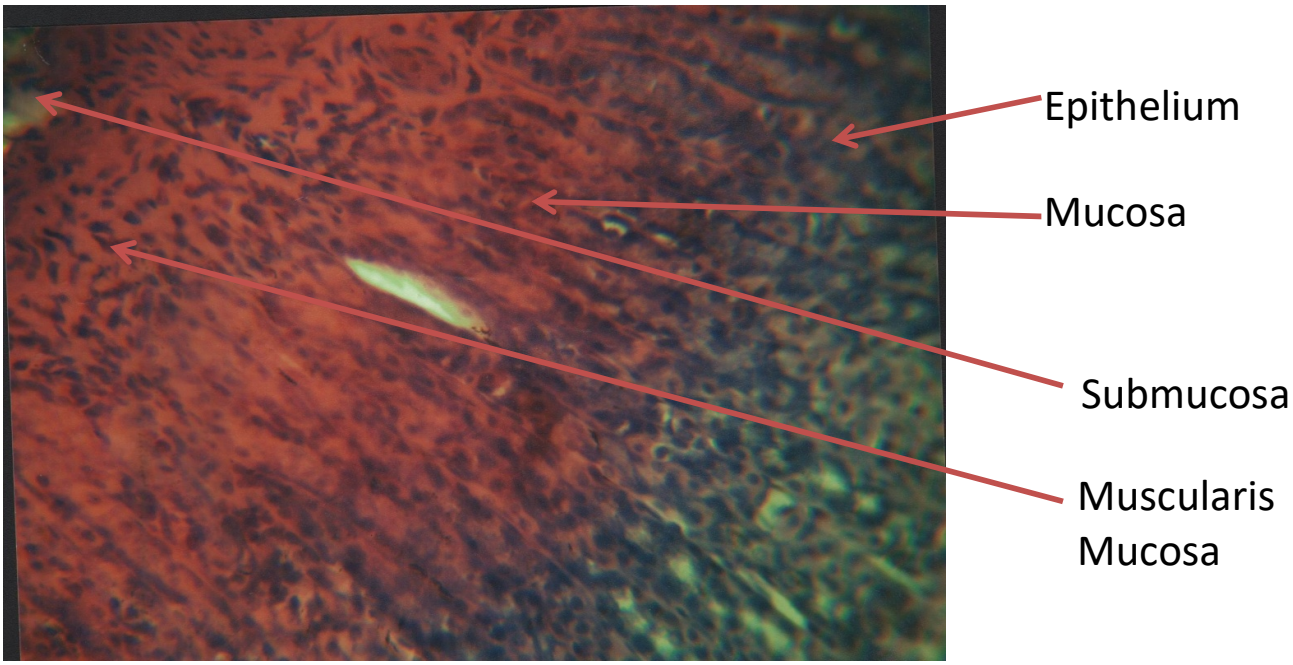


Plate 5 (Stomach of rat intoxicated with Aspirin to induce ulcer + treatment with Standard drug, Omeprazole 20mg/kg.b.wt): There is regeneration of the gastric mucosa, submucosa and muscularis mucosa but little ulceration was still evident. (x400), stain: H and E.

4.19 DISCUSSION

The present study evaluated the protective role and antioxidant potentials of Aqueous Anti-Ulcer Drug (AQAUD) on aspirin induced gastric ulcers in albino rats with the possible involvement of antioxidants. AQAUD is an anti-ulcer herbal medicamenet which is composed of juice from *Citrus aurantifolia* (lime), Gbogbonise, *Strophantus hispidus* (root) and *Ipomoea mauritana* (root). The phytochemical analysis showed that the "AQAUD" contains flavonoids, phenols, alkaloids, glycosides and tannins. These compounds have been found to inhibit bacterial growth and are capable of protecting certain plants against bacterial infections (Clark 1981, Okwu, 2004). Phenolic compounds and flavonoids are well known antioxidants and scavenging agents against free radical associated with oxidative damage and ulcers (Ferguson *et al.,* 2006). The presence of these compounds in AQAUD may give credence to its local usage in the treatment of peptic ulcers. Flavonoids are important secondary metabolites in plants, modulating lipid peroxidation involved in artherogenesis, thrombosis and carcinogenesis. It has been confirmed that pharmacological effect of flavonoids is correlating with their antioxidant activities (Shi *et al.,* 2006).

Nitric oxide is a key signaling molecule that plays a crucial role in the pathogenensis of various diseases associated with inflammation (Gates *et al.,* 2008). It is a free radical generated from sodium nitroprusside in aqueous solution at physiological PH and reacts with oxygen to form oxides of nitrogen. Nitrite is one of the oxides of nitrogen which was significantly inhibited by plant extracts through direct competition with oxygen in the reaction medium (Alisi and Onyeze, 2008). The scavenging activity of AQAUD against nitric oxide formation was comparable to those of the standard drug (quercetin) used in this study. The IC_{50} and IC_{80} for the AQAUD and quercetin were 114.66±5.73µg/ml and 38.44±1.92 µg/ml and 383.73±19.19µg/ml and 124.31±6.22 µg/ml respectively. However, Quercetin scavenged nitric oxide better than AQAUD. It can be inferred that the presence and the quantity of antioxidant compounds in AQAUD could justify the observed results and thus may give support to its traditional use in the treatment of diseases caused by inflammation and cellular damage like ulcers. The mechanism of action of flavonoids and phenolic compounds are through scavenging process (Cook and Samman, 1996). Nitric oxide scavenging effect of AQAUD could be due to the phenolic and flavonoid compounds present in it.

Hydroxyl radical has been implicated in the oxidative damage of DNA, proteins and lipids (Spencer *et al.,*1994). The hydroxyl radicals generated by Fe^{2+}-ascorbic acid and EDTA-H_2O_2 system (Fenton's reaction) was scavenged by the AQAUD and quercetin in a concentration dependent manner. The IC_{20} for the both were 146.28±7.31µg/ml and 236.36±11.82µg/ml respectively. This observation showed that quercetin is a better hydroxyl radical scavenger. However, AQAUD can be used as a remedy to combat the oxidative activity of hydroxyl radical in the absence of quercetin.

In gastric secretion parameters, aspirin significantly increased ($P<0.05$) the free and total acidity of the gastric contents. AQAUD 250mg/kg b.wt treatment did not show a significant difference in free acidity, while AQAUD 500mg/kgb.wt significantly decreased ($P<0.05$) the free acidity. Also, AQAUD 250mg/kg b.wt.and 500mg/kg b.wt did not show a significant difference in total acidity of the gastric content. Aspirin induced gastric ulcers with the erosion of the gastric mucosa. In ulcer index, aspirin also significantly increased ($P<0.05$) the ulcer index of the gastric mucosa. AQAUD significantly reduced the ulcer index of the gastric mucosa with attendant regeneration of gastric mucosa. These results could be explained by the fact that prostaglandins normally protect the gastrointestinal mucosa from damage by maintaining blood flow and increasing mucosal secretion of mucous and bicarbonate (Voultilalnen *et al.,* 2001). NSAID's like aspirin cause mucosal damage by interfering with prostaglandins synthesis, increasing acid secretion and block the diffusion of H+ (Roa *et al.,* 1999). Aspirin blockade of cyclooxygenase-1 (COX-1) and (COX II) results in reduction of prostaglandins synthesis. This interruption of prostaglandins synthesis results in impairment of mucosal damage repair, thus facilitating mucosal injury (Burke *et al.,* 2006).

Free radicals are involved in the development of gastric ulcers. If the generation of free radicals exceeds the ability of these free radical scavenging enzymes, gastric mucosa may be injured by the free radicals resulting to the development of gastric ulcer (Vanisree *et al.,* 1996). From the experimental results, aspirin administration significantly decreased the SOD and CAT activities in the stomach homogenates, which is likely to be due to free radicals generation. AQAUD 250mg/kg b.wt. and 500mg/kg b.wt significantly raised up decreased SOD and CAT activities to almost normal. These observations may be attributed to the flavonoid and phenolic contents of AQAUD which are known antioxidants and scavenging agents against free radicals associated oxidative damage and ulcers (Ferguson *et al.,* 2006).

Reduced glutathione (GSH) is a tripeptideand superoxide radical scavenger. It protects thiol protein groups required for maintaining the integrity of cells against oxidation (Masella *et al.,* 2005). GSH is present in the stomach at high concentration and plays an important role in maintaining the integrity of the gastric mucosa (Altinkaynak *et al.,* 2003). From the results, aspirin did not reduced GSH concentration, when compared to the control. This may be as a result of other compensatory mechanisms. AQAUD 250mg/kg b.wt significantly ($P<0.05$) increased the GSH concentration when compared to the control. This could be linked to the flavonoid and phenolic content of the AQAUD as well as its radical scavenging ability. It is possible that flavonoid and phenolic compounds had a sparing effect on the GSH concentration or administration increased the GSH biosynthetic capability of the cells from the cystein amino acids present in the body. AQAUD 500mg/kg b.wt. and omeprazole increased GSH concentration but not significant when compared to the control. The result of the acute toxicity test showed that AQAUD is generally safe at a concentration of 5000 mg/kg b.wt since no death was observed up to the concentration of 5000mg/kg b.wt in mice.

Malondialdehyde (MDA) concentration can be used to assess lipid peroxidation used as an index to quantify the damage that occurs in membranes of tissues as a result of free radical generation (Dianzani, 1985). Gastric mucosal lipid peroxidation has been reported to increase in incidence of experimental ulcers (Sairam *et al.,* 2001). From the results, oral administration of aspirin significantly ($P<0.05$) increased the MDA concentration in the stomach homogenates. This significant elevation of MDA concentration in aspirin treated rats is possibly due to the generation of free radicals through metal ion or superoxide catalyzed peroxidation process.

Also from the results, AQAUD 250mg/kg b.wt and 500mg/kg b.wt reduced the elevated MDA concentration but not significantly when compared to the control. This reduction may be linked to the flavonoids and phenolic compound content of AQAUD which function as antioxidants that scavenged free radicals. It has been shown that both lime juice and peels (*Citrus aurantifolia*) present in AQAUD demonstrated antioxidant properties (Maryam and Jamal, 2001). AQAUD 500mg/kg b.wt. was comparable to omeprazole (the standard antiulcer drug) in reducing MDA concentration.

In this study also, significant elevation of nitrite concentration in gastric mucosa was observed in aspirin induced gastric ulceration. AQAUD 250mg/kg b. wt and 500mg/kg b. wt significantly ($P<0.05$) reduced the elevated nitrite level in the gastric mucosa. Nitric oxide is

a mediator of gastro-intestinal mucosal defense but paradoxically, it also contributes to mucosal damage. The synthesis of nitric oxide is mediated by the enzyme nitric oxide synthase (NOS), which is present in gastric mucosa in two constitutive (cNOS) isoforms, namely endothelial (eNOS) and neuronal (nNOS) (Cho, 2001). The inducible (iNOS) enzyme is found in macrophages and neutrophils (Wallace and Miller, 2000). In the digestive system, nitric oxide produced by cNOS is cytoprotective and nitric oxide produced by iNOS is cytotoxic (Motawi *et al.,* 2007). Nitric oxide at low concentration (from cNOS) plays a role in protecting the integrity of epithelial tissues by improving the mucosal blood flow in the GIT system (Whittle, 1994). This protective effect of nitric oxide is because of the inhibition of activation, adhesion and migration of leucocytes in the inflammatory reaction (Banick *et al.,* 1997).

NSAIDs (indomethacin) have been reported to decrease tissue cNOS-derived nitric oxide (Motawi *et al.,*2007, Motawi*et al.,*2008). Souza *et al.,*(2004) used genetic inducible nitric oxide synthase (iNOS) deficient mice to prove that iNOS-generated nitric oxide is involved in gastric damage. Nitric oxide produced from activation of iNOS reacts directly with superoxide to form peroxynitrite, a potent autotoxic oxidant that causes gastric damage (Lanas, 2008). This could explain the increase in nitrite concentration (as an indicator of nitric oxide production) in gastric mucosa of aspirin treated rats compared with control which may be due to increased production of iNOS in gastric tissues. This result is consistent with those of many investigations (Prasenjit *et al., 2014.*) reporting that aspirin-induced gastropathy is associated with significant increase in gastric mucosal nitrite/nitrate levels.

Histological results showed that aspirin caused severe ulcerations of the surface epithelium, Lamina proporia and Muscularis mucosa of the gastric mucosa. This observation is in line with the biochemical parameters evaluated and could be as a result of free radicals generated and excess production of gastric acid that eroded the gastric mucosa. The significant regeneration of cells of the gastric mucosa and healing of the ulcerations observed with "AQAUD" 250mg/kg b.wt. could be due to efficient scavenging of the free radicals generated as a result of aspirin administration as well as its adequate antioxidant property. "AQAUD" 500mg/kg b.wt. showed significant regeneration of cells of the gastric mucosa but still had ulceration. It is possible that the anti-ulcer effect of "AQAUD" may be due to other mechanisms not well understood in this scope of study. This is because "AQAUD" 250mg/kg b.wt. was more protective than "AQAUD" 500mg/kg b.wt., yet "AQAUD" 500mg/kg b.wt. had a better antioxidant capacity."AQAUD" may be combining other mechanisms (not yet

clear) with antioxidative, blocking of Acid secretion and interference with cyclooxygenase activity. Omeprazole 20mg/kg b.wt.also showed regeneration of the cells of the gastric mucosa with little ulceration still evident. These observations showed that “AQAUD” 250mg/kg b.wt. might be the most appropriate dosage or concentration to be taken.

CHAPTER FIVE

5.0 CONCLUSION AND RECOMMENDATIONS

5.1 CONCLUSION

Aqueous Anti-Ulcer Drug (AQAUD) offered some protection against aspirin-induced gastric mucosal damage. The antioxidant compounds present in AQAUD played protective roles against the production of free radicals. The present study revealed that AQAUD administered at 250mg/kg b.wt. has promising potentals for the development of alternative treatment against gastric ulcers.

5.2 RECOMMENDATIONS

Based on the outcome of this study, the following recommendations were made:

1. Toxicological profile in this study was limited to the gastric mucosa. It is possible that AQAUD may have deleterious toxic effects on other organs like the liver, kidney, heart etc. Therefore, more histopathology should be carried out on these organs to ascertain if there is any toxic effect on them.
2. Also, the effect of AQAUD on haematological parameters should be studied to determine if there is any effect on them.
3. Chronic toxicity study should be carried out to know if the long term usage of AQAUD in the treatment of gastric ulcers can exert any toxic effect in animals.

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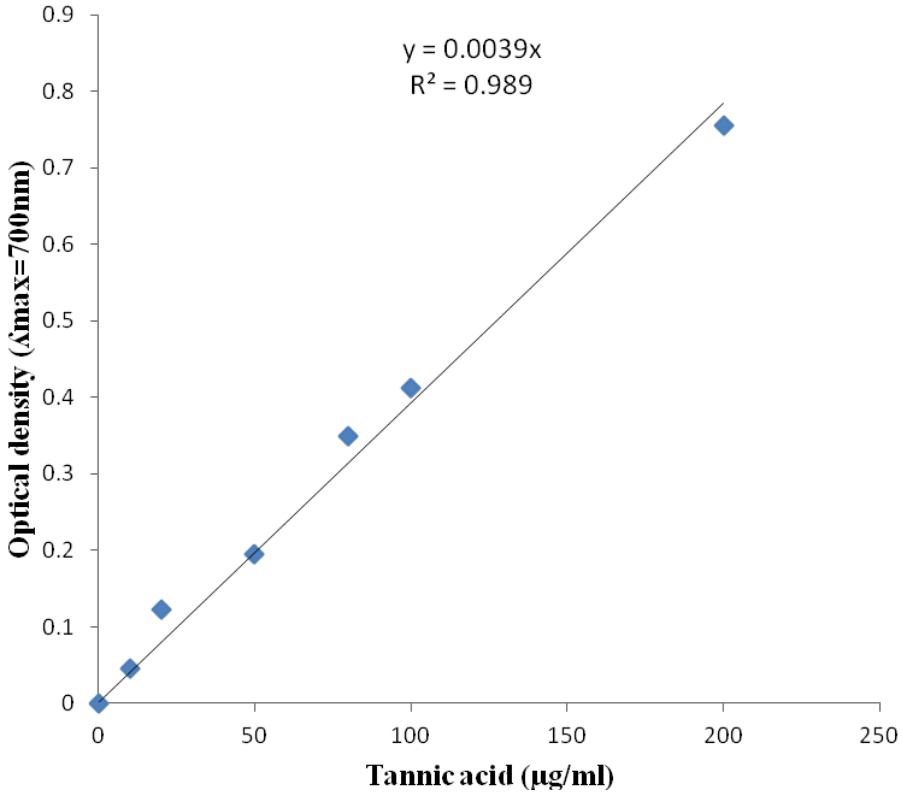
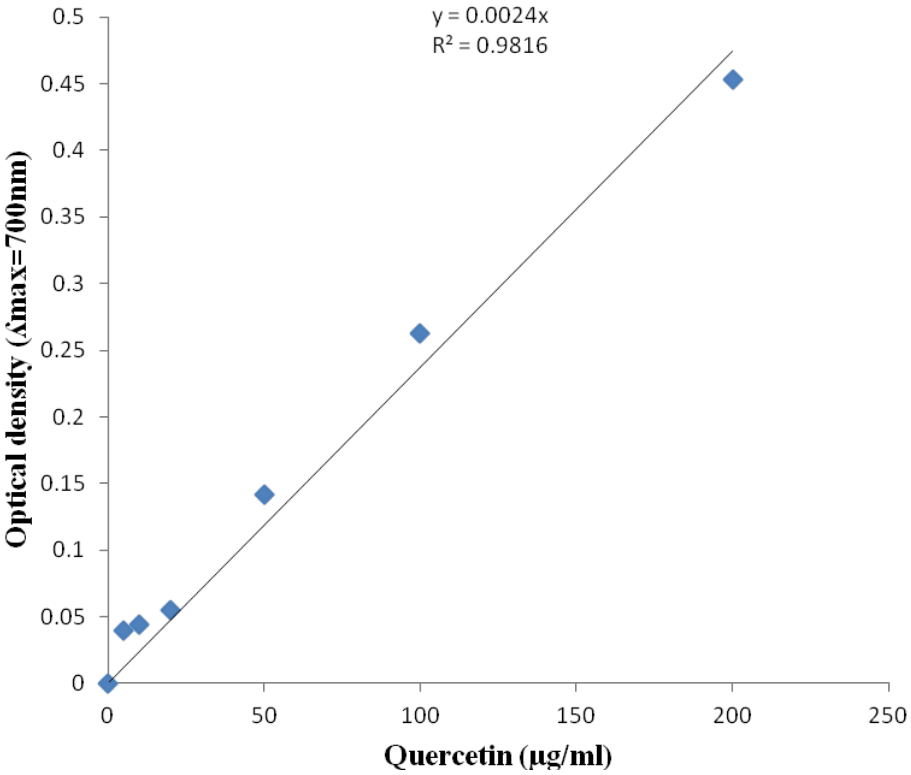
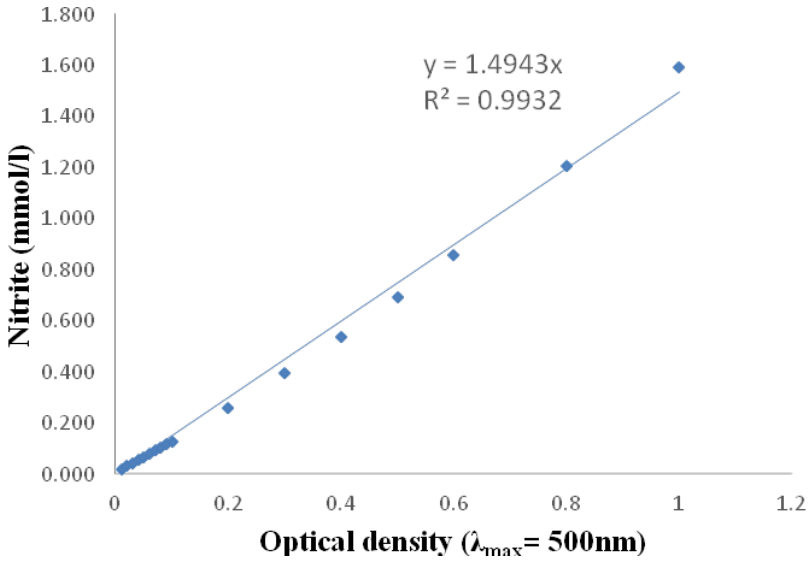
Appendix I



Calibration curve for calculation of total phenolic content



Calibration curve for calculation of flavonoid content



Nitrite Calibration curve for calculation of nitrite content

```
ONEWAY PROTECTION BY AntiulcerPropertiesOfAQAUDInAspirinInducedUlcer
  /STATISTICS DESCRIPTIVES HOMOGENEITY
  /MISSING ANALYSIS
  /POSTHOC=TUKEY DUNCAN ALPHA(0.05).
```

Oneway

Descriptives

PROTECTION

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
NC	6	.0000	.00000	.00000	.0000	.0000	.00	.00
ASP	6	.0000	6.44379	2.63067	-6.7623	6.7623	-5.88	5.88
AQAUD 250mg/kgbwt	6	70.5882	12.33893	5.03735	57.6393	83.5371	52.94	88.24
AQAUD 500mg/kgbwt	6	90.1961	11.56697	4.72219	78.0573	102.3349	76.47	100.00
OMEP 20mg/kgbwt	6	84.3137	17.71230	7.23102	65.7258	102.9016	52.94	100.00
Total	30	49.0196	42.53997	7.76670	33.1349	64.9043	-5.88	100.00

Test of Homogeneity of Variances

PROTECTION

Levene Statistic	df1	df2	Sig.
4.586	4	25	.006

ANOVA

PROTECTION

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	49273.356	4	12318.339	96.043	.000
Within Groups	3206.459	25	128.258		
Total	52479.815	29			

Post Hoc Tests

Multiple Comparisons

Dependent Variable: PERCENTAGE PROTECTION

	(I) AntiulcerPropertiesOfAQAUDInAspirinInducedUlcer	(J) AntiulcerPropertiesOfAQAUDInAspirinInducedUlcer	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Tukey HSD	NC	ASP	.00000	6.53856	1.000	-19.2029	19.2029
		AQAUD 250mg/kgbwt	-70.58824*	6.53856	.000	-89.7911	-51.3853
		AQAUD 500mg/kgbwt	-90.19608*	6.53856	.000	-109.3990	-70.9932
		OMEP 20mg/kgbwt	-84.31373*	6.53856	.000	-103.5166	-65.1108
	ASP	NC	.00000	6.53856	1.000	-19.2029	19.2029
		AQAUD 250mg/kgbwt	-70.58824*	6.53856	.000	-89.7911	-51.3853
		AQAUD 500mg/kgbwt	-90.19608*	6.53856	.000	-109.3990	-70.9932
		OMEP 20mg/kgbwt	-84.31373*	6.53856	.000	-103.5166	-65.1108
	AQAUD 250mg/kgbwt	NC	70.58824*	6.53856	.000	51.3853	89.7911
		ASP	70.58824*	6.53856	.000	51.3853	89.7911

AQAUD 500mg/kgbwt	AQAUD 500mg/kgbwt	-19.60784*	6.53856	.044	-38.8108	-.4049
	OMEP 20mg/kgbwt	-13.72549	6.53856	.252	-32.9284	5.4774
	NC	90.19608*	6.53856	.000	70.9932	109.3990
	ASP	90.19608*	6.53856	.000	70.9932	109.3990
	AQAUD 250mg/kgbwt	19.60784*	6.53856	.044	.4049	38.8108
OMEP 20mg/kgbwt	OMEP 20mg/kgbwt	5.88235	6.53856	.894	-13.3206	25.0853
	NC	84.31373*	6.53856	.000	65.1108	103.5166
	ASP	84.31373*	6.53856	.000	65.1108	103.5166
	AQAUD 250mg/kgbwt	13.72549	6.53856	.252	-5.4774	32.9284
	AQAUD 500mg/kgbwt	-5.88235	6.53856	.894	-25.0853	13.3206

*. The mean difference is significant at the 0.05 level.

Homogeneous Subsets

PROTECTION

	AntiulcerPropertiesOfAQAUDInAspirinInduced Ulcer	N	Subset for alpha = 0.05		
			1	2	3
Tukey HSD[a]	NC	6	.0000		
	ASP	6	.0000		
	AQAUD 250mg/kgbwt	6		70.5882	
	OMEP 20mg/kgbwt	6		84.3137	84.3137
	AQAUD 500mg/kgbwt	6			90.1961
	Sig.		1.000	.252	.894
Duncan[a]	NC	6	.0000		
	ASP	6	.0000		
	AQAUD 250mg/kgbwt	6		70.5882	
	OMEP 20mg/kgbwt	6			84.3137

AQAUD 500mg/kgbwt	6			90.1961
Sig.		1.000	1.000	.377

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 6.000.

```
ONEWAY FREEACIDITY BY AntiulcerPropertiesOfAQAUDInAspirinInducedUlcer
  /STATISTICS DESCRIPTIVES HOMOGENEITY
  /MISSING ANALYSIS
  /POSTHOC=TUKEY ALPHA(0.05).
```

Oneway

Descriptives

FREEACIDITY

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Normal Control	5	8.8000	2.38747	1.06771	5.8356	11.7644	5.00	11.00
Asp	5	13.6000	2.30217	1.02956	10.7415	16.4585	10.00	16.00
AQAUD 250mg/kg b.wt	4	12.0000	1.82574	.91287	9.0948	14.9052	10.00	14.00
AQAUD 500mg/kg b.wt	4	8.7500	1.50000	.75000	6.3632	11.1368	7.00	10.00
OMEP 20mg/kg b.wt	5	11.0000	1.22474	.54772	9.4793	12.5207	10.00	13.00
Total	23	10.8696	2.59903	.54193	9.7457	11.9935	5.00	16.00

Test of Homogeneity of Variances

FREEACIDITY

Levene Statistic	df1	df2	Sig.
.856	4	18	.509

ANOVA

FREEACIDITY

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	81.859	4	20.465	5.519	.004
Within Groups	66.750	18	3.708		
Total	148.609	22			

Post Hoc Tests

Multiple Comparisons

Dependent Variable: FREEACIDITY

Tukey HSD

(I) AntiulcerPropertiesOfAQAUDInAspirinInducedUlcer	(J) AntiulcerPropertiesOfAQAUDInAspirinInducedUlcer	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Normal Control	Asp	-4.80000*	1.21792	.007	-8.4827	-1.1173
	AQAUD 250mg/kg b.wt	-3.20000	1.29180	.140	-7.1061	.7061
	AQAUD 500mg/kg b.wt	.05000	1.29180	1.000	-3.8561	3.9561
	OMEP 20mg/kg b.wt	-2.20000	1.21792	.400	-5.8827	1.4827
Asp	Normal Control	4.80000*	1.21792	.007	1.1173	8.4827
	AQAUD 250mg/kg b.wt	1.60000	1.29180	.730	-2.3061	5.5061
	AQAUD 500mg/kg b.wt	4.85000*	1.29180	.011	.9439	8.7561
	OMEP 20mg/kg b.wt	2.60000	1.21792	.249	-1.0827	6.2827
AQAUD 250mg/kg b.wt	Normal Control	3.20000	1.29180	.140	-.7061	7.1061
	Asp	-1.60000	1.29180	.730	-5.5061	2.3061
	AQAUD 500mg/kg b.wt	3.25000	1.36168	.164	-.8674	7.3674
	OMEP 20mg/kg b.wt	1.00000	1.29180	.935	-2.9061	4.9061
AQAUD 500mg/kg b.wt	Normal Control	-.05000	1.29180	1.000	-3.9561	3.8561
	Asp	-4.85000*	1.29180	.011	-8.7561	-.9439
	AQAUD 250mg/kg b.wt	-3.25000	1.36168	.164	-7.3674	.8674
	OMEP 20mg/kg b.wt	-2.25000	1.29180	.435	-6.1561	1.6561

OMEP 20mg/kg b.wt	Normal Control	2.20000	1.21792	.400	-1.4827	5.8827
	Asp	-2.60000	1.21792	.249	-6.2827	1.0827
	AQAUD 250mg/kg b.wt	-1.00000	1.29180	.935	-4.9061	2.9061
	AQAUD 500mg/kg b.wt	2.25000	1.29180	.435	-1.6561	6.1561

*. The mean difference is significant at the 0.05 level.

Homogeneous Subsets

FREEACIDITY

Tukey HSD[a,b]

AntiulcerPropertiesOfAQAUDInAspirinInducedUlcer	N	Subset for alpha = 0.05	
		1	2
AQAUD 500mg/kg b.wt	4	8.7500	
Normal Control	5	8.8000	
OMEP 20mg/kg b.wt	5	11.0000	11.0000
AQAUD 250mg/kg b.wt	4	12.0000	12.0000
Asp	5		13.6000
Sig.		.124	.290

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 4.545.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

```
ONEWAY TOTALACIDITY BY AntiulcerPropertiesOfAQAUDInAspirinInducedUlcer
  /STATISTICS DESCRIPTIVES HOMOGENEITY
  /MISSING ANALYSIS
  /POSTHOC=TUKEY ALPHA(0.05).
```

Oneway

Descriptives

TOTALACIDITY

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean	Minimum	Maximum

					Lower Bound	Upper Bound		
Normal Control	5	18.8000	4.60435	2.05913	13.0829	24.5171	12.00	24.00
Asp	5	27.6000	2.30217	1.02956	24.7415	30.4585	25.00	31.00
AQAUD 250mg/kg b.wt	4	23.5000	2.64575	1.32288	19.2900	27.7100	21.00	27.00
AQAUD 500mg/kg b.wt	4	21.5000	3.41565	1.70783	16.0649	26.9351	18.00	26.00
OMEP 20mg/kg b.wt	5	21.6000	4.61519	2.06398	15.8695	27.3305	14.00	26.00
Total	23	22.6087	4.56009	.95085	20.6368	24.5806	12.00	31.00

Test of Homogeneity of Variances

TOTALACIDITY

Levene Statistic	df1	df2	Sig.
.600	4	18	.668

ANOVA

TOTALACIDITY

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	210.278	4	52.570	3.828	.020
Within Groups	247.200	18	13.733		
Total	457.478	22			

Post Hoc Tests

Multiple Comparisons

Dependent Variable: TOTALACIDITY

Tukey HSD

(I) AntiulcerPropertiesOfAQAUDInAspirinInducedUlcer	(J) AntiulcerPropertiesOfAQAUDInAspirinInducedUlcer	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Normal Control	Asp	-8.80000*	2.34379	.011	-15.8871	-1.7129

Asp	AQAUD 250mg/kg b.wt	-4.70000	2.48596	.357	-12.2170	2.8170
	AQAUD 500mg/kg b.wt	-2.70000	2.48596	.811	-10.2170	4.8170
	OMEP 20mg/kg b.wt	-2.80000	2.34379	.754	-9.8871	4.2871
	Normal Control	8.80000*	2.34379	.011	1.7129	15.8871
AQAUD 250mg/kg b.wt	Asp	4.10000	2.48596	.487	-3.4170	11.6170
	AQAUD 500mg/kg b.wt	6.10000	2.48596	.146	-1.4170	13.6170
	OMEP 20mg/kg b.wt	6.00000	2.34379	.121	-1.0871	13.0871
	Normal Control	4.70000	2.48596	.357	-2.8170	12.2170
AQAUD 500mg/kg b.wt	Asp	-4.10000	2.48596	.487	-11.6170	3.4170
	AQAUD 500mg/kg b.wt	2.00000	2.62043	.938	-5.9237	9.9237
	OMEP 20mg/kg b.wt	1.90000	2.48596	.938	-5.6170	9.4170
	Normal Control	2.70000	2.48596	.811	-4.8170	10.2170
OMEP 20mg/kg b.wt	Asp	-6.10000	2.48596	.146	-13.6170	1.4170
	AQAUD 250mg/kg b.wt	-2.00000	2.62043	.938	-9.9237	5.9237
	OMEP 20mg/kg b.wt	-.10000	2.48596	1.000	-7.6170	7.4170
	Normal Control	2.80000	2.34379	.754	-4.2871	9.8871
Normal Control	Asp	-6.00000	2.34379	.121	-13.0871	1.0871
	AQAUD 250mg/kg b.wt	-1.90000	2.48596	.938	-9.4170	5.6170
	AQAUD 500mg/kg b.wt	.10000	2.48596	1.000	-7.4170	7.6170

*. The mean difference is significant at the 0.05 level.

Homogeneous Subsets

TOTALACIDITY

Tukey HSD[a,b]

AntiulcerPropertiesOfAQAUDInAspirinInducedUlcer	N	Subset for alpha = 0.05	
		1	2
Normal Control	5	18.8000	
AQAUD 500mg/kg b.wt	4	21.5000	21.5000
OMEP 20mg/kg b.wt	5	21.6000	21.6000
AQAUD 250mg/kg b.wt	4	23.5000	23.5000
Asp	5		27.6000
Sig.		.346	.139

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 4.545.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

```
ONEWAY GSH BY AntiulcerPropertiesOfAQAUDInAspirinInducedUlcer
  /STATISTICS DESCRIPTIVES HOMOGENEITY
  /MISSING ANALYSIS
  /POSTHOC=TUKEY ALPHA(0.05).
```

Oneway

Descriptives

GSH

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Normal Control	5	7.3685	1.19268	.53338	5.8876	8.8494	6.33	9.10
Asp	5	6.9224	1.75212	.78357	4.7469	9.0979	5.06	9.72
AQAUD 250mg/kg b.wt	4	12.3033	2.93959	1.46980	7.6257	16.9808	9.02	15.68
AQAUD 500mg/kg b.wt	4	10.4552	1.31406	.65703	8.3642	12.5462	8.70	11.86
OMEP 20mg/kg b.wt	5	6.7369	.94119	.42091	5.5683	7.9056	5.34	7.96
Total	23	8.5293	2.69794	.56256	7.3626	9.6959	5.06	15.68

Test of Homogeneity of Variances

GSH

Levene Statistic	df1	df2	Sig.
2.541	4	18	.076

ANOVA

GSH

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	107.519	4	26.880	9.195	.000
Within Groups	52.617	18	2.923		
Total	160.136	22			

Post Hoc Tests

Multiple Comparisons

Dependent Variable: GSH

Tukey HSD

(I) AntiulcerPropertiesOfAQAUDInAspirinInducedUlcer	(J) AntiulcerPropertiesOfAQAUDInAspirinInducedUlcer	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Normal Control	Asp	.44610	1.08132	.993	-2.8236	3.7158
	AQAUD 250mg/kg b.wt	-4.93479*	1.14692	.003	-8.4028	-1.4667
	AQAUD 500mg/kg b.wt	-3.08670	1.14692	.095	-6.5547	.3813
	OMEP 20mg/kg b.wt	.63155	1.08132	.976	-2.6382	3.9013
Asp	Normal Control	-.44610	1.08132	.993	-3.7158	2.8236
	AQAUD 250mg/kg b.wt	-5.38089*	1.14692	.002	-8.8489	-1.9128
	AQAUD 500mg/kg b.wt	-3.53280*	1.14692	.045	-7.0008	-.0648

	OMEP 20mg/kg b.wt	.18545	1.08132	1.000	-3.0843	3.4552
	Normal Control	4.93479*	1.14692	.003	1.4667	8.4028
	Asp	5.38089*	1.14692	.002	1.9128	8.8489
AQAUD 250mg/kg b.wt	AQAUD 500mg/kg b.wt	1.84809	1.20896	.558	-1.8076	5.5037
	OMEP 20mg/kg b.wt	5.56634*	1.14692	.001	2.0983	9.0344
	Normal Control	3.08670	1.14692	.095	-.3813	6.5547
	Asp	3.53280*	1.14692	.045	.0648	7.0008
AQAUD 500mg/kg b.wt	AQAUD 250mg/kg b.wt	-1.84809	1.20896	.558	-5.5037	1.8076
	OMEP 20mg/kg b.wt	3.71825*	1.14692	.032	.2502	7.1863
	Normal Control	-.63155	1.08132	.976	-3.9013	2.6382
	Asp	-.18545	1.08132	1.000	-3.4552	3.0843
OMEP 20mg/kg b.wt	AQAUD 250mg/kg b.wt	-5.56634*	1.14692	.001	-9.0344	-2.0983
	AQAUD 500mg/kg b.wt	-3.71825*	1.14692	.032	-7.1863	-.2502

*. The mean difference is significant at the 0.05 level.

Homogeneous Subsets

GSH

Tukey HSD[a,b]

AntiulcerPropertiesOfAQAUDInAspirinInducedUlcer	N	Subset for alpha = 0.05		
		1	2	3
OMEP 20mg/kg b.wt	5	6.7369		
Asp	5	6.9224		
Normal Control	5	7.3685	7.3685	
AQAUD 500mg/kg b.wt	4		10.4552	10.4552
AQAUD 250mg/kg b.wt	4			12.3033
Sig.		.980	.090	.499

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 4.545.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

```
ONEWAY MDA BY AntiulcerPropertiesOfAQAUDInAspirinInducedUlcer

  /STATISTICS DESCRIPTIVES HOMOGENEITY
  /MISSING ANALYSIS
  /POSTHOC=TUKEY ALPHA(0.05).
```

Oneway

Descriptives

MDA

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Normal Control	5	.0008	.00003	.00001	.0007	.0008	.00	.00
Asp	5	.0010	.00005	.00002	.0009	.0011	.00	.00
AQAUD 250mg/kg b.wt	4	.0009	.00006	.00003	.0008	.0010	.00	.00
AQAUD 500mg/kg b.wt	4	.0009	.00003	.00001	.0008	.0009	.00	.00
OMEP 20mg/kg b.wt	5	.0007	.00018	.00008	.0005	.0009	.00	.00
Total	23	.0008	.00015	.00003	.0008	.0009	.00	.00

Test of Homogeneity of Variances

MDA

Levene Statistic	df1	df2	Sig.
2.968	4	18	.048

ANOVA

MDA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.000	4	.000	9.340	.000

Within Groups	.000	18	.000		
Total	.000	22			

Post Hoc Tests

Multiple Comparisons

Dependent Variable: MDA

Tukey HSD

(I) AntiulcerPropertiesOfAQAUDInAspirinInducedUlcer	(J) AntiulcerPropertiesOfAQAUDInAspirinInducedUlcer	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Normal Control	Asp	-.00026*	.00006	.004	-.0004	-.0001
	AQAUD 250mg/kg b.wt	-.00019	.00006	.055	-.0004	.0000
	AQAUD 500mg/kg b.wt	-.00012	.00006	.351	-.0003	.0001
	OMEP 20mg/kg b.wt	.00006	.00006	.847	-.0001	.0002
Asp	Normal Control	.00026*	.00006	.004	.0001	.0004
	AQAUD 250mg/kg b.wt	.00007	.00006	.819	-.0001	.0003
	AQAUD 500mg/kg b.wt	.00014	.00006	.246	-.0001	.0003
	OMEP 20mg/kg b.wt	.00032*	.00006	.000	.0001	.0005
AQAUD 250mg/kg b.wt	Normal Control	.00019	.00006	.055	.0000	.0004
	Asp	-.00007	.00006	.819	-.0003	.0001
	AQAUD 500mg/kg b.wt	.00007	.00007	.844	-.0001	.0003
	OMEP 20mg/kg b.wt	.00025*	.00006	.008	.0001	.0004
AQAUD 500mg/kg b.wt	Normal Control	.00012	.00006	.351	-.0001	.0003

OMEP 20mg/kg b.wt	Asp	-.00014	.00006	.246	-.0003	.0001
	AQAUD 250mg/kg b.wt	-.00007	.00007	.844	-.0003	.0001
	OMEP 20mg/kg b.wt	.00018	.00006	.070	.0000	.0004
	Normal Control	-.00006	.00006	.847	-.0002	.0001
	Asp	-.00032*	.00006	.000	-.0005	-.0001
	AQAUD 250mg/kg b.wt	-.00025*	.00006	.008	-.0004	-.0001
	AQAUD 500mg/kg b.wt	-.00018	.00006	.070	-.0004	.0000

*. The mean difference is significant at the 0.05 level.

Homogeneous Subsets

MDA

Tukey HSD[a,b]

AntiulcerPropertiesOfAQAUDInAspirinInducedUlcer	N	Subset for alpha = 0.05		
		1	2	3
OMEP 20mg/kg b.wt	5	.0007		
Normal Control	5	.0008	.0008	
AQAUD 500mg/kg b.wt	4	.0009	.0009	.0009
AQAUD 250mg/kg b.wt	4		.0009	.0009
Asp	5			.0010
Sig.		.065	.052	.236

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 4.545.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

PREPARATION OF REAGENTS

17% $NaCO_3$: This was prepared by dissolving 17g of $NaCO_3$ in some distilled water, then making up to the total volume of 100ml.

2% HCl: This was prepared by diluting 2ml of HCl in some distilled water, then making up to the volume of 100ml.

10% $NaCO_3$: This was prepared by dissolving 10g of $NaCO_3$ in some distilled water , then making up the total volume to 100ml.

1% $AlCl_3$: This was prepared by dissolving 1g of $AlCl_3$ in some distilled water, then making up the total volume to100ml.

10% $AlCl_3$: This was prepared by dissolving 10g of $AlCl_3$ in some distilled water, then making up the total volume to100ml.

4% NaOH: This was prepared by dissolving 4g of NaOH in some distilled water, then making up the total volume to100ml.

5% $NaNO_3$: This was prepared by dissolving 5g of $NaNO_3$ in some distilled water, then making up to the total volume of 100ml.

0.01N NaOH: This was prepared by dissolving 0.2g of NaOH in some distilled water, then it was made up to the total volume of 500ml.

10% Formal Saline: This was prepared by diluting 25ml of conc Formaline in some distilled water, adding 2.25g of NaCl, then making up to the total volume of 250ml.

Phosphate buffer (150Mm, PH 7.4): This was prepared by dissolving 2.106g of $NaH_2PO_4.2H_2O$ and 8.733g Na_2HPO_4 in some water. It was made up to the total volume of 500ml and it was adjusted to PH 7.4 with dilute NaOH.

5% Phosphoric acid (v/v): This was prepared by diluting 5ml of conc phosphoric acid in some distilled water, and then made up to total volume 500ml**.**

1% Sulphanilamide: This was prepared by dissolving 1g of sulphanilamide in 5% phosphoric acid, then it was made up to 100ml with 5% phosphoric acid.

0.1% Naphthylethylenediamine-dihydrochloride (NED): This was prepared by dissolving 0.1g of NED in some distilled water, then making up to 100ml with distilled.

Griess reagent: This was prepared by mixing equal volumes of 1% sulphanilamide in 5% phosphoric acid with 0.1% Naphthylethylenediamine-dihydrochloride (NED).

20% Acetic acid: This was prepared by diluting 20ml of acetic acid in some distilled water, then making up to the total volume of 100ml.

0.8% Thiobarbituric acid (TBA): This was prepared by dissolving 0.8g of thiobarbituric acid in dilute HCl, and making up to the total volume of 100ml with distilled water.

8.1% Sodium dodecyl sulphate (SDS): This was prepared by dissolving 0.81g of SDS in some distilled water, then making up to 10ml with distilled water.

1% Thiobarbituric acid (TBA): This was prepared by dissolving 1g of thiobarbituric acid in dilute HCl, then making up to 100ml with distilled water.

10% Trichloroacetic acid (TCA): This was prepared by dissolving 10g of TCA in some distilled water, then making up to the final volume of 100ml with distilled water.

0.1Mm $Fecl_3$: This was prepared by dissolving 0.127g of $Fecl_3$ in some distilled water, then making up to the total volume of 1000ml with distilled water.

Standard GSH: 400mg of GSH was dissolved in phosphate buffer and made up to 100ml with the buffer. This served as the GSH stock solution (0.4%).

Ellman's reagent (5,5-Dithiobis-2-nitrobenzoic acid): This was prepared by dissolving 0.2g of Ellman's reagent in phosphate buffer, then making up to the total volume of 500ml with the buffer.