# *Aframomum sceptrum* AQUEOUS EXTRACT: ANTIDOTE FOR CYANIDE INTOXICATION OF SELECTED OXIDATIVE STRESS MARKERS IN RATS

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**DELTA STATE UNIVERSITY,**
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**A DISSERTATION SUBMITTED TO POST GRADUATE SCHOOL IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF MASTER'S IN SCIENCE (M.Sc) DEGREE IN BIOCHEMISTRY, DELTA STATE UNIVERSITY, ABRAKA,**

## CERTIFICATION

I declare that this research was independently carried out by me **ATINAYA, David Uruemu** in the Department of Biochemistry, Faculty of Science Delta State University Abraka for the award of M.Sc Degree in Biochemistry and has not been carried out by any one for the award of any Diploma or Degree.

_____ _____

**ATINAYA, David Uruemu Sunday** **Date**
**(Researcher)**

## APPROVAL PAGE

This is to certify that this research was carried out by Atinaya, Uruemu Sunday David in the Department of Biochemistry, Delta State University, Abraka, under the supervision of Prof. B.O. George.

_____ _____

PROF. B.O. GEORGE DATE
SUPERVISOR

_____ _____

DR. N. TONUKARI DATE
HEAD OF DEPARTMENT

## DEDICATION

This research is dedicated to the Almighty God, my dearest wife, Mrs. Cynthia Elohor Atinaya, my family and to my friends.

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## ABSTRACT

Cyanide-induced oxidative stress is known to play a key role in mediating cell death in rat. This study determined the effect of *Aframomum sceptrum* aqueous extract on oxidative stress in cyanide poisoned rats. Thirty rats weighing between 100-160g were randomly divided into six groups of five rats as follows; group A: were given normal water, group B: cyanide was added to their water, group C and D; were given cyanide contaminated water and 10mg/Kg and 20mg/Kg body weight aqueous extract of *A. sceptrum* respectively, group E and F: were given normal water and 10mg/Kg and 20mg/Kg body weight aqueous extract of *A. sceptrum* respectively. *A. sceptrum* aqueous extract was administered three times per day orally using intragastric tube, and potassium cyanide (KCN) solution at concentration of 9.0mg/Kg in the drinking tap water daily for a period of 4 weeks. A significant ($p<0.05$) decrease in haematocrit (PCV), haemoglobin (Hb), total protein (TP) and albumin in the serum of cyanide control group compared with the normal control. The results also showed a significant ($p<0.05$) higher activities of aspartate aminotransaminase (AST), alanine aminotransaminase (ALT), alkaline phosphatase (ALP) and decrease activities of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) in the serum and liver of the cyanide control group compared with the normal control. A significant ($p<0.05$) decrease in reduced glutathione (GSH), and increase malondialdehyde (MDA) level was observed in the serum and liver of the cyanide control group compared with the normal control. Treatment of the cyanide exposed rats with the spice extract improved the indices of antioxidant status but did not affect the group not given cyanide contaminated water thus making the *A. sceptrum* extract a potential source of natural antioxidant for cyanide poisoning.

# CHAPTER ONE

# INTRODUCTION

Cyanide is a potent cytotoxic agent found in so many places and it is known for its quick detrimental action and toxic effect (Ghodsi and Baghshani, 2013). As a consequence of infusion of certain therapeutic drugs, smoke inhalation, industrial accidents, or terrorism, consumption of cyanide-containing foods, cyanide exposure can take place (Alcorta, 2004). The toxic effect of cyanide is as a result of its increased potency as a poison of respiration in all forms of life that is aerobic (Ghodsi and Baghshani, 2013). Apart from acute cyanide intoxication, in recent years, chronic toxic effect has frequently been reported and it is said that chronic dietary, industrial and environmental sources are the most widespread problems emanating from cyanide (Barillo, 2009). The haematocrit also known as packed cell volume (PCV) or erythrocyte volume fraction (EVF), is the volume percentage (%) of red blood cells in blood. Decrease of haemoglobin, with or without a complete decrease of red blood cells might lead to symptoms of anaemia. Glutathione protects the cellular system against harmful effect of lipid peroxidation. Decreased level of reduced glutathione detected in the blood of malaria mice could represent its increased consumption due to oxidative stress and the low haematocrit (George *et al.*, 2012). Aspartate transaminase (AST) also called serum glutamate oxaloacetate transaminase (SGOT) or aspartate aminotransferase (ASAT) is similar to ALT in that it is another enzyme associated with liver parenchyma cells. It is raised in acute liver damage, but is also present in red blood cells and skeletal and cardiac muscle and is therefore not specific to the liver. Increase in AST levels, can however occur in connection with damages of heart or skeletal muscle as well as of liver parenchyma (Vozarova *et al*., 2002).

Albumins are a family of globular proteins, the most common of which are serum albumins. The albumin family consists of all proteins that are water soluble, are moderately soluble in concentrated salt solutions, and experience heat denaturation. Albumin and globulin are produce in the liver (Ghodsi and Baghshani, 2013). Low total protein levels can suggest a liver disorder, a kidney disorder, or a disorder in which protein is not digested or absorbed properly. Ghodsi and Baghshani (2013) reported decreased in serum albumin and total protein in rats drinking water contaminated with cyanide.

In some tissues such as liver, catalase is found predominantly in peroxisomes (Quan *et al.*, 1986). Fortunately, the body itself has its own free radical defense system. Every cell

produces antioxidant enzymes called superoxide dismutase (SOD), catalase and glutathione peroxidase (Menvielle-Bourg, 2005). Malondialdehyde (MDA) is therefore an end-product generated by decomposition of arachidonic acid and larger PUFAs, through enzymatic or nonenzymatic processes. MDA production by enzymatic processes is well known but its biological functions and its possible dose-dependent dual role have not been studied although MDA is known as more chemically stable and membrane-permeable than ROS and less toxic than 4-HNE and methylglyoxal (MG) (Zarkovic *et al.*, 2013).

Dietary antioxidant is a substance (commonly found in foods) that greatly reduces the detrimental effects of "reactive species", such as reactive oxygen and nitrogen molecules, that hampers normal physiological activities on a cellular level in humans (Aruoma, 1994). Spices have been acknowledged not only to have properties that make food more pleasant but also important preservative and antioxidant properties (Shobana and Naidu, 2000). Spices have been used for medicinal purposes as well as food additives over centuries (Wilson and Demmig-Adams, 2007).

*A. sceptrum*, a well known local spice, is consumed in south-south part of Nigeria, has been analyzed for it chemical and antioxidant composition (Erukainure *et al.*, 2011). The presence of bioactive metabolites such as flavonoids, phenols, tannins alkaloids and saponins are responsible for it antioxidant activity and this affirms the use of *A. sceptrum* in the management of various ailments (George *et al.*, 2013).

## 1.1 Aim of the Study

The aim of this study is to investigate the effects of *A. sceptrum* aqueous extract on oxidative stress induced by cyanide toxicity in rats

## 1.2 Objective of the Study

The specific objectives of the study are;

- To compare the effects of *A. sceptrum* on heamatocrit (PCV) and heamoglobin (Hb) levels in rats exposed to cyanide.
- To compare the level of blood reduced glutathione (GSH) of rats exposed to cyanide treated with aqueous extract of *A. sceptrum.*
- To compare the effects of *A. sceptrum* on serum alanine amino transaminase (ALT), aspartate amino transaminase (AST), alkaline phosphatise (ALP) activities in rats exposed to cyanide.

- To compare changes in serum total protein and albumin levels in rats exposed to cyanide treated with aqueous extract of *A. sceptrum.*
- To compare changes in superoxide dismutase (SOD) and catalase (CAT) activities in the serum of rats exposed to cyanide treated with aqueous extract of *A. sceptrum.*
- To compare changes in reduced glutathione (GSH) and malonyldialdehyde (MDA) levels in the liver of rats exposed to cyanide treated with aqueous extract of *A. sceptrum.*

## 1.3 Significance of the Study

As a result of the intake of cyanide through various means, various diseases conditions manifest. Thus this study will reveal the ameliorating effect of *Aframomun sceptrum* on oxidative stress in cyanide exposed rats and to show its dose responses.

## 1.4 Scope of the Study

This study focuses on the evaluation of the effect of *Aframomun sceptrum* on rats exposed to cyanide.

## CHAPTER TWO

# LITERATURE REVIEW

## 2.1 Cyanide

Cyanide is a chemical compound that contains monovalent combining group CN. This group, known as the cyano group, consists of a carbon atom triple-bonded to a nitrogen atom. In inorganic cyanides, such as potassium cyanide, KCN, this group is present as the negatively charged cyanide ion ($CN^-$); these compounds, which are regarded as salts of hydrocyanic acid, are highly toxic. The cyanide ion is isoelectronic with carbon monoxide and with molecular nitrogen (Greenwood and Earnshaw, 1997). Most cyanides are highly toxic ( Morocco, 2005).

Organic cyanides are usually called nitriles. The CN group in such compounds is linked by a covalent bond to carbon-containing group such as methyl ($CH_3$) in methyl cyanide (acetonitrile). Hydrocyanic acid, also known as hydrogen cyanide, or HCN, is highly volatile liquid used to prepare acrylonitrile, which is used in the production of acrylic fibers, synthetic rubber, and plastics. Cyanides are employed in a number of chemical processes, including fumigation, case hardening of iron and steel, electroplating and the concentration of ores. In nature, substances yielding cyanide are present in certain seeds, such as apricot, the pit of the cherry and the seeds of apples (Akyildiz *et al.*, 2010).

### 2.1.1 Nomenclature

In IUPAC nomenclature, organic compounds that have a –CN functional group are called nitriles. Thus nitriles are organic compound and usually do not releases cyanide ions. A functional group with a hydroxyl and cyanide bonded to the same carbon is called cyanohydrins. An example of cyanohydrins is aceto cyanohydrins [$(CH_3)_2C(OH)CN$]. Unlike nitriles, cyanohydrins do release hydrogen cyanides. In inorganic chemistry, salts containing the $CN^-$ ion are referred to as cyanides (Morocco, 2005).

### 2.1.2 Occurrence and Reactions in Nature

Cyanides are produced by certain bacteria, fungi and algae and are found in a number of plants. Cyanides are found in substantial amounts in certain seeds and fruits stones, e.g.; those of apricots, apples, and peaches. In plants, cyanides are usually bound to sugar molecules in the form of cyanogenic glycosides and defend the plant against herbivores. Cassava roots (also called manioc), an important potato-like food grown in

tropical countries (and the base from which tapioca is made), also contain cyanogenic glycosides (Vetter, 2000; Jones, 1998).

### 2.1.3 Toxicity

Most cyanides are highly toxic and the cyanide anion is an inhibitor of the enzyme cytochrome C oxidase in the fourth complex of the electron transport chain (found in the membrane of the mitochondria of eukaryotic cells). It attaches to the iron within this protein. The binding of cyanide to this enzyme prevents transport of electrons from cytochrome C to oxygen. As a result, the electron transport chain is disrupted, meaning that the cell can no longer aerobically produce ATP for energy (Nelson and Cox, 2000). Tissues that depend highly on aerobic respiration, such as the central nervous system and the heart, are particularly affected. This is an example of histotoxic hypoxia (Biller, 2007).

Hydrogen cyanide (HCN), a gas at ambient temperature and pressure which can be inhaled is the most hazardous cyanide compound. When working with it, it is very important to wear an air respirator supplied by an external source to avoid inhaling the gas. Hydrogen cyanide is produce when a solution containing labile cyanide is made acidic, because HCN is a weak acid. Alkaline solutions are safer to use because they do not evolve hydrogen cyanide gas. Hydrogen cyanide may be produced in the combustion of polyurethanes; for this reason, polyurethanes are not recommended for use in domestic and aircraft furniture. Oral ingestion of a small quantity of solid cyanide or a cyanide solution as little as 200mg, or to airborne cyanide of 270ppm is sufficient enough to cause death within minutes (Biller, 2007).

Organic nitriles do not readily release cyanide ions, and so have low toxicities. By contrast, compounds such as trimethylsilyl cyanide [$(CH_3)_3SiCN$] readily release HCN to the cyanide ion upon contact with water (Livinghouse, 1990).

### 2.1.4 Antidote

Antidote is a substance which counteract or controls the effect of a poison or disease. Cyanide is a poison that can be controlled using an antidote. Antidotes for cyanide include amyl nitrite, sodium nitrite and sodium thiosulfate. Hydroxocobalamin reacts with cyanide to form cyanocobalamin, which can be safely eliminated by the kidneys. This method has the advantage of avoiding the formation of methemoglobin (Shepherd and Velez, 2008). An older cyanide antidote include administration of three substances namely

amyl nitrite pearls (administered by inhalation), sodium nitrite and sodium thiosulfate. The goal of the antidote was to generate a large pool of ferric iron ($Fe^{3+}$) to compete for cyanide with cytochrome C oxidase so that cyanide will bind to the antidote rather than the enzyme. The nitrites oxidize haemoglobin to methmoglobin, which competes with cytochrome oxidase for the cyanide ion. Cyanmethmoglobin is formed and the cytochrome oxidase enzyme is restored. The major mechanism to remove the cyanide from the body is the enzymatic conversion to thiocyanate by the mitochondrial enzyme rhodonase. Thiocyanate is relatively non-toxic molecule and is excreted by the kidneys. To accelerate this detoxification, sodium thiosulfate is administered to provide a sulfur donor for rhodonase, needed in order to produce thiocyanate (Chaudhary and Gupta, 2012).

### 2.1.5 Potassium Cyanide

Potassium cyanide (KCN), a cyanide salt, is used in numerous industrial processes such as electroplating of metals, chemical synthesis, and extraction of gold and silver. KCN is also use in photography; in plastic, paper and textile processing; metal coating; cleaning or polishing; and so on. Furthermore KCN can be used as fumigant in agriculture, and as a poison against rats and pest (Haddad and Winchester, 1990).

### 2.1.6 Human Toxicity

Acute poisoning with KCN is severe, dramatic and acute, and can result to death with minutes. Lower exposure doses of cyanide poisoning in the initial phase may result in nausea, vomiting, abdominal pain, confusion, hyperventilation, anxiety, circulatory collapse, tachycardia, hypertension, headache, etc., while in the later phase, metabolic acidosis, seizure, pulmonary edema, apnea, convulsions, bradycardia, hypertension, coma and death may occur. Death occurs mainly by cardiac arrest (Soto-Blanco *et al*., 2002).

### 2.1.7 Metabolism and Excretion of Potassium Cyanide

KCN decomposes slowly in water and rapidly in acids, releasing HCN gas which is highly toxic. In the body, cyanides mixed with water form so called “prussic acid” (hydrocyanic acid, HCN), which can rapidly penetrate mucous and cell membranes.

Cyanide by reaction with sodium thiosulphate, which occur in the body in low concentration, is converted to thiocyanate which can be easily excreted (Haddad and Winchester 1990; Lundquist *et al.*, 1985). About 80% of a cyanide dose is detoxified via the liver to thiocyanate, which is excreted in the urine (Baselt and Cravey, 1995).

### 2.1.8 Toxicological Mechanism

Cyanide prevent the cells of the body from getting oxygen, causing hypoxia, (very low level of oxygen), or anoxia (lack of oxygen), which may eventually result in cell death. The mechanism of cyanide poisoning is that, the $CN^-$ ion binds to the ferric iron ($Fe^{3+}$) of mitochondrial cytochrome oxidase. This results in inhibition of oxidative phosphorylation, anaerobic metabolism, lactic acid accumulation, and decreased ATP production. Cyanide is more harmful to the heart and brain than to other organs because the heart and brain use a great deal of oxygen (Hall *et al.*, 1987). KCN, like all other cyanides, is a potent poison inhibiting cytochrome oxidase as its binds to complex (iv) of the electron transport chain, ETC, and thereby inhibiting respiration by forming a permanent bind with the iron atom in heme of cytochrome (Ekwall *et al.,* 1998).

### 2.2.0 *Aframomum sceptrum*

*Aframomum sceptrum* is among the local spices consumed and used to enhance cooking flavour, aroma and palatability in Nigeria mostly the South-South part. It is commonly known as Guinea grains, grains of paradise, or black amomum in English (Burkill, 1985). The Urhobo's in the southern part of Nigeria call it Atiko. *A. sceptrum* belong to the family of Zingiberaceae, which constitute a family of terrestrial rhizomal herbs with over 1400 species distributed in over 50 genera (Hepper, 1996). They are mostly found in tropical areas (Asia and Africa) (Koechin, 1965). They are closely allied to the Amomum of Asia and indeed some species of Amomum are used ethno medicinally in South-East Asia (Perry, 1980). Several species from the genus Aframomum are major food plants and their antiparasitc, antifungi, antibacterial and antiviral properties have been reported (Cousins and Huffman, 2002). Bioassays of the extract of *A. danielli* have revealed active growth inhibitors of *Salmonela enteriditis*, *Pseudomonas fragi*, *P. flourescens*, *Proteus vulgaris*, *Streptococcus aureus*, *Aspergillus flavus*, *A. parasiticus*, *A. ochraceus* and *A. niger* (Adegoke and Skura, 1994). *A. sceptrum* has also been revealed to possess antioxidant properties and can be by food and pharmaceutical industries as new potent source of natural antioxidant (George and Osioma, 2011). *A. sceptrum* is rich in crude protein and crude fibre. The presence of bioactive compounds is an affirmation of the use of this spice in the management of various ailments. The total phenolic and flavonoids contents may be responsible for its observed antioxidant activities. Consumption of this spice can therefore act as primary and/or secondary antioxidants (Erukainure *et al.*,

2011). *A. sceptrum* is wide spreading inflorescences at foot of leafy shoots or at some distance away. The whole plant has been reported to be used for ethno dietary, medicinal and spiritual purposes (Burkill, 1985). Locally, the pods are crushed to remove the seeds which are then fermented. The fermented seed are dried, then blended and used as spices in cooking.

Bioactive metabolites from other Aframomum species have been reported, but there exist little or no information on that of *A. sceptrum*.

### 2.2.1 Phytochemical Composition of *Aframomum sceptrum*

Phytochemical screening of the spice showed the presence of alkaloids, flavonoids, phenols, tannins and saponins (Erukainure *et al.*, 2011). Alkaloids, comprises of a large group of nitrogenous compounds and widely used as cancer chemotherapeutic agents. Alkaloids have also been reported to interfere with cell division (Valero and Salmeroj, 2003). The flavonoid content was relatively high compared to fresh thyme, but lower than those of dried parsley and fresh dill weed (USDAARS, 2003).The best-described property of almost every group of flavonoids is their capacity to act as antioxidants. The flavones and catechins seem to be the most powerful flavonoids for protecting the body against ROS (Nijveldt *et al*., 2001). Studies have revealed that consumption of flavonoids can be used in the management of coronary heart disease (Knekt *et al*., 1996). The presence of flavonoids in the spice may be the reason for its antioxidant activities and healing effects. Dietary tannins have been reported to affect protein digestibility and metal ion availability, but recent studies suggests that free or protein-complex condensed and hydrolysable tannins are more effective than small phenolics (Hagerman, 2002). Tannin imports an astringent taste that affects palatability of food. The tannin content of *A. sceptrum* may contribute to its antioxidant activities. The relationship between total phenol contents and antioxidant activity has been widely studied in different foodstuffs (Jayaprakasha *et al*., 2008). Antioxidant activity of foodstuff significantly increases with the presence of high concentration of total phenol and flavonoid contents (Erukainure *et al*., 2011).

### 2.3.0 Antioxidants

Antioxidants are substances that may protect cells from the damage caused by unstable molecules known as free radicals. Antioxidants interact with and stabilize free radicals and may prevent some of the damage free radicals might otherwise cause. Free

radical damage may lead to cancer. Examples of antioxidants include beta-carotene, lycopene, vitamins C, E, A and other substances (Sies, 1997).

Antioxidant can also be defined as any substance that when present at low concentrations compare with those of the oxidizable substrate, significantly delays or inhibits oxidation of that substrate (Antolovich *et al.*, 2002). An antioxidant is a molecule capable of slowing or preventing the oxidation of other molecules. Oxidation is a chemical reaction that transfers electrons from a substance to an oxidizing agent. Oxidation reactions can produce free radicals, which start chain reactions that damage cells. Antioxidants terminate these chain reactions by removing free radical intermediates and inhibit other oxidation reactions by being oxidized themselves (Duarte and Lunec, 2005). As a result, antioxidants are often reducing agents such as thiols, ascorbic acid or polyphenols (Sies, 1997).

Although oxidation reactions are crucial for life, they can also be damaging; hence, plants and animals maintain complex systems of multiple types of antioxidants, such as glutathione, vitamin C and vitamin E as well as enzymes such as catalase, superoxide dismutase and various peroxidases. Low levels of antioxidants, or inhibition of the antioxidant enzymes, causes oxidative stress and may damage or kill cells (Valko *et al.*, 2007). As oxidative stress might be an important part of many human diseases, the use of antioxidants in pharmacology is intensively studied, particularly as treatments for stroke and neurodegenerative diseases. However, it is unknown whether oxidative stress is the cause or the consequence of disease. Antioxidants are also widely used as ingredients in dietary supplements in the hope of maintaining health and preventing diseases such as cancer and coronary heart disease (Sies, 1997). Although initial studies suggested that antioxidant supplements might promote health, later large clinical trials did not detect any benefit and suggested instead that excess supplementation may be harmful (Baillie et al., 2009). In addition to these uses of natural antioxidants in medicine, these compounds have many industrial uses, such as preservatives in food and cosmetics and preventing the degradation of rubber and gasoline. For many years chemists have known that free radicals cause oxidation which can be controlled or prevented by a range of antioxidants substances (Bjelakovic *et al.*, 2007). It is vital that lubrication oils should remain stable and liquid should not dry up like paints. For this reason, such oil usually has small quantities of antioxidants such as phenol or amine derivatives, added to them. Although plastics are

often formed by free radical action, they can also be broken down by the same process, so they too, require protection by antioxidants like phenols or naphthol. Low density polythene is also of protected by carbon black which absorbs the ultraviolet light which causes radical production (Sies, 1997).

### 2.3.1 Catalase (EC 1.11.1.6)

Catalase is a common enzyme found in nearly all living organisms exposed to oxygen. It catalyzes the decomposition of hydrogen peroxide to water and oxygen (Chelikani *et al.*, 2004). It is a very important enzyme in reproductive reactions. Likewise, catalase has one of the highest turnover numbers of all enzymes; one catalase molecule can convert millions of molecules of hydrogen peroxide to water and oxygen each second (Goodsell, 2004).

Catalase is a tetramer of four polypeptide chains, each over 500 amino acids long (Boon *et al.*, 2007). It contains four porphyrinheme (iron) groups that allow the enzyme to react with the hydrogen peroxide. The optimum pH for human catalase is approximately 7 (Maehly and Chance, 1954), and has a fairly broad maximum (the rate of reaction does not change appreciably at pHs between 6.8 and 7.5) (Aebi, 1984). The pH optimum for other catalases varies between 4 and 11 depending on the species. The optimum temperature also varies by species (Toner *et al.*, 2000).

#### 2.3.1.1 Molecular Mechanism of Catalase

While the complete mechanism of catalase is not currently known (Boon *et al.*, 2007), the reaction is believed to occur in two stages:

$$H_2O_2 + Fe^{3+}\text{-}E \rightarrow H_2O + O{=}Fe^{4+}\text{-}E^{\cdot+}$$

$$H_2O_2 + O{=}Fe^{3+}\text{-}E^{\cdot+} \rightarrow H_2O + Fe^{3+}\text{-}E + O_2$$

Here $Fe^{+}$-E represents the iron center of the heme group attached to the enzyme. $Fe^{4+}$-$E^{\cdot+}$ is a mesomeric form of $Fe^{5+}$-E, meaning the iron is not completely oxidized to +5, but receives some "supporting electrons" from the heme ligand. This heme has to be drawn then as a radical cation (+).

As hydrogen peroxide enters the active site, it interacts with the amino acids Asn147 (asparagine at position 147) and His74, causing a proton (hydrogen ion) to transfer between the oxygen atoms. The free oxygen atom coordinates, freeing the newly formed

water molecule and $Fe^{4+}$=O. $Fe^{4+}$=O reacts with a second hydrogen peroxide molecule to reform $Fe^{3+}$-E and produce water and oxygen (Boon *et al.*, 2007). The reactivity of the iron center may be improved by the presence of the phenolate ligand of Tyr357 in the fifth iron ligand, which can assist in the oxidation of the $Fe^{3+}$ to $Fe^{4+}$. The efficiency of the reaction may also be improved by the interactions of His74 and Asn147 with reaction intermediates (Boon *et al.*, 2007). In general, the rate of the reaction can be determined by the Michaelis-Menten equation. Catalase can also catalyze the oxidation, by hydrogen peroxide, of various metabolites and toxins, including formaldehyde, formic acid, phenols, acetaldehyde and alcohols. It does so according to the following reaction:

$H_2O_2 + H_2R \rightarrow 2H_2O + R$

The exact mechanism of this reaction is not known.

#### 2.3.1.2 Cellular Role of Catalase

Hydrogen peroxide is a harmful byproduct of many normal metabolic processes; to prevent damage to cells and tissues, it must be quickly converted into other, less dangerous substances. To this end, catalase is frequently used by cells to rapidly catalyze the decomposition of hydrogen peroxide into less-reactive gaseous oxygen and water molecules (Gaetani *et al.*, 1996).

The true biological significance of catalase is not always straightforward to assess: Mice genetically engineered to lack catalase are phenotypically normal, indicating this enzyme is dispensable in animals under some conditions (Ho *et al.*, 2004). A catalase deficiency may increase the likelihood of developing type 2 diabetes (László *et al.*, 2001; László, 2008). Some humans have very low levels of catalase (acatalasia), yet show few ill effects. The predominant scavengers of $H_2O_2$ in normal mammalian cells are likely peroxiredoxins rather than catalase.

Catalase is usually located in a cellular, bipolar environment organelle called the peroxisome (Alberts *et al.*, 2002). Peroxisomes in plant cells are involved in photorespiration (the use of oxygen and production of carbon dioxide) and symbiotic nitrogen fixation (the breaking apart of diatomic nitrogen ($N_2$) to reactive nitrogen atoms). Hydrogen peroxide is used as a potent antimicrobial agent when cells are infected with a pathogen. Catalase-positive pathogens, such as *Mycobacterium tuberculosis*, *Legionella pneumophila*, and *Campylobacter jejuni*, make catalase to deactivate the peroxide radicals, thus allowing them to survive unharmed within the host (Srinivasa *et al.*, 2003).

### 2.3.2 Superoxide Dismutase (EC 1.15.1.1)

Superoxide dismutase (SOD) is a metalloenzyme whose active center is occupied by copper and zinc, sometimes manganese and iron (Rotilio, 1974). The enzyme superoxide dismutase catalyzes the dismutation of superoxide into oxygen and hydrogen peroxide. SOD is endogenously produced intracellular enzyme present in essentially every cell in the body. There are at least three forms of superoxide dismutase in nature. Human erythrocytes contain an SOD enzyme with divalent copper and divalent zinc. Chicken liver mitochondria and E. coli contain a form with trivalent manganese. E. coli also contain a form of the enzyme with trivalent iron. The Cu-Zn enzyme is dimer of molecular weight 32,500. The two subunits are joined by a disulfide bond. SODs are enzymes that play major roles in the protection of cells against oxidative damage (Shabnum, 2014).

Cellular SOD is actually represented by a group of metalloenzymes with various prosthetic groups. The prevalent enzyme is the cupro-zinc (CuZn) SOD, which is a stable dimeric protein (Cass, 1985). Superoxide dismutase is formed when reduced flavins present, for example, in xanthine oxidase, are reoxidizedunivalently by molecular oxygen.

$$\text{Enz-flavin-}H_2 + O_2 \longrightarrow \text{Enz-flavin-H} + O_2^- + H^+$$

Superoxide can reduce oxidized cytochrome c or be removed by superoxide dismutase.

$$O_2^- + \text{cyt c } (Fe^{3+}) \longrightarrow O_2 + \text{cyt c } (Fe_{2+})$$

$$O_2^- + O_2 + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2$$

#### 2.3.2.1 Role of Superoxide Dismutase in Health

Superoxide dismutase plays an extremely important role in the protection of cells against oxidative damage. The two major forms of superoxide dismutase in humans are the mitochondrial manganese SOD and the cytosolic copper/zinc SOD. A copper/zinc SOD, isolated from beef liver, has been used intra-articularly for degenerative joint disorders as an anti-inflammatory agent. Superoxide dismutase is also marketed as a nutritional supplement (Beyer *et al.*, 1991).

Superoxide dismutase is also an effective defense weapon and Mycobacteria and Nocadia have SOD which enables them to resist the injection of superoxide by phagocytes. When these organisms cause serious disease, it takes the body a very long time to win, and depending on the strength of the patient, the bacteria may win. Although the enzyme isn't

especially fast relative to the spontaneous dismutation of superoxide, the ability of the enzyme to provide some protection to organisms is shown by the existence of a motor neuron disease in individuals who have point mutations in SOD and by the finding that the absence of superoxide dismutase may lead to a form of anaemia (Nicholls and Budd, 2000). It is said that SOD protects the lens of the eyes by guiding against free radical damage.

### 2.3.3 Glutathione Peroxidase (GPx) (EC 1.11.1.9)

Glutathione peroxidase (GPx) (EC 1.11.1.9) is the general name of an enzyme family with peroxidase activity whose main biological role is to protect the organism from oxidative damage. The biochemical function of glutathione peroxidase is to reduce lipid hydroperoxides to their corresponding alcohols and to reduce free hydrogen peroxide to water (Muller *et al.*, 2007). Several isozymes are encoded by different genes, which vary in cellular location and substrate specificity. Glutathione peroxidase 1(GPx1) is the most abundant version, found in the cytoplasm of nearly all mammalian tissues, whose preferred substrate is hydrogen peroxide. Glutathione peroxidase 4 (GPx4) has a high preference for lipid hydroperoxides; it is expressed in nearly every mammalian cell, though at much lower levels. Glutathione peroxidase-2 is an intestinal and extracellular enzyme, while glutathione peroxidase-3 is extracellular, especially abundant in plasma. So far, eight different isoforms of glutathione peroxidase (GPx1-8) have been identified in humans (Muller *et al.*, 2007).

The main reaction that glutathione peroxidase catalyzes is:

$$2GSH + H_2O_2 \longrightarrow GS–SG + 2H_2O$$

Where GSH represents reduced monomeric glutathione and GS–SG represents glutathione disulfide. The mechanism involves oxidation of the selenol of a selenocysteine residue by hydrogen peroxide. This process gives the derivative with a selenenic acid (RSeOH) group. The selenenic acid is then converted back to the selenol by a two step process that begins with reaction with GSH to form the GS-SeR and water. A second GSH molecule reduces the GS-SeR intermediate back to the selenol, releasing GS-SG as the by-product. A simplified representation is shown below (Bhabak and Mugesh, 2010):

$$RSeH + H_2O_2 \rightarrow RSeOH + H_2O$$

$RSeOH + GSH \rightarrow GS\text{-}SeR + H_2O$

$GS\text{-}SeR + GSH \rightarrow GS\text{-}SG + RSeH$

Glutathione reductase then reduces the oxidized glutathione to complete the cycle:

$GS–SG + NADPH + H^+ \longrightarrow 2GSH + NADP^+$

#### 2.3.3.1 Structure of Glutathione Peroxidase

Mammalian GPx1, GPx2, GPx3, and GPx4 have been shown to be selenium-containing enzymes, whereas GPx6 is a selenoprotein in humans with cysteine-containing homologues in rodents. GPx1, GPx2, and GPx3 are homotetrameric proteins, whereas GPx4 has a monomeric structure. As the integrity of the cellular and subcellular membranes depends heavily on glutathione peroxidase, its antioxidative protective system itself depends heavily on the presence of selenium (Muller *et al.*, 2007).

#### 2.3.3.2 Clinical Significance of Glutathione Peroxidase

It has been shown that low levels of glutathione peroxidase as measured in the serum may be a contributing factor to vitiligo (Zedah *et al.*, 2015). Lower plasma glutathione peroxide levels were also observed in patients with type 2 diabetes with macroalbuminuria and this was correlated to the stage of diabetic nephropathy (Sedighi *et al.*, 2014). In one study, the activity of glutathione peroxidase along with other antioxidant enzymes such as superoxide dismutase and catalase was not associated with coronary heart disease risk in women (Yang *et al.*, 2014). Glutathione peroxidase activity was found to be much lower in patients with relapsing-remitting multiple sclerosis (Socha *et al.*, 2014). One study has suggested that glutathione peroxidase and superoxide dismutase polymorphisms play a role in the development of celiac disease (Katar *et al.*, 2014).

### 2.4.0 Glutathione (GSH)

Glutathione (GSH) is an important antioxidant in plants, animals, fungi, and some bacteria and archaea, preventing damage to important cellular components caused by reactive oxygen species such as free radicals, peroxides, lipid peroxides and heavy metals. It is a tripeptide. It contains an unusual peptide linkage between the amine group of cysteine and the carboxyl group of the glutamate side chain. GSH, an antioxidant, helps to protect cells from reactive oxygen species such as free radicals and peroxides (Pompella *et al.*, 2003).

Thiol groups are reducing agents, existing at a concentration around 5 mM in animal cells. Glutathione reduces disulfide bonds formed within cytoplasmic proteins to cysteines by serving as an electron donor. In the process, glutathione is converted to its oxidized form, glutathione disulfide (GSSG) (Shabnum, 2014). Once oxidized, glutathione can be reduced back by glutathione reductase, using NADPH as an electron donor (Couto *et al.*, 2013). The ratio of reduced glutathione to oxidized glutathione within cells is often used as a measure of cellular toxicity (Pastore *et al.*, 2003).

### 2.4.1 Function of Glutathione

Glutathione exists in both reduced (GSH) and oxidized (GSSG) states. In the reduced state, the thiol group of cysteine is able to donate a reducing equivalent ($H^+ + e^-$) to other unstable molecules, such as reactive oxygen species. In donating an electron, glutathione itself becomes reactive, but readily reacts with another reactive glutathione to form glutathione disulfide (GSSG). Such a reaction is probable due to the relatively high concentration of glutathione in cells (up to 5 mM in the liver).GSH can be regenerated from GSSG by the enzyme glutathione reductase (GSR). For every GSSG, two reduced GSH molecules are gained, which can again act as antioxidants scavenging reactive oxygen species in the cell.

In healthy cells and tissue, more than 90% of the total glutathione pool is in the reduced form (GSH) and less than 10% exists in the disulfide form (GSSG). An increased GSSG-to-GSH ratio is considered indicative of oxidative stress (Halprin, 1967).

Glutathione has multiple functions:

- It is the major endogenous antioxidant produced by the cells, participating directly in the neutralization of free radicals and reactive oxygen compounds, as well as maintaining exogenous antioxidants such as vitamins C and E in their reduced (active) forms (Scholz *et al.*, 1989).
- Regulation of the nitric oxide cycle is critical for life, but can be problematic if unregulated (Clementi *et al*., 1999).
- It is used in metabolic and biochemical reactions such as DNA synthesis and repair, protein synthesis, prostaglandin synthesis, amino acid transport, and enzyme activation. Thus, every system in the body can be affected by the state of the glutathione system,

especially the immune system, the nervous system, the gastrointestinal system, and the lungs (Shabnum, 2014).

- It has a vital function in iron metabolism. Yeast cells depleted of or containing toxic levels of GSH show an intense iron starvation-like response and impairment of the activity of extra mitochondrial ISC enzymes, followed by death (Kumar *et al.*, 2011).

#### 2.4.1.1 Function of Glutathione in Animals

GSH is known as a substrate in both conjugation reactions and reduction reactions, catalyzed by glutathione S-transferase enzymes in cytosol, microsomes, and mitochondria. However, it is also capable of participating in non-enzymatic conjugation with some chemicals. In the case of *N*-acetyl-*p*-benzoquinone imine (NAPQI), the reactive cytochrome P450-reactive metabolite formed by paracetamol (acetaminophen), which becomes toxic when GSH is depleted by an overdose of acetaminophen, glutathione is an essential antidote to overdose. Glutathione conjugates to NAPQI and helps to detoxify it. In this capacity, it protects cellular protein thiol groups, which would otherwise become covalently modified; when all GSH has been spent, NAPQI begins to react with the cellular proteins, killing the cells in the process. The preferred treatment for an overdose of this painkiller is the administration (usually in atomized form) of *N*-acetyl-L-cysteine (often as a preparation called Mucomyst), which is processed by cells to L-cysteine and used in the *de novo* synthesis of GSH.

Glutathione (GSH) participates in leukotriene synthesis and is a cofactor for the enzyme glutathione peroxidase. It is also important as a hydrophilic molecule that is added to lipophilic toxins and waste in the liver during biotransformation before they can become part of the bile. Glutathione is also needed for the detoxification of methylglyoxal, a toxin produced as a byproduct of metabolism (Shabnum, 2014).

This detoxification reaction is carried out by the glyoxalase system. Glyoxalase I (EC 4.4.1.5) catalyzes the conversion of methylglyoxal and reduced glutathione to *S*-D-lactoyl-glutathione. Glyoxalase II (EC 3.1.2.6) catalyzes the hydrolysis of *S*-D-lactoyl-glutathione to glutathione and D-lactic acid (Al-Jassabi and Al-Bataina, 2005; Kawase *et al.*, 1996).

Glutathione has recently been used as an inhibitor of melanin in the cosmetics industry. In countries such as Japan and the Philippines, this product is sold as a skin-

whitening soap. Glutathione competitively inhibits melanin synthesis in the reaction of tyrosinase and L-DOPA by interrupting L-DOPA's ability to bind to tyrosinase during melanin synthesis. The inhibition of melanin synthesis was reversed by increasing the concentration of L-DOPA, but not by increasing tyrosinase. Although the synthesized melanin was aggregated within one hour, the aggregation was inhibited by the addition of glutathione. These results indicate glutathione inhibits the synthesis and agglutination of melanin by interrupting the function of L-DOPA" (Matsuki *et al.*, 2008).

Glutathione, along with oxidized glutathione (GSSH) and S- nitrosoglutathione (GSNO), have been found to bind to the glutamate recognition site of the NMDA and AMPA receptors (via their γ-glutamyl moieties), and may be endogenous neuromodulators. At millimolar concentrations, they may also modulate the redox state of the NMDA receptor complex (Varga *et al.*, 1997). In addition, glutathione has been found to bind to and activate ionotropic receptors that are different from any other excitatory amino acid receptor, and which may constitute glutathione receptors, potentially making it a neurotransmitter (Oja, 2000).

### 2.5.0 Alkaline Phosphatase (EC 3.3.3.1)

Alkaline phosphatase (ALP) (EC 3.1.3.1) is a hydrolase enzyme responsible for removing phosphate groups from many types of molecules, including nucleotides, proteins, and alkaloids. The process of removing the phosphate group is called dephosphorylation. As the name suggests, alkaline phosphatases are most effective in an alkaline environment. It is sometimes used synonymously as basic phosphate (Tamas *et al.*, 2002). Alkaline phosphatase is an enzyme found in your bloodstream. ALP helps break down proteins in the body and exist in different forms, depending on where it originates. It is mostly produced in the liver, but some is also made in the bones, intestines, and kidneys. In pregnant women, ALP is made in the placenta.

### 2.5.1 Human Physiology

In humans, alkaline phosphatase is present in all tissues throughout the entire body, but is particularly concentrated in liver, bile duct, kidney, bone, intestinal mucosa and the placenta. Humans and most other mammals contain the following alkaline phosphatase isoenzymes;

- ALPI – Intestinal (molecular weight of 150kDa)

- ALPL – Tissue – nonspecific (liver/bone/kidney)
- ALPP – Placental (Regan isozyme)

### 2.5.2 Clinical Significance of Alkaline Phosphatase

Normal ALP levels in adults are approximately 20 to 140IU/L, though levels are significantly higher in children and pregnant women. Blood test should always be interpreted using the reference range from the laboratory that performed the test. High ALP levels can occur if the bile ducts are obstructed. Also, ALP increases if there is active bone formation occurring, as ALP is a byproduct of osteoblast activity (such as the case in Paget's disease of bone). Level are also elevated in people with untreated Coeliac disease (Preussner, 1998). Lower levels of ALP are less common than elevated levels. The sources of elevated levels can be deduced by obtaining serum levels of gamma glutamyltranferase (GGT). Concomitant increases of ALP with GGT should raise the suspicion of hepatobiliary diseases (Dugdale, 2014).

Placenta alkaline phosphatase is elevated in seminomas (Lange *et al.*, 1982) and active forms of rickets, as well as in bilary obstruction, bone condition, osteoblastic bone tumors, liver diseases or hepatitis, leukemia, lymphoma, Paget's disease, sarcoidosis, hyperthyroidism, hyperparathyroidism, pregnancy etc (Dugdale, 2014). The following conditions or diseases may lead to reduced levels of alkaline phosphatase:

- Hypophosphatasia, an autosomal recessive disease
- Postmenopausal women receiving estrogen therapy because of osteoporosis
- Men with recent hear surgery, malnutrition, magnesium deficiency, hypothyroidism, or severe anaemia.
- Children with achondroplasia and cretinism
- Children after a severe episode of enteritis
- Pernicious anemia
- Aplastic anemia
- Chronic myelogenous leukemia
- Wilson's disease

In addition, the following drugs have been demonstrated to reduce alkaline phosphatase:

- Oral contraceptives (Schiele *et al.*, 1998).

### 2.6.0 Lipid Peroxidation

Lipid peroxidation refers to the oxidative degradation of lipids. It is the process in which free radicals "steal" electrons from the lipids in cell membranes, resulting in cell damage. This process proceeds by a free radical chain reaction mechanism. It most often affects polyunsaturated fatty acids, because they contain multiple double bonds in between which lie methylene bridges (-CH2-) that possess especially reactive hydrogen. As with any radical reaction, the reaction consists of three major steps: initiation, propagation, and termination (Dalle-Donne et al., 2006). The chemical products of this oxidation are known as lipid peroxides or lipid oxidation products (LOPs)

### 2.6.1 Mechanism of Lipid Peroxidation

1. Initiation

Initiation is the step in which a fatty acid radical is produced. The most notable initiators in living cells are reactive oxygen species (ROS), such as superoxide ($\bullet O_2^-$), peroxides ($H_2O_2$) and hydroxyl radical ($\bullet OH$), which combines with a hydrogen atom to make water and a fatty acid radical.

2. Propagation

The fatty acid radical is not a very stable molecule, so it reacts readily with molecular oxygen, thereby creating a peroxyl-fatty acid radical. This radical is also an unstable species that reacts with another free fatty acid, producing a different fatty acid radical and lipid peroxide, or cyclic peroxide if it had reacted with itself. This cycle continues, as the new fatty acid radical reacts in the same way.

3. Termination

When a radical reacts with a non-radical, it always produces another radical, which is why the process is called a "chain reaction mechanism". The radical reaction stops when two radicals react and produce a non-radical species. This happens only when the concentration of radical species is high enough for there to be a high probability of collision of two radicals. Living organisms have different molecules that speed up termination by catching free radicals and, therefore, protecting the cell membrane (Yin *et al*., 2011). One important molecule which is an antioxidant is vitamin E. Other anti-oxidants made within the body include the enzymes superoxide dismutase, catalase, and peroxidase (Valko *et al.*, 2007).

The end product of lipid peroxidation are reactive aldehydes, such as malondialdehyde (MDA) and 4 – hydroxynonenal (HNE), the second one being known also as "second messenger of free radicals" and major bioactive marker of lipid peroxidation, due to its numerous biological activities resembling activities of reactive oxygen species (Zarkovic,2003).

### 2.6.2 Biological roles of Lipid Peroxidation Products

It has been known that lipid peroxidation gives complex products including hydroperoxides ($H_2O_2$), cleavage products such as aldehydes (-CHO-), and polymeric materials, and that these products exert cytotoxic and genotoxic effects (Esterbauer, 1993). Lipid peroxidation products and modified proteins have been found in human atherosclerotic lesions, although their pathological significance, such as cause or consequence, has not yet been fully elucidated. More recently, the role of lipid peroxidation products as signaling messengers has received a great deal of attention (Poli et al., 2004). For example, 9- and 13-HODE have been shown to act as activators and ligand of PPARc, leading to the induction of CD36 scavenger receptors and foam cell formation (Nagy et al., 1998). Oxysterols are involved in the regulation of gene expression, and cholesterol metabolism and homeostasis (Repa and Mangelsdorf, 2002). Furthermore, it has been found that cyclopentenone prostaglandins, 15-deoxy-D12, 14-prostaglandin J2 in particular, induce phase II detoxification enzymes (Kawamoto et al., 2000), and exert a complex array of neurodegenerative, neuroprotective, and anti-inflammatory effects (Musiek et al., 2005). Cells exhibit a broad spectrum of responses to oxidative stress, depending on the stress type and level encountered. Oxidative stress exceeding the antioxidant capacity level may induce oxidative damage, but low-level stress may enhance the defense capacity. Such an adaptive response has been observed in several instances, particularly in low-dose irradiation (Matherset al., 2004). It was reported that the pretreatment of human umbilical vein endothelial cells with 15d-PGJ2 protected the cells from subsequent 4-HNE-induced apoptosis (Levonenet al., 2001). It was recently found that a sub lethal level of 4-HNE exerted a cyto-protective effect primarily through the induction of thioredoxinreductase 1 against subsequent oxidative stress.

### 2.7.0 Aminotransferases

Aminotransferases, also called transaminases are present in most of the tissues of the body. They catalyze the interconversions of the amino acids and 2-oxacids by transfer of amino groups. Transaminases are specific for the amino acid from which the amino group has to be transferred to a keto acid. 2-oxoglutarate and glutamate couple serves as one amino group acceptor and donor pair in all amino transfer reactions (Pratt and Kaplan, 2000).

### 2.7.1 Alanine Aminotransferase (EC 2.6.1.2)

Alanine aminotransferase (ALT), also known as glutamate pyruvate transaminase (GPT), is a pyridoxal enzyme which belongs to the class-I pyridoxal-phosphate-dependent aminotransferase family, Alanine aminotransferase subfamily. Alanine aminotransferase / Gpt1 / ALT catalyses the reversible interconversion of L-alanine and 2-oxoglutalate to pyruvate and L-glutamate, and plays a key role in the intermediary metabolism of glucose and amino acids (Ghouri *et al*., 2010).


Fig. 2.1 Reaction of L-alanine and 2-oxoglutamate
Source: Ghouri et al, 2010

Alanine aminotransferase / Gpt1 / ALT is expressed in Liver, kidney, heart, and skeletal muscles and it expresses at moderate levels in the adipose tissue. As a key enzyme for gluconeogenesis, Alanine aminotransferase is a widely-used serum marker for liver injury (Ghouri *et al*., 2010). Two ALT isoenzymes have been identified, ALT1 and ALT2 (GPT1 and GPT2), which are encoded by separate genes and share significant sequence homology, but differ in their expression patterns. GPT1/Alanine aminotransferase is widely distributed and mainly expressed in intestine, liver, fat tissues, colon, muscle, and heart, in the order of high to low expression level (Goessling *et al*., 2008; Schindhelm *et al*., 2006). Serum activity levels of this enzyme are routinely used as a biomarker of liver injury caused by drug toxicity, infection, alcohol, and steatosis (Ghouri *et al*., 2010; Wang *et al*., 2012).

### 2.7.2 Aspartate Aminotransferase (EC 2.6.1.1)

Aspartate aminotransferase (AspAT/ASAT/AAT) or serum glutamic oxaloacetic transaminase (SGOT), is a pyridoxal phosphate (PLP)-dependent transaminase enzyme

(EC 2.6.1.1). Aspartate aminotransferase catalyzes the reversible transfer of an α-amino group between aspartate and glutamate and, as such, is an important enzyme in amino acid metabolism (Hayashi *et al.*, 1990). AST is found in the liver, heart, skeletal muscle, kidneys, brain, and red blood cells, and it is commonly measured clinically as a marker for liver health (Goessling *et al*., 2008; Schindhelm *et al*., 2006).

Aspartate transaminase catalyzes the interconversion of aspartate and α-ketoglutarate to oxaloacetate and glutamate.

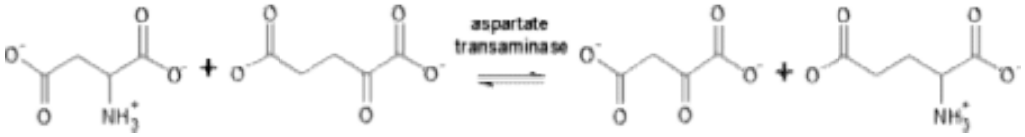



Fig 2.2 Reaction of aspartate and α-ketoglutarate
Source: (Kochhar and Christen, 1992).

As a prototypical transaminase, AST relies on PLP as a cofactor to transfer the amino group from aspartate or glutamate to the corresponding ketoacid. In the process, the cofactor shuttles between PLP and the pyridoxamine phosphate (PMP) form (Kirsch *et al*., 1984). The amino group transfer catalyzed by this enzyme is crucial in both amino acid degradation and biosynthesis. In amino acid degradation, following the conversion of α-ketoglutarate to glutamate, glutamate subsequently undergoes oxidative deamination to form ammonium ions, which are excreted as urea. In the reverse reaction, aspartate may be synthesized from oxaloacetate, which is a key intermediate in the citric acid cycle (Berg *et al*., 2006).

### 2.7.3 Mechanism of Aspartate Aminotransferase

Aspartate transaminase, as with all transaminases, operates via dual substrate recognition; that is, it is able to recognize and selectively bind two amino acids (Asp and Glu) with different side-chains (Hirotsu *et al*., 2005). In either case, the transaminase reaction consists of two similar half-reactions that constitute what is referred to as a ping-pong mechanism. In the first half-reaction, amino acid 1 (e.g., L-Asp) reacts with the enzyme-PLP complex to generate ketoacid 1 (oxaloacetate) and the modified enzyme-PMP. In the second half-reaction, ketoacid 2 (α-ketoglutarate) reacts with enzyme-PMP to produce amino acid 2 (L-Glu), regenerating the original enzyme-PLP in the process. Formation of a racemic product (D-Glu) is very rare (Kochhar and Christen, 1992).

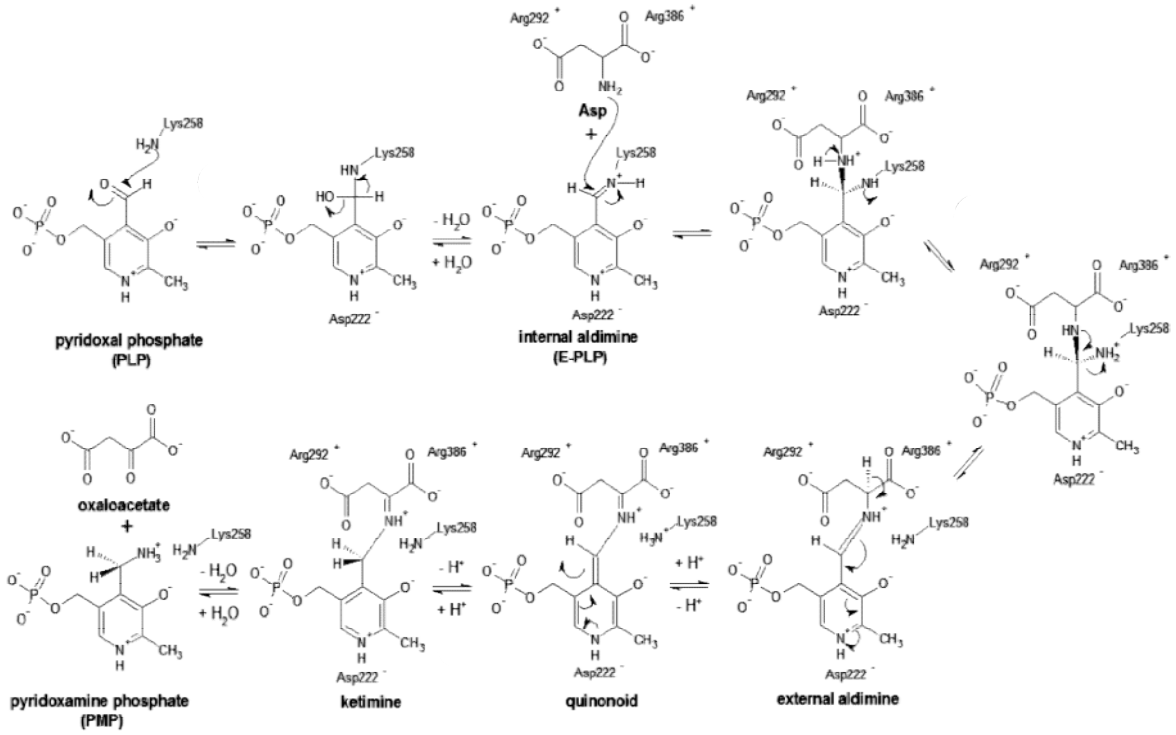



**Fig 2.3:** Reaction Mechanism for Aspartate Aminotransferase
Source: (Kochhar and Christen, 1992).

### 2.7.4 Clinical Significance of Aminotransferases

The activities of both AST and ALT are high in tissues especially liver, heart, and muscles. Any damage or injury to the cells of these tissues may cause release of these enzymes along with other intracellular proteins/enzymes into the circulation leading to increase activities of these enzymes in the blood. Some increases in the activities of both the enzymes are seen after alcohol intake (Goessling *et al*., 2008; Schindhelm *et al*., 2006).

Determinations of activities of AST and ALT in serum in patients with liver diseases like viral hepatitis and other forms of liver diseases with necrosis, give high values even before the appearance of clinical signs and symptoms like jaundice (Gaze, 2007). Activity levels of 20 to 50 fold higher than normal are frequently seen in liver cells damage but it may reach as high as 100 times in severe damage to cells. In myocardial infarction high activity of AST is seen in serum. ALT activity is within normal range or slightly increased in uncomplicated myocardial infarction. Rise in AST is seen within 6 to 8 hours of the onset of chest pain, highest level at 18 to 24 hours and returns to pre-infarction levels by 4th to 5th day (Bhagavan *et al*., 1998). There are other superior markers available for myocardial infarction as AST lacks the tissue specific characteristics, as its activity may

also increased in diseases of other tissues like liver and skeletal muscles (Bhagavan *et al*., 1998; Pratt and Kaplan, 2000). In other conditions like pulmonary emboli, acute pancreatitis, hemolytic disease and gangrene the activity of AST is found to be 2 to 5 times higher than the normal activity (Bhagavan *et al*., 1998).

### 2.8.0 Haematocrit (PCV)

The haematocrit, also known as packed cell volume (PCV) or erythrocyte volume fraction (EVF) is the volume of red blood cells in relation to the volume of plasma (Rodak, 2007). It is normally 45% for men and 40% for women (Purves *et al.*, 2004). It is considered an integral part of a person's complete blood count results, along with haemoglobin concentration, white blood cell count, and platelet count. Because the purpose of red blood cells is to transfer oxygen from the lungs to body tissues, a blood sample's haematocrit – the red blood cell volume percentage – can become a point of reference of its capability of delivering oxygen (Dailey, 2001). Additionally, the measure of a subject's blood sample's haematocrit levels may expose possible diseases in the subject. Anaemia refers to an abnormally low haematocrit, as opposed to polycythemia, which refers to an abnormally high haematocrit. Anaemia results in a decrease in oxygen transport due to reduced oxygen carrying capacity of blood (Birchard, 1997). Anaemia is not a disease, but rather an indicator of a disease somewhere in the body (Marieb, 2003). Iron deficiency, autoimmune diseases, rupturing of the red cell membrane and vitamin deficiency are all causes of anaemia. For a condition such as anaemia that goes unnoticed, one way it can be diagnosed is by measuring the haematocrit in the blood. Both are potentially life – threatening disorder (Dailey, 2001).

### 2.8.1 Variance of Heamatocrit value within the Population

Haematocrit value can vary according to age, sex, ethnicity, illness and environment with normal variation occurring between healthy individuals. Male have a range of 40% to 54%, whereas for female the range is 35% to 40% (Radak, 2007).

It is difficult to obtain a "normal range" or "normal value" for haematology results obtained from a population. This problem arises due to the variation in health among individuals at the time of blood donation (Dacey and Lewis 1991). It is for this reason that the terms 'reference value" and "reference ranges" are often used in association with the physiological variables of a sample population.

Alcoholics, drug and steroid abusers, women following miscarriages, child birth or abortions, malnourished people and the elderly can have decreased haematocrit value ranging from 29% to 15%. When the total red blood cell amount, mass, heamoglobin and haematocrit values decreases by more than 10%, a person is said to be anaemic (Radak, 2007).

Conversely, raised haematocrit values indicate excessive number of red blood cells, which make the blood thick, increases blood viscousity and impairs circulation (Marieb, 2003). These raised values can occur if an individual is dehydrated, in shock, living at high altitude, experiencing or about to experience a heart attack, suffering from hypothermia and following extreme physical activity (Wonder, 2001).

### 2.9.0 Haemoglobin

Haemoglobin is present in red blood cells and is an essential chemical which carries oxygen from lungs to other parts of the body. It is metalloprotein having quarternary structure which contains iron and performs the important function of transporting oxygen via Red Blood Cells (RBCs) in blood in mammals as well as other animals (Maton *et al.*, 1993). It also fulfills different effect modulation and gas transport duties, although which differ from species to species and most probably is altogether different in invertebrates. Some oxygen is dissolved in blood while some bound to haemoglobin. But the more amount of oxygen molecules bounded to haemoglobin, the more oxygen in reached to every part of the body. Hence this pack of chemicals known as haemoglobin performs the most vital duty of binding oxygen to it so that it reaches each and every body part. Hemoglobin has an oxygen-binding capacity of 1.34 mL $O_2$ per gram (Dominguez *et al.*, 1981), which increases the total blood oxygen capacity seventy-fold compared to dissolved oxygen in blood. The mammalian hemoglobin molecule can bind (carry) up to four oxygen molecules (Constanzo, 2007).

The name haemoglobin is made from the blend of heme and globin. Globin is the globular protein in which heme-an iron atom is embedded which is the main chemical binding oxygen. The common type of haemoglobin consists of four subunits:

- Two alpha (α) subunits or globins
- Two beta (β) subunits or globins

All these units are made up of long protein stretch which usually is coiled in the form of eight alpha helices. The heme group is a ring of carbon atoms having an iron atom embedded in the center. This iron atom is safely protected in the centre because this atom is capable of holding the oxygen by forming chemical bond. Iron ion binds to six various things whereas the oxygen forms a coordinate covalent bond with it and gets released in blood at the right place. Iron is a transition metal having red color and that is the reason why our blood is red in color (Sadava *et al.*, 2008).

When we breathe, oxygen in the lungs passes through the thin-walled blood vessels and into the red blood cells, where it binds to the hemoglobin, turning it into the bright red oxy-haemoglobin. The blood then passes around the body until it reaches cells and tissues which require oxygen to sustain their processes. These cells are rich in $CO_2$, which is a waste product of these processes. The $CO_2$ displaces the weakly-bound $O_2$ and forms carbaminohaemoglobin, which then travels in the bloodstream back around to the lungs where it is again displaced by oxygen (Gupta, 2014).

Both oxygen ($O_2$) and carbon (iv) oxide ($CO_2$) bind reversibly to haemoglobin, but certain other molecules, like carbon monoxide (CO), are small enough to fit into the protein crevice, but form such strong bonds with the iron that the process is irreversible. Thus high concentrations of CO rapidly use up the body's limited supply of haemoglobin molecules, and prevent them from binding to oxygen. This is why CO is poisonous - the affected person rapidly dies of asphyxiation because his blood is no longer able to carry enough oxygen to keep the tissues and brain supplied. Hemoglobin binding affinity for CO is 200 times greater than its affinity for oxygen, meaning that small amounts of CO dramatically reduces haemoglobin’s ability to transport oxygen. When haemoglobin combines with CO, it forms a very bright red compound called carboxyhaemoglobin. When inspired air contains CO levels as low as 0.02%, headache and nausea occur. If the CO concentration is increased to 0.1%, unconsciousness will follow. In heavy smokers, up to 20% of the oxygen active sites can be blocked by CO. Another poisonous molecule that binds to hemoglobin is hydrogen cyanide (HCN). Once cyanide is taken into the blood stream the majority (92-99%) is found bound to hemoglobin in red blood cells. From there it is taken to the body's tissues where it binds to an enzyme called cytochrome oxidase and stops cells from being able to use oxygen (Gupta, 2014).

### 2.9.1 Structure of Haemoglobin

Haemoglobin is a tetramer molecule in which the alpha globin chain comprises of 141 amino acids whereas the beta globin chain constitutes 146 amino acids. Both these globin proteins have the same tertiary and secondary structures having 8 helical segments each. Also each chain of globin consists of 1 heme molecule which is made up of porphyrin ring containing 4 pyrrole molecules linked together cyclically along with an iron ion ligand that is bound at the center. This heme molecule is placed in between helix E and helix F of the globin protein. The subunits of globin chains are present in two dimmers and are strongly bonded to each other (Bunn and Forget, 1986).

### 2.9.2 Function of Haemoglobin

The haemoglobin molecules with the help of external chemical factors take up oxygen molecules in the lungs and then send them to the various tissues of our body. The biggest regulator of oxygen affinity in the haemoglobin is the oxygen itself. If in lungs, the oxygen levels are high the haemoglobin shows greater affinity towards the oxygen molecules and as it bounds to more oxygen, this property of affinity increases and vice versa. When the oxyhaemoglobin binds to the maximum capacity, it becomes saturated but its affinity towards oxygen increases whereas when this binding loose oxygen molecule the affinity decreases (Perutz, 1990). This regulation activity is called as cooperativity and is an important function as it allows maximum amount of haemoglobin to be carried to the tissues and also allows deoxyhaemoglobin which is releasing the oxygen tissue. The external chemical factors which help in regulation of oxyhaemoglobin affinity includes pH, DPG (2, 3-diphosphoglycerate) and carbon dioxide (Ackers *et al.*, 1992).

### 2.9.3 Clinical significance of Haemoglobin

Many anaemias are detected by routine laboratory screening performed before the patient is symptomatic. When the patient does have symptoms from an abnormality in the hemoglobin level, the symptoms are often a nonspecific weakness or fatigue. The only finding on physical examination may be pallor; additional changes in the nail beds (such as spooning), glossitis (red tongue), or hepatosplenomegaly (enlarged liver or spleen) may

give a clue to the etiology of the anaemia. Symptoms are usually related to the level of haemoglobin, its abruptness of onset and its duration. A patient with pernicious anaemia may feel well at the same level of haemoglobin that would cause severe weakness in a patient with acute gastrointestinal hemorrhage. This is due to volume compensation by plasma and shifts in the oxygen dissociation curve which occurs over time (Scott *et al.*, 1981).

Anaemia is a very common blood condition which is associated with the decrease in amount of haemoglobin and very predominant in women. It can also be defined as a lowered ability of the blood to carry oxygen (Radak, 2007). The haemoglobin percentage required by the body of both women and men is different and hence when the blood tests reveal less hemoglobin percentage, the person is said to be anaemic. Apart from this there are various other functions and activities related to this component which is also known to give our blood the red colour. Studies reveal that each red blood cell comprises of around 280 million haemoglobin molecules (Griffith and Kaltashov, 2006).

### 2.10.0 Protein

` Proteins are large biomoloecules, or macromolecules, consisting of one or more long chains of amino acid residues. Proteins perform a vast array of functions within living organisms, including catalyzing metabolic reactions, DNA replication, responding to stimuli, and transporting molecules from one location to another.

Proteins are the end products of most information pathways. A typical cell requires thousands of different protein at any given moment. These must be synthesized in response to the cell's current needs, transported (targeted) to their appropriate cellular locations` 1, and degraded when no longer needed (Nelson and Cox, 2013). Proteins are of primary importance in cellular tissues and body structures and functions. Proteins are made of different combinations of about twenty naturally occurring amino acid. They are also different from one another by the amino acids forming them. Amino acids are the building blocks from which all proteins or polypeptides chains are built or synthesized. The amino acid combination in a polypeptide chain and the specific or final structure of the polypeptide or protein is determined by genetic information that is found in the nucleic acid that includes the Deoxyribonucleic Acid (DNA) and Ribonucleic Acid (RNA) (Bolarin, 2013).

Amino acids vary in structures. They have a common features; the presence of an amino ($-NH_2$) group and a carboxyl (-COOH) group. The amine group, which is the nitrogen component, is a major component of amino acid. Amino acids are joined together by peptide bonds forming peptide chains - hence there may be:

a. Two amino acids joined to form a dipeptide
b. Four amino acids to form tetrapeptide
c. "Oligo" (few) to form oligopeptides
d. Many amino acids will form polypeptides

Proteins are mainly large polypeptides of one, two or more chains. The uniqueness of individual protein lies in the arrangement or sequence of its amino acids (Bolarin, 2013).

The body synthesizes non-essential amino acids from other compounds or substances. The synthesis or metabolism and catabolism of proteins take place mainly in the liver while the excretion of the end products of protein metabolism (urea and creatinine, etc.) is carried out mainly in the kidneys.

Essential amino acids cannot be synthesized by the body and must be provided or supplied in diet or foodstuff. The diet must not only contain sufficient protein (i.e. quantitatively sufficient) but also the protein intake must be qualitatively adequate or nourishing. The essential amino acids are very essential for growing children and not very essential for adults. A negative nitrogen balance can result if they are absent from the diet of infants (Bolarin, 2013).

The liver is the major active site in all aspects of amino acid metabolism. It is responsible for the synthesis of urea, which is the main compound in which nitrogen of amino is excreted from the body. Urea is produced through the deamination of amino acid to ammonium ion, which is then converted to urea via a series of reactions requiring specific enzymes in the liver. Urea accounts for about 95 percent of the nitrogen excreted by the kidney (Bolarin, 2013).

### 2.10.1 Biosynthesis of Protein

Protein biosynthesis is the process whereby biological cells generate new proteins; it is balanced by the loss of cellular proteins via degradation or export. Translation, the assembly of amino acids by ribosomes, is an essential part of the biosynthetic pathway, along with generation of messenger RNA (mRNA), aminoacylation of transfer RNA (tRNA), co-translational transport, and post-translational modification.

Protein biosynthesis is strictly regulated at multiple steps. They are principally during transcription (phenomena of RNA synthesis from DNA template) and translation (phenomena of amino acid assembly from RNA).

Proteins are assembled from amino acids using information encoded in genes. Each protein has its own unique amino acid sequence that is specified by the nucleotide sequence of the gene encoding this protein. The genetic code is a set of three-nucleotide sets called codons and each three-nucleotide combination designates an amino acid, for example AUG (adenine-uracil-guanine) is the code for methionine. Because DNA contains four nucleotides, the total number of possible codons is 64; hence, there is some redundancy in the genetic code, with some amino acids specified by more than one codon. Genes encoded in DNA are first transcribed into pre-messenger RNA (mRNA) by proteins such as RNA polymerase. Most organisms then process the pre-mRNA (also known as a primary transcript) using various forms of Post-transcriptional modification to form the mature mRNA, which is then used as a template for protein synthesis by the ribosome. In prokaryotes the mRNA may either be used as soon as it is produced, or be bound by a ribosome after having moved away from the nucleoid. In contrast, eukaryotes make mRNA in the cell nucleus and then translocate it across the nuclear membrane into the cytoplasm, where protein synthesis then takes place. The rate of protein synthesis is higher in prokaryotes than eukaryotes and can reach up to 20 amino acids per second (Dobson, 2000).

The process of synthesizing a protein from an mRNA template is known as translation. The mRNA is loaded onto the ribosome and is read three nucleotides at a time by matching each codon to its base pairing anticodon located on a transfer RNA molecule, which carries the amino acid corresponding to the codon it recognizes. The enzyme aminoacyl tRNA synthetase "charges" the tRNA molecules with the correct amino acids. The growing polypeptide is often termed the *nascent chain*. Proteins are always biosynthesized from N-terminus to C-terminus. The size of a synthesized protein can be measured by the number of amino acids it contains and by its total molecular mass, which is normally reported in units of *daltons* (synonymous with atomic mass units), or the derivative unit kilodalton (kDa). Yeast proteins are on average 466 amino acids long and 53 kDa in mass (Lodish *et al.*, 2004). The largest known proteins are the titins, a

component of the muscle sarcomere, with a molecular mass of almost 3,000 kDa and a total length of almost 27,000 amino acids (Fulton and Isaac, 1991).

### 2.10.2 Cellular function of Protein

Proteins are the chief actors within the cell, said to be carrying out the duties specified by the information encoded in genes (Lodish *et al.*, 2004). With the exception of certain types of RNA, most other biological molecules are relatively inert elements upon which proteins act. Proteins make up half the dry weight of an *Escherichia coli* cell, whereas other macromolecules such as DNA and RNA make up only 3% and 20%, respectively (Voet and Voet, 2004). The set of proteins expressed in a particular cell or cell type is known as its proteome.

The chief characteristic of proteins that also allows their diverse set of functions is their ability to bind other molecules specifically and tightly. The region of the protein responsible for binding another molecule is known as the binding site and is often a depression or "pocket" on the molecular surface. This binding ability is mediated by the tertiary structure of the protein, which defines the binding site pocket, and by the chemical properties of the surrounding amino acids' side chains. Protein binding can be extraordinarily tight and specific. Extremely minor chemical changes such as the addition of a single methyl group to a binding partner can sometimes suffice to nearly eliminate binding; for example, the aminoacyl tRNA synthetase specific to the amino acid valine discriminates against the very similar side chain of the amino acid isoleucine (Sankaranarayanan and Moras, 2001)..

Proteins can bind to other proteins as well as to small-molecule substrates. When proteins bind specifically to other copies of the same molecule, they can oligomerize to form fibrils; this process occurs often in structural proteins that consist of globular monomers that self-associate to form rigid fibers. Protein–protein interactions also regulate enzymatic activity, control progression through the cell cycle, and allow the assembly of large protein complexes that carry out many closely related reactions with a common biological function. Proteins can also bind to, or even be integrated into, cell membranes. The ability of binding partners to induce conformational changes in proteins allows the construction of enormously complex signaling networks. Importantly, as interactions

between proteins are reversible, and depend heavily on the availability of different groups of partner proteins to form aggregates that are capable to carry out discrete sets of function, study of the interactions between specific proteins is a key to understand important aspects of cellular function, and ultimately the properties that distinguish particular cell types (Copland *et al.*, 2009).

### 2.10.3 Clinical significance of Protein

A decrease in serum total protein may reflect decreased protein synthesis or increased protein loss. Nearly all proteins are synthesized in the liver; hence, hepatic failure is a cause of decreased serum protein. However, serum total protein is not a sensitive measure of hepatic failure because most proteins have biological half-lives of days to weeks. Therefore, inadequate production of proteins by a failing liver may not be reflected in low serum protein until after other symptoms of hepatic failure are already present, such as jaundice, hyperammonemia and coagulopathy.

Protein synthesis requires dietary amino acids that cannot be synthesized (ie, the essential amino acids). Thus, decreased serum protein levels may also result from malnutrition. As in hepatic failure, the decrease in total protein resulting from malnutrition does not appear until existing proteins are degraded, which may take several weeks. A group of malabsorptive disorders, such as celiac disease, Crohn syndrome, and short-bowel syndrome cause hypoproteinemias, often known as protein-losing enteropathy. This is a misnomer because the protein is not lost; rather, the inability to absorb proteins causes a deficiency in essential amino acids, resulting in deficient protein synthesis even with adequate protein intake.

A decrease in total protein is also observed when hepatic function is normal but the proteins are lost in the urine. In healthy kidneys, the glomerular membrane excludes most proteins from crossing into the Bowman's capsule, and the small amount of protein that is filtered is mostly reabsorbed by the renal tubules. Damage to the glomerular membrane from toxins or inflammation causes it to become leaky and the greater concentration of proteins in the filtrate overwhelms the capacity of the renal tubules to reabsorb protein. Loss of proteins in the urine results in a decrease in total serum protein.

Elevation of serum protein concentration has 2 principal causes: dehydration, in which there is less water in the body and the blood volume decreases, thereby concentrating the proteins, and overproduction of specific proteins, which is more

common. The most commonly overproduced proteins are immunoglobulins, the levels of which can be elevated in infections and in hematological neoplasms.

Disorders associated with high or low serum concentrations of specific proteins other than albumin are numerous. Transferrin and ferritin are used to assess iron status; ceruloplasmin reflects copper transport and storage; cardiac troponins reveal myocardial damage; tumor markers such as prostate-specific antigen (PSA), alpha fetoprotein (AFP), carbohydrate antigen (CA) markers, carcinoembryonic antigen (CEA), and so forth are used to detect, and monitor treatment of, cancer; fibrinogen and coagulation factors are used to assess hemostatic function; and various enzymes reveal tissue damage and necrosis (Roger, 2014).

### 2.11.0 Albumin

Albumin is a protein produced by the liver. It is most abundant in human plasma. Normally it constitutes about 55% of all plasma proteins. Albumin performs multiple functions, including transport of many small molecules in the blood, such as bilirubin, calcium, and magnesium. Albumin also binds to toxins and heavy metals, thereby preventing damage they might otherwise cause to your body. One of albumin's major roles is in the maintenance of "osmotic or oncotic pressure" that causes fluid to remain within the blood stream instead of leaking out into the tissues (Peter, 1996).

Total serum protein is made up of albumin and globulins. Albumin is the farthest-migrating protein toward the anode at 6.8 pH followed by $\alpha_1$- globulin, $\alpha_2$-globulin, beta-(β-) globulins and gamma-(ɣ-) globulins. Albumin is relatively a small protein – the molecular weight is about 66.3kDa (Bolarin, 2013).

### 2.11.1 Synthesis and function of Albumin

Albumin is synthesized in the liver as preproalbumin which has an N-terminal peptide that is removed before the nascent protein is released from the rough endoplasmic reticulum. The product, proalbumin, is in turn cleaved in the Golgi vesicles to produce the secreted albumin.

Albumin is the most abundant plasma or serum protein in the vascular or extra-vascular medium. Albumin is responsible for the transport of important blood constituent such as drugs, hormones (e.g. thyroid hormones, etc.), bilirubin, lipids (for example fatty acids), enzymes, metals (e.g. protein-bound calcium, etc.), injected dyes (e.g.

Bromosulphthalein, etc) and others. It acts as reservoir for some hormones and as a source of amino acid for peripheral tissues because it acts as a carrier for these hormones and absorbed dietary amino acids (Bolarin, 2013). Albumin also plays a major role in stabilizing extracellular fluid volume by contributing to oncotic pressure (known also as colloid osmotic pressure) of plasma. Because smaller animals (for example rats) function at a lower blood pressure, they need less oncotic pressure to balance this, and thus need less albumin to maintain proper fluid distribution.

### 2.11.2 Clinical significance of Albumin

Albumin test can help determine if a patient has liver disease or kidney disease, or if the body is not absorbing enough protein. The normal range is between 3.4 to 5.4 g/dL. Normal value ranges may vary slightly among different laboratories (McPherson, 2011).

Low albumin levels can signal a number of health conditions, including:

- Liver disease
- Inflammation
- Shock
- Malnutrition
- Nephritic syndrome
- Crohn's disease
- Celiac disease
- Burns (Rachel, 2012).

A diseased liver might produce lesser albumin. In kidney disease, albumin can escape into the urine in large amounts. Severe malnutrition or a very low protein diet can also reduce the albumin level. If the concentration of albumin gets very low, fluid moves from the blood vessels into the tissues, resulting in swelling in the ankles (edema). This fluid can also accumulate in the abdomen (ascites) and in the lungs (pulmonary edema) (Peter, 1996).

Increased blood albumin may be due to:

- Dehydration
- High protein diet
- Having a tourniquet on for a long time when giving a blood sample

## CHAPTER 3

# MATERIALS AND METHODS

### 3.1 *Aframomum sceptrum*

The *Aframomum sceptrum* (Atiko) were obtained from Abraka main market, Delta State and were identified at the Department of Botany, Delta State University, Abraka.

### 3.2 Preparation of *Aframomum sceptrum* extract

*Aframomum spectrum* seeds were sun-dried to obtain a constant weight for two weeks. They were then ground to fine powder using Warren blender. Twenty five grams (25g) of the ground seed was soaked in 100ml of distilled water boiled for five minutes (5 mins). This was then shaken for ten minutes (10 mins) and allowed to cool then filtered. The extract was then concentrated using rotary evaporator at 40 – 50$^{o}$C under reduced pressure. The extracts were stored frozen in a deep freezer until required.

### 3.3 Preparation of Cyanide solution

Potassium cyanide (KCN) solution was prepared at a concentration of 9.0mg/kg in the drinking tap water (0.09g/L). Every day the amount of KCN administered in the drinking water was adjusted to body weight and the water consumption and fresh solution of cyanide provided daily. The stability of KCN in the drinking water is stable for at least four (4) days after preparation.

### 3.4 Experimental animals and procedure

A total of thirty (30) albino rats eight weeks old male weighing between 100g and 160g were obtained from the animal house Delta State University Abraka. The rats were fed on growers mash and were given water ad libitum. The rats were housed in cages constructed of wood and wire guaze under controlled condition of 12 hours light and 12 hours dark cycle. The rats were divided into six (6) groups of 5 rats per group as follows:

**Group 1:** Normal control: Rats in this group received grower mash and tap water daily throughout the experiment

**Group2:** Cyanide control: Animals in this group were given KCN solution at concentration 9.0mg/kg in the drinking tap water every day in addition to their food.

**Group 3:** Cyanide + *A. sceptrum* extract (ASE) Dose 1: The rats in this group were given KCN solution and 10mg/kg of ASE plus growers mash.

**Group 4:** Cyanide + *A. sceptrum* extract (ASE) Dose 2: Animals in this group were given KCN solution and 20mg/kg of ASE plus growers mash.

**Group 5:** Normal + *A. sceptrum* extract (ASE) Dose 1: The rats in this group received normal tap water and 10mg/kg of ASE plus growers mash.

**Group 6:** Normal + *A. sceptrum* extract (ASE) Dose 2: Animals in this group were given tap water and 20mg/kg of ASE plus growers mash.

### 3.5 Administration of *Aframomum sceptrum* extracts

The oral administration of the *A. sceptrum* extracts were carried out using intragastric tube, three times per day for four (4) weeks. On the last day the rats were allowed to fast overnight and sacrificed by cervical decapitation.

### 3.6 Biochemical Analysis

#### 3.6.1 Determination of Packed Cell Volume (PCV)

Packed cells volume is a measure of the relative of red blood cells in a certain amount of blood sample. It is aimed at calculating the mean cell haemoglobin (red blood cell) concentration to plasma in a given blood sample. The method of Jones (1961) was used to determine PCV.

**Principle:**

The packed cell volume is that proportion of whole blood occupied by red cells. Anti-coagulated blood in a heparinised capillary tube is centrifuged and the PCV value is read with a microhaematocrit reader.

**Method**

Microhaematocrit technique

**Procedures:**

About ¾ of a heparinize capillary tube was filled with blood. One end was sealed using plasticine. The capillary tube is then carefully located in a groove of the numbered grooves

with the sealed end against the ring gasket. The number of the groove is written out. It was then centrifuged for 3-5 minutes (at 3000rpm). The PCV value was read from the scale of microhaematocrit reader immediately after centrifuging and then the result recorded.

### 3.6.2 Determination of Heamoglobin

Heamoglobin was determined by Tietz (1976) method using commercially available heamoglobin kit (TECO Diagnostics, U.S.A.).

**Principle:**

In the cyanmethemoglobin method, erythrocytes are lysed by a stromatolytic agent in the present of a surfactant and release their heamoglobin into solution. Heamoglobin is oxidized to methemoglobin by ferricyanide and the methemoglobin is converted into the stable cyanmethemoglobin by addition of KCN. The absorbance of cyanmethemoglobin is measured at 540nm and colour intensity is proportional to heamoglobin concentration (Tietz, 1976).

**Procedure:**

Two milliliters (2.0ml) of heamoglobin reagent was dispened into test tubes labeled "blank", "Standard" and "Test". 0.01ml of sample was added into respective tubes. They were mixed and allow to stand for 3 minutes at room temperature. 2.0ml of standard reagent was added to the test tube labeled standard. The spectrophotometer was set to 540nm and zero with the reagent blank. The absorbance values of all tubes was read and recorded.

**Calculation:**

$$\text{Heamoglobin (g/dl)} = \frac{\text{Absorbance of Test}}{\text{Absorbance of Standard}} \times \text{Conc. of standard (g/dl)}$$

### 3.6.3 Determination of Serum Total Protein

The serum protein was determined using the method of Tietz (1995) using commercially available Total protein kit (RANDOX Laboratory Ltd, United Kingdom).

**Principle:**

Cupric ions, in an alkaline medium, interact with protein peptide bonds resulting in the formation of a coloured complex

**Procedure:**

To three test tubes labeled reagent blank, standard and sample, 0.02ml of distilled water, 0.02ml of standard solution and 0.02ml of sample were added respectively. 1.0ml of reagent 1 (R1) was then added to each of the test tubes. They were mixed well and incubated for 30 minutes at 20 to 25$^{o}$C. The absorbance of the sample ($A_{sample}$) and the standard ($A_{standard}$) was measured against the reagent blank at 570 nm wavelength.

**Calculation:**

$$\text{Total protein conc. (g/dl)} = \frac{A_{sample}}{A_{standard}} \times \text{Standard conc.}$$

### 3.6.4 Determination of Serum Albumin

Serum albumin was determined using the method of Doumas *et al.* (1971) using commercial available albumin reagent set (TECO Diagnostics, U.S.A.).

**Principle:**

Serum albumin binds selectively to the dye bromocresol green at pH 4.2. The increase in absorbance of the resulting albumin-dye complex, read at 630nm, is proportional to the albumin concentration.

**Procedure:**

Into three test tubes labeled blank, standard and test was added 1.5ml of reagent. Thereafter 0.01ml of sample was added to respective tubes, mixed and allowed to stand at room temperature for 5 minutes. The spectrophotometer was zeroed with blank at 630nm and the absorbance of the tubes were read and recorded.

**Calculation:**

$$\text{Albumin (g/dl)} = \frac{\text{Absorbance of test}}{\text{Absorbance of standard}} \times \text{concentration of standard}$$

### 3.6.5 Assay of Enzymes

#### 3.6.5.1 Assay for Aspartate Aminotransferase (AST) Activity

Estimation of Aspartate Aminotransferase (AST) activity in the serum was carried out by the method of Reitman and Frankel (1957) using AST test kit (RANDOX Laboratories, United Kingdom). The assay is based on the following reaction of the enzyme.

$$\text{L - Aspartate} + \alpha\text{-oxoglutarate} \xrightarrow{\text{GOT}} \text{Oxaloacetate} + \text{L- glutamate}$$

Aspartate Aminotransferase was measure by monitoring the concentration of oxaloacetate hydrazone formed with 2,4 – dinitrophenylhydrazine.

**Procedure:**

Reagents were prepared according to instructions. Into 2 test tubes labeled sample and blank were dispersed 0.5ml of reagent 1. Into sample test tube, 0.1ml of sample was added and 0.1ml of distilled water into blank test tube. Mix and incubated for exactly 30 minutes at 37$^{o}$C. 0.5ml of reagent 2 was then added to both test tubes. The resulting solution was allowed to stand for exactly 20 minutes at 20 to 25$^{o}$C before addition of 5.0ml of sodium hydroxide to both test tubes. Mix and read the absorbance of the sample ($A_{sample}$) against the reagent blank at 546nm after 5 minutes.

**Calculation:**

The activity of AST in the serum was obtained from standard AST plot as shown in Appendix III.

### 3.6.5.2 Assay for Alanine Aminotransferase (ALT) Activity

Estimation of Alanine Aminotransferase (ALT) activity in the serum was carried out by the method of Reitman and Frankel (1957) using ALT test kit (RANDOX Laboratories, United Kingdom). The assay is based on the following reaction of the enzyme.

$$\text{L - Alanine} + \alpha\text{-oxoglutarate} \xrightarrow{\text{GPT}} \text{pyruvate} + \text{L- glutamate}$$

Alanine Aminotransferase is measure by monitoring the concentration of pyruvate hydrazone formed with 2,4 – dinitrophenylhydrazine.

**Procedure:**

Reagents were prepared according to instructions. Into 2 test tubes labeled sample and blank were dispersed 0.5ml of reagent 1. Into sample test tube, 0.1ml of sample was added and 0.1ml of distilled water into blank test tube. Mix and incubated for exactly 30 minutes at 37$^{o}$C. 0.5ml of reagent 2 was then added to both test tubes. The resulting solution was allowed to stand for exactly 20 minutes at 20 to 25$^{o}$C before addition of 5.0ml of sodium hydroxide to both test tubes. Mix and read the absorbance of the sample ($A_{sample}$) against the reagent blank at 546nm after 5 minutes.

**Calculation:**

The activity of ALT in U/l in the serum was obtained from standard plot as shown in Appendix IV.

### 3.6.5.3 Assay for Alkaline Phosphatase (ALP) Activity

The activity of Alkaline Phosphatase (ALP) in serum was estimated spectrophotometrically by kinetic method using a commercial available diagnostic kit (TECO Diagnostics, U.S.A).

**Principle:**

The alkaline phosphatase acts upon the AMP buffered sodium thymolphthalein monophosphate. The addition of an alkaline reagent stops enzyme activity and simultaneously develops a blue chromogen, which is measured photometrically.

**Procedure:**

For each sample, 0.5ml of Alkaline Phosphatase substrate was dispensed into labeled test tubes and equilibrates to 37$^{o}$C for 3 minutes. At timed intervals, 0.05ml each of standard, control and sample was added to its respective test tube. It gently mixed. Deionized water was used as reagent blank. This was then incubated for exactly 10 minutes at 37$^{o}$C. Following the same time interval as above, 2.5ml of alkaline phosphatase colour developer was added and mixed well. The spectrophotometer was zeroed using the reagent blank at 590nm. The absorbance of the sample was then read.

**Calculation:**

$$\frac{\text{Absorbance of Sample}}{\text{Absorbance of standard}} \times \text{Value of standard (IU/L)}$$

### 3.6.5.4 Assay for Superoxide Dismutase (SOD) Activity

The activity of SOD in the serum and liver was determined spectrophotometrically using the method of Misra and Fredorich (1972).

**Principle:**

The assay of SOD is an indirect method based on the inhibitory effect of SOD in the initial rate of epinephrine auto-oxidation. One unit of SOD was given as the amount of SOD necessary to cause 50% inhibition of the oxidation of epinephrine to adrenochrome during 60 seconds.

**Procedure:**

The homogenate supernatant or serum (0.2ml) was added to 2.5ml of 0.05M carbonate buffer (pH 10.2) to equilibrate in the spectrophotometer and the reaction started

by the addition of 0.3ml freshly prepared epinephrine (0.3mM) as the substrate to the buffer-supernatant mixture and quickly mixed by inversion.

The reference cuvette contains 2.5ml of the buffer, 0.3ml of substrate and 0.2ml of deionized water. The increase absorbance at 480nm due to adrenochrome formed was monitored every 30 seconds for 120 seconds.

**Calculation:**

a. % Inhibition = $100 - 100\left(\frac{\text{Epinephrine oxidation in the presence of SOD`}}{\text{Epinephrine oxidation in the absence of SOD}}\right)$

Where 1 unit of SOD activity = amount of SOD giving 50% inhibition

b. Indirect method for calculating SOD activity

$$\text{Units/g wet tissue} = \frac{\%\ \text{Inhibition x 1 x 1000}}{X^1 \text{ x } 50}$$

Where, $X^1$ = mg of tissue in reaction mixture

1/50 converts to 50% inhibition

1000 converts to g of wet tissue

### 3.6.5.5 Assay of Catalase (CAT) Activity

The activity of catalase was determined in the serum by the method of Kaplan (1972).

**Principle:**

In the assay, excess potassium permanganate was added and then residual unreacted permangantate was measured spectrophotometrically. It has been shown that the decomposition of hydrogen peroxide by catalase follows first order kinetics (Haber and Weiss, 1934).

**Procedure:**

This was carried out by pipetting 1.0ml phosphate buffer into a reference cuvette. Then, 2.0ml of sample was added into the reference cuvette and test cuvette respectively. Enzymatic reaction was initiated by adding 1.0ml of cold 10mM $H_2O_2$ into the test cuvette and mixing thoroughly. The reaction was carried out in an ice-water bath (0-2$^o$C). After exactly 3 minutes, the substrate concentration was measured at 240nm.

**Calculation:**

The mathematical determination of the activity was done using the formula below:

$$K = \frac{S_o}{} \text{ x } 2.3$$

$$S_3 \quad \overline{t}$$

Where K = First order rate constant

t = time interval over which the reaction is measured (viz 3 mins)

$S_o$ = Substrate concentration at zero time

$S_3$ = Substrate concentration at 3 minutes

### 3.6.5.6 Assay for Glutathione Peroxidase (GPx) Activity

The activity of Glutathione Peroxidase (GPx) was determined using the method of Khan *et al.*, 2009.

**Principle:**

$$2GSH + H_2O_2 \xrightarrow{\text{Glutathione Peroxidase}} GSSG + 2H_2O$$

$$GSSG + \beta\text{-NADPH} \xrightarrow{\text{Glutathione Peroxidase}} \beta\text{-NADP} + GSH$$

Glutathione peroxidase activity was measured by monitoring the disappearance of β-NADPH.

**Procedure:**

The reagents, 1.49ml phosphate buffer (0.1M; pH 7.4), 0.1ml EDTA (1mM), 0.1ml sodium azide (1mM), 0.05ml glutathione reductase (1 IU/ml), 0.05ml GSH (1mM), 0.1ml NADPH (0.2mM), 0.01ml $H_2O_2$ (0.25mM) were mixed with 0.1ml of liver homogenate in a total volume of 2ml. Using a spectrophotometer at 340nm the disappearance of NADPH was recorded at 25$^oC$. The activity of the enzyme was calculated as nM NADPH oxidized per minutes per mg protein using molar extinction of 6.22 x $10^3$ $M^{-1}cm^{-1}$.

### 3.6.6 Determination of Serum Reduced Glutathione

The serum reduced glutathione level was estimated using the method of Beulter *et al.* (1963).

**Principle:**

The sulfhydryl group of reduced glutathione reacted with DTNB (5,5 - dithiobisnitro benzoic acid, Elman's reagent) and produce a yellow colour, 5 – thio – 2 – nitrobenzoic acid (TNB). The mixed disulphide, GSTNB (between GSH and TNB) that is concomitantly produced is reduced by glutathione reductase to recycle the GSH and produce more TNB. The rate of TNB produced is directly proportional to the concentration of GSH in the sample.

**Procedure:**

Two tenth milliliters of serum was added to 1.8ml of distilled water. 3ml of the precipitating solution was mixed with the hemolysate. The mixture was allowed to stand for approximately 5 minutes and then filtered. 2ml of the filtrate was added to 8ml of the phosphate solution in a test tube. 1ml of DTNB in a solution was then added. A blank was prepared with 8ml of phosphate solution, 2ml of precipitating solution and 1ml of the DTNB reagent. The absorbance reading was measured at 412nm wavelength using spectrophotometer.

**Calculation:**

Serum reduced GSH (%mg in blood) = 310.4 x E x Absorbance

Where E = the correction factor = 0.542

The liver reduced glutathione was estimated using the method of Ellman, (1959).

**Procedure:**

To 0.5ml of tissue homogenate, added 2ml 10% trichloroacetic acid and centrifuged. 1ml of supernatant was treated with 0.5ml of Ellman's reagent and 3ml of phosphate buffer. The colour developed was read at 412nm. A series of standard were treated in similar manner along with a blank containing 3.5ml of buffer.

**Calculation:**

The concentration of reduced GSH in μmole of GSH/0.5g of wet tissue will be extrapolated from a standard curve plotted.

### 3.6.7 Determination of Lipid Peroxidation

A breakdown product of lipid peroxidation thiobarbitoric acid reactive substance (TBARS) was determined in the serum by the method of Gutteridge and Wilkins (1982).

**Principle:**

Malondialdehyde (MDA) formed from the breakdown of polyunsaturated fatty acid (PUFA) served as a convenient index for the determination of the extent of peroxidation reaction. A malondialdehyde will be identified with the barbituric acid to give a red species which will be assayed at 532nm.

**Procedure:**

Into 2ml of glacial acetic acid was added 0.2ml of sample followed by 2ml 1% TBA in 0.05M NaOH. The loosely stopped tubes was immersed in boiling water bath for 15 minutes, allowed to cool and centrifuged at 800rpm for 15 minutes. The clear

supernatant was carefully transferred into a cuvette and absorbance read at 532nm against a reagent blank. A molar extinction coefficient of 1.56 x $10^5$ $m^{-1}cm^{-1}$ was used according to the expression of Adam-Vizi and Seregi (1982).

**Calculation:**

$$\text{MDA (unit/g tissue)} = \Delta A \times \frac{V_T}{E \times V_s \times X^1}$$

Where $\Delta A$ = Change in absorbance (nm)

$V_T$ = Total volume of reaction = 4.2ml

$V_s$ = Volume of sample (0.2ml)

$X^1$ = Weight of tissue in reaction mixture (g)

E = Molar absorbance index = 1.56 x $10^5 m^{-1}cm^{-1}$

### 3.6.8 Histology of the Liver

The histological analysis of the liver was carried out in the Histology Laboratory, Central Hospital, Warri Delta State.

### 3.6.9 Statistical analysis

The data for various biochemical parameters were analyzed using analysis of variance (ANOVA) and the group mean were compared by Tukey Multiple Range Test (TMRT). Values are given as mean $\pm$ S.D.

## CHAPTER FOUR

## 4.0 RESULTS

The results in Table 4.1 shows the effect of A. sceptrum on Heamatocrit (PCV) and Heamoglobin (Hb) levels in rats exposed to cyanide. The heamatocrit and haemoglobin values of Group B (cyanide control) was significantly ($p<0.05$) lower than all other groups. No significant difference was observed in the The heamatocrit and haemoglobin values of Group E and F when compared with group A. A significant increase was observed in the heamatocrit and haemoglobin values in group C and D when compared with group B.

**Table 4.1: The effect of *A. sceptrum* on Heamatocrit (PCV) and Heamoglobin (Hb) levels in rats exposed to cyanide**.

| Parameters | Experimental Groups | | | | | |
|---|---|---|---|---|---|---|
| | A | B | C | D | E | F |
| PCV (%) | $30.2\pm2.464^{a}$ | $14.20\pm1.75^{b}$ | $19.06\pm0.60^{c}$ | $23.46\pm0.96^{d}$ | $30.36\pm1.17^{a}$ | $32.56\pm2.87^{a}$ |
| Hb (mg/dl) | $12.14\pm2.62^{a}$ | $5.25\pm1.71^{b}$ | $8.56\pm1.17^{c}$ | $9.26\pm0.61^{cd}$ | $13.16\pm0.28^{a}$ | $13.46\pm0.52^{a}$ |

Values are expressed as Mean ± SD, n = 5. Values with different alphabet superscripts in the same row differ significantly ($p<0.05$).

**Key**

Group A= Normal Control

Group B= Cyanide Control

Group C = Cyanide + 10 mg /kg body weight aqueous extract of *A. sceptrum*

Group D = Cyanide + 20 mg /kg body weight aqueous extract of *A. sceprum*

Group E = Normal + 10 mg /kg body weight aqueous extract of *A. sceptrum*

Group F = Normal + 20 mg /kg body weight aqueous extract of *A. sceptrum*

The Table 4.2 shows changes in serum total protein and albumin levels in rats exposed to cyanide treated with aqueous extract of *A. sceptrum.* The serum total protein and albumin levels in group B (cyanide exposed rats) were significantly ($p<0.05$) lower compared with group A. Group C and D showed significant increased in serum total protein and albumin levels when compared with group B respectively. The rats not exposed cyanide receiving treatment with 10mg/kg body weight of *A. sceptrum* (group E) and 20mg/kg body weight of *A. sceptrum* (group F) showed no significant in serum total protein and albumin levels when compared with group A .

**Table 4.2: Changes in serum Total Protein and Albumin levels in rats exposed to cyanide treated with aqueous extract of *A. sceptrum.***

| Parameters | Experimental Groups | | | | | |
|---|---|---|---|---|---|---|
| | A | B | C | D | E | F |
| Total Protein (g/dl) | 14.66±7.64[a] | 7.32±1.33[b] | 13.69±0.78[ab] | 14.82±1.11[a] | 15.66±1.88[a] | 16.76±2.31[a] |
| Albumin(g/dl) | 9.48±1.46[a] | 2.30±0.72[b] | 4.82±0.81[c] | 6.36±1.11[c] | 9.17±0.42[a] | 10.36±1.31[a] |

Values are expressed as Mean ± SD, n = 5. Values with different alphabet superscripts in the same row differ significantly ($p<0.05$).

Table 4.3 indicates the effect of *A. sceptrum* on serum ALT, AST and ALP activities in rats exposed to cyanide. The results show that the ALT, AST and ALP activities in the serum of group B rats was significantly ($p<0.05$) higher when compared with all the experimental groups. Treatment with 10mg/kg body weight of *A. sceptrum* (group C) and 20mg/kg body weight of *A. sceptrum* (group D) there were significant ($p<0.05$) decreased in serum ALT, AST and ALP activities when compared with group B respectively. No significant difference was observed in the serum ALT, AST and ALP activities in group E and F when compared with group A respectively.

**Table4. 3: The effect of *A. sceptrum* on serum AST, ALT and ALP activities in rats exposed to cyanide.**

| Parameters | Experimental Groups |
|---|---|

| | A | B | C | D | E | F |
|---|---|---|---|---|---|---|
| AST(u/l) | 30.14±1.64[a] | 50.10±4.42[b] | 40.68±3.52[c] | 35.76±1.59[ac] | 29.26±2.34[ad] | 28.66±3.81[a] |
| ALT(u/l) | 20.28±0.79[a] | 41.38±2.40[b] | 33.16±2.54[c] | 27.54±2.17[d] | 19.68±0.80[a] | 18.84±0.63[a] |
| ALP(u/l) | 110.02±14.58[a] | 160.26±3.86[b] | 141.92±7.90[c] | 121.88±4.22[ad] | 106.56±4.06[ad] | 100.26±9.90[a] |

Values are expressed as Mean ± SD, n = 5. Values with different alphabet superscripts differ significantly ($p<0.05$).

Table 4.4 shows the effect of *A. sceptrum* on ALT, AST and ALP activities in the liver of rats exposed to cyanide. The ALT, AST and ALP activities in the liver of group B rats was significantly ($p<0.05$) higher when compared with all the experimental groups. Treatment with 10mg/kg body weight of *A. sceptrum* (group C) and 20mg/kg body weight of *A. sceptrum* (group D) there were significant ($p<0.05$) decreased in serum ALT, AST and ALP activities when compared with group B respectively. No significant difference was observed in the serum ALT, AST and ALP activities in group E and F when compared with group A respectively.

**Table 4.4: The effect of *A. sceptrum* on liver AST, ALT and ALP activities in rats exposed to cyanide.**

| Parameters | Experimental Groups | | | | | |
|---|---|---|---|---|---|---|
| | A | B | C | D | E | F |
| AST(u/l) | 44.22±2.84[ad] | 60.30±4.29[b] | 50.88±5.04[ac] | 45.48±2.67[a] | 42.58±3.31[ad] | 41.54±0.98[ad] |
| ALT(u/l) | 35.40±3.64[a] | 56.20±4.61[b] | 48.20±5.21[c] | 42.58±1.87[ac] | 32.86±3.78[a] | 30.76±3.78[a] |
| ALP(u/l) | 130.89±2.13[a] | 180.50±7.82[b] | 168.68±1.95[cd] | 159.62±2.62[d] | 127.82±5.43[a] | 126.04±4.99[a] |

Values are expressed as Mean ± SD, n = 5. Values with different alphabet superscripts differ significantly ($p<0.05$).

Table 4.5 shows changes in superoxide dismutase (SOD) and catalase (CAT) activities in the serum of rats exposed to cyanide treated with aqueous extract of *A. sceptrum.* The results indicate that the serum SOD and CAT activities in group B were significantly lower when compared with all other experimental groups (group A, C, D, E and F). Treatment

with 10mg/kg body weight of *A. sceptrum* (group C) and 20mg/kg body weight of *A. sceptrum* (group D) showed significant increased in serum SOD and CAT activities when compared with group B respectively. The rats not exposed cyanide receiving treatment with 10mg/kg body weight of *A. sceptrum* (group E) and 20mg/kg body weight of *A. sceptrum* (group F) showed no significant difference in serum SOD and CAT activities when compared with group A.

**Table 4.5: Changes in Superoxide dismutase (SOD) and Catalase (CAT) activities in the serum of rats exposed to cyanide treated with aqueous extract of *A. sceptrum*.**

| Parameters | Experimental Groups | | | | | |
|---|---|---|---|---|---|---|
| | A | B | C | D | E | F |
| SOD (units/g wet tissue) | 74.80±3.88[a] | 26.56±4.48[b] | 40.78±1.19[c] | 51.99±4.76[d] | 74.68±3.19[a] | 76.52±3.60[a] |
| CAT(units/g wet tissue) | 38.33±2.22[a] | 15.30±5.05[b] | 23.94±2.55[c] | 34.40±3.71[a] | 39.46±3.68[a] | 40.07±3.98[a] |

Values are expressed as Mean ± SD, n = 5. Values with different alphabet superscripts in the same row differ significantly ($p<0.05$).

Table 4.6 indicates the changes in superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) activities in the liver of rats exposed to cyanide treated with aqueous extract of *A. sceptrum*. The activity of SOD, CAT, and GPx in the liver of group B were significantly lower ($p<0.05$) when compared to all the other experimental groups (group C, D, E and F) respectively. The results also revealed that treatment with 10mg/kg body weight of *A. sceptrum* (group C) and 20mg/kg body weight of *A. sceptrum* (group D) there were significant increased in the liver SOD, CAT, and GPx activities when compared with the cyanide control group (group B) respectively. There were no significant difference in group E and group F in liver SOD, CAT, and GPx activities when compared with group A respectively.

**Table 4.6: Changes in Superoxide dismutase (SOD), Catalase (CAT) and Glutathione peroxidase (GPx) activities in the liver of rats exposed to cyanide treated with aqueous extract of *A. sceptrum*.**

Values are expressed as Mean ± SD, n = 5. Values with different alphabet superscripts in the same row differ significantly ($p<0.05$).

In Table 4.7 below, the blood reduced glutathione level was significantly ($p < 0.05$) lower in group B (cyanide control) as compared to group A (normal control) and all other groups. Group C and D have a significant increase in blood reduced glutathione level as compared with cyanide control group. No significant difference was observed in the blood GSH level in group E and F when compared with group A.

**Table 4.7: The level of blood reduced glutathione (GSH) of rats exposed to cyanide treated with aqueous extract of *A. sceptrum*.**

| Parameters | Experimental Groups | | | | | |
|---|---|---|---|---|---|---|
| | A | B | C | D | E | F |
| SOD(units/g wet tissue) | 85.33±7.73$^{a}$ | 37.40±5.03$^{b}$ | 48.04±2.35$^{c}$ | 54.50±1.27$^{cd}$ | 86.20±3.83$^{a}$ | 87.76±2.17$^{a}$ |
| CAT(units/g wet tissue) | 45.43±2.88$^{a}$ | 25.38±3.62$^{b}$ | 34.64±3.83$^{c}$ | 42.48±1.82$^{a}$ | 47.16±1.84$^{a}$ | 48.48±1.29$^{ad}$ |
| GPX(units/g wet tissue) | 11.27±3.66$^{a}$ | 4.35±1.96$^{b}$ | 7.04±1.12$^{b}$ | 8.96±0.96$^{c}$ | 12.02±0.65$^{a}$ | 12.48±0.52$^{a}$ |
| Parameters | Experimental Groups | | | | | |
| | A | B | C | D | E | F |
| GSH (mg % of blood) | 7.41±2.44$^{a}$ | 0.914±0.22$^{b}$ | 4.86±0.17$^{c}$ | 6.14±0.66$^{ac}$ | 7.75±0.58$^{a}$ | 8.33±0.57$^{ad}$ |

Values are expressed as Mean ± SD, n = 5. Values with different alphabet superscripts in the same row differ significantly ($p<0.05$).

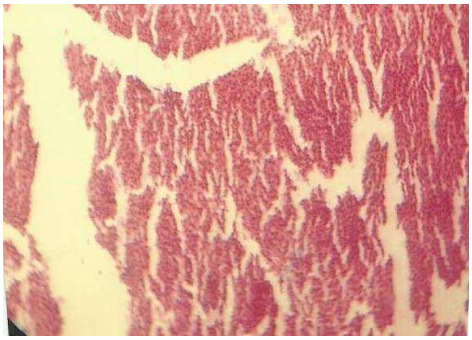
**Table 4.8: Changes in reduced glutathione (GSH) and Malonyldialdehyde (MDA) levels in the liver of rats exposed to cyanide treated with aqueous extract of *A. sceptrum*.**

| Parameters | Experimental Groups |
|---|---|

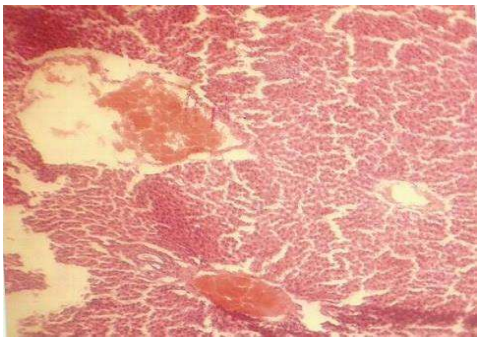
| | A | B | C | D | E | F |
|---|---|---|---|---|---|---|
| GSH(units/g wet tissue) | $17.43\pm3.46^{a}$ | $9.18\pm2.89^{b}$ | $12.36\pm1.65^{bc}$ | $14.65\pm1.09^{ac}$ | $18.08\pm0.93^{a}$ | $18.28\pm1.01^{a}$ |
| MDA(units/g wet tissue) | $0.57\pm0.12^{a}$ | $2.15\pm0.78^{b}$ | $1.83\pm0.52^{b}$ | $1.53\pm0.12^{b}$ | $0.52\pm0.97^{a}$ | $0.49\pm0.03^{a}$ |

Values are expressed as Mean ± SD, n = 5. Values with different alphabet superscripts in the same row differ significantly ($p<0.05$).

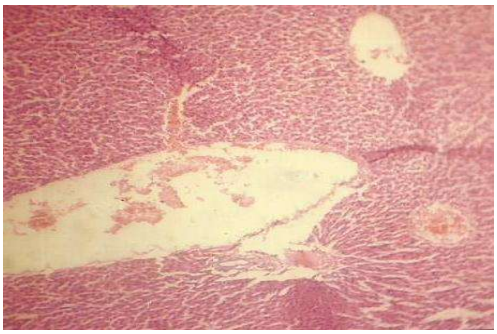
In Table 4.8, the reduced glutathione level in the liver was significantly ($p < 0.05$) lower in group B as compared to group A and all other groups. Group C and D have a significant increase in liver reduced glutathione level as compared with cyanide control group. No significant difference was observed in the liver GSH level in group E and F when compared with group A. The MDA level in the liver was significantly ($p < 0.05$) higher in group B (cyanide control) as compared to all the groups. No significant difference was observed in liver MDA level in group A when compared to group E and F.



**Normal control showing hepatic cells and broad central vein**

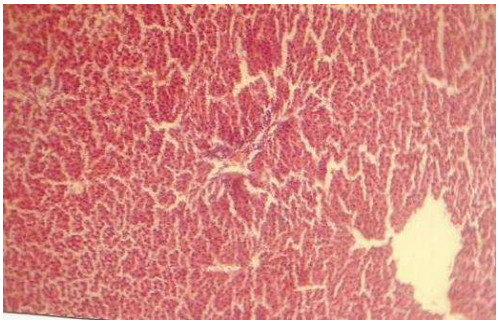


**Cyanide control showing ballooning degradation and severe hepatic necrosis**



**Cyanide + 10mg/kg body weight of *A. sceptrum*, showing reduction in hepatic necrosis and ballooning degradation**

**Cyanide + 20mg/kg body weight of *A. sceptrum* , showing broad central vein and hepatic cell**



**Normal + 10mg/kg body weight of *A. sceptrum*, showing hepatic cells prominent nucleus, no hepatic necrosis**

**Normal + 20mg/kg body weight of *A. sceptrum*, showing preserved nucleus, hepatic cells and no hepatic necrosis**

Figure 4.1 **Liver histology of rat exposed to cyanide treated with aqueous extract of *A. sceptrum*.**

# CHAPTER FIVE

# DISCUSSION, CONCLUSION, RECOMMENDATIONS AND CONTRIBUTION TO KNOWLEDGE

## 5.1 Discussion

Cyanide, in the environment, has been associated with many intoxication episodes in humans and animals resulting from the ingestion of foods, environmental pollution, chemical war, suicide, homicide, occupational factors and use in some drugs such as nitroprusside and laetrile (Tulswani *et al.*, 2005). Cyanide concentrates in erythrocytes through binding to methemoglobin (Tulswani *et al.*, 2005). Subacute oral administration of cyanide in rats produced changes in several biochemical indices and pathology in various organs (Tylleskar *et al.*, 1991). The purpose of this study was to determine the antioxidant property of aqueous extract *A. sceptrum* in some oxidative stress markers and antioxidants biochemical parameters in rats exposed to cyanide.

The results in the study from Table 4.1 above revealed that the PCV and Hb level were significantly ($p < 0.05$) decreased in cyanide control rats as compared to normal control and all other groups. The decreased in haematocrit value, in cyanide exposed animals indicate destruction of erythrocytes (Dede *et al.*, 2002). Fariss (1991) had earlier reported that antioxidants are useful in protecting against chemical toxicity. The supplementation of the food with *A. sceptrum* extracts decrease the toxic effect of cyanide on the haematological values and has a protective role in anaemia and illness (Table 4.1). This is because administration of the *A. sceptrum* extract tends to restore haematological parameters (PCV and Hb) to control values. The protective role was more effective with 20mg/kg body weight of *A. sceptrum* extract as simultaneously compare to 10mg/kg body weight of *A. sceptrum*.

The decreased serum levels of albumin and total protein in the present study (Table 4.2) would indicate possible hepato-toxic effects of cyanide exposure in rats that is

reminiscent of previously reported pathological and biochemical findings in several animal species exposed to cyanide (Okolie and Iroanya, 2003; Tulsawani *et al.,* 2005). As shown in the results, the observed changes were mitigated in the rats treated with the aqueous extract of *A. sceptrum* in comparison to the group not treated (cyanide control), which indicates that the *A. sceptrum* extract has capability of providing some protection against cyanide induced tissue damage.

In this study, alkaline phosphatase (ALP), aspartate aminotransferase (AST), and alanine amino transferase (ALT) activities were found to be increased following cyanide exposure (Table 4.3 and Table 4.4). This is in line with Okolie and Osagie (1999) who stated that sublethal cyanide poisoning increases serum AST activity in rats. Similar results were found in study conducted by Elsaid and Elkomy (2006) which showed significant increases of ALP, AST and ALT enzyme activities in rats drinking water contaminated with cyanide. It is well known that the ALP, AST and ALT are very active in the liver, hence they are marker enzyme and damage to the liver may lead to its increase in the serum. From the results it may suggest that *A. sceptrum* is an effective antidote for cyanide toxicity because it decreases the level of serum and liver ALP, AST and ALT activities after administration to cyanide poisoned rats in comparison to rats poisoned with only cyanide. However, oral administration of *A. sceptrum* to the rats not exposed to cyanide, the ALP, AST and ALT activities in the serum and the liver was also enhanced but this were not significant when compared to the normal control.

There are many documents in the literature on the effect of cyanide on the antioxidant enzymes systems in different cells and tissues of animals. SOD catalyses the dismutation of superoxide radical to hydrogen peroxide, which is then converted to water by GPx or by catalase (Nikoli-Kokic *et al.,* 2010). Based on the present study results prolonged sublethal cyanide administration caused significant decrease in the mean value of serum and liver SOD, CAT and liver GPx, activities as compared to control group. The reduction of SOD and CAT in the serum and liver of the cyanide treated rats (Table 4.5 and Table 4.6)is somewhat similar with the previous reports indicating the reduction of SOD and catalase activities in some tissues of cyanide toxified rats and rabbits (Okolie and Osobase, 2005). The decreased SOD activity may be attributed to irreversible inactivation of this enzyme by its product, $H_2O_2$, due to cyanide-induced increase in superoxide anion

generation ((Nikoli-Kokic *et al.,* 2010) or directly to its irreversible inhibition by cyanide (Okolie and Osobase, 2005).

On the other hand, the higher activity of serum and liver SOD, CAT and liver GPx in cyanide exposed rats treated with the aqueous extract of *A. sceptrum* might be as a compensatory response to enhanced ROS formation and may supply more protection against free radical-mediated oxidative damages. GSH help in the removal of hydrogen peroxide by the GPx-catalysed reaction (Konukoglu *et al.,* 1998). It has been reported that cyanide poisoning in rats significantly decreased reduced glutathione in the blood, liver and brain (Mathangi *et al.,* 2011). Also, acute exposure of lethal dose of cyanide was found to decline GSH levels in the liver of rats (Mathangi *et al.*, 2011). However, the lower blood reduced GSH in the cyanide toxified rats in comparison with the normal controls (Table 4.7) is in line with the work of Mathangi *et al.,* 2011 who indicated significant reduction in erythrocytic GSH concentration in cyanide toxified rats.

Based on the present results, significant enhancement of MDA values were observed in liver of cyanide-intoxicated rats. In line with the findings, the results of Mathangi *et al.* (2011) showed that oral administration of cyanide to rats for 90 days caused increased lipid peroxidation in the brain and liver. The administration of *A. sceptrum* aqueous extract there was lower liver MDA level when compared with normal control group. No significant difference was observed in the liver MDA level in 10mg/kg body weight of *A. sceptrum* aqueous extract and 20mg/kg body weight of *A. sceptrum* aqueous extract in comparison with the normal control. This is in agreement with previous investigation conducted by George *et al.* (2012). The administration of A sceptrum tend to normalized liver MDA level (George *et al.,* 2013; George *et al.,* 2012).

In figure 4.1, the normal control showed normal hepatic cells and broad central vein, the cyanide control showed ballooning degradation and severe hepatic necrosis. Treatment of the Cyanide exposed rats with 10mg/kg body weight and 20mg/kg body weight aqueous extract of *A. sceptrum*, showed a reduction in hepatic necrosis and ballooning degradation. Treatment of the Normal rats with 10 and 20 mg/kg body weight of aqueous extract of *A. sceptrum*, showing hepatic cells prominent nucleus, no hepatic necrosis The basis of the results of this study, may be due to the possible mechanism of hepatoprotective effect through antioxidant activity of *A. sceptrum* (George *et al.,* 2012).

Erukainure *et al*., 2011 stated that the antioxidant activity of *A. sceptrum* might be due to the presence of active constituents such as flavonoids, and quinones.

## 5.2 Conclusion

Aqueous extract of *A. sceptrum* exerts effects not only by modulating lipid peroxidation but it also acts as enhancing the antioxidant and detoxifying enzyme systems. Oral administration of aqueous extract of *A. sceptrum* 10mg/kg bwt and 20mg/kg bwt three time per week for 28 days have a promising role and it worth to be considered as an antidote for cyanide toxicity.

## 5.3 Recommendation

Further, investigation should be carried out on the characterization of the aqueous extract of *A. sceptrum* to determine the exact phytoconstituents that is responsible for the protective and antioxidant activity of the aqueous extract of *A. sceptrum*.

## 5.4 Contribution to Knowledge

Studies have shown that the aqueous extract of *A. sceptrum* may help in prevention of oxidative stress. It was observed that the aqueous of the aqueous extract of *A. sceptrum* could be an antidote against oxidative damage in cyanide toxicity.

The antioxidant activity of the aqueous extract of *A. sceptrum* in serum and liver of cyanide toxicity in rats is evident in this study from the fact that:

i. The liver glutathione peroxidase and reduced glutathione content, catalase and superoxide dismutase were increased after the administration of the aqueous extract of *A. sceptrum*.

ii. The administration of the aqueous extract of *A. sceptrum* reduced the lipid peroxidation level since the malondialdelyde values after treatment were close to the normal control values.

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## APPENDIX I

### *Aframomum sceptrum* EXTRACT PREPARATION /ORAL TOXICITY STUDY

**1. Calculation of the preparation of *A. sceptrum***

A. sceptrum = 25g

Distilled water = 100ml

Residue of filtrate = 9.5

:. 25g – 9.5g = 15.5

Original preparation 15.5g/100ml

= 0.155g/ml

= 155mg/ml (Stock)

***2. A. Sceptrum* Oral Toxicity Study**

The acute oral toxicity was estimated using Lorke's method (1983)

The $LD_{50}$ value was calculated using the formula

$LD_{50}$ = a x b

Where a = Least dose that killed a rat and b = Highest dose that did not kill any rat

$LD_{50}$ Dosage

Dose 1 : 10mg/kg body weight

Dose 2: 50mg/kg body weight

Dose 3: 1000mg/kg body weight

Dose 4: 2000mg/kg body weight

| No of rats | Dose (mg/kg) | Clinical signs | Mortality |
|---|---|---|---|
| 4 | 10 | None | Zero |
| 4 | 50 | None | Zero |
| 4 | 1000 | None | Zero |
| 4 | 2000 | None | Zero |

The administration of *A. sceptrum* extract intraperetually to rats up to 2000mg/kg body weight for seven (7) days did not record any mortality as shown in the Table above. Thus the $LD_{50}$ is greater than 2000mg/kg body weight.

## APPENDIX II

## APPARATUS/REAGENT

**1. Apparatus/Reagents for Haemoglobin**

i. Heparinized capillary tube
ii. Microhaematocrit centrifuge
iii. Microhaematocrit reader
iv. Plasticine, sterile lancet and cotton wool (dry and swab)

**2. Reagent composition for the determination of Total protein**

**R1. Biuret reagent**

| | |
|---|---|
| Sodium hydroxide | 100 mmol/l |
| Na-K-tartrate | 16 mmol/l |
| Potasium iodide | 15 mmol/l |
| Cupric sulphate | 6 mmol/l |

**R2. Blank reagent**

| | |
|---|---|
| Sodium hydroxide | 100 mmol/l |
| Na-K-tartrate | 16 mmol/l |

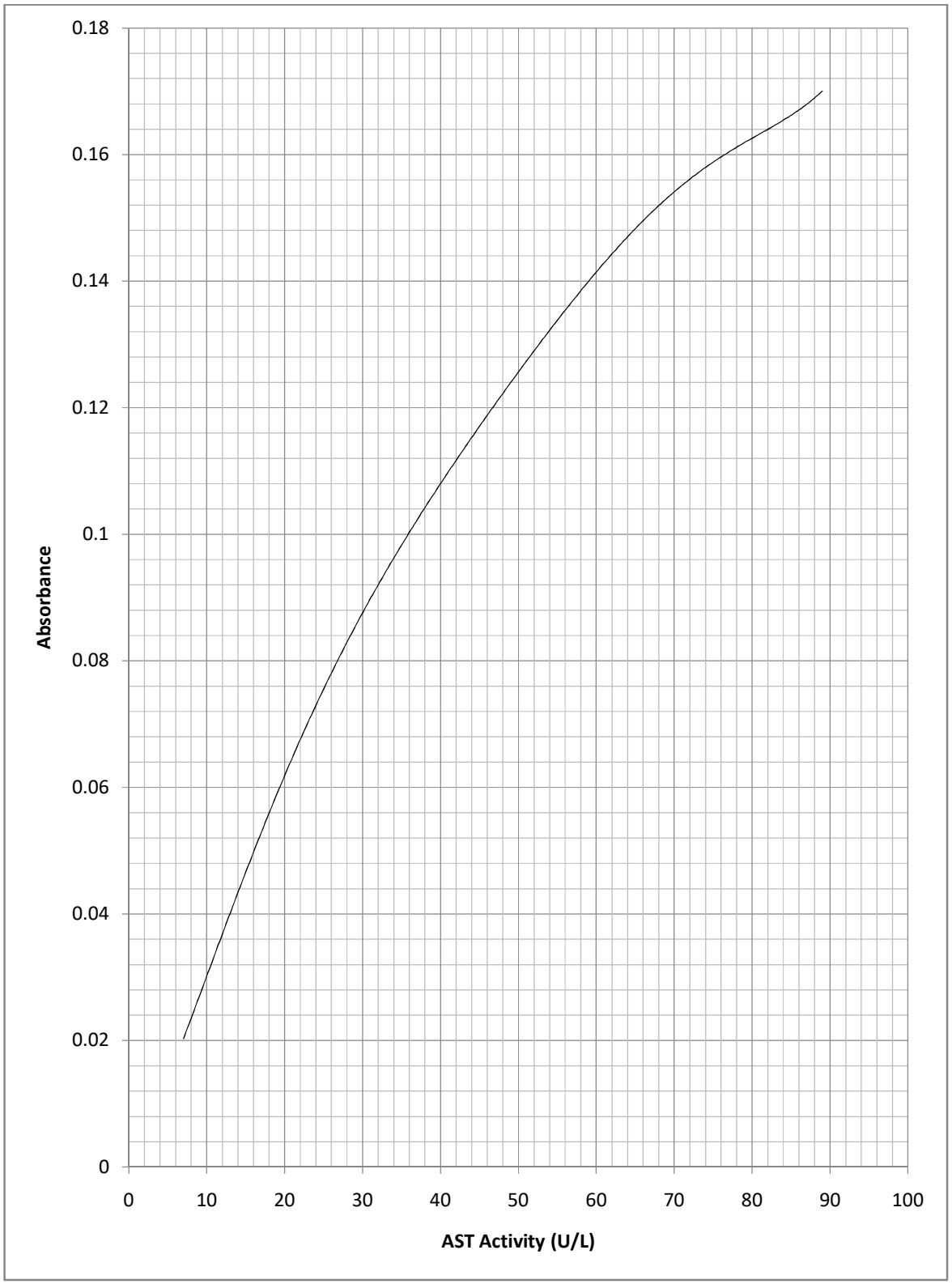
**CAL. Standard**

Protein

**3. Reagent for GPx**

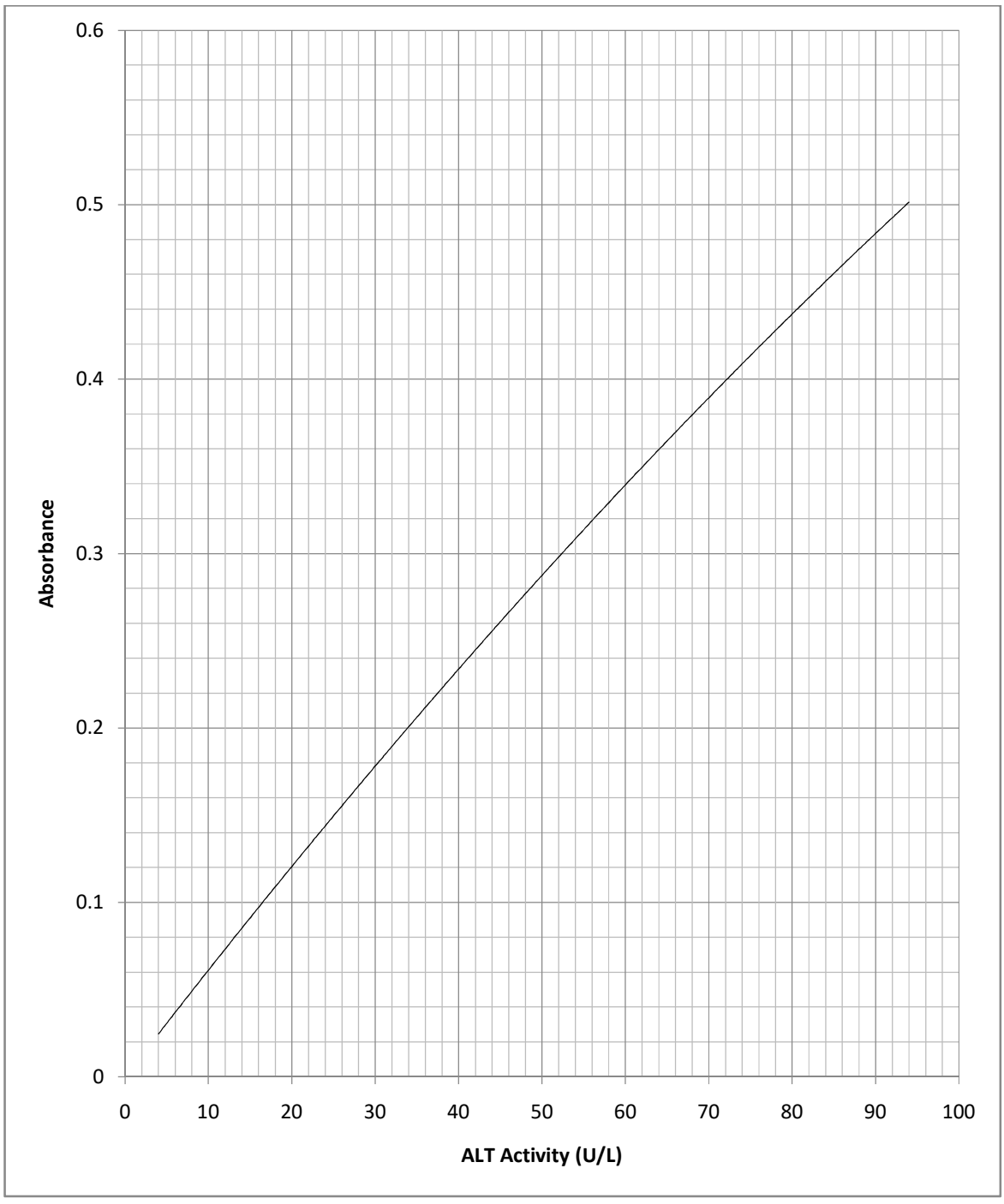
i. Phosphate buffer
ii. Ethylenediaminetetraacetic Acid (EDTA)
iii. Sodium Azide solution
iv. β-Nicotinamide Adenine Nucleotide Phosphate, reduced form (β-NADPH)
v. Glutatione Reductase enzyme solution (GR)
vi. Glutathione, reduced (GSH)
vii. Hydrogen peroxide ($H_2O_2$)

## APPENDIX III
## STANDARD PLOT FOR AST ACTIVITIES


0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
Absorbance
0
10
20
30
40
50
60
70
80
90
100
AST Activity (U/L)

## APPENDIX IV

## STANDARD PLOT FOR ALT ACTIVITIES




## APPENDIX V

## STATISTICAL EVALUATION OF DATA

The values obtained from the assays were analyzed using mean and standard deviations.

The statistical formulas are given below

1. Mean (x) = $\frac{\sum x}{n}$
2. Standard deviation (δ) = $\sqrt{\frac{\sum(x-x)^2}{n-1}}$

Where ∑ = Summation

x = individual samples

n = number of sample activity

Analysis of variance was calculated in each case using the formulae below.

1. Correction Term (CT)

   CT = $\frac{(\sum\sum x)^2}{N}$

2. Total Sum of Squares (TSS)

   TSS = $\sum\sum x^2 - CT$

3. Treatment Sum of Squares (TrSS)

   TrSS = $\frac{\sum(Treatment\ Totals)^2}{No\ of\ replicates} - CT$

4. Error Sum of Squares (ESS)

   ESS = TSS – TrSS

5. Mean Square (MS)

   MS = $\frac{Sum\ of\ Square\ (SS)}{Degree\ of\ Freedom\ (DF)}$

   Treatment MS (TrMS) = $\frac{TrSS}{TrDF}$

   Error MS (EMS) = $\frac{ESS}{EDF}$

6. Degree of Freedom

   Total DF (TDF) = N -1

   N = Total number of observation

   Treatment DF (TrDF) = Number of Treatment – 1

   Error DF (EDF) = TDF –TrDF

**Anova Summary Table**

| Source of Variance | DF | SS | MS | F-value | F-critical |
|---|---|---|---|---|---|
| Total | TDF | TSS | | | |
| Treatment | TrDF | TrSS | TrMS | F-value | F-critical |
| Error | EDF | ESS | EMS | | |

**Least significant difference (LSD) test**

LSD = $(Sx_1 - x_2)t$

t = tabular or critical $t_{0.05}$, df (error)

$Sx_1 - x_2$= Standard error of difference between means

$Sx_1 - x_2 = \sqrt{\frac{2 \times EMS}{N}}$

N = number of replicates per treatment