EFFECT OF CHLOROFORM- METHANOL EXTRACT OF *Tetracarpidium conophorum* NUTS (WALNUTS) ON OXIDATIVE STRESS MARKERS IN HYDROGEN PEROXIDE INDUCED WISTAR ALBINO RATS

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TITLE PAGE

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A PROJECT REPORT SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR AWARD OF DEGREE OF MASTER OF SCIENCE (M.Sc) IN NUTRITIONAL BIOCHEMISTRY, UNIVERSITY OF NIGERIA, NSUKKA

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AUGUST, 2014.

CERTIFICATION

Onuorah, Ndidiamaka Sussan, a postgraduate student with Registration Number PG/M.Sc/11/58601 in the Department of Biochemistry has satisfactorily completed the requirement for the course work and research for the award of degree of Master of Science (M.Sc) in Nutritional Biochemistry. The work embodied in this report is original and has not been submitted in part or full for any other diploma or degree of this or any other University.

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EXTERNAL EXAMINER

DEDICATION

This work is dedicated to the Almighty God, who is my Indomitable help in ages past and my hope for years to come.

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ABSTRACT

This work was done to ascertain the efficacy of the seed of *Tetracarpidium conophorum* on hydrogen peroxide induced oxidative stress in Wistar albino rats. Thirty five (35) male wistar albino rats weighing (120- 140g) were distributed into seven (7) groups of five rats each. Groups 2-6 were administered with hydrogen peroxide (1.0 ml/kg), while group 1 served as the normal control while group 2 serves as positive control Groups 3, 4, 5 and 6 were treated with vitamin C (100 mg/kg), 200, 400 and 800 mg/kg of the extract respectively for five days, Group 7 was administered 800 mg/kg b.w of the extract only. Blood was collected from the animals on the 7^{th} day through occular puncture for assay of some biochemical parameters. The qualitative and quantitative analysis of the seed extract were determined using standard methods and showed that the extract contained terpenoids (4.36 ± 0.06 mg/g), Tannins (1.89 ± 0.11 mg/g), alkaloids (20.31 ± 0.30mg/g) and cardiac glycosides (12. 45 ± 0.08 mg/g), anthraquinones, saponins and steroids were not detected in the extract. Vitamins constituents of the extract were vitamin A (10.55 ± 2.67 mg/ 100g), vitamin C (13.09 ± 0.23 mg/ 100g) and vitamin E (5.77 ± 0.08 mg/ 100g). The mineral constituents indicated the presence of mg (133.59 ± 0.11 mg/ 100g), Ca (118.90 ± 0.01 mg/ 100g), Fe (3.67 ± 0.07 mg/ 100g), Zn (2.22 ± 0.01 mg/ 100g), Cu (1.54 ± 0.78 mg/ 100g). The acute toxicity test of the extract showed no toxicity up to 5000 mg/ kg b.w. Serum ALT activity significantly decreased ($p< 0.05$) in all the test groups compared to group 2. Serum AST and ALP activity decreased significantly ($p< 0.05$) in all the test groups except group 6 for ALP activity compared to the enzyme activities of normal and positive controls. A significant decrease ($p< 0.05$) was observed in the serum MDA concentration of rats in the test groups when compared to the group 2. A significant increase ($p < 0.05$) was observed in the serum GPx activity of groups 4, 6 and 7 compared to the GPx activity of group 2 rats. There was a significant increase ($p < 0.05$) in groups 4 and 7 compared to group 2. The serum cholesterol concentration showed a significant decrease ($p < 0.05$) in the test groups relative to those of the controls. There was a significant decrease ($p < 0.05$) in the serum LDL and TAGs of rats in the test groups when compared to the controls. The serum HDL of groups 5 and 7 increased significantly ($p < 0.05$) compared to the normal control and the positive control. The effect of the extract on lipid profile showed that it increased HDL at a concentration of 400mg/kg body. These antioxidant enzymes results support the claims made by several scientists that the plant could be used to scavenge free radicals in the system which often lead to the risk of various diseases.

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A.O.A.C:	Association of Analytical Chemists
ALP:	Alkaline Phosphatase
ALT:	Alanine Aminotransferase
ANOVA:	Analysis of variance
AST:	Aspartate Aminotransferase
CAT:	Catalase
DNA:	Deoxyribonucleic acid
EDTA:	Ethylene Diammine Tetraacetic acid
Fe:	Iron
GPx:	Glutathione peroxidase
GR:	Glutathione Reductase
GSH:	Reduced Glutathione
HDL:	High density lipoproteins
LD:	Lethal Dose
LDL:	Low density lipoproteins
MDA:	Malondialdehyde
Mg:	Magnesium
NADPH:	Nicotinamide adenine dinucleotide phosphate
PUFAs:	Poly unsaturated fatty acids
RNA:	Ribonucleic acids
ROS:	Reactive oxygen species
SDS:	Sodium Dodecyl sulphate
SOD:	Superoxide dismutase
SPSS:	Statistical package for social sciences
TAGs:	Triacylglycerol
TBA:	Thiobarbituric acid
TCA:	Trichloroacetic acid
UV:	Ultra violet
VLDL:	Very low density lipoproteins
b. w:	body weight

CHAPTER ONE
INTRODUCTION

1.1 Background of the study

The use of various fruits, vegetables, nuts and various parts of plants in traditional medicine is as old as man (Evans, 2002). This lies outside the mainstream of orthodox or Western medicine, it has been estimated that two thirds of the world population (mainly in developing countries) rely on traditional medicine as their primary form of health care (Sumner, 2000). The use of traditional medicine cannot fade out in the treatment and management of diseases in the African continent and this could be attributed to the socio-cultural, socio-economic, lack of basic health care and qualified personnel (Elujoba *et al.*, 2005). Plants contain active components such as anthraquinones, flavonoids, glycosides, saponins, tannins, etc which posses medicinal properties that are harnessed for the treatment of different diseases (Chevalier, 2000). The active ingredients for a vast number of pharmaceutically derived medications contain the healing properties known as the active principles and are found to differ from plant to plant (Chevalier, 2000).Nuts vary considerably in their nutrient content and are sources of vitamins, antioxidants, proteins, essential amino acids, etc. (Fasuyi, 2006). They are included in meals mainly for their nutritional values. However, some are reserved for their medicinal values such as increase in brain health, decreased depression, increase in antioxidant levels; thus, helping to mop up free radicals which have been implicated in a number of diseases (Oladiyi *et al*., 2007).

In a normal cell, there is an appropriate pro-oxidant/antioxidant balance. However this balance can be shifted towards the prooxidant following the ingestion of certain chemicals or drugs when the levels of antioxidants are low; this gives rise to oxidative stress and results in cell damage if prolonged or massive (Murray *et al.,* 2009). Thus oxidative stress is a metabolic perturbation of homeostasis. On the other hand, antioxidants are a complex and diverse group of molecules that protect key biological sites from oxidative damage (Murray *et al*., 2009).Lipid peroxidation is a degenerative process involving peroxidative decomposition of unsaturated fatty acids mediated by free radical or reactive oxygen species (Gutteridge and Halliwell, 1995).

Recently oxidative stress has been linked to many age associated diseases including heart diseases, cancer, atherosclerosis as well as brain disorders (Singh *et al*., 1995). It can also lead to inhibition of some metabolic enzymes (Devasagayam *et al.,* 2004).

Fortunately, aerobic organism have evolved very effective defense system against oxidative assault, this is due to the consistency of both hydrophilic (GSH, Vit C) and lipophilic (Vit E,

Carotenoid pigment) antioxidant compounds or scavengers and specific antioxidant enzymes including superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase. Epidemiological studies have shown strong correlation between plasma antioxidant vitamin levels and mortality rates from heart disease (Schafer and Buettner, 2001).

Hence, oxidative stress is one of the common causes of health disorders posing a great threat to global health care. Medicinal plants are currently being used in various parts of the world especially in the civilized world in the treatment of several diseases such as artherosclerosis, heart disease, brain disorder etc (Ajaiyeoba and Fadare, 2006).

Most nuts contain antioxidant enzymes as well as antioxidant vitamins (A, C, E). *Tetracarpidium conophorum* nuts used as snack in various countries of the world have been shown to have positive effects on oxidative stress (Oke, 1995).Due to their ability to increase poly unsaturated fatty acids, good cholesterol (HDL) antioxidant vitamins in several parts of the world.Hence there is need to investigate the effect of chloroform-methanol extract of *T. conophorum* nuts on hydrogen peroxide induced oxidative stress markers and possibly advocate their inclusion in food preparations for everyone and especially for the elderly and this has necessitated this research.

1.2 *Tetracarpidium conophorum*

Tetracarpidium conophorum (Walnut) consists of families of Juglandaceae (English Walnut), Euphorbiaceae (African Walnut) and Olacaceae (African Walnut (Dalziel) 1937). Each family has its own peculiar characteristics but they have some things in common such as the nuts. Juglandaceae, is mostly found in Southeast Europe to Japan and more widely in the New world. *Tetracarpidium conophorum* (family Euphorbiaceae) is found in Nigeria and Cameroun while coula edulis (family Olacaceae) which is also referred to as African Walnut is found in Congo, Gabon and Liberia (Wikipedia, 2008).

Tetracarpidium conophorum is a climbing shrub 10-20 feet long, it is known in the Southern Nigeria as Ukpa (Igbo), Western Nigeria as awusa or asala (Yoruba). It is known in the littoral and the Western Cameroun as Kaso or ngak (Dalziel, 1937). It is found in Uyo, Akamkpa, Akpabuyo, Lagos, Kogi, Ogbomoso and Ibadan. The plant is cultivated principally for the nuts which are cooked and consumed as snacks (Oke, 1995).

The plant is glabrous with deciduous male flowers leaving the females at the base of the raceme (Petrova, 1980).

A bitter taste is usually observed upon drinking water immediately after eating the nuts. This could be attributed to the presence of chemical substances such as alkaloid (Ayodele, 2003). The seeds contain ascorbic acid and heavy metals, amino acids and fatty acids (Oyenuga, 1997) reported on the amino acid and fatty acid compositions of the nuts and on the use of its leaf juice have been used for the treatment of prolonged and constant hiccups.

1.2.1 Scientific classification of *Tetracarpidium conophorum*

Kingdom: *Plantae*
Division: *Magnoliophyta*
Class: *Magnoliopsida*
Order: *Malpighiales*
Family: *Janiroidea*
Genus: *Tetracarpidium*
Species: *conophorum*
Govarts (2003)

Fig 1: Pods of *Tetracarpidium conophorum* Ayoola *et al.* (2011)

Fig 2: Seeds of *Tetracarpidum conophorum* Ayoola *et al.* (2011)

1.2.2 Medicinal, Nutritional and Industrial importance of *Tetracarpidium conophorum*

1.2.2.1 Medicinal uses of *Tetracarpidum conophorum* (Walnuts)

Tetracarpidium conophorum is a medicinal plant widely cultivated for the production of its seeds. The seed have been implicated in Southern Nigeria ethno medicine as a male fertility agent (Ajaiyeoba and Fadare, 2006). The seed is used in the treatment of indigestion, constipation and diarrhea (Wolters, 2009). The seed is a good source of vitamins. Alkaloids are the most efficient plant substances used therapeutically. Pure isolated alkaloids and the synthetic derivatives are used as the basic medicinal agent because of their analgesic, antispadomic and bacterial properties. This is why the seed is believed to stop asthma and is prescribed to be taken between bouts of asthma, but not for acute asthma. It is used for the elderly as a constipation cure (Wikipedia, 2009). The presence of tannins in the seed of *Tetracarpidium conophorum* can support its strong use for healing of haemorrhoids, frost bite and varicose ulcers in herbal medicine (Igboko, 1983, Maduiyi, 1983).

Walnuts have been reported as having Chelating ability which in turn could account for its high antioxidant activity which have been compared to the use of dimercapto- succinic acid (DMSA). 2, 3- dimercapto -1- propanesulfonic acid (DMPS) and alpha lipoic acid (ALA) (Muanya, 2012).

Walnuts are a good source of protein, vitamin C, folic acid and vitamin E, they also have an extremely high level of polyunsaturated fat and are a good source of omega 3- fatty acids (Cortes *et al.,* 2006) such as linoleic acid, alpha-linolenic acid (ALA) and arachidonic acids. Regular intake of Walnuts in the diet helps to lower, total as well as LDL or ôbad cholesterolô and increases HDL or ñgood cholesterolò levels in the blood. Walnuts are a rich source of many phytochemical substances that may contribute to their overall anti- oxidant activity, including melatonin, ellagic acid, Vitamin E, Carotenoid and poly phenolic compounds. These Compounds have potential health effects against Cancer, aging, inflammation and neurological diseases (Reiter *et al.*, 2005).Walnuts Oil has flavourful nutty aroma and excellent astringent properties, applied locally, it helps to keep the skin well protected from dryness. It has also been used in cooking and as ócarrier or base oilô in traditional medicines in massage therapy, aromatherapy in pharmaceutical and cosmetic industry (Fortin, 1996).

1.2.2.2 Nutritional uses of *Tetracarpidium conophorum* (Walnuts)

Walnuts are excellent sources of vitamin E, not in the alpha tocopherol but in the gamma-tocopherol, particularly in studies of cardio vascular health of men, this gamma-

tocopherol form has been found to provide significant protection from heart problems and maintaining the integrity of cell membranes of mucous membranes and skin by protecting it from harmful oxygen radicals. (Blomhoff *et al*., 2006).

Some phytonutrients found in walnut for example, the quinine juglone are found in virtually no other commonly eaten food. Othersô such as the tannin-tellimagrandin or the flavonol morin are also rare and valuable as antioxidants and anti inflammatory nutrients. These anti- inflammatory and anti- oxidant phytonutrients also help explain the decreased risk of certain cancers- including prostate cancer and breast cancer (Fukuda *et al.*, 2003).
Walnut are packed with many important B- Complex groups of vitamins such as riboflavin, niacin, thiamine, pantothenic acid, Vitamin B_6, and Folates.

They are also a rich source of mineral salts such as mangenese, copper, potassium, calcium, iron, magnesium, zinc and selenium. Copper is a cofactor for many vital enzymes, including cytochrome c- oxidase and superoxide dismutase. Zinc is a co- factor in many enzymes that regulate growth and development, sperm generation, digestion and nucleic acid synthesis. Selenium is an important micro nutrient which functions as a co-factor for anti-oxidant enzymes such as glutathione peroxidases. (Esminger *et al*., 1983). Walnuts oil has flavourful nutty aroma used in salad dressings and also used as an edible oil in cooking.

1.2.2.3 Industrial uses of *Tetracarpiduim conophorum* (Walnuts)

Locally, the oil has been used as a moisturizer to keep the skin well protected from dryness. The bark is used as dye in clothing and textile industry because it contains a juice that will readily stain anything it comes into contact with. Walnut hulls contain phenolic compounds (ferulic acid, vanillic acid, coumaric acid, syringic acid, myricetrin juglone (Cosmulesc *et al.,* 2010) and regiolone (Liu *et al.,* 2007). Black Walnut heartwood is heavy, hard strong and durable with a chocolate brown colour. Walnut shells are used as thickener in paint and plastic industry, a filler in explosives and for cleaning and polishing, used as abrasive element in home soap making (Liu *et al*., 2007).The floor of the Globe Theatre in Elizabethan London was made of Walnut shells and compacted down to a very hard and polishable surface.

1.3 Oxidative Stress

Oxidative stress is an imbalance between the systemic manifestation of reactive oxygen species and a biological systemôs inability to readily detoxify the reactive intermediates or to repair the resulting damage (Murray *et al*.,2009). Disturbances in the

normal redox state of cells can cause toxic effects through the production of peroxides and free radicals that damage all components of the cell including proteins, lipids and DNA.

In humans and animals, oxidative stress is thought to be involved in the development of many diseases or may exacerbate their symptoms (Proctor *et al.,* 1984, Proctor, 1989) These include cancer, (Halliwell, 2001), Parkinsonôs disease, Alzeihmerôs disease, atherosclerosis, heart failure, myocardial infarction, schizophrenia, Bipolar disorder, fragile X syndrome, sickle cell disease, autism and chronic fatigue syndrome (Gwen *et al*.,2005).

Oxidative stress is a term used to refer to the shift towards the pro-oxidants in the pro-oxidative/ antioxidants balance that can occur as a result of an increase in oxidative metabolism (Manda *et al.,* 2009). ROS reactions with biomolecules such as lipid, protein and DNA, produce different types of secondary radicals like lipids radicals, non-sugar and base derived radicals, amino acid radicals depending upon the nature of the ROS (Niki *et al.,* 2005). These radicals in the presence of oxygen are converted to peroxyl radicals. Peroxyl radicals are critical in biosystems, these reactions exert oxidative stress on the cells, tissues and organs of the body. The biological implications of such reactions depends on several factors like site of generation, nature of the substrate, activation of repair mechanisms, redox status among many others (Koppeno, 1993; Goldstein *et al.,* 1993).

1.3.1 Chemical and biological effects of oxidative stress.

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

Production of reactive oxygen species is a particularly destructive aspect of oxidative stress. Such species include free radicals and peroxides. Some of the less reactive of these species (such as superoxide) can be converted by oxidoreduction reactions with transition metals or other redox cycling compounds (including quinines) into more aggressive radicals that can cause extensive cellular damage (Valko *et al*., 2005). The major portion of long term effects is inflicted by damage on DNA (Evans and Cooke, 2004). Most of these oxygen ï derived species are produced at a low level by normal aerobic metabolism. Normal cellular defense mechanisms destroy most of these. Likewise, any damage to cells is constantly

repaired. However, under the severe levels of oxidative stress that cause necrosis, the damage causes ATP depletion, preventing controlled apoptotic death and causing the cell to simply fall apart (Lelli *et al*., 1998, Lee and Shacter, 1999).

1.3.2 Oxidative stress and diseases

Oxidative stress is suspected to be implicated in neurodegenerative diseases including Lou Gehrigôs disease, Parkinsonôs disease, Alzheimerôs disease, and Huntingtonôs disease (Patel and Chu 2011). Indirect evidence via monitoring biomarkers such as reactive oxygen species, and reactive nitrogen species production, antioxidant defense mechanism indicates that oxidative damage may be involved in the pathogenesis of these diseases (Nunomura *et al*., 2005), while cumulative oxidative stress with disrupted mitochondrial respiration and mitochondrial damage are related with Alzheimerôs disease, Parkinsonôs disease, and other neurodegenerative diseases (Ramalingam and Kim, 2012).

Oxidative stress is thought to be linked to certain cardiovascular disease, since oxidation of LDL in the vascular endothelium is a precursor to plaque formation. Oxidative stress also plays a role in the ischemic cascade due to oxygen reperfusion injury following hypoxia. This cascade includes both strokes and heart attacks. Oxidative stress has also been implicated in chronic fatigue syndrome (Nijs *et al*., 2006). Oxidative stress also contributes to tissue injury following irradiation and hyperoxia as well as in diabetes.

1.3.3 Free Radical generations / Reactive oxygen species.

Free radicals can be defined as those atoms or molecules containing one or more unpaired electrons in their outer most shell and mostly is very reactive due to the presence of these unpaired electron(s) (Knight, 1998), Reactive oxygen species (ROS) is a collective name given to both oxygen free radicals and non oxygen free radicals (Mittler, 2002). Reactive oxygen species can also be used to refer to a group of oxidants.

1.3.3.1 Sources of Free radicals

1. Electron Transport Chain

Production of superoxide and hydrogen peroxide usually takes place in the mitochondria of a cell (Valko *et al*., 2004; Nelson *et al*., 2006). The mitochondria electron transport chain is the main source of ATP in the mammalian cell; hence, it is essential for life. During energy transduction, a small number of electrons ñleakò to oxygen prematurely,

forming the oxygen free radical superoxide, which has been implicated in the pathophysiology of a variety of diseases (Valko *et al.,* 2007).

2. Stress responses and defence pathways. (Phagocytosis)

Reactive oxygen species are natureôs response to external and internal stimuli. This is done in most cases to defend the body against foreign pathogenic and or parasitic invasion for instance; the killing of parasites during disease and infection states has been hypothesized. The hydroxyl radical, OH, is the neutral form of the hydroxide ion. The hydroxyl radical has a high reactivity, making it a very dangerous radical with a very short in vivo half- life of approximately 10^{-9} s (Pastor *et al.,* 2000). Thus, when produced in vivo, OH reacts close to its site of formation. Cellular productions of these ROS are enhanced during stress and can posed threat to cells, but it is also thought that ROS act as signals for the activation of stress-response and defence pathways (Mittler, 2002). Thus, ROS can be viewed as cellular indicators of stress and as secondary messengers involved in the stress- response signal transduction pathway (Valko *et al.,* 2005). Over- accumulation of ROS can result in cell death (Toykuni, 1999). ROS-induced cell death can result from oxidative processes such as membrane lipid peroxidation, protein oxidation, enzyme inhibition and DNA and RNA damage (Etsuo *et al.,* 1991).

3. Metal catalysts

Metals such as iron, copper, chromium, vanadium and cobalt are capable of redox cycling in which a single electron may be accepted or donated by the metal. This action catalyzes reactions that produce reactive radicals and can produce reactive oxygen species (Pratviel, 2012). The most important reactions are probably Fentonôs reaction and the Haber-Weiss reaction, in which hydroxyl radical is produced from reduced iron and hydrogen peroxide. The hydroxyl radical then can lead to modifications of amino acids (e.g. meta-tyrosine and ortho-tyrosine formation from phenylalanine), carbohydrates, initiate lipid peroxidation, and oxidized nucleobases. Most enzymes that produce reactive oxygen species contain one of these metals. The presence of such metals in biological systems in an uncomplexed form (not in a protein or other protective metal complex) can significantly increase the level of oxidative stress. In humans, hemochromatosis is associated with increased tissue iron levels, Wilsonôs disease with increased tissue levels of copper and chronic manganism with exposure to manganese ores. The reaction of transition metals with proteins oxidated by reactive oxygen species or reactive nitrogen species can yield reactive

products that accumulate over time and contribute to aging and disease. For example, in Alzheimerôs patients, peroxidized lipids and proteins accumulate in lysosomes of the patientôs brain cells (Devasagayam *et al.,* 2004)

4. Non- metal catalysts

Certain organic compounds in addition to metal redox catalysts can also produce reactive oxygen species. One of the most important classes of these are the quinines. Quinines can redox cycle with their conjugate semiquinones and hydroquinones, in some cases catalyzing the production of superoxide from dioxygen or hydrogen peroxide from superoxide. Oxidative stress generated by the reducing agent uric acid may be involved in the Lesch- Nyhan syndrome, stroke, and metabolic syndrome. Likewise, production of reactive oxygen species in the presence of homocysteine may figure in homocystinuria, as well as atherosclerosis, stroke, and Alzheimers.

1.4 Hydrogen Peroxide (H_2O_2)

Hydrogen peroxide is a lipid soluble radical or oxidant formed by dismutation by the enzyme SOD in the inactivation of destructive superoxide ions by converting them to hydrogen peroxide which is in turn transformed into water and oxygen by the enzyme catalase. Peroxisomes are known to produce H_2O_2, but not O_2, under physiologic conditions (Valko *et al.,* 2004). Peroxisomes are major sites of oxygen consumption in the cell and participate in several metabolic functions that use oxygen. Oxygen consumption in the peroxisome leads to H_2O_2 production, which is then used to oxidize a variety of molecules (Forman *et al*., 2010). This organelle also contains catalase, which decomposes hydrogen peroxide and presumably prevents accumulation of this toxic compound. Thus, the peroxisome maintains a delicate balance with respect to the relative concentrations or activities of these enzymes to ensure no net production of ROS (Juranek and Bezek, 2005). When peroxisomes are damaged and their H_2O_2 consuming enzymes down regulated, H_2O_2 releases into the cytosol which is significantly contributing to oxidative stress (Juranek and Bezek, 2005). Proteins can undergo direct and indirect damage following interaction with ROS resulting into peroxidation, changes in their tertiary structure, proteolytic degradation, protein- protein cross linkages and fragmentation (Yu, 1994). Although, DNA is a stable, well- protected molecule, ROS can interact with it and cause several types of damage such as modification of DNA bases, single and double strand DNA breaks, loss of purines (apurinic

sites), damage to the deoxyribose sugar, DNA- protein cross linkage and damage to the DNA repair system (Droge, 2002).

1.5 Lipid Peroxidation

Lipid peroxidation is a well-established mechanism of cellular injury in both plants and animals, and is used as an indicator of oxidative stress in cells and tissues. It is the process in which free radicals ñstealò electrons from the lipids in cell membranes, resulting in cell damage. This process proceeds by a free radical chain reaction mechanism (Marnett, 1999). Lipid peroxidation is an autocatalytic free radical- medicated destructive process whereby poly-unsaturated fatty acids in cell membranes undergo degradation to form lipid hydroperoxides (Moore and Robert, 1998). Lipid peroxidation of cellular structures, a consequence of increased oxygen free radicals, is thought to play an important role in atherosclerosis and micro vascular complications of diabetes mellitus which is consequent from oxidative stress (Soliman, 2008).Lipid peroxidation triggers the loss of membrane integrity, causing increased cell permeability, enzyme inactivation, structural damage to DNA and cell death (Halliwell, 1992). 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 OH and HO, which combines with a hydrogen atom to make water and a fatty acid radical. 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 too is an unstable specie 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 (Koppeno, 1993; Goldstein *et al.,* 1993). This cycle continues, as the new fatty acid radical reacts in the same way. Lipid peroxides are unstable and decompose to form a complex series of compounds including reactive carbonyl compounds.

When a radical reacts with a non- radical, it always produces another radical, which is why the process is called a ñchain reaction mechanismò. The radical reaction stops when two radicals react and produce a non- radical specie (Juranek and Bezek, 2005). This happens only when the concentration of radical species is high enough for there to be a high probability of collision of two radicals. Hence, the generation of free radicals lead to lipid peroxidation and formation of severe damage in tissues (Soliman, 2008). Cellular membranes are vulnerable to the oxidation by ROS due to the presence of high concentration of unsaturated fatty acids in their lipid components. ROS reactions with membrane lipids cause lipid peroxidation, resulting in formation of lipid hydroperoxide (LOOH) which can further

decompose to an aldehyde such as malondialdehyde, 4- hydroy nonenal (4-HNE) or form cyclic endoperoxide, and hydrocarbons (Trangvarasittichai *et al.,* 2009).

Living organisms have evolved different molecules that speed up termination by catching free radicals and, therefore, protecting the cell membrane. One important such antioxidant is vitamin E. Other anti- oxidants made within the body include the enzymes superoxide dismutase, catalase and perioxidase. If not terminated fast enough, there will be damage to the cell membrane, which consists mainly of lipids (Seiler *et al.,* 2008).

1.5.1 Malondialdehyde

By-products of lipid peroxidation such as conjugated dienes and malondialdehyde (MDA). MDA is generated as a relatively stable end product from the oxidative degradation of poly- unsaturated fatty acids (PUFA). This free radical- driven lipid peroxidation has been causatively implicated in the aging process, atherosclerosis, Alzheimerôs disease and cancer (Niki *et al*., 2005). Serum MDA has been used as a biomarker of lipid peroxidation and has served as an indicator of free radical damage (Tangvarasittichai *et al.,* 2009). Malondialedehyde is a highly reactive three carbon dialdehyde that occurs naturally and exits primarily in an enol form. It is a toxic compound that reacts with DNA to form covalently-bonded adducts with deoxyadenosine and deoxyguanosine, an event that can cause a mutagenic transformation within DNA (Nordberg and Amer, 2001).

Additionally, Malondialedehyde can interact with several functional groups on proteins and lipoproteins, altering their chemical behaviour and possibly contributing to carcinogenesis and mutagenesis (Ogugua and Ikejiaku, 2005). Due to its highly reactive nature, Malondialedehyde also functions as an electrophile that can cause toxic stress within the cell and is, therefore, a potent marker for measuring the overall level of oxidative stress within an organism (Conn, 1995; Del- Rio *et al*., 2005; Soliman, 2008; Tangvarasittichai *et al*., 2009).

1.5.2 Antioxidants

Antioxidants are the bodyôs first line of defense against damage, and are critical for maintaining optimum health and wellbeing. Free radicals are capable of attacking the healthy cells of the body, causing them to lose their structure and function (Mittler, 2002). Fortunately, free radical formation is controlled naturally by various beneficial compounds known as antioxidants. It is when the availability of antioxidants is limited that this damage can become cumulative and debilitating (Cheeseman and Slater, 1993). Antioxidants are

capable of stabilizing, or deactivating, free radicals before they attack cells. Antioxidants are absolutely, critical for maintaining optimal cellular and systemic health and well being (Traber and Atkinson, 2007). To protect the cells, organ and systems of the body against reactive oxygen species, humans have evolved a highly sophisticated and complex antioxidant protection system (Vertuani *et al*., 2004). It involves a variety of components, both endogenous and exogenous in origin, that function interactively and synergistically to neutralize free radicals.

These components include:

- Nutrient ï derived antioxidants like ascorbic acid (vitamin C) , tocopherols and tocotrienols (vitamin E), carotenoids, and other low molecular weight compound such as glutathione and lipoic acid (Waggaiallah and Alzohairy, 2011).
- Antioxidant enzymes, e.g. superoxide dismutase, catalase, glutathione peroxidase, and glutathione reductase, which catalyze free radical quenching reactions (Switala and Loewen, 2002).
- Metal binding proteins, such as ferritin, lactoferrin, albumin, and ceruloplasmin that sequester free iron and copper ions that are capable of catalyzing oxidative reactions (Sies, 1997).
- Numerous other antioxidant phytonutrients present in a wide variety of plant foods (Oke, 1995).

1.5.2.1 Enzymatic antioxidants

In addition to dietary antoxidants, the body relies on several endogenous defense mechanisms to help protect against free radical- induced cell damage. The antioxidant enzymes- glutathione peroxidase , catalase, and superoxide dismutase (SOD) ï metabolize oxidative toxic intermediates (Niki *et al*., 2005; Soliman, 2008; Tangvarasittichai *et al*., 2009) and require micronutrient cofactors such as selenium, iron, copper, zinc and manganese for optimum catalytic activity (Okezie, 1996). Intensive agricultural methods have also resulted in significant depletion of these valuable trace minerals in our soils and the foods grown in them (Vlietinck *et al.,* 1995).

1.5.2.2 Superoxide dismutase

Superoxide dismutase (SOD) is a prime antioxidant enzyme found in two forms. One, complexed with zinc and copper, is localized in the cytosol, while the other, bound with manganese, is found in the mitochondrial matrix. Both forms of this metalloenzyme catalyze the inactivation of destructive superoxide ion by converting them to hydrogen peroxide which is then transformed to water and oxygen by the enzyme catalase (Sies, 1997; Soliman, 2008). Reactive forms of oxygen such as superoxide, leak from respiratory enzymes and wreak havoc on the cell. This superoxide can then cause mutations in DNA or attack enzymes that make amino acids and other essential molecules (Vertuani *et al.*, 2004). To combat this potential danger, most cells make superoxide dismutase, an enzyme that detoxifies superoxide (Sies 1997). $2O_2 + 2H \xrightarrow{SOD} H_2O_2 + O_2$

1.5.2.3 Catalase

Catalase is an antioxidant enzyme found in aerobically active cells that catalyses the conversion of the ROS hydrogen peroxide to molecular oxygen and two molecules of water, according to the reaction: $H_2O_2 \longrightarrow O_2 + 2H_2O$ (Nelson *et al*., 2006)

Catalases are some of the most efficient enzymes found in cells (Switala and Loewen, 2002). These enzymes, since they must fight against reactive molecules, are also unusually stable enzymes. Catalase performs its rapid destruction of hydrogen peroxide in two steps. First, a molecule of hydrogen peroxide binds and is broken apart. One oxygen atom is extracted and attached to the iron atom, and the rest is released as harmless water. Then, a second hydrogen peroxide molecule binds. It is also broken apart and the pieces are combined with the iron- bound oxygen atom, releasing water and oxygen gas (Switala and Loewen, 2002).

1.5.2.4 Glutathione

Glutathione (GSH) can be used to ñdetoxifyò reactive oxygen species such as hydrogen peroxide H_2O_2, a process in which glutathione is oxidized to the dimmer glutathione disulfide in a reaction catalyzed by glutathione peroxidase (GPx). Reduced glutathione in turn is regenerated from glutathione disulfide by glutathione reductase (GR) in a reaction using NADPH as a cofactor (Ukeda *et al*., 1997). Glutathione peroxidase enzyme protects the erythrocyte against peroxides that are generated intracellularly or exegenously (Waggiallah and Alzohairy, 2011). Glutathione reductase plays an important role in protecting hemoglobin, red cell enzymes, and biological cell membranes against oxidative damage by

increasing the level of reduced glutathione in the process of aerobic glycolysis (Traber and Atkinson, 2007). Deficiency of the enzymes may result in mild to moderately severe hemolytic anemia upon exposure to certain drugs or chemicals (Okezie, 1996).

1. $H_2O_2 + 2GSH \xrightarrow{GPx} GSSG + 2H_2O$
2. $GSSG + NADPH + H^+ \xrightarrow{GR} 2GSH + NADP^+$
3. $Rx + GSH \xrightarrow{GT} xH + GSR$

1.5.3 Antioxidant Vitamins

Vitamin A, Vitamin C and Vitamin E are among the most widely studied antioxidant vitamins.

1.5.3.1 Carotenoids (Pro Vitamin A)

Carotenoids are natureôs most widespread pigments and have also received substantial attention because of both their provitamin and antioxidant roles. More than 600 different carotenoids have been identified in nature. They occur widely in plants, microorganisms, and animals. Carotenoids have a 40-carbon skeleton of isoprene units. The structure may be cyclized at one or both ends, may have various hydrogenation levels, or may possess oxygen-containing functional groups. Lycopene and ß-carotene are examples of acyclized and cyclized carotenoids, respectively (Ommen *et al.*, 1996). Carotenoid compounds most commonly occur in nature in the all-trans form. The most characteristics feature of carotenoids is the long series of conjugated double bonds forming the central part of the molecule. This gives them their shape, chemical reactivity, and light- absorbing properties. ß-Carotene, Ũ-carotene, and ß-cryptoxanthin are able to function as provitamin A. Zeaxanthin and lutein are the major carotenoids in the macular region (yellow spot) of the retina in humans (Britton, 1995). Vegetables and fruits, including carrots, orange, sweet potatoes, winter squash, pumpkin, papaya, mango, and cantaloupe, are rich sources of the carotenoid; ß- carotene. Tomatoes, watermelons, pink grapefruits, apricots, and pink guavas are the most common sources of lycopene in-take comes from processed tomato products such as ketchup, tomato paste, and tomato soup. Carotenoid pigments play important functions in photosynthesis and photoprotection in plant tissues. The photoprotection role of carotenoids originates from their ability to quench and inactivate reactive oxygen species such as singlet oxygen formed from exposure of light and air. This photoprotection role is also associated with its antioxidant activity in human health. Carotenoids can react with free radicals and become radicals themselves. Their reactivity depends on the length of the chain of conjugated

double bonds and the characteristics of the end groups. Carotenoid radicals are stable by virtue of the delocalization of the unpaired electron over the conjugated polyene chain of the molecules. This delocalization also allows addition reactions to occur at many sites on the radical (Britton, 1995). Astaxanthin, zeaxanthin, and lutein are excellent lipid- soluble antioxidants that scavenge free radicals, especially in a lipid- soluble environment. Carotenoids at sufficient concentrations can prevent lipid oxidation and related oxidative stress.

1.5.3.2. Vitamin C (Ascorbic Acid)

Vitamin C is a carbohydrate by virtue of its structure (see fig.3). And the presence of double bond in the enediol group confers it with a powerful reducing property.

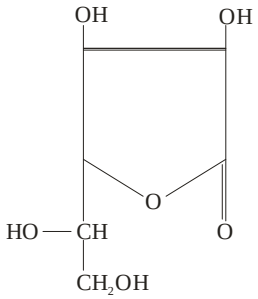


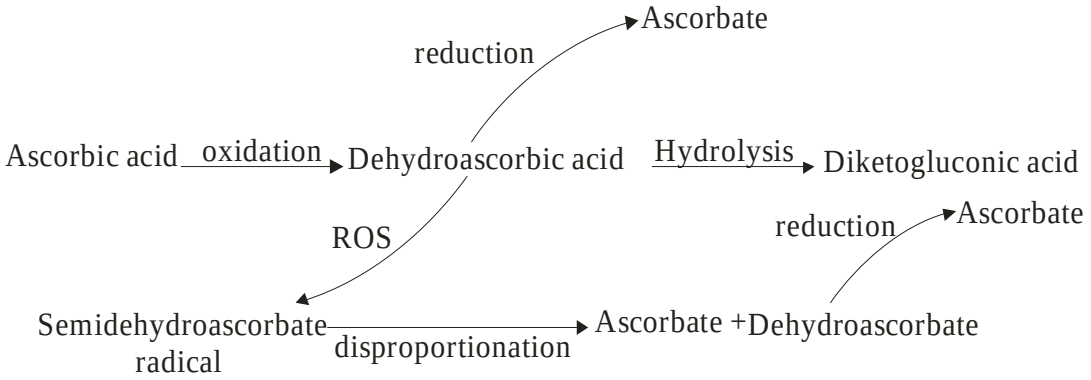
Fig. 3: Structure of L- ascorbic acid

Because of the genetical absence of the enzyme L-glucono-ɔ-lactose oxidase (EC. 1.1.3.8), the last enzyme in the synthesis of Vitamin C, it is impossible for most mammals to synthesize this vitamin de novo. Thus, the entire human requirement must come from diet. This means that vital functions of this vitamin will be lacking in individuals and affected animals that donôt include this vitamin in their diets.

Ascorbic acid was first isolated from plant and animal tissues by Szent Gyorgy in 1928 and later synthesized chemically in 1937. In fact, vitamin C has been known since 1753 when a British Sailor cured scurvy with oranges and lemons. Since then the protective importance of Vitamin C has been emphasized and utilized. Sources of the vitamin include liver, milk, green, vegetables, turnip green, green pepper, tomatoes, potatoes, lemons, guava and other citrus fruits.

Ascorbic acid in diet is absorbed into red blood cells in its oxidized form- dehydroascorbic acid. This is followed by its reduction to ascorbate or hydrolysis to diketogluconic acid. On the other hand, the oxidation product (dehydroascorbic acid) can react with free radical to form semidehydroascorbate radical which undergoes

disproportionation to yield ascorbate and dehydroascorbate. The dehydro ascorbate is unstable and is reduced back to ascorbate with reduced glutathione as an electron or hydrogen donor or hydrolysed irreversibly to diketo-L-gluconic acid (see the scheme below). The product of vitamin C oxidation is extracted completely through transepithelial Na-dependent active transport of the brush border.



Note that dehydorascorbic should be reduced to ascorbate because the former is toxic to the system.

1.5.3.3 Vitamin E

Vitamin E has been recognized as the oldest antioxidant in biological systems. It has been reported to scavenge, prevent, inhibit and or delay oxidative stress mediated generation of ROS and related reactions. Vitamin E stabilizes the phosphatidylserine and ethanolamine responsible for the stability of membranes. Ajaiyeoba and Fadare, (2006) and several other workers report that in various models of atherosclerosis, administration of vitamin E attenuates the progression of the disease. Again, in their work, Harding *et al.,* (1995) found that vitamin E reduced neuropathy, retinopathy and heart diseases. The functions of vitamin E have been attributed to the possession of a hydroxyl group (-OH) in its structure. This possession of OH helps it to quench and prevent lipid peroxidation and related reactions by transferring a hydrogen atom with its single electron to the radical, thus rendering the radical inactive.

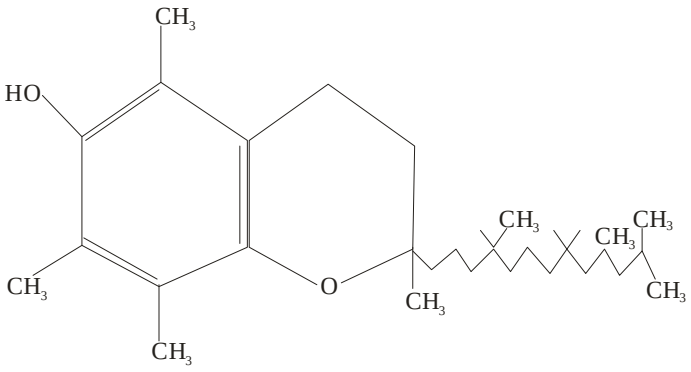


Fig. 4: Structure of α-tocopherol

Vitamin E plays a crucial role in cell membranes and this is probably the reason it is found in higher concentrations than any other antioxidants in plasma lipoproteins including low density lipoproteins, mitochondrial membrane and endoplasmic reticulum. Vitamin E inhibits platelets aggregation, production of prostaglandins and decreases thromboxane production. These workers also reported a protective action of vitamin E in red blood cell against superoxide anion (O_2) attack. Many scientists believe that some compounds including antioxidant vitamin E may help protect against many common diseases by taming harmful molecules called free radicals.

1.5.4 Cholesterol

Cholesterol is a waxy substance that is present in the blood plasma and in all animal tissues. Chemically, Cholesterol is an organic compound belonging to the steroid family; its molecular formular is $C_{27}H_{46}O$ (Abell *et al.,* 1952). Cholesterol is essential to life; it is a primary component of the membrane that surrounds each cell, and it is the staring material or an intermediate compound from which the body synthesizes bile acids, steroid hormones, and vitamin D (Olson, 1998). Cholesterol circulates in the bloodstream and is synthesized by the liver and several other organs. Human beings also ingest considered amounts of cholesterol synthesized by the liver with the increased dietary intake of cholesterol resulting in the liverôs decreased synthesis of the compound (Olson, 1998).

Cholesterol is insoluble in the blood; it must be attached to certain protein complexes called lipoproteins in order to be transported through the bloodstream. Low- density lipoproteins (LDLs) transport cholesterol from its site of synthesis in the liver to the various tissues and body cells, where it is separated from the lipoprotein and is used by the cell (Gordon *et al*., 1989). Cholesterols attached to LDLs are primarily those that builds up in

atherosclerotic deposits in the blood vessels hence LDLs are termed ôbadô cholesterol (Olson, 1998). High- density lipoprotein (HDLs) may possibly transport excess or unused cholesterol from the tissues back to the liver, where it is broken down to bile acids and is then excreted thereby serving to retard or reduce atherosclerotic build up, thus, it is termed ógoodô cholesterol (Lewis and Rader, 2005).

1.5.5 Phytochemicals

Phytochemicals simply mean plant chemicals. They are naturally occurring components in fruits, vegetables, legumes and grains. They give plants its colour, flavour, smell and are part of a plantôs natural defense system (disease resistance). According to Liu (2004), phytochemicals are bioactive, non- nutrient plant compounds in fruits, vegetables, grains and other plant foods that have been linked to reducing the risk of major degenerative diseases. Anderson, (2004) defined phytochemicals as plant- derived chemicals, which are beneficial to human health and disease prevention which are examples of phytochemicals, are known also to reduce the cholesterol production in the body and through that keep the blood pressure down (Anderson, 2004; Akpanyung, *et al*., 1995). They do this either by working alone or in the combination of vitamins and other nutrients in foods (Liu, 2004).
Reports show that the greatest sources of these phytochemicals are seeds, fruits and vegetables (Willet, 2002; Liu; 2004, Altar and Adeogun, 1995). Hence we need to discover the potentials of our local seeds in possessing these phytonutrients. This will help to provide vital data for food processors and nutrition workers as well as the consumers for selection of these seeds. In plants phytochemicals attract beneficial and repel harmful organisms, serve as photoprotectants, and respond to environmental changes. For instance, isoflavones, anthocyanin and flavonoids do function as phytoalexins, substances that assist a plant to resist pathogens (Agte *et al.,* 2000). Carotenoids help in light collection under conditions of low light or help to dissipate excess absorbed energy as heat under conditions of high sun exposure. Basically, plants are nutritionally important by their content of protein, carbohydrate, fats and oils, minerals, vitamins and water responsible for growth and development in man and animals. Much more than these, researchers have come up with the fact that some plant chemicals which have been regarded as anti- nutritional or anti- nutrients have potentials in helping to reduce the risk of several deadly diseases in man (Williamson *et al*., 1997; Stahl *et al.,* 1998; Agte *et al.*, 2000). Reports show that these phytochemicals reduce LDL i.e. the cholesterol involved in depositing fat in the arteries (Anderson, 2004),

prevent blood clotting which can reduce the risk for a heart attack or a stroke. Sulphur compounds,

Some phytonutrients found in walnut for example, the quinine juglone are found in virtually no other commonly eaten food. Othersô such as the tannin-tellimagrandin or the flavonol morin are also rare and valuable as antioxidants and anti inflammatory nutrients. These anti- inflammatory and anti- oxidant phytonutrients also help explain the decreased risk of certain cancers- including prostate cancer and breast cancer (Fukuda *et al.*, 2003).

1.5.5.1 Phytochemical Constituents of Plants

(a) Terpenoids

Terpenoids, also known as Isoprenoids are the major families of natural compounds, comprising of more than 40,000 different molecules (McCaskill and Croteau, 1998). The isoprenoids biosynthetic pathway produces both primary and secondary metabolites that are of great significance to plant growth and persistence (Haudenschild and Croteau, 1998) Terpenoids are secondary metabolites that have molecular structures comprising carbon backbones that are made up of isoprene (2- methylbuta-1, 3- diene) units. The terpenoids comprise of two isoprene units, containing ten carbon atoms. Among the primary metabolites produced by this pathway are: the phytohormones ï abscisic acid (ABA); gibberellic acid (Gas) and cytokinins; the carotenoids; plastoquinones and chlorophylls involved in photosynthesis; the ubiquinones required for respiration; and the sterols that impact membrane structure (Harborne, 1998). Many of the terpenoids are important for the quality of agricultural products, such as the flavour of fruits and the fragrance of flowers like linalool (Pichersky *et al.,* 1994). In addition, terpenoids can have medicinal properties such as anti-carcinogenic (e.g. Taxol and perilla alcohol), antimalarial (e.g. artemisinin), anti- ulcer, hepaticidal, antimicrobial or diuretic (e.g. glycyrrhizin) activity (Haudenschild and Croteau, 1998; McCaskill and Croteau, 1998; Rodriguez-Concepcion, 2004; Bertea *et al.*, 2005). The steroids and sterols in animals are biologically produced from precursors of terpenoids and sometimes terpenoids are added to proteins to increase their attachment to the cell membrane, a process known as isoprenylation (Harborne, 1998).

(b) Flavonoids

Flavonoids are polyphenolic compounds that are ubiquitous in nature and are categorized according to their chemical structure into flavones, anthocyanidins, isoflavones, catechins, flavanols, chalcones and flavanones (Dakora, 1995). They occur mostly in

vegetables, fruits and beverages like tea, coffee and fruits drinks. They accumulate in plants as phytoalexins defending them against microbial attack (Grayer and Harborne, 1994; Harborne and Williams, 2000) and fungal attack (Jensen *et al*., 1998).

Flavonoids have been found to possess many useful effects on human health. They have been shown to have several biological properties including anti- inflammatory activity, enzyme inhibition, antimicrobial activity, oestrogenic activity (Havsteen, 1983; Harborne and Williams, 2000,), antioxidant and free radical scavenging ability (Robak and Grylewski, 1988). Flavonoids have also been shown to interact with cytochrome P_{450} (Ng *et al.,* 1996) have anti-leukemic properties and mild vasodilatory properties useful for the treatment of heart disease.

(c) Saponins

Saponins are group secondary metabolites found widely distributed in the plant kingdom as plant glycosides, characterized by a skeleton resulting from the 30-carbon precursor oxidosqualene to which glycosyl residues are attached. They have sturdy foaming property (Harborne, 1998) and are subdivided into triterpenoid and steroid glycosides. Saponins are stored in plants cell as inactive precursor but are readily converted into biologically active antibiotics by plant enzymes in reply to pathogenic attack. (Okwu, 2005).Saponins protect plants against attack by pathogens and pests (Price *et al.,* 1978). They are molecules that have substantial markable value and are processed as drugs and medicines, foaming agents, sweeteners, taste converters and cosmetics (Hostettmann and Marston, 1995).

Saponin containing plants are used as traditional medicines, especially in Asia, and are intensively used in food, veterinary and medical industries (Hostettmann and Marston, 1995). The pesticide activity of saponins has long been reported (Irvine, 1961). Saponin- glycosides are very lethal to cold-blooded organisms, but not to mammals (Hostettmann and Marston, 1995). Plant extracts containing a high percentage of saponins are commonly used in Africa to treat water supplies and wells contaminated with disease vectors; after treatment, the water is safe for human drinking (Hostettmann and Marston, 1995). Saponins induce strong cytotoxic CD8+ lymphocyte responses and potentate the response to mucosal antigens (Kensil, 1996). They have both stimulatory effects on the components of specific immunity and non-specific immune reactions such as inflammation (de Oliveira *et al*., 2001) and monocyte proliferation (Delmas *et al*., 2000)

Saponins have long been known to possess lytic action on erythrocyte cell membranes and this property has been used in their detection. The haemolytic actions of saponins are alleged to be due to their affinity for the aglycone moiety of membrane sterols, mainly cholesterol with which they form undissolvable complexes (Glauert *et al*., 1962).

(d) Tannins

Tannins are an exceptional group of water soluble phenolic metabolites of relatively high molecular weight and having to complex strongly with carbohydrates and proteins (Petridis, 2010). Tannins are astringent, bitter plant polyphenols and the astringency from tannins is what causes the dry and pucker feeling in the mouth following the consumption of unripened fruit or red wine (Serafini *et al.,* 1994). They are grouped into two forms hydrolysable and condensed tannins (Nityanand, 1997). Hydrolysable tannins are potentially toxic and cause poisoning if large amounts of tannin- containing plant material such as leaves of oak (Quercus spp.) and yellow wood (Terminalia oblongata) are consumed (Garg *et al*., 1982) and as such seen as one of the anti- nutrients of plant origin because of their capability to precipitate proteins, inhibit the digestive enzymes and decline the absorption of vitamins and minerals (Khattab *et al.,* 2010).

Several health benefits have been assigned to tannins and some epidemiological associations with the decreased frequency of chronic diseases have been established (Serrano *et al*., 2009). Several studies have shown significant biological effects of tannins such as antioxidant or free radical scavenging activity as well as inhibition of lipid peroxidation and lipoxygenases in vitro (Amarowicz *et al*., 2000). They have also been shown to posses antimicrobial, antiviral, antimutagenic and antidiabetic properties (Dolara *et al*., 2005). The antioxidant activity of tannins results from their free radical and reactive oxygen species-scavenging properties, as well as the chelation of transition metal ions that modify the oxidation process (Serrano *et al*., 2009).

(e) Steroids

Sterols are triterpenes which are based on the cyclopentane hydrophenanthrene ring system (Harborne, 1998). Sterols in plants are generally described as phytosterols with three known types occurring in higher plants: sitosterol (formerly known as β- sitosterol), stignasterol and campsterol (Harborne, 1998). These common sterols occur both as free and as simple glucosides. Sterols have essential functions in all eukaryotes. Free sterols are integral components of the membrane lipid bilayer where they play important role in the

regulation of membrane fluidity and permeability (Corey *et al.,* 1993). While cholesterol is the major sterol in animals, a mixture of various sterols is present in higher plants, with sitosterol usually predominating. However, certain sterols are confined to lower plants such as ergosterol found in yeast and many fungi while others like fucoterol, the main steroid of many brown algae is also detected in coconut (Harborne, 1998).

(f) Alkaloids

Alkaloids play a very important role in organism metabolism and functional activity. They are metabolic products in plants, animals and micro-organisms. They occur in both vertebrates and invertebrates as endogenous and exogenous compounds. Many of them have a distributing effect on the nervous systems of animals. Alkaloids are the oldest successfully used drugs throughout the historical treatment of many diseases (Wink, 1998) and are one of the most diverse groups of secondary metabolite found in living organism. They have an array of structural types, biosynthetic pathways, and pharmacological activities (Roberts and Wink, 1998). In plants and insects, toxic alkaloids are sequestered for use as a passive defense mechanism by acting as deterrents for predating insects (Schmeller and Wink, 1998). Alkaloids have been used throughout history in folk medicine in different regions around the world. They have been a constituent part of plants used in phytotherapy. Many of the plants that contain alkaloids are just medicinal plants and have been used as herbs. Some alkaloids that have played an important role in this sense include aconitine, atropine, colchicines, coniine, ephedrine, ergotamine, mescaline, morphine, strychnine, psilocin and psilocybin (Schmeller and Wink, 1998).

Many alkaloids are known to have effect on the central nervous system and some act as antiparasitic agents (such as morphine, a pain killer). For example, quinine was widely used against Plasmodium falciparum. In this respect, it is found from the phytochemical screening that most plants traditionally used to treat malaria contain alkaloids among other things (Jeruto *et al*., 2011).

1.5.6 Minerals and Vitamins Constituents of Chloroform-Methanol Extract of *Tetracarpidium conophorum* Seeds

A. Minerals Constituents

The roots of the plant have been shown to contain, Ca, Mg, Cu, Zn, K, Na though in little quantity. The low sodium content in the root of *Tetracarpidium conophorum* is an added advantage because of the direct relationship of sodium intake with hypertension in humans

(Dahl, 1972).This may be the reason why the plant is used to prevent and control high blood pressure (James, 2000).It is rich in copper which is a cofactor for many vital enzymes, including cytochrome C- oxidase and superoxide dismutase (manganese and zinc also perform the same function) (Claude and Paule,1979).The presence of zinc indicates that the seed has a role in nerve functions, male & female fertility (ovaries and testes), it ensures normal sexual development, formation of red and white blood cells and for nucleic acid synthesis.

B. Vitamins Constituents

In 2010, the Journal of the American college of Nutrition reported that Walnut & walnut oil improve reaction to stress. Carotenoid pigments play important functions in photosynthesis and photoprotection in plant tissues. The photoprotection role of carotenoids originates from their ability to quench and inactivate reactive oxygen species such as singlet oxygen formed from exposure of light and air. This photoprotection role is also associated with its antioxidant activity in human health. Carotenoids can react with free radicals and become radicals themselves. Their reactivity depends on the length of the chain of conjugated double bonds and the characteristics of the end groups. Carotenoid radicals are stable by virtue of the delocalization of the unpaired electron over the conjugated polyene chain of the molecules.

Dô Amelio (1999) reported the presence of ascorbic acid in the roots of *Tetracarpidium conophorum* which can be used in herbal medicine for the treatment of skin conditions such as eczema, pruritus, psoriasis and parasitic skin infections. It is also interesting to note that as an antioxidant, ascorbic acid prevents or at least minimizes the formation of carcinogenic substances from dietary sources (Hunt *et al*., 1980). The kind of vitamin E found in walnut seeds is somewhat unusual, and particularly beneficial. Instead of having most of its vitamin E present in the alpha tocopherol form, it provides an unusually high level of vitamin E in the form of gamma tocopherol about 21g per 100 g (Blomhoff *et al.,* 2006). Particularly, in studies on the cardiovascular health of men. Vitamin E is a powerful lipid soluble antioxidant, required for maintaining the integrity of cell membranes of mucus membranes and skin protecting it from harmful oxygen free radicals.

Management of Oxidative Stress

(1) Taking whole foods, natural fruits, seeds and vegetables

(2) Taking diets rich in antioxidant vitamins

(3) Preventing and avoiding external stimuli that will result in internal stress

(4) Taking medications under strict medical prescription.

(5) Having enough knowledge of ones body chemistry.

1.5.7 The Liver

The liver is a self regenerating organ that plays important roles in the body. It functions not only in metabolism and removal of exogenous toxins and therapeutic agents responsible for metabolic derangement but also in the biochemical regulation of fats, carbohydrates, amino acids, protein, blood coagulation and immunomodulation (Ram and Goel, 1999). Due to its ability to regenerate, a moderate cell injury is not reflected by measurable change in its metabolic function. However, damage caused by lipid peroxidation on the membrane of the hepatocyctes allows the leakage of some cytosolic enzymes of the liver into the blood stream.

1.5.7.1 Serum enzyme markers involved in hepatic disorder

When the integrity of the membrane of the hepatocytes is compromised, certain enzymes located in the cytosol are released into the blood. Their estimation in the serum is a useful quantitative marker for the evaluation of liver damage (Ram and Goel, 1999). Glutamate dehydrogenase activity is not found in normal serum but moderate elevation is found in most cases of acute hepatitis indicating cellular damage. Another demonstrable type of membrane damage involves injury to lysosomes which leads to the release of acid ribonuclease and acid phosphatases, and other liver enzymes such as alanine trasaminase, aspartate transaminase and alkaline phosphatase, into the blood stream. These enzymes are elevated to distinguish and assess the extent and type of hepatocellular injury (Ram and Goel, 1999).

1.6 Aim and Objectives of the Study

1.6.1 Aim of the Study

This study was aimed at investigating the effect of chloroform-methanol extract of *Tetracarpidium conophorum* seeds on hydrogen peroxide-induced oxidative stress markers in Wistar rats.

1.6.2 Specific Objectives of the study

- To determine qualitatively and quantitatively the phytochemical constituents of chloroform-methanol extracts of *Tetracarpidium conophorum* seeds.
- To determine the acute toxicity (LD_{50}) of the chloroform- methanol extract of *Tetracarpidium conophorum* seeds.
- To determine the vitamin and mineral levels of the chloroform-methanol extract of *Tetracarpidium conophorum* seeds.
- To determine the effect of the chloroform- methanol extract of *Tetracarpidium conophorum* seeds on oxidative stress markers.
- To determine the effect of the chloroform- methanol extract of *Tetracarpidium conophorum* seeds on some liver markers enzymes such as ALT,ALP and AST.
- To determine the effect of the chloroform-methanol extract of *Tetracarpidium conophorum* on serum lipid peroxidation index (malondialdehyde concentration).
- To determine the effect of the chloroformï methanol extract of *Tetracarpidium conophorum* on serum lipid indices such as Total cholesterol, HDL,LDL and TAGs.

CHAPTER TWO
MATERIALS AND METHODS

2.1 Materials

2.1.1 Plant Materials

Tetracarpiduim conophorum seeds (Walnut) were obtained from the Bridge Head Market, Onitsha in Onitsha South L.G.A. of Anambra State, Nigeria and was identified by Mr. Paulinus Ugwuozor of the Department of Botany, University of Nigeria, Nsukka.

2.1.2 Animals

Thirty five (35) male albino Wistar rats weighing between 120 and 140g were used for the studies and eighteen (18) adult albino mice were used for the acute toxicity (LD_{50}) test. The animals used in this study were obtained from the Animal House of the Department of Zoology University of Nigeria, Nsukka. The rats were fed with standard growers mash rat pellets (Grand Cereals Ltd, Enugu) and water. The guide for the care and use of laboratory animals procedure were followed in this study (Indian Council of medical Research, 2001).

2.1.3 Equipment

The equipment used were obtained from the Department of Biochemistry and other scientific shops in Nsukka. They include.

	Manufacturer
Glasswares	Pyrex, England
Centrifuge	PAC, Pacific, England
Micro pipette	Pyrex, England
Water bath	Gallenkamp, England
Refrigerator	Thermocool, Engla
Weighing balance	Mettler HAS, USA
Spectrophotometer	Spectronic 20D, USA
Thermometer	Zeal, England
Electrical Oven	Gallenkamp, England

2.1.4 Chemicals and Reagents

Hydrochloric acid	BDH, England
Potassium iodide	East Anglia, England
Sulphuric acid	BDH, England

Glacial acetic acid	Sigma, USA
Naphthlene diamine dihydrochloride	BDH, England
Trichloroacetic acid	Sigma Aldrich, Germany
Sodium Duodecyl Sulphate	DDA, England
2, 4- dinitrophenyl hydrazine	Marck Darmstad, Germany
Thiourea solution	Randox, USA
Cupric Sulphate Solution	Marck Darmstad, Germany
Hydrogen peroxide	BDH, England
Dichomate acetic acid	May & Baker, England
Molischôs reagent	DCA, Spain
Mayerôs reagent	BDH, England
Dragendorffôs reagent	May & Baker, England
Wagnerôs reagent	Random, USA
Picric acid	Marck Darmstad, Germany
Ammonia solution	BDH, England
Ethyl acetate	BDH, England
Aluminum Chloride solution	BDH, England
1% Thiobarbituric acid	BDH, England
Chloroform	Sigma Aldrich, Germany
Methanol	Sigma Aldrich, Germany
Dimethyl Sulphoxide	BDH, England

2.2 Methods

2.2.1 Preparation of plant material

The nuts were washed, cooked, deshelled, cut, sun dried and pulverised to powder and then stored in an air tight container prior to analysis.

2.2.2 Extraction of plant material

A known weight, (500g) of *Tetracarpidium conophonum* was macerated in chloroform and methanol in the ratio 2:1 for 48hours after which it was filtered with Whatman No 1 filter paper, the filtrate was concentrated into a slurry in an oven at 60^{o}C and was stored in the refrigerator for subsequent uses.

2.2.3 Preparation of Reagents for Phytochemical Analysis

5% (w/v) Ferric chloride solution

A quantity 5.0 g of ferric chloride was dissolved in 100 ml of distilled water.

Ammonium solution

A measured volume, 187.5 ml of the stock concentrated ammonium solution was diluted in 31.25 ml of distilled water and then made up to 500 ml with distilled water.

45% (v/v) ethanol

Absolute ethanol (45 ml) was mixed with 55ml of distilled water.

Aluminium chloride solution

A known weight, 0.5 g of aluminium chloride was dissolved in 100 ml of distilled water.

Dilute sulphuric acid

A measured volume, 10.9 ml of concentrated sulphuric acid was mixed with 5 ml of distilled water and made up to 100 ml.

Lead sub acetate solution

A quantity, 45 ml of 15% lead acetate (i.e. 15.0 g of lead acetate in 100 ml of distilled water) was dissolved in 20 ml of absolute ethanol and made up to 100 ml with distilled water.

Wagner's reagent

Iodine crystals (2.0 g) and potassium iodide (3.0 g)were dissolved in 10 ml of water and then made up to 100 ml with distilled water.

Mayer's reagent

Mercuric chloride (13.5 g) was dissolved in 50 ml of distilled water. Also 5.0 g of potassium iodide was dissolved in 20 ml of distilled water. The two solutions were mixed and the volume made up to 100 ml with distilled water.

Dragendorff's reagent

A measured quantity, 0.85 g of bismuth carbonate was dissolved in 100 ml of glacial acetic acid and 40 ml of distilled water to give solution A. Another solution called solution B was prepared by dissolving 8.0 g of potassium iodide in 20 ml of distilled water. Both solutions were mixed to give a stock solution.

Molisch reagent

A known weight, 1.0 g of Ŭ-naphthol was dissolved in 100 ml of absolute ethanol.

2% (v/v) Hydrochloric acid

Concentrated hydrochloric acid (2.0)ml was distilled with some distilled water and made up to 100 ml.

1 % (w/v) Picric acid

A quantity, 1.0 g of picric acid was dissolved in 100 ml of distilled water.

Preparation of 25 % Trichloroacetic Acid (TCA)

A known weight, 25 g of TCA was dissolved in distilled water and made up to the 100 ml mark with distilled water in a measuring cylinder.

Preparation of 1 % Thiobarbituric acid (TBA)

A quantity, 1g of TBA was dissolved in distilled water and made up to the 100 ml mark with distilled water in a measuring cylinder.

Preparation of 0.3 ml Sodium Hydroxide (NaOH)

A quantity, 1.3g of NaOH was dissolved in a little amount of water and made up to 100 ml mark with distilled water in a measuring cylinder.

Preparation of 20% Sodium Duodecyl Sulphate (SDS)

SDS (20 g) was dissolved in some quantity of distilled water and made up to the 100 ml mark with distilled water in a measuring cylinder with distilled water.

Preparation of 2% Glacial Acetic Acid

A known quantity, 2 g of glacial acetic acid was dissolved in distilled water and made up to 100 ml with distilled water in a measuring cylinder.

2.2.4 Qualitative Phytochemical analysis of *Tetracarpidium conophorum*

The phytochemical analysis of the seeds of *Tetracarpidium conophorum* was carried out according to the method of Harborne (1998) and Trease and Evans (2002). The methods are shown below:

2.2.4.1 Test for alkaloids

The sample (0.2 g) is boiled with 5ml of 2% HCl on a steam bath. The mixture was filtered and 1ml of the filtered is treated with 2 drops of the following reagents

(i) Dragendorffôs reagent: An orange precipitate indicated the presence of alkaloids.

(ii) Mayerôs reagent: A creamy- white precipitate indicated the presence of alkaloids.

(iii) Wagnerôs reagent: a reddish-brown precipitate indicated the presence of alkaloids

(iv) Picric acid (1%) : a yellow precipitate indicated the presence of alkaloids.

2.2.4.2 Test for flavonoids

A quantity, 0.2 g of the sample was heated with 10 ml ethyl acetate in boiling water for 3 minutes. The mixture was filtered, and the filtrate used for the following tests.

(i) Ammonium test: 4 ml of the filtrate was shaken with 1 ml of dilute ammonium solution to obtain two layers. The layers were allowed to separate. A yellow precipitate observed in the ammonium layer indicates the presence of flavonoids.

(ii) Aluminium chloride test: 4 ml of the filtrate was shaken with 1ml of 1% aluminium chloride solution and observed for light yellow colouration that indicates the presence of flavonoids.

2.2.4.3 Test for glycosides

The sample (2.0 g) was mixed with 30 ml of distilled water and 15ml of dilute sulphuric acid respectively and heated in a water bath for 5 minutes. The mixtures was filtered and the filtrates used for the following test.

(i) To 5ml of each of the filtrates, 1.0 ml of Fehlingôs solutions A and B was added until it turned alkaline (tested with litmus paper) and heated on a water bath for 2 minutes. A brick- red precipitate indicates the presence of glycosides.

2.2.4.4 Test for saponins

A known quantity, 0.1 g of the sample was boiled with 5 ml of distilled water for 5 minutes. The mixture was filtered while still hot. The filtrate was used for the following tests.

(i) Emulsion test: A quantity, 1 ml of the filtrate was added to two drops of olive oil. The mixture was shaken and observed for the formation of emulsion.

(ii) Frothing test: A quantity, 1 ml of the filtrate was diluted with 4 ml of distilled water. The mixture was shaken vigorous and then observed on standing for a stable froth.

2.2.4.5 Test for tannins

A quantity, 2 g of the sample was boiled with 5 ml of 45% ethanol for 5 minutes. The mixture was cooled and then filtered and the filtrate was treated with the following solutions.

(i) Lead sub acetate solution: to 1 ml of the filtrate, 3 drops of lead sub acetate solution was added. A gelatinous precipitate indicates the presence of tannins.

(ii) Bromine water: To 1 ml of the filtrate was added 0.5 ml of bromine water and then observed for a pale brown precipitate.

(iii) Ferric chloride solution: A quantity, 1 ml of the filtrate was diluted with distilled water and then 2 drops of ferric chloride solution was added. A transient greenish to black colour indicates the presence of tannins.

2.2.4.6 Test for terpenoids and steroids

A measured volume, 9 ml of ethanol was added to 1.0 g of the sample and refluxed for a few minutes and filtered. The filtrate was concentrated to 2.5 ml on a boiling water bath, and 5 ml of hot water was added. The mixture was allowed to stand for 1 hour, and the waxy matter filtered off. The filtrate was extracted with 2.5 ml of chloroform using a separating funnel. To 0.5 ml of the chloroform extract in a test tube was carefully added 1ml of concentrated sulphuric acid to form a lower layer. A reddish- brown interface shows the presence of steroids.

Another 0.5 ml aliquot of the chloroform extract was evaporated to dryness on a water bath and heated with 3 ml of concentrated sulphuric acid for 10 minutes on water. A grey colour indicates the presence of terpenoids.

2.2.5 Quantitative Phytochemical Analysis of the Seeds of *Tetracarpidium conophorum*

2.2.5.1 Alkaloids determination

The determination of alkaloids was as described by Harborne (1973). A portion, (5 g) of the sample was weighed into a 250 ml beaker and 200 ml of 10% acetic acid in ethanol was added, covered and allowed to stand for 2 hours. This was filtered and the extract was concentrated in a water bath to one quarter of the original volume. Concentrated ammonium hydroxide was added drop- wise to the extract till a precipitate was formed. The precipitate was collected and washed with dilute ammonium hydroxide and then filtered. The residue is the alkaloid, which was dried and weighed.

2.2.5.2 Flavonoids determination

This was determined according to the method of Harborne (1973). A measured quantity, 5g of the sample was boiled in 50 ml of 2M HCl solution for 30 minutes under reflux. It was allowed to cool and then filtered through Whatman No. 1 filter paper. A

measured volume of the extract was treated with equal volume of ethyl acetate starting with drop. The solution was filtered into a weighed crucible. The filtrate was heated to dryness in an oven at 60^0 C. The dried crucible was weighed again and the difference in the weight gave the quantity of flavonoid present in the sample.

2.2.5.3 Determination of Steroids

This was determined by the method described by Edeoga *et al.* (2005). A known weight of each sample was dispersed in 100ml freshly distilled water and homogenized in a laboratory blender. The homogenate was filtered and the filtrate was eluted with normal ammonium hydroxide solution (pH 9). The elute (2 ml) was put in test tube and mixed with 2ml of chloroform. Ice-cold acetic anhydride (3 ml) was added to the mixture in the flask and 2 drops of conc. H_2SO_4 were cautiously added. Standard sterol solution was prepared and treated as described above. The absorbances of standard and prepared sample were measured in a spectrophotometer at 420 nm.

2.2.6. Determination of vitamin contents of the seeds of *Tetracarpidium conophorum*

The vitamin determination was performed with the method of Pearson (1976).

2.2.6.1 Vitamin A

Ground sample was macerated with 20 ml of petroleum ether. This was decanted into a test tube and then evaporated to dryness. A volume 0.2 ml of chloroform- acetic anhydride (1:1, v/v) was added to the residue. 2 ml of TCA- chloroform in (1:1, v/v) was added to the resulting solution and absorbance was measured at 620 nm. Vitamin A standard was prepared in like manner and the absorbance taken at 620 nm, the concentration of vitamin A in the sample was extrapolated from the standard curve.

2.2.6.2 Vitamin C

A known quantity, 1.0 g of the sample was macerated with 20 ml of 0.4% oxalic acid. This was filtered and to 1 ml of filtered was added 9 ml of indolephenol reagent. The standard solution of vitamin C was prepared similarly and the absorbance of the standard solution and the sample were read at 520 nm. The concentration of vitamin C was extrapolated from the standard curve of vitamin C.

2.2.6.3 Vitamin E

A measured quantity, 1.0 g of the sample was macerated with 20 ml of ethanol and then filtered 0.2% (0.2 g in 100 ml) ferric chloride in ethanol and 1ml of 0.5% Ŭ- dipyridine to 1ml of the filtrates. This was diluted to 5 ml with distilled water. Absorbance was taken at 520 nm. The standard solutions were prepared similarly and the concentration of vitamin E extrapolated from the standard curve.

2.2.7 Determination of Mineral Contents of the Seeds of *Tetracarpidium conophorum*

The method of AOAC (1970) was used. Two grammes of sample were weighed into a crucible and ashed into a furnace at 550 ^{0}C for 6 hours. The ash was cooled and 6N HCl added and boiled for 10minutes, while covering the crucible with a watch glass. After boiling the sample, allow to cool and filter into 100 ml volumetric flask. The crucible was washed with distilled water and the washings added to the ash filtered. The ash filtered was then made up to 100 ml with distilled water. An aliquot of the filtrate was aspirated into the atomic absorption spectrophotometer and the absorbance values corresponding to different minerals recorded. The percentage of the elements in the samples was calculated from the absorbance values of the samples and standard solutions.

A. Determination of Magnesium

The standard method of AOAC (1995) was used to determine Calcium and Magnesium. The sample solution was preparation by wet digestion. Ten millilitre (10 ml) of the test solution was pipetted into a 250 ml conical flask and also add to it 25 ml of NN3-NH buffer solution twenty five millilitre (25 ml) of water was added to the mixture followed by 2-3 drops of Enriochrome Black T indicator and titrated against 0.0 1N EDTA. Solution the volume of EDTA used was the volume equipment of calcium & magnesium in the mixture.

Volume of Mg = (Volume of Ca and Mg ï Volume of Ca).

B. Determination of copper

Pipette suitable aliquot of sample digest (containing not more than 50 ε g of copper) into a short stem separator, add 2N H_2SO_4 to make total volume of 25 ml and add 10 ml of citrate EDTA reagent. Add two drops of thymol blue indicator and 6N NH_4OH drop wise until solution turns green or blue- green. Cool and add 1 ml of carbamate solution and 15 ml of CCl_4. Shake vigorously for 2 minutes. let layers separate and drain CCl_4 through cotton pledget into glass stoppered tube. Determine absorbance ñAò at 400nm.

C. Determination of Zinc

The dithizone method used as described by AOAC (1976)

Principles:

Zinc was separated from other metals by extraction with dithizone and then determined by measuring the colour of zinc-dithizone complex in carbontetrachloride. The separation was achieved by extracting at pH of 4.0- 5.0. Set by the addition of sufficient sodium thiosulphate zinc also forms a metal thiosulphate. Zinc also forms a weak thiosulphate complex that tend to retard the slow and incomplete between zinc and dithol zone.

Procedure:

A known volume, 5 ml of the digested sample was pipeted into a test tube and 5 ml of acetate buffer was added. 1 ml of sodium thiosulphate sodium was added and mixed after which 10 ml of dithizone solution was added. The mixture was shaken vigorously for 4 minutes. The absorbance was taken at 535 nm. The standard was prepared and the concentration of the sample found.

Calculation:

$$\text{Concentration of Zn} = \frac{\text{Absorbance of test sample}}{\text{Absorption of standard}} \times \frac{\text{Concentration of Standard}}{\text{wt of sample used.}}$$

2.2.8 Acute Toxicity Test of Chloroform-Methanol Extract of *Tetracarpidium conophorum*

The method of Lorke (1983) was used for the acute toxicity test of the chloroform-methanol extract of *Tetracarpidium conophorum*. Eighteen (18) albino mice were used for the study. The test involved two stages. In stage one, the animals were grouped into three (3) groups of three rats each and were given 10, 100 and 1000 mg/kg body weight of the extracts respectively and in the second stage 1,600, 2,900 and 5,000 mg/ kg body weight of the extracts were administered to the animals. The administration of the extracts was done orally. The median lethal dose (LD_{50}) was calculated from the second phase.

2.2.9 Experimental Design and Induction of Oxidative Stress

Thirty five (35) male wistar albino rats weighing (120-140g) were used for the study. They were acclimatized for seven (7) days with free access to feed and water. After acclimatization, they were evenly distributed into seven (7) groups of five rats each. The rats were given extracts according to their groupings except group 1and 2, group 3 was given 100mg/kg body weight of vitamin C for five days, groups 4, 5 and 6 were treated with 200mg/kg, 400mg/kg and 800mg/kg body weight of the extract respectively for five days, on

the sixth day they were withdrawn from feed and water, and they were induced with 1.0ml/kg body weight of hydrogen peroxide intraperitonealy. The procedure used is a modification of Suja *et al.,* (2004).The route of administration of the extract was via oral route with the aid of an oral intubation tube. The blood was collected on the 7^{th} day through occular puncture. The groups and doses administered are summarized below:

Group 1: Vehicle only. (Normal control)

Group 2: H_2O_2 induced rats no treatment (Positive control)

Group 3:H_2O_2 induced +100mg/kg body weight of vitamin C (Negative control)

Group 4:H_2O_2 induced + 200mg/kg body weight of *Tetracarpidium conophorum* seed Extract

Group 5:H_2O_2 induced +400 mg/kg body weight of *Tetracarpidium conophorum* seed Extract

Group 6:H_2O_2 induced + 800mg/kg body weight of *Tetracarpidium conophorum* seed Extract

Group 7: Vehicle + 800mg/kg body weight of *Tetracarpidium conophorum* seed Extract

2.2.10 Liver Function Test of Rats Treated with Chloroform- Methanol Extract of *Tetracarpidium conophorum* Seeds

2.2.10.1 Assay for Alanine Aminotransferase (ALT) Activity

Principle: ALT is measured by monitoring the concentration of pyruvate hydrazone formed with 2,4-dinitrophenylhydrazine. The colour intensity is measured against the blank at 540nm.

Method: The blank and sample test tubes were set up in duplicates. 0.1ml of serum was pipetted into the samples tubes. To these were added 0.5ml buffer solution containing phosphate buffer, L-alanine and Ŭ-oxoglutarate. The mixtures were thoroughly mixed and incubated for exactly 30 minutes at 37^0 C ml and pH 7.4. A volume, 0.5 ml of reagent containing 2,4- dinitrophenylhydrazine was later added to both tubes while 0.1 ml of sample was added to sample blank tube. The tubes were mixed thoroughly and incubated for exactly 20 minutes at 25^0 C. Five milliliter (5.0 ml) of sodium hydroxide solution was then added to each tube and mixed. The absorbed was read against the blank after 5 minutes at 540 nm.

2.2.10.2 Assay for Aspartate Amino Transferase (AST) Activity

Principle: AST or SGOT is measured by monitoring the concentration of oxaloacetate hydrazone formed with 2, 4-dinitrophenylhydrazine. The colour intensity is measured against the blank at 546 nm.

Method: The blank and sample test tubes were set up in duplicates. A volume 0.1 ml of serum was pipetted into the sample tubes and 0.5 ml of Reagent 1 was pipette into both sample and blank tubes. The solution were thoroughly mixed and incubated for exactly 30

minutes at 37^0 C ml and pH 7.4. A known volume, 0.5ml of reagent 2 containing 2,4-dinitropheylhydrazine was added into all the test tubes followed by 0.1 ml of sample into the blank tubes. The tubes were mixed thoroughly and incubated for exactly 20 minutes at 25^0 C and 5.0 ml of sodium hydroxide solution was then added to each tube and mixed. The absorbance was read against the blank after 5 minutes at 546 nm.

2.2.10.3 Assay for Alkaline Phosphatase (ALP) Activity

Principle: The principle of this method is based on the reaction involving serum alkaline phosphatase and a colourless substrate of phenolphthalein monophosphate, giving rise to phosphoric acid and phenolphthalein which at alkaline pH values turn pink that can be determined spectrophotometrically.

P-nitrophenylphosphate + H_2O $\longrightarrow$ ALP $\longrightarrow$ PO_4^{2-} + P-nitrophenol (pink at PH=9.8)

Method: The blank and sample test tubes were set up in duplicate. A volume 0.05 ml of sample was pipetted into the sample test tubes and 0.05 ml of distilled water was pipette into the blank tube. Three milliliters (3.0 ml) of substrate was pipetted into each tube respectively, which was then mixed and the initial absorbance taken at 405 nm. The stop watch was started and the absorbance of the sample and the blank read again three more times at one minute intervals.

Calculation: alkaline phosphatase activity was calculated as follows:

$$\text{Activity of ALP (in U/L)} = \frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \times \frac{3300}{1}$$

2.2.11 Determination of Lipid Peroxidation (Malondialdehyde Concentration)

Lipid peroxidation was determined spectrophotometrically by measuring the level of the lipid peroxidation product, malondialdehyde (MDA) as described by Wallin *et al.* (1993). Lipid degradation occurs forming such products as malondialdehyde (from fatty acids with three or more double bonds), ethane and pentane (from the n-terminal carbons of 3 and 6 fatty acids, respectively). Malondialdehyde appears in the blood and urine and is used as an indicator of free radical damage. Malondialdehyde (MDA) is an index of lipid peroxidation (Juranek and Bezek, 2005). Volume 0.1 ml of the serum was mixed with 0.9 ml of H_2O in a beaker. A known volume, 0.5 ml of 25% TCA (trichloroacetic acid) in 0.3% NaOH were also added to the mixture. The mixture was boiled for 40 minutes in water-bath and then cooled in cool water, then 0.1 ml of 20% sodium duodecyl sulphate (SDS) was also added to the cooled

solution and mixed properly. Then, the absorbance was taken at wavelength 532 nm and 600 nm against a blank.

$$\% \text{ TBARS} = \frac{\text{A532 ï A600} \times 100}{0.5271 \times 0.1} \quad \text{(mg/dl)}$$

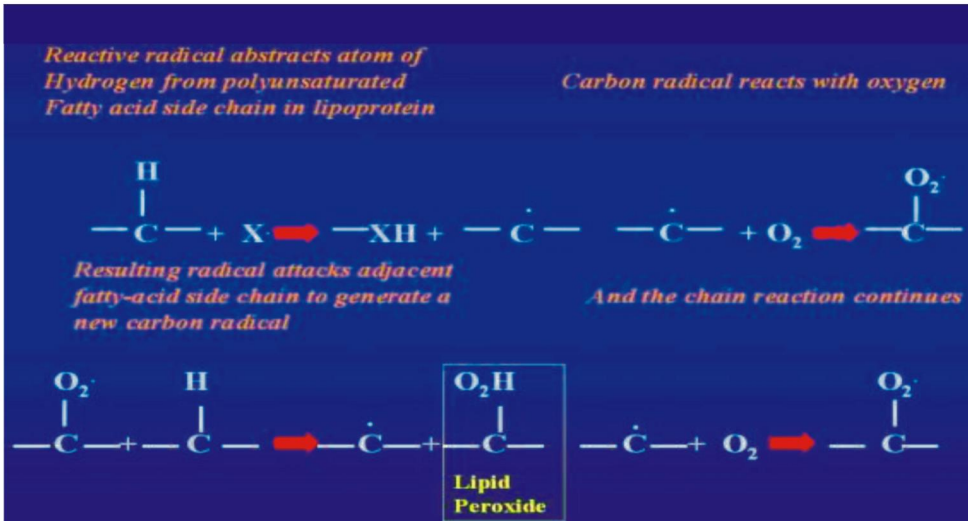


Fig 5: Reactions of lipid peroxidation

2.2.12 Determination of Glutathione Concentration

This was determined according to the method of King and Wootton (1959). A known volume, 0.1 ml of the sample was mixed with 0.9 ml of distilled water in a beaker 0.02 ml of sodium sulphate was also added, shaken and allowed to stand for 2 minutes at room temperature. A known volume 0.02 ml of Lithium Sulphate (20%), 0.2 ml of 20% $NaCO_3$ and 0.2 ml of phosphor-18-tungstic acid were also added to the beaker, it was shaken and allowed to stand for 4 minutes while observing for maximum colour development. A volume 2.5ml of 2% sodium sulphate was also added and the absorbance was taken at 680 nm within 10 minutes. A blank (0.1M H_2O) was also set up and commenced with 0.1ml of water instead of the sample glutathione concentration was calculated from a standard cystein curve.

2.2.13 Assay of Catalase Activity

This was done according to the method of Aebi (1983).

Principle:

The ultra violet absorption of hydrogen peroxide can be easily measured at 240 nm. On the decomposition of hydrogen peroxide (H_2O_2) with catalase the absorption decreases with time and from this decrease catalase activity was measured.

Procedure: A quantity, 2 millilitres (2 ml) of hydrogen peroxide and 2.5 ml of phosphate buffer were added to a beaker. Adequately, 0.5 ml of the sample was also added and mixed. One milliliter (1 ml) portion of the reaction mixture was added to 2 ml of dichromate acetic acid reagent. The absorbance was read at 570 nm at a mixture interval into four (4) places. Catalase activity was calculated using the following equation:

$$\text{Catalytic concentration (unit/L)} = 0.23 \times \log \frac{\text{Abs 1}}{\text{Abs 2}} / 0.00693$$

2.2.14 Assay of Superoxide (SOD) Activity

This was determined using the method of Xin *et al*. (1991).

Superoxide dismutases (SOD) are Dismutase enzymes that catalyse the conversion of two superoxides into hydrogen peroxide and oxygen.

$$O_2 - + O_2 - \xrightarrow{SOD} O_2 + H_2O_2$$

Erythrocyte superoxide dismutase (SOD) serve as antioxidant enzymes. The principle of SOD activity assay was based on the inhibition of nitroblue tetrazolium (NBT) reduction.

A known weight 0.01 g of adrenalin was dissolved in 17 ml of distilled water 0.1 ml of serum + 0.9 ml of phosphate buffer (pH 7.8), Take 0.2 ml in triplicate + 2.5 ml buffer. Add 0.3ml adrenalin solution inside the cuvette. Mix, take absorbance at 500 nm at 30 seconds interval for five (5) times. The changing rate of absorbance is used to determine superoxide dismutase activity.

2.2.15 Determination of Total Cholesterol Concentration

The method of Kameswara *et al.* (1999) was used in the determination of total Cholesterol concentration. In clinical chemistry, over the last decade however, lipids have become associated with lipoprotein metabolism and atherosclerosis. The method of Abell *et al.* (1952) involves hydrolysis of the cholesterol by organic solvents and subsequent alkaline hydrolysis of the cholesterol esters.

Assay principle The cholesterol is determined after enzymatic hydrolysis and oxidation. The indicator quinoneimine is formed from hydrogen peroxides and 4- aminoantipyrine in the presence of phenol and peroxidase.

Test procedure

Three (3) test tubes were set up in a test tube rack and labeled blank, standard and simple respectively. To the blank, it was added (10µl) distilled H_2O, add 10µl standard specimen to

the standard test tube and 10µl sample (serum) to the sample test tube. To each of these test tubes was added 1000µl of the cholesterol reagent. It was thoroughly mixed and incubated for 10 minutes at room temperature (20- 25^0C) or 5 minutes at 37^0C in water bath. Absorbance of the sample against the blank was read within 60 minutes.

2.2.16 Determination of Low density Lipoprotein (LDL-C) Concentration

Principle: LDL-C can be determined as the difference between total cholesterol and the cholesterol content of the supernatant after precipitation of the LDL fraction by polyvinyl sulphate (PVS) in the presence of polyethyleneglycol monomethyl ether.

Procedure:

The serum samples were kept at 2-8 ^{0}C, 0.1 ml of the precipitant solution to 0.2 ml of the serum sample was added and mixed thoroughly and allowed to stand for 15 minutes. Centrifuge at 2,000 x g for 15minutes. Determine the cholesterol concentration in the supernatant. Determination of the concentration of the serum total cholesterol as described by Kameswara *et al.*, (1999).

Calculation:

LDL-C (mmol/L) = Total Cholesterol (mmol/L - 1.5 x Supernatant Cholesterol (mmol/L).

2.2.17 Determination of High Density Lipoprotein (HDL-C) Concentration

Principle:

LDL and VLDL (low and very low density lipoproteins) are precipitated from serum by the action of a polysaccharide in the presence of divalent cations. Then, high density lipoproteins cholesterol (HDL-C) presence in the supernatant determined.

Procedure: The serum samples were kept at 2-8^{0}C. Add 0.1 ml of the precipitant solution to 0.3 ml of the serum sample and mixed thoroughly and allowed to stand for 15 minutes. Centrifuge at 2,000 x g for 15 minutes. Determine the cholesterol concentration in the supernatant. Determine the concentration of the serum total HDL- cholesterol as described by Kameswara *et al.,* (1999).

2.2.18 Determination of Triacylglycerol Concentration

This was determined by the method of Tietz (1990).

Principle: The triacylglycerols are determined after enzymatic hydrolysis with lipases. The indicator is a quinoneimine formed from hydrogen peroxide, 4- aminophenazone and 4-chlorophenol under the catalytic influence of peroxidase.

Triacylglycerol + H_2O $\xrightarrow{\text{lipases}}$ Glycerol + fatty acids

Glycerol + ATP $\xrightarrow{\text{GK}}$ Glycerol-3-phosphate + ADP

Glycerol-3-phosphate + O_2 $\xrightarrow{\text{GPO}}$ Dihydroxyacetone + phosphate + H_2O_2

$2H_2O_2$ + 4 - aminophenazone + 4 chlorophenol $\xrightarrow{\text{POD}}$ Quinoneimine +HCl +$4H_2O$

Method: A known volume 0.1 ml of the sample was pipetted into a clean labelled tube and 0.1 ml of trichloroacetic acid (TCA) was added to it, mixed and then centrifuge at 250 rpm for 10 minutes. The supernatant was decanted and reserved for use. The assay procedure was carried out as shown below:

S/N		Blank	Standard	Sample
1	Distilled water	0.5	-	-
2	Standard solution (ml)	-	0.5	-
3	TCA (ml)	0.5	0.5	-
4	Supernatant (ml)	-	-	1.0
5	Reagent mixture (ml)	1.0	1.0	1.0

The mixtures were allowed to stand for 20minutes at 25^0C and the absorbance of the sample and standards read against the blank at 540nm

Calculation: the concentration of creatinine in serum was calculated as follows:

$$\frac{\text{Absorbance of sample}}{\text{Absorbance of standard.}} \times \text{Standard concentration (mmol/1)} = \text{mmol/1}$$

2.3 Statistical Analysis

The data obtained were analyzed using Statistical Package for Social Sciences (SPSS), version 16.0 and the results expressed as mean ± SD. Significant differences of the result were established by one way ANOVA and the acceptance level of significance was ($p < 0.05$) for all the results.

CHAPTER THREE

RESULTS

3.1 Qualitative phytochemical constituents of the Chloroform-Methanol Extracts of *Tetracarpidium conophorum* Seeds

Table 1 shows the result of the qualitative phytochemical constituents of the chloroform-methanol extract of *Tetracarpidium conophorum* seeds, containing terpenoids, tannins, alkaloids and cardiac glycoside; anthraquinones, saponins and steroids were not detected.

3.2 Quantitative Phytochemical Constituents of Chloroform-Methanol Extract of *Tetracarpidum conophorum* Seeds

Table 2 shows the quantitative phytochemical components of the chloroform-methanol seed extract of *Tetracarpidium conophorum.* The result showed the relative presence of terpenoids (4.36±0.06 mg/g), tannins (1.89±0.11mg/g), alkaloids (20.31±0.30mg/g) and cardiac glycosides (12.45±0.08mg/g ; anthraquinones, saponins and steroids were not detected.

3.3 Vitamin Composition of the Chloroform-methanol Seed Extract of *Tetracarpidium conophorum*

As shown in Table 3, Vitamins A, C and E showed relative amount of 10.55±2.67, 13.09±0.23 and 5.77±0.08 mg/100g respectively

3.4 Mineral Constituents of the Chloroform-Methanol Extract of *Tetracarpidium conophorum* Seeds

Table 4 shows the result of the mineral composition of the chloroform-methanol extract of the *Tetracarpidium conophorum* seeds. The extract contains Mg(133.59 ± 0.11mg/100g), Ca(118.90±0.01mg/100g), Fe(3.67±0.07mg/100g), Zn(2.22±0.01mg/100g) and Cu(1.54±0.78mg/100g).

Table 1: Qualitative Phytochemical constituents of Chloroform-Methanol extract of *Tetracarpidium conophorum* seeds

Phytochemicals	Seed Extract Bioavailability
Terpenoids	+ +
Tannins	+
Alkaloids	+ + +
Cardiac glycosides	+ + +
Anthraquinones	ND
Saponins	ND
Steroids	ND

Key: + = Slightly present
+ + = Moderately present
+ + + = Highly present
ND = Not Detected

Table 2: The result of the quantitative phytochemical constituents of chloroform methanol extract of *Tetracarpidium conophorum* seeds

Phytochemical Constituents	Quantitative Bioavailability Mean ± SD of triplicate analysis
Terpenoids (mg/g)	4.36 ± 0.06
Tannins (mg/g)	1. 89 ± 0.11
Alkaloids (mg/g)	20.31 ± 0.30
Cardiac glycosides (mg/g)	12.45 ± 0.08
Anthraquinones	ND
Saponins	ND
Steroids	ND

ND = Not Detected

Table 3: Vitamin Constituents of chloroform methanol extract of *Tetracarpidium conophorum* seeds.

Vitamin Constituents analysis	Extract Mean ± SD of triplicate
A (mg/100g)	10.55 ± 2.67
C (mg/ 100g)	13. 09 ± 0.23
E (mg/ 100g)	5.77 ± 0.08

Table 4: Quantitative mineral constituents of the chloroform-methanol seed extract of the *Tetracarpidium conophorum*

Mineral Constituents (mg/100g)	Seed extract Mean ± SD of triplicate analysis
Mg	133.59 ± 0.11
Ca	118. 90 ± 0.01
Fe	3.67 ± 0.07
Zn	2.22 ± 0.01
Cu	1.54 ± 0.78

3.5 Acute Toxicity (LD_{50}) Test of the Chloroform-Methanol Extract of *Tetracarpidium conophorum* Seeds

The acute toxicity test of chloroform methanol extracts of the seed of *Tetracarpidium conophorum* seeds and showed no death up to 5000mg/kg body weight of the extract.

	Dosage mg/ kg Body weight	**Mortality**
Phase 1		
Group 1	10	0/3
Group 2	100	0/3
Group 3	1000	0/3
Phase II		
Group 1	1600	0/3
Group 2	2900	0/3
Group 3	5000	0/3

3.6 Effect of Chloroform ï Methanol Extract of *Tetracarpidium conophorum* Seeds on Serum Alanine Aminotransferase (ALT) Activity of Hydrogen Peroxide-Induced Wistar Albino Rats

The result in Figure 6 shows that there was a significant increase ($p<0.05$) in the serum ALT activity of rats in group 2 representing hydrogen peroxide-induced untreated rats when compared with the other control groups 1 and 3 representing normal rats and hydrogen peroxide-induced and treated with 100 mg/kg body weight of vitamin C respectively and test groups treated with *Tetracarpidium conophorum* seed extract. On the other hand a significant decrease ($p<0.05$) was observed in the serum ALT activity of the test groups. The test groups 4 and 6 representing hydrogen peroxide-induced and treated with 200 and 400 mg/kg body weight was comparable to the normal control rats of group 1.

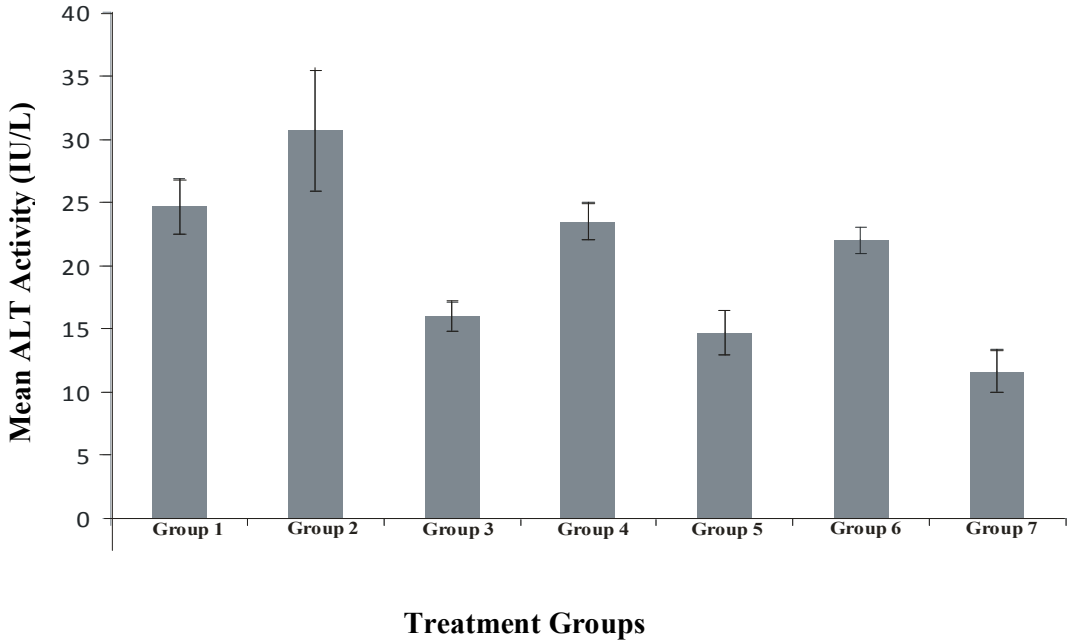


Fig.6: Effect of chloroformï methanol extract of *Tetracarpidium conophorum* seeds on serum alanine aminotransferase (ALT) activity of H_2O_2 - induced rats

Group 1: Control (Normal Rats)
Group 2: Positive Control (hydrogen peroxide induced untreated rats)
Group 3: Hydrogen peroxide induce d + 100mg/kg body weight of vitamin C
Group 4: Hydrogen peroxide induced + 200mg/kg body weight of chloroform ï methanol seed extract.
Group 5: Hydrogen peroxide induced + 400mg/kg body weight of chloroform ï methanol seed extract.
Group 6: Hydrogen peroxide induced + 800mg/kg body weight of chloroform ï methanol seed extract.
Group 7: Non induced rats + 800mg/kg body weight of chloroform - methanol seed extract

3.7. Effect of Chloroform-Methanol Extract of *Tetracarpidium conophorum* Seeds on Serum Alkaline Phosphatase (ALP) Activity of Hydrogen Peroxide-Induced Wistar Albino Rats

Non- significant decrease ($p>0.05$) was observed in the serum ALP activity of group 2 rats administered hydrogen peroxide only compared with the ALP activity of group 1 rats representing normal control group as shown in Fig. 7. There was also a non- significant decrease ($p>0.05$) in groups 4 and 5 (induced and treated with 200 and 400mg/kg of extract respectively when compared to group 2. Except in group 6 (induced and treated with 800 mg/kg of Extract) which showed a significant increase ($p<0.05$) when compared to all the controls and test groups.

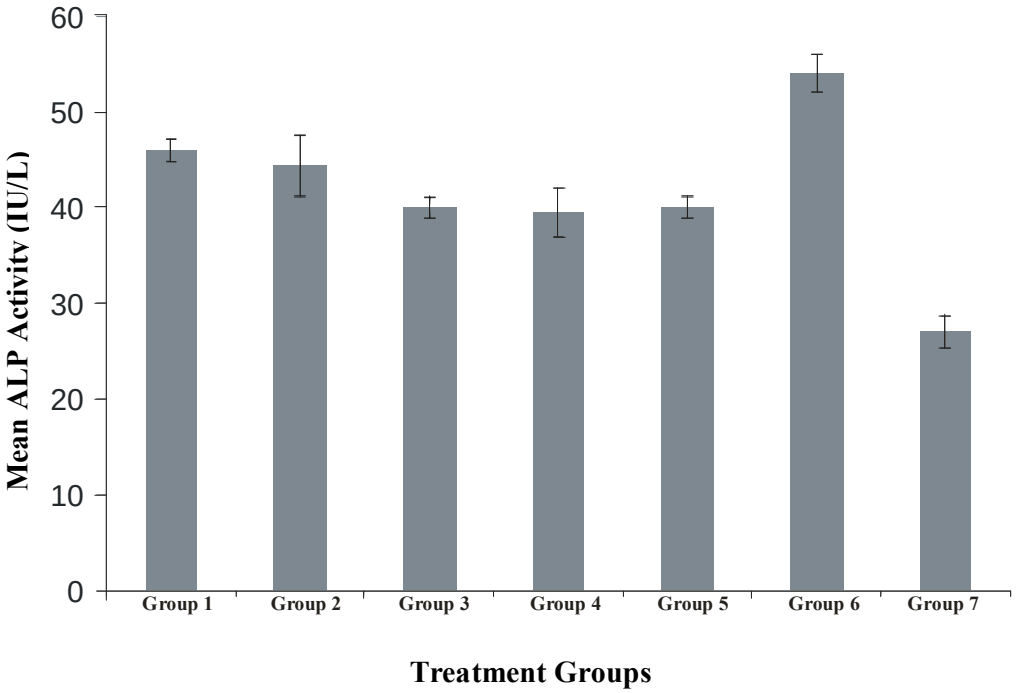


Fig.7: Effect of chloroformï methanol extract of *Tetracarpidium conophorum* seeds on serum alkaline phosphatase (ALP) activity of H_2O_2- induced rats

Group 1: Control (Normal Rats)
Group 2: Positive Control (hydrogen peroxide induced untreated rats)
Group 3: Hydrogen peroxide induce d + 100mg/kg body weight of vitamin C
Group 4: Hydrogen peroxide induced + 200mg/kg body weight of chloroform ï methanol seed extract.
Group 5: Hydrogen peroxide induced + 400mg/kg body weight of chloroform ï methanol seed extract.
Group 6: Hydrogen peroxide induced + 800mg/kg body weight of chloroform ï methanol seed extract.
Group 7: Non induced rats + 800mg/kg body weight of chloroform - methanol seed extract

3.8 Effect of Chloroform-Methanol Extract of *Tetracarpidium conophorum* Seeds on Aspartate aminotransferase (AST) Activity of Hydrogen Peroxide-Induced Wistar Albino Rats

Fig 8 shows non- significant decrease ($p>0.05$) in the serum AST activity of the rats in the different test groups(groups 4,5 and 6) representing hydrogen peroxide-induced rats treated with 200, 400 and 800 mg/kg body weight of extract respectively compared to group 2 representing hydrogen peroxide-induced untreated rats. Also a significant decrease ($p<0.05$) was observed in the serum AST activity of groups 4,5 and 6 when compared with normal rats of group 1.

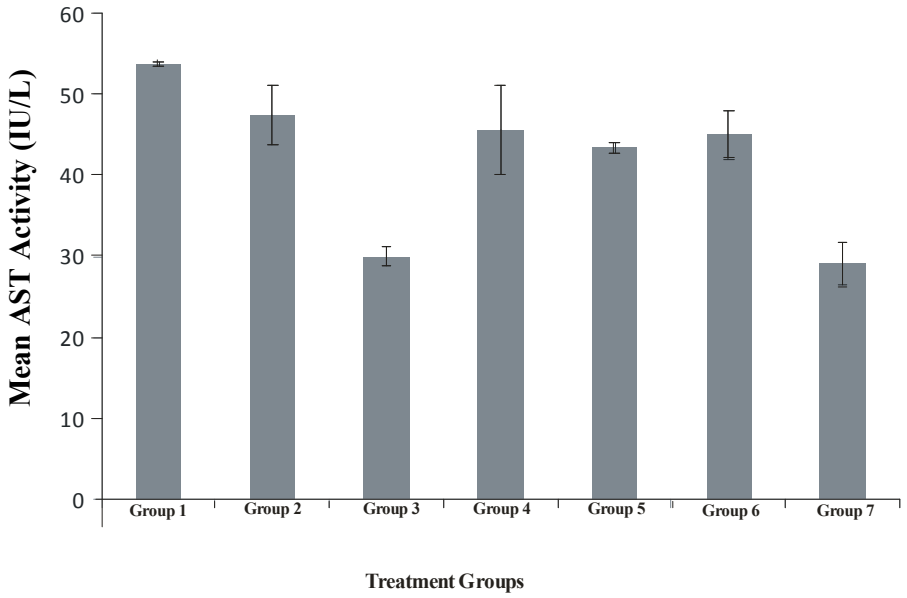


Fig.8: Effect of chloroform ï methanol extract of *Tetracarpidium conophorum* seeds on serum aspartate amino transferase activity of H_2O_2 -induced rats.

Group 1: Control (Normal Rats)
Group 2: Positive Control (hydrogen peroxide induced untreated rats)
Group 3: Hydrogen peroxide induce d + 100mg/kg body weight of vitamin C
Group 4: Hydrogen peroxide induced + 200mg/kg body weight of chloroform ï methanol seed extract.
Group 5: Hydrogen peroxide induced + 400mg/kg body weight of chloroform ï methanol seed extract.
Group 6: Hydrogen peroxide induced + 800mg/kg body weight of chloroform ï methanol seed extract.
Group 7: Non induced rats + 800mg/kg body weight of chloroform - methanol seed extract

3.9 Effect of Chloroform-Methanol Extract of *Tetracarpidium conophorum* Seeds on Serum Superoxide dismutase Activity (SOD) of Hydrogen-peroxide Induced Wistar Albino Rats

A significant increase ($p<0.05$) was observed in groups 4 and 5(hydrogen peroxide induced, treated with 200 and 400 mg/kg b.w respectively) when compared with groups 2 representing hydrogen peroxide-induced untreated rats. A significant decrease ($p<0.05$) was observed in Fig. 9 in the control groups: 1, 2 and 3 representing normal rats, hydrogen peroxide-induced untreated and hydrogen peroxide-induced and treated with 100 mg/kg body weight of vitamin C when compared with the test groups 4 and 7 representing hydrogen peroxide-induced and treated with 200 mg/kg body weight of *Tetracarpidium conophorum* seed extract and non-induced rats administered 800 mg/kg body weight of extract. On the contrary a significant increase ($p<0.05$) was observed on the SOD activity of groups 4 and 7 representing hydrogen peroxide-induced and treated with 200 mg/kg body weight of *Tetracarpidium conophorum* seed extract and non-induced rats administered 800 mg/kg body weight of extract when compared with other test groups 5 and 6 (induced, treated with 400 and 800mg/kg body weight of extract respectively).

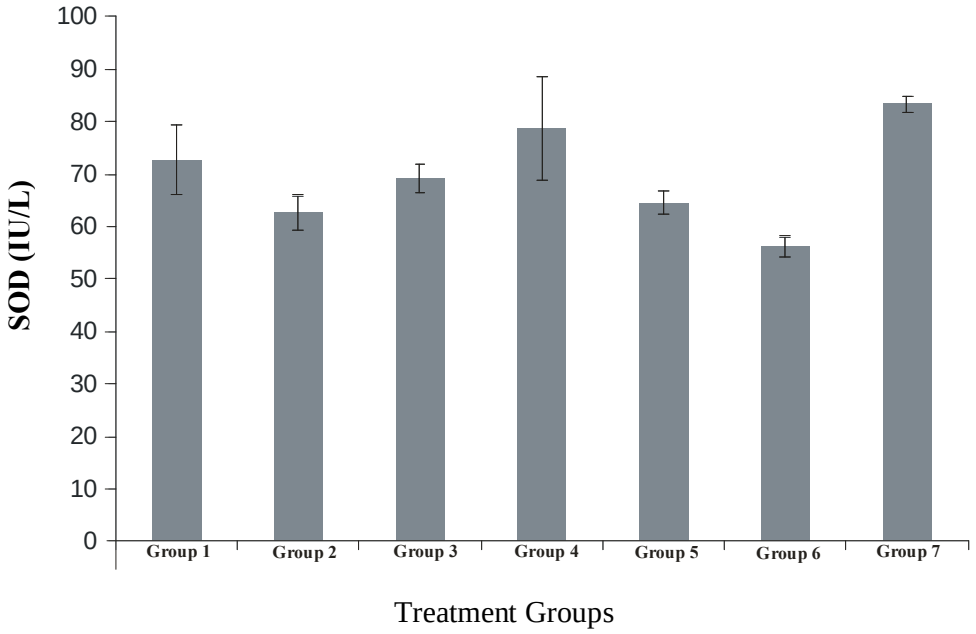


Fig.9: Effect of chloroformï methanol extract of *Tetracarpidium conophorum* seeds on serum superoxide dismutase (SOD) activity of H_2O_2-induced rats

Group 1: Control (Normal Rats)
Group 2: Positive Control (hydrogen peroxide induced untreated rats)
Group 3: Hydrogen peroxide induced + 100mg/kg body weight of vitamin C
Group 4: Hydrogen peroxide induced + 200mg/kg body weight of chloroform ï methanol seed extract.
Group 5: Hydrogen peroxide induced + 400mg/kg body weight of chloroform ï methanol seed extract.
Group 6: Hydrogen peroxide induced + 800mg/kg body weight of chloroform ï methanol seed extract.
Group 7: Non induced rats + 800mg/kg body weight of chloroform -methanol seed extract

3.10.1 Effect of Chloroform-Methanol Extract of *Tetracarpidium conophorum* Seeds on Serum Catalase Activity of Hydrogen peroxide-Induced Wistar Albino Rats

Fig. 10 shows a significant increase ($p<0.05$) in the serum catalase activity of group 6 representing hydrogen peroxide induced administered 800 mg/kg body weight seed extract of *Tetracarpidium conophorum* when compared with the group 2. The test groups of 5 and 6 are comparable to the normal rats in group 1.

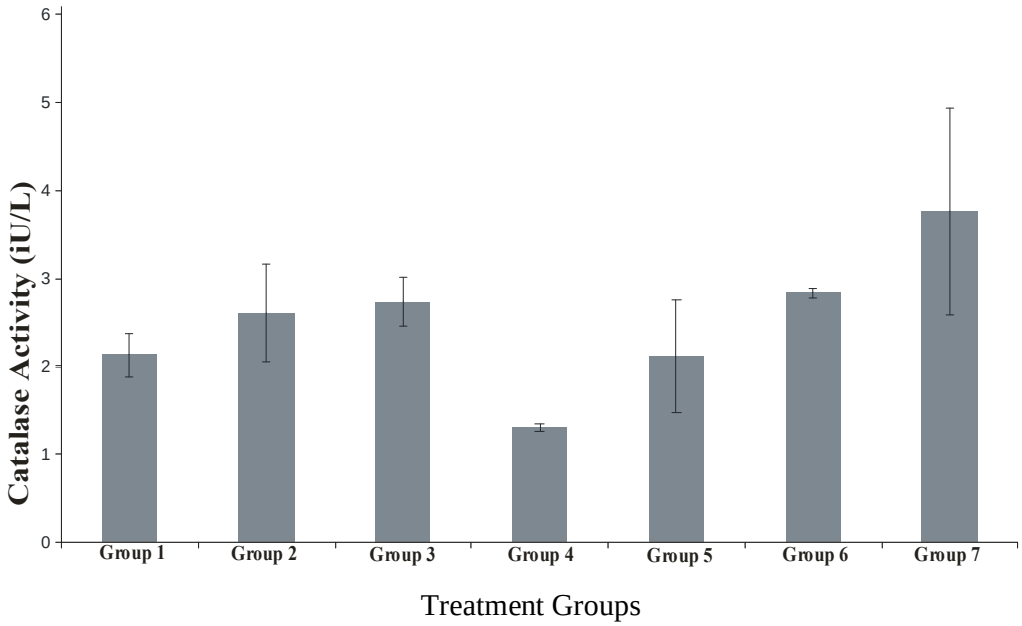


Fig.10: Effect of chloroformï methanol extract of *Tetracarpidium conophorum* seeds on serum catalase activity of H_2O_2- induced rats

Group 1: Control (Normal Rats)
Group 2: Positive Control (hydrogen peroxide induced untreated rats)
Group 3: Hydrogen peroxide induce d + 100mg/kg body weight of vitamin C
Group 4: Hydrogen peroxide induced + 200mg/kg body weight of chloroform ï methanol seed extract.
Group 5: Hydrogen peroxide induced + 400mg/kg body weight of chloroform ï methanol seed extract.
Group 6: Hydrogen peroxide induced + 800mg/kg body weight of chloroform ï methanol seed extract.
Group 7: Non induced rats + 800mg/kg body weight of chloroform ï methanol seed extract

3.11 Effect of Chloroform-Methanol Extract of *Tetracarpidium conophorum* Seeds on Serum Glutathione Peroxidase (GPx) Activity of Hydrogen Peroxide-Induced Wistar Albino Rats

A significant increase ($p<0.05$) was observed in the normal rats of group 1 when compared with the other controls 2 and 3 representing hydrogen peroxide-induced untreated rats and hydrogen peroxide-induced rats treated with 100 mg/kg body weight of vitamin C as seen in Fig. 11. A significant decrease ($p<0.05$) was observed in group 2 representing hydrogen peroxide-induced, untreated rats) when compared with the test groups. Group 5 representing hydrogen peroxide-induced and treated with 400mg/kg body weight extract showed a significant decrease ($p<0.05$) when compared with the other test groups.

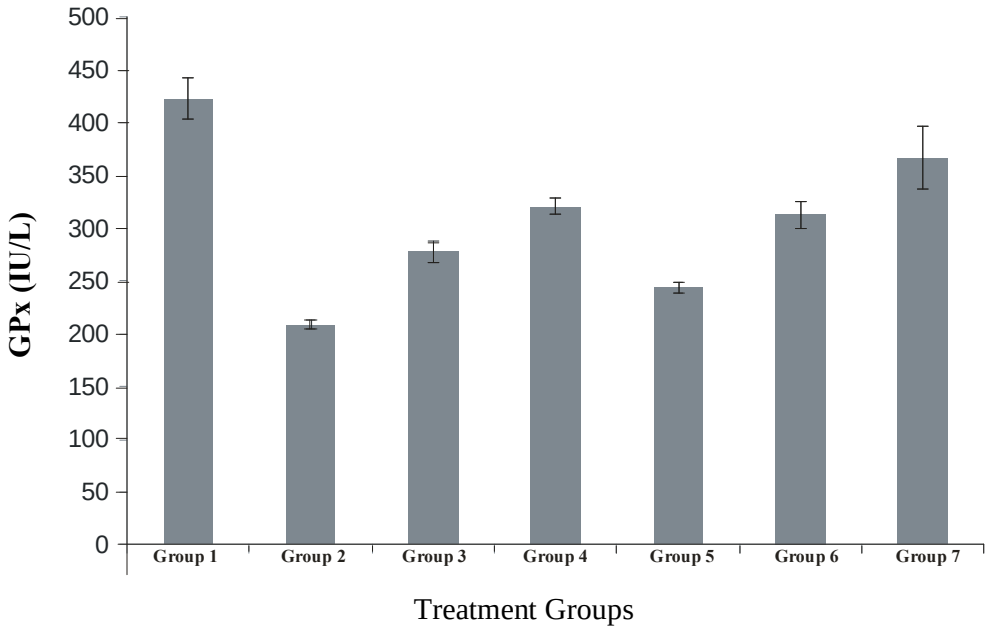


Fig 11: Effect of chloroformï methanol extract of *Tetracarpidium conophorum* seeds on serum Glutathione peroxidase (GPx) activity of H_2O_{2-} induced rats

Group 1: Control (Normal Rats)
Group 2: Positive Control (hydrogen peroxide induced untreated rats)
Group 3: Hydrogen peroxide induce d + 100mg/kg body weight of vitamin C
Group 4: Hydrogen peroxide induced + 200mg/kg body weight of chloroform ï methanol seed extract.
Group 5: Hydrogen peroxide induced + 400mg/kg body weight of chloroform ï methanol seed extract.
Group 6: Hydrogen peroxide induced + 800mg/kg body weight of chloroform ï methanol seed extract.
Group 7: Non induced rats + 800mg/kg body weight of chloroform - methanol seed extract

3.12 Effect of Chloroform- Methanol Extract of *Tetracarpidium conophorum* Seeds on Serum Malondialdehyde (MDA) Concentration of Hydrogen Peroxide-Induced Wistar Albino Rats

There was a non significant increase ($p>0.05$) in the serum MDA activity of rats in group 2 that is hydrogen peroxide- induced untreated rats compared to other controls groups 1 and 3 representing normal rats and hydrogen peroxide induced rats treated with 100 mg/kg body weight of vitamin C respectively as shown in Fig 12. A significant decrease ($p<0.05$) was observed in groups 4 and 7 representing hydrogen peroxide-induced treated with 200 mg/kg body weight of *Tetracarpidium conophorum* seed extract and non induced, treated with 800 mg/kg body weight of extract respectively when compared with group 2.

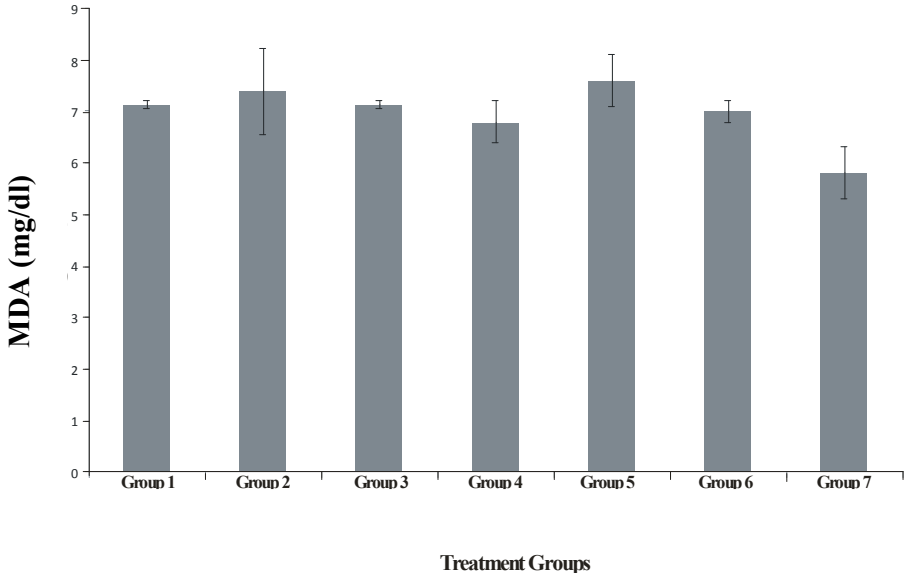


Fig.12: Effect of chloroform-methanol extract of *Tetracarpidium conophorum* seeds on serum malondialdehyde (MDA) concentration of H_2O_2-induced rats

Group 1: Control (Normal Rats)
Group 2: Positive Control (hydrogen peroxide induced untreated rats)
Group 3: Hydrogen peroxide induce d + 100mg/kg body weight of vitamin C
Group 4: Hydrogen peroxide induced + 200mg/kg body weight of chloroform ï methanol seed extract.
Group 5: Hydrogen peroxide induced + 400mg/kg body weight of chloroform ï methanol seed extract.
Group 6: Hydrogen peroxide induced + 800mg/kg body weight of chloroform ï methanol seed extract.
Group 7: Non induced rats + 800mg/kg body weight of chloroform - methanol seed extract

3.13 Effect of Chloroform-Methanol Extract of *Tetracarpidium conophorum* Seeds on Serum Cholesterol Concentration of Hydrogen Peroxide Induced Wistar Albino Rats

There was a significant increase ($p<0.05$) in serum cholesterol concentration of group 2 (induced and untreated rats) when compared to groups 1 and 3 normal rats, induced and treated with 100 mg/kg body weight of vitamin C. There was a significant decrease ($p<0.05$) in serum cholesterol concentration of rats in groups 4 and 7 that is hydrogen peroxide induced and treated with 200mg/kg body weight and non-induced and treated with 800mg/kg body weight respectively when compared with the group 2.

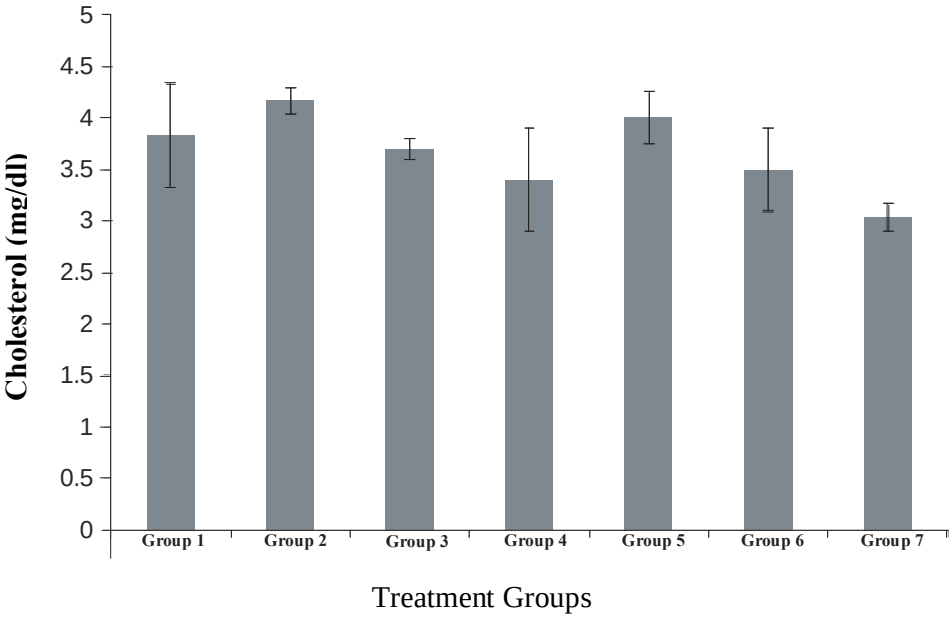


Fig.13: Effect of chloroformï methanol extract of *Tetracarpidium conophorum* seeds on serum cholesterol concentration of H_2O_2- induced rats

Group 1: Control (Normal Rats)
Group 2: Positive Control (hydrogen peroxide induced untreated rats)
Group 3: Hydrogen peroxide induce d + 100mg/kg body weight of vitamin C
Group 4: Hydrogen peroxide induced + 200mg/kg body weight of chloroform ï methanol seed extract.
Group 5: Hydrogen peroxide induced + 400mg/kg body weight of chloroform ï methanol seed extract.
Group 6: Hydrogen peroxide induced + 800mg/kg body weight of chloroform ï methanol seed extract.
Group 7: Non induced rats + 800mg/kg body weight of chloroform - methanol seed extract

3.14 Effect of Chloroform-Methanol Extract of *Tetracarpidium conophorum* Seeds on Serum Low Density Lipoproteins (LDL) Concentration of Hydrogen peroxide-Induced Wistar Albino Rats

Significant increase ($p<0.05$) was observed in the serum low density lipoprotein (LDL) concentration of group 2 rats that were induced with hydrogen peroxide but untreated compared with the LDL concentrations of rats in other control groups and test groups as shown in Fig.14. On the contrary trend, group 4 hydrogen peroxide- induced rats treated with 200mg/kg body weight of the extract showed significant decrease ($p<0.05$) in the LDL compared with that of the test groups.

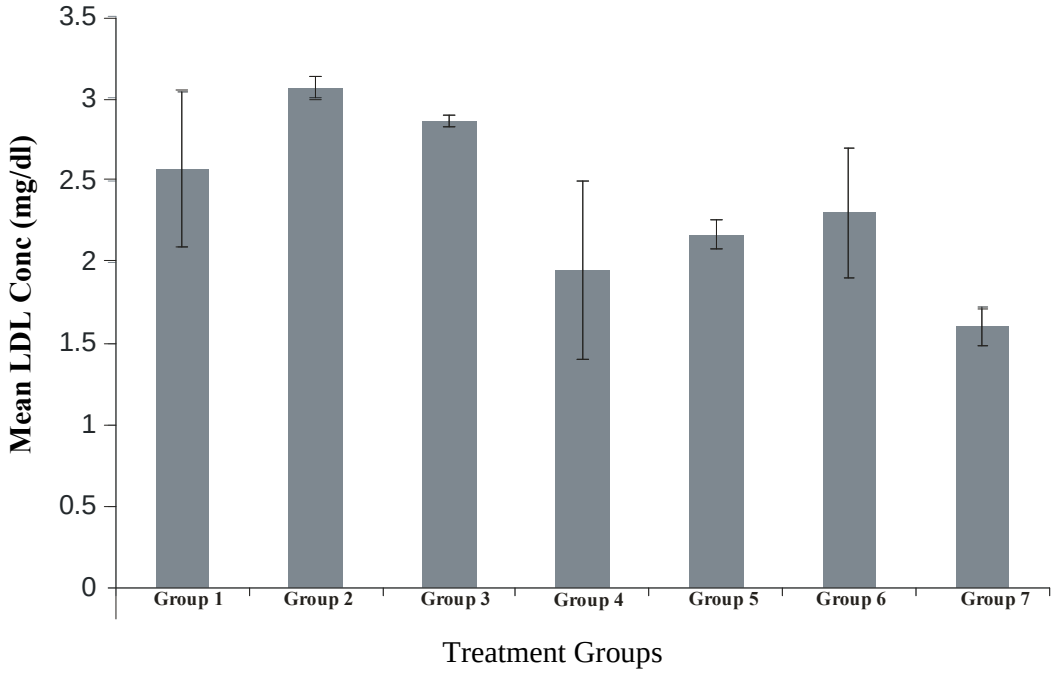


Fig.14: Effect of chloroformï methanol extract of *Tetracarpidium conophorum* seeds on serum Low density Lipoproteins (LDL) concentration of H_2O_{2-} induced rats

Group 1: Control (Normal Rats)
Group 2: Positive Control (hydrogen peroxide induced untreated rats)
Group 3: Hydrogen peroxide induce d + 100mg/kg body weight of vitamin C
Group 4: Hydrogen peroxide induced + 200mg/kg body weight of chloroform ï methanol seed extract.
Group 5: Hydrogen peroxide induced + 400mg/kg body weight of chloroform ï methanol seed extract.
Group 6: Hydrogen peroxide induced + 800mg/kg body weight of chloroform ï methanol seed extract.
Group 7: Non induced rats + 800mg/kg body weight of chloroform - methanol seed extract

3.15 Effect of Chloroform-Methanol Extract of *Tetracarpidium conophorum* Seeds on Serum Triacylglycerol (TAG) Concentration of Hydrogen peroxide-Induced Wistar Albino Rats

Group 2 which is hydrogen peroxide-induced untreated rats showed significant increase ($p<0.05$) in the serum TAG concentration when compared with groups 1 and 3 representing normal rats and hydrogen peroxide-induced, treated with 100mg/kg body weight vitamin C respectively as shown in Fig 15. There was a significant increase ($p<0.05$) in TAG concentration of rats in group 4 hydrogen peroxide-induced, treated with 200mg/kg body weight of extract when compared to all the controls and all other test groups.

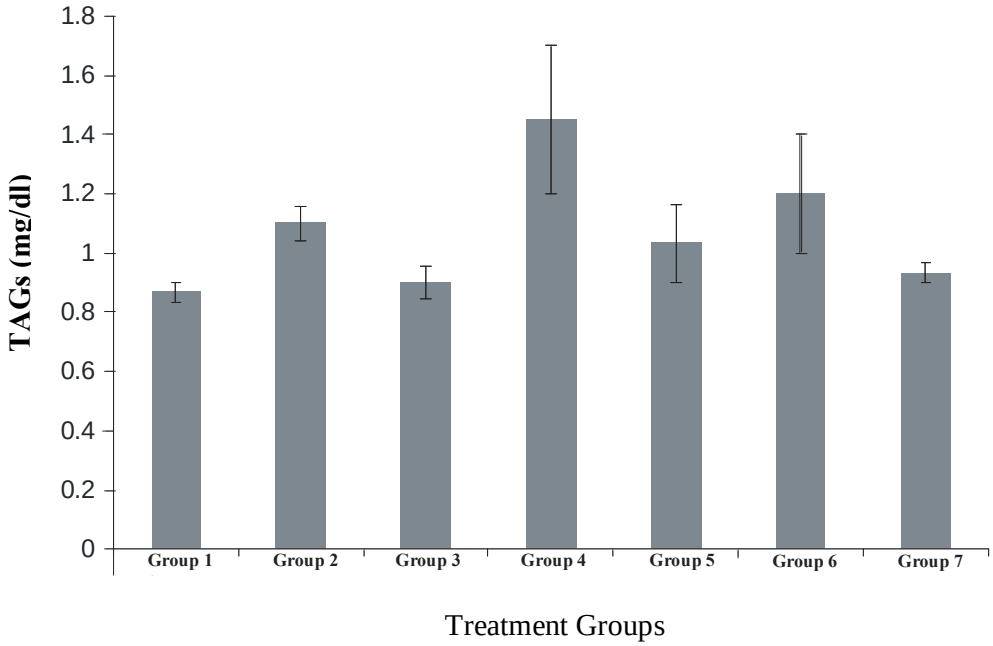


Fig.15: Effect of chloroformï methanol extract of *Tetracarpidium conophorum* seeds on serum triacylglycerol (TAG) concentration of H_2O_{2-} induced rats

Group 1: Control (Normal Rats)
Group 2: Positive Control (hydrogen peroxide induced untreated rats)
Group 3: Hydrogen peroxide induce d + 100mg/kg body weight of vitamin C
Group 4: Hydrogen peroxide induced + 200mg/kg body weight of chloroform ï methanol seed extract.
Group 5: Hydrogen peroxide induced + 400mg/kg body weight of chloroform ï methanol seed extract.
Group 6: Hydrogen peroxide induced + 800mg/kg body weight of chloroform ï methanol seed extract.
Group 7: Non induced rats + 800mg/kg body weight of chloroform - methanol seed extract

3.16 Effect of Chloroform- Methanol Extract of *Tetracarpidium conophorum* Seeds on Serum High Density Lipoprotein (HDL) Concentration of Hydrogen peroxide-Induced Wistar Albino Rats

There was a significant increases ($p<0.05$) in the serum HDL concentration of rats in group 3 which is hydrogen peroxide induced, treated with 100mg/kg body weight of vitamin C when compared with the normal rats in group 1 and hydrogen peroxide- induced untreated rats in group 2. There was a significant increase ($p<0.05$) in group 5 representing hydrogen peroxide-induced rats, treated with 400mg/kg body weight of the extract when compared to groups 4 and 6 representing hydrogen peroxide- induced treated with 200 and 800mg/kg body weight of extract respectively as shown in Fig 16.

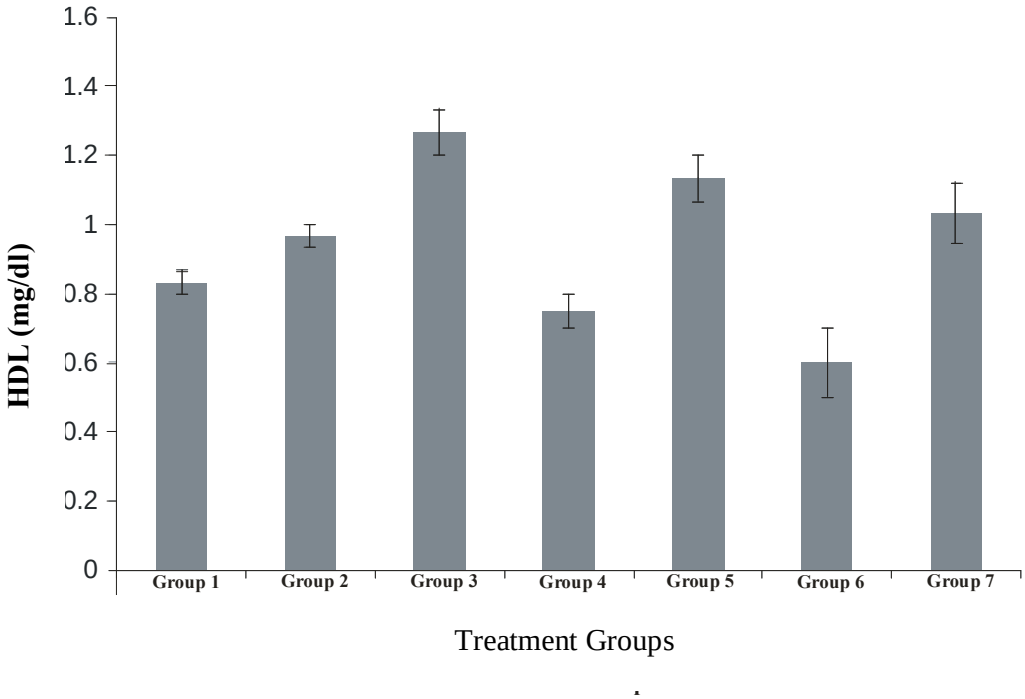


Fig.16: Effect of chloroformï methanol extract of *Tetracarpidium conophorum* seeds on serum High density Lipoproteins (HDL) concentration of H_2O_2- induced rats

Group 1: Control (Normal Rats)
Group 2: Positive Control (hydrogen peroxide induced untreated rats)
Group 3: Hydrogen peroxide induce d + 100mg/kg body weight of vitamin C
Group 4: Hydrogen peroxide induced + 200mg/kg body weight of chloroform ï methanol seed extract
Group 5: Hydrogen peroxide induced + 400mg/kg body weight of chloroform ï methanol seed extract.
Group 6: Hydrogen peroxide induced + 800mg/kg body weight of chloroform ï methanol seed extract.
Group 7: Non induced rats + 800mg/kg body weight of chloroform - methanol seed extract

CHAPTER FOUR

DISCUSSION

It is of utmost importance that we investigate the usefulness of walnut and plant foods grown in Nigeria for biologically useful natural compounds based on ethnomedical uses Ajaiyeoba (2002). Qualitative and quantitative phytochemical screenings of the seeds of *Tetracarpidium conophorum* were analyzed as well as their mineral and vitamin constituents. The qualitative phytochemical analysis of chloroform-methanol seed extract of *Tetracarpidium conophorum* showed the presence of terpenoids, tannins, alkaloids and cardiac glycosides while anthraquinones, saponins and steroids were not detected.

Reports show that the greatest sources of these phytochemicals are seeds, fruits and vegetables (Liu, 2004). The quantitative phytochemical analysis indicated the terpenoid content to be 4.36 ± 0.06 mg/100g, alkaloids; 20.31 ± 0.30 mg/ 100g, tannins; 1.89 ± 0.11 mg/ 100g, cardiac glycosides; 12.45 ± 0.08 mg/ 100g. The very high content of alkaloids in the seed extract may indicate its usefulness as a cardio preventive and potent *in vitro* antiproliferative activity (Negi *et al.,* 2011). Alkaloids are the most efficient plant substances used therapeutically, Pure isolated alkaloids and the synthetic derivatives are used as basic medicinal agents because of their analgesic, antispasmodic and antibacterial properties. This is why the seed is believed to stop asthma but not for acute asthma (Wikipedia, 2009). These results indicate that walnut seeds could be used as a potential source of drugs. Ayoola *et al*. (2011) found alkaloids and tannins to be present in the seed extract of *Tetracarpidium conophorum* while cardiac glycosides and terpenoids were absent. Ajaieyoba and Fadare (2006) reported the presence of alkaloids, saponins and tannins and absence of cardiac glycosides. The presence of alkaloids and tannins in all the above mentioned reports was in line with the result of this research and could be responsible for its use in the treatment of dysentery.

The vitamin and mineral content of *Tetracarpidium conophorum* seed extract were analyzed. The vitamin analysis showed that vitamin A was 10.55 ± 2.67 mg/ 100g, vitamin C; 13.09 ± 0.23 and vitamin E; 5.77 ± 0.08 mg / 100g. These vitamins mentioned above are useful antioxidants that help to protect the body from free radicals by scavenging them thus curbing the incidence of oxidative stress in humans and animals (Temple, 2000). The mineral content of the seed extract were found as follows: Magnesium (133. 59 ± 0.11) mg/ 100g, iron (3. 67 ± 0.07) mg/ 100g , copper (1. 54 ± 0.78) mg / 100g, zinc (2. 22 ± 0.01) mg/ 100g and calcium (118.90 ± 0.01) mg/100g. The presence of Copper may be responsible for the absorption of iron, it is therefore often seen with Iron naturally. Copper is important for

cellular protection of the mucous membrane, antianaemic and essential for the formation of haemoglobin from Iron (Claude and Paule, 1979). The presence of zinc is an indication that the seed has some effect on nerve function and male fertility, normal sexual development, especially for the development of testes and ovaries, RBC, WBC, healthy heart and normal growth (Elizabeth, 1994).

The result of the acute toxicity (LD_{50}) test of the chloroform methanol extract of *Tetracarpidium conophorum* showed no toxicity on mice even when the concentration of the extract was increased to 5000mg/kg body weight. This is an indication that the cooked seeds are safe for human and livestock consumption. This agreed with the findings of (Oke, 1995) who reported that the plant is cultivated principally for the nuts which are cooked and consumed as snacks.

There was a significant increase ($p < 0.05$) in the serum ALT activity of rats in group 2 which were hydrogen peroxide-induced untreated rats when compared with other controls and test groups when compared with group 2 as shown in Fig. 6, this may indicate liver damage, muscle injury and hepatic necrosis (Roper, 1987). The marked decrease in serum ALT activity of the test groups more especially group 5 (Hydrogen peroxide-induced, treated with 800 mg/kg body weight of *T. conophorum* extract) may indicate the ability of the extract to curb hepatotoxicity. Also the serum ALP activity followed the same trend except that groups 1and 6 representing normal and hydrogen peroxide-induced treated rats with 800 mg/kg body weight of extract respectively where the serum ALP activity was high. Alkaline phosphatase is not specific to the liver, an increase could also be seen during period of active bone formation. Therefore, an increase in its activity in groups 1 and 6 does not necessarily indicate hepatotoxicity as seen in Fig. 7. The serum AST activity from figure 8 showed that there was a non- significant increase ($p>0.05$) in groups 4,5 and 6 representing hydrogen peroxide-induced rats treated with 200, 400 and 800mg/kg body weight of extract respectively compared to group 2 representing hydrogen peroxide-induced untreated rats. A number of scientific reports indicated that certain flavonoids, terpenoids and steroids have protective effects on the liver due to their antioxidant properties (Jeruto *et al*., 2011).

A significant decrease ($p<0.05$) was observed in groups 4 and 7 representing hydrogen peroxide-induced treated with 200 mg/kg body weight of *Tetracarpidium conophorum* seed extract and non induced, treated with 800 mg/kg body weight of extract respectively when compared with the other test groups as well as control groups. This increased serum MDA activity in group 2 as shown in Fig. 12 was in line with the works of Ganie *et al*. (2011) who reported that administration of hydrogen peroxide (0.1%) in drinking

water of the rats for 25 weeks increased the malondialdehyde levels, but in this present study there was a decrease in the MDA levels of those treated with 200mg/kg body weight. The decreased lipid peroxidation in animals of group 4 agrees with the fact that this extract was efficient in the management of oxidative stress.

In the serum levels of glutathione peroxidase a significant increase ($p < 0.05$) was observed in all the test groups compared to group 2. The selenium dependent GPx removes both H_2O_2 and lipid peroxides by catalyzing the conversion of lipid hydroperoxide to hydroxyl acids in the presence of GSH. The serum catalase activity showed that there is a dose-dependent increase in CAT activity from groups 4 to 6. The SOD activity showed that there is significant increase ($p < 0.05$) between groups 4 and 7 when compared to the control groups and at a dose of 200 mg/ kg body weight the SOD activity is optimal.

There is a significant decrease ($p < 0.05$) in the serum cholesterol and LDL, of the test groups relative to those of the controls, this indicated that the seed extract of *Tetracarpidium conophorum* have a cholesterol lowering ability. This is in agreement with the work of Banel and Hu (2009) which reported that high walnut enriched diets significantly decrease total and LDL Cholesterol for the duration of short term trials. LDL is mostly referred to as bad Cholesterol and have been associated with cardiovascular Diseases (CVD) such as heart attack, arther osclerosis and ischemic heart disease etc. A reduction in the serum total cholesterol level of the animals in all the test groups except group 5 may be explained by impaired or diminished beta oxidation of fatty acids (Rang *et al.,* 1995). It may also be associated with low incorporation into chylomicrons, VLDL-C and LDL (Smith *et al.,* 2000) and because studies have revealed that polyunsaturated fatty acids (PUFA) decreased total cholesterol levels in animals (Grundy and Denke, 1990; Diniz *et al*., 2004).

A significant increase ($p < 0.05$) was noticed in the serum TAGs of the test groups except in group 4 and 7 which showed a significant decrease compared with the controls. Triacylglycerol measurements are used in the diagnosis and treatment of diseases involving lipid metabolism and various endocrine disorders e.g. diabetes mellitus, nephrosis and liver obstruction.Studies have revealed that cardiovascular diseased patients had markedly elevated levels of TAGs and reduced HDL-C (Miller *et.al*.,1981) but in group 5 there was a decrease in TAGs concentration which is favourable. The HDL levels of the groups 5 and 7 were significant ($p < 0.05$) compared to the controls 1 and 2, but a dose of 400 mg/kg body weight became optimal for serum HDL levels. HDL is an anti-atherogenic lipoprotein, it does this by transporting Cholesterol from peripheral tissues into the liver where they are detoxified thereby acting as a protection from cardiovascular Diseases. Cholesterol measurements are

used in the diagnosis and treatments of lipid lipoprotein measurement disorders. Lipids play an important role in the body; they serve as hormones or hormone precursor, as in digestion, provide energy, storage and metabolic fuels, acts as functional and structural components in biomembranes and form insulation to allow nerve conduction and prevent heat loss.

4.2 Conclusion

It is very important to investigate the usefulness of plants and plant foods grown in Nigeria for anti disease agents and for biological useful natural compounds based on ethnomedical uses (Ajaiyeoba, 2002). The result of this study shows that the chloroform-methanol extract of the seeds of *Tetracarpidium conophorum* may serve as an ameliorative or protective agent on oxidative stress which exposes the body to high risk of diseases than ever before because at a dose of 200mg/kg body weight of the rats, most antioxidant enzymes were optimal (superoxide dismutase and glutathione peroxidase). Malondialdehyde was also reduced at 200 mg/kg body weight. These antioxidant enzymes may have worked synergistically with the various antioxidant vitamins and minerals together with the phytochemicals present in them in a dose dependent manner. This gives scientific evidence to the claims in different part of the country and beyond that the plant is used in the treatment and prevention of various ailments. This also implies that the extract has hepatoprotective properties by restoring the hepatic marker enzymes and reversing the oxidant/ antioxidant imbalance that is characteristic of oxidative stress.

4.3 Suggestion for Further Studies

- The seed should be used on anaemic rats to ascertain the use as a haematinic agent.
- Different solvents may be used to extract the seeds to know the effect on hydrogen peroxide-induced oxidative stress rats.
- The seed extract posed no toxic effect on the liver during the short period of treatment, prolonged treatment of the seed extract is then suggested. This is to ascertain its prolonged effect on the liver of rats.
- Further studies are required to evaluate the effect of *Tetracarpidium conophorum* on the functional indices of rat organs and histopathological examination**.**

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