ANTIOXIDANT AND HEPATOPROTECTIVE STUDIES OF *ANNONASENEGALENSIS*PERS. (ANNONACEAE) STEM BARK.

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JULY, 2015.

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A PROJECT PRESENTED IN PARTIAL FULFILMENT OF THE REQUIREMENT FOR THE AWARD OF THE MASTERS DEGREE OF PHARMARCY (M. PHARM)

UNIVERSITY OF NIGERIA, NSUKKA.

PROJECT SUPERVISOR: PROF. C. O. EZUGWU.

TITLE PAGE

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APPROVAL PAGE

This project titled **"Antioxidant and Hepatoprotective Studies of *Annonasenegalensis* Pers. (Annonaceae) Stem Bark"** is hereby certified as having met the requirement for the award of Masters of Pharmacy (M. Pharm) degree in the Department of Pharmacognosy and Environmental Medicine, Faculty of Pharmaceutical Sciences, University of Nigeria, Nsukka.

……..	**…….**
Prof. C. O. Ezugwu	**Prof. C. O. Ezugwu**
Supervisor	**Head of department**

……..

External Examiner

DEDICATION

I dedicate this work to my lovely Ifeyinwa, Chi, Kosi, Ify, Oluebube, and Chelu, and also to the evergreen Memory of my late father Omeke Joseph who died before I could express my appreciation for the role he played in my life. May his soul rest in ñthe bosom of Abrahamò (ef. Lk: 16:22)

AKNOWLEDGEMENT

The completion of this work was made feasible through the instrumentality of a good number of people. My first gratitude goes to God almighty whose priceless gift of life and wisdom sustained this debut. My immense gratitude goes to my supervisor Prof. C. O. Ezugwu for his good supervision, encouragement and advice. I also remain grateful to Dr. Michel Tchimene whose hardworking abilities have inspired me a lot.

My appreciation goes to the following friends who offered me a helping hand in one way or the other towards the success of this work. They include Rev. Fr. JB Okechukwu, Pharm (Dr.) Mrs. Collete Elechi, Pharm Madu, Pharm Emeka Ezea, Dr. Ugwoke, Okorie Austine, Mrs. Ezugwu J.C, Obasi Linus, Obasi Simon, Pharm Tobi Sode, Ogochukwu Eze,Pharm. Oluchi, and others.

My special thanks go to my beloved wife Mrs. Omeke Ifeyinwa Emmanuela and my children Chi, Kosi, Ify, Oluebube, and Cheluchi as well as my elder and younger ones Caroline, Giovanni, Fes, Pauly, Glo, Gera for their constant prayers, support, patience and encouragement throughout the period of my M.Pharm program. I am highly indebted to you all.

Pharm Omeke C. P. E.

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Abstract

The study was carried out to determine pharmacognostic standards, phytochemical constituents, evaluate the antioxidant and hepatoprotective activities of the extract and fractions of *Annona senegalensis* Pers. (Annonaceae) by employing both the *in vitro* and *in vivo* experimental models. The acute toxicity tests result showed the drug is safe at 5000mg/kg doses.

The effect of DPPH free radical scavenging, ABTS radical scavenging, Hydroxyl radical scavenging, Hydrogen peroxide scavenging, lipid peroxidation assay, assay of catalase, superoxide Dismutase assay, total protein, ɓ-carotene bleaching, FRAP scavenging, liver and superoxide anion radical scavenging activities were evaluated. Hepatoprotective effects of the extract was evaluated against CCl_4 induce liver damage. Carbon tetrachloride (CCl_4) induce hepatotoxicity was evaluated by significant increase ($p < 0.05$) in serum AST, ALT, ALP activity and bilirubin level accompanied by significant decrease ($p > 0.05$) lipid peroxidation, and catalase activity in liver tissue. All these parameters were also evaluated using the nï hexane, ethyl acetate, methanol fractions.

The results showed that the extract/fractions of stem bark of *A. senegalensis* had better antioxidant activities at high concentrations when compared to the standard. Co-administration of the extract/fractions (400mg/Kg) protects the CCl_4 ï induced lipid peroxidation, restored altered serum elevated enzymes. It showed that it is dose dependent.

The results obtained in the present study indicate that the stem bark is a potential source of natural antioxidants.

CHAPTER ONE

1.0 INTRODUCTION

There is amazing abundance of plant life in rain forest and nature has blessed the mankind with a treasure of herbal remedy secrets that offer new approaches to health and healing. It is quite interesting to discover that different herbs can be indicated for a vast number of health problems. Therefore, a lot of scientific screening and research have been going on into investigating the various constituents of plants that are responsible for a particular activity or more, despite all odds. Many drugs have been discovered by the exploitation of traditional medicine since the early dates of human existence (Nwaogu, 1997). Plants have invariably been a rich source for new drugs and some antioxidant drugs in use today were either obtained from plants or developed using their chemical structures as templates(Nwaogu, 1997).

Currently, there is an increasing awareness of the value of traditional medicine and the necessity for improving its standard. Indeed, the Organization of African Unity (O.AU) has in the last few years, held lots of international symposia and these were on a particular aspect of a subject, notably medicinal plants. It has been observed that many plants contain a variety of phytochemical substances, which have appreciable physiological and pharmacological actions on man and animals. Researches on natural products over the years have revealed enormous potentials of plants as source of medicinal agents. Plants are no longer being cultivated for food alone, but also as sources of drugs.

Herbal medicine, which is the oldest form of healthcare known to mankind, involves the use of herbs (medicinal plants) for therapeutic or medicinal purposes. Herbal medicine can be broadly classified into various systems: traditional Chinese herbalism, which is part of the traditional

oriental medicine; Ayurveda herbalism, which is derived from Ayurveda; and Western herbalism, which originally came from Greece and Rome to Europe and then spread to North and South America.

The medicinal plants which may be leaves, stems, roots, flowers, seeds, fruits or whole plant or any combination of these parts are prepared in various forms for therapeutic purposes. From a scientific approach, most of the preparations are considered unscientific since they are not pharmacologically authenticated or standardized and are seen as unrefined.

Many plants have varied pharmacological effects which have been confirmed. Extracts of *Digitalis* spp, *Colchicumautomnale, Catharanthusroseus* and *Peyotecactus* had cardio ï active, anti ï inflammatory, anti ï neoplastic and central nervous system actions respectively. It is already estimated that 122 drugs from 94 plants species have been discovered throughethno botanical leads. Plants commonly used in traditional medicines assumed to be safe due to their long usage in the treatment of disease according to knowledge accumulated over centuries. However, recent scientific findings had shown that many plants used as food or in traditional medicine are potentially toxic, mutagenic and carcinogenic (Schimmer *et al*., 1994).

Cancer chemoprevention by using antioxidant approaches has been suggested to offer a good potential in providing important fundamental benefits to public health, and is now considered by many clinicians and researchers as a key strategy for inhibiting, delaying, or even reversal of the process of carcinogenesis. The cancer chemopreventive activities of naturally occurring phytocompounds are of great interest.

Liver diseases such as jaundice, cirrhosis and fatty liver diseases are very common and large public health problem in the world. Jaundice and hepatitis are two major hepatic disorders that

account for a high death rate. There is no rational therapy available for treating liver disorders and management of liver diseases is still a challenge to the modern medicine. The modern medicines have little to offer for alleviation of hepatic ailments whereas most important representatives are of phytoconstituents .The traditional system of medicine like Ayurveda and Siddha system of medicine, Unani system, Chinese system of medicine, Kampoo (Japanese) system of medicine have a major role in the treatment of liver ailments.

Some medicinal plants are used in treatment of hepatobiliary pathologies. Many Nigerian ethno botanic traditions propose a rich repertory of medicinal plants used by the population for treatment of liver diseases. However, there were not enough scientific investigations on the hepatoprotective activities conferred to these plants. One of such plant from Nigerian flora is *Annona senegalensis*Pers.It is believed in folkore that the fruit obtained from this multipurpose plant is widely used locally in the treatment of two commonly energy deficiency syndrome known as kwashiorkor and marasmus. Dalziel, (1995) made report about the plant to be of great medicinal value and its used in native medicine to treat headache and body ache, eyelid swelling. The stem bark of *A. senegalensis* is used by local populations all over Africa in treating guinea worms, diarrhea and especially in northern Nigeria, gastroenteritis, snake bites, toothache, respiratory infections and malaria. Awa and colleagues (2012) reported the use of leaves in the treatment of pneumonia, and as a stimulant to improve health. A decoction from the roots is used to stop chest colds, venereal diseases, stomach ache and dizziness (Jiofack. *et al.,* 2010).

Many indigenous herbal plants of regional interest have been used popularly as folk medicines in Nigeria or other African countries; however, their bioactivities or pharmacological effects are to be investigated.

1.1 PLANT PHARMACOGNOSTIC PROFILE

1.1.1 TAXONOMY

Kingdom: Plantae

Division: Magnoliophyta

Class: Magnoliopsida

Order: Magnoliales

Family: Annonaceae

Genus: *Annona*

Species*:* *senegalensis*

Authority: Pers

Fig 1: ***A. senegalensis***

1.2.0 BOTANICAL DESCRIPTION

Annona senegalensis is a shrub or small tree 2 ï 6m tall but may reach 11m under favorable conditions, It has a bark smooth to roughish, silvery-grey or grey-brown, with leaf scars and roughly circular flakes exposing paler patches of under bark. Young branches with dense, brown, yellow or grey hairs that are lost later. The leaves are alternate, simple, oblong, ovate or elliptic, 6 ï 18.5cm x 2.5 ï 11.5cm, green to bluish green, almost without hairs on top, but after with brownish hairs or underside. They have net veination which may be green or reddish on both surfaces. The apex is round or slightly notched with base square to slightly lobed base. The margined is entire; petiole short, 0.5 ï 2.5cm thick set (Ketende et al. 1995). Flowers up to 5cm in diameter, on stalk, 2cm long, solitary or in groups of 2 ï 4, arising above the leaf axils; 6 fleshly cream to yellow petals in 2 whorls, greenish outside, creamy or crimson, 0.8 ï 1.5cm x 0.9 ï 1.1cm, glabrous or minutely papillose within; 3 in number, free, smaller than the petals, 3-4x4 - 5cm; stamens 1.7 - 2.5mm long. Fruits formed from many fused compels, fleshy, lumpy, egg shaped, 2.5 - 5x2.5 - 4cm, ovoid or globose; unripe fruit green turning yellow to orange or ripening stalk 1.5 ï 5cm long; seeds numerous, cylindrical, oblong, orange brown. The genus name, ñ*Annona*ò, is from the Latin word ñanonò, meaning ñyearly produceò, referring to the production habits of fruits of the various species in the genus. The specific name means ñof Senegalò, which is where the type specimen was collected (Beentje, 1994).

1.2.1 REPORTED ACTIVE CONSTITUENTS

A. senegalensis has been shown to contain a lot of constituents which are responsible for its various pharmacological properties. These secondary metabolites which include; alkaloids (-); roemerine, an aporphine), tannins, flavonoids, resins, glycosides, carbohydrates and saponins.Others constituents reported include aliphatic ketones, alkanes, fatty acids, and sterols from the leaves, monoterpenoids and sesquiterpenoids from the essential oil of the leaves and fruits, amino acids from the stem bark; and ent-kaurenoids from the root back (Silva,*et al,* 1995).

1.2.2 ETHNOMEDICINAL USES OF *A. SENEGALENSIS*

Several plant parts of A. senegalensis are used in traditional medicine in various countries of tropical Africa for the treatment of many diseases and symptoms including: cancer, convulsions, diarrhea, dysentery, Malaria fever and filariasis, male impotency, pain of the chest and intestines, inflammations, trypanosomiasis, venereal diseases and snake bite. Root extracts of *A. senegalensis* have been found to exhibit antineoplastic activity in mice bearing sarcoma 180 ascites tumor cell, and antiprotozoal activity in mice infected with *Trypanosomabrucei* (Silva,1995).

. The leaves are sometimes used as vegetables, while the edible white pulp of the ripe fruit has a pleasant, pineapple like taste (FAO, 1983). An effective insecticide is obtained from the bark. The bark is used for treating guinea worms and other worms, gastroenteritis, toothache and respiratory infections. Gum from the bark is used in sealing cuts and wounds. The leaves are used for treating pneumonia and as a tonic to promote general wellbeing. Roots are used for stomach-ache, chest colds and dizziness. Various plant parts are combined for treating dermatological diseases and ophthalmic disorders. In South Africa, roots are said to cure

madness, and in Mozambique, they are fed to small children to induce them to forget the breast and thus hasten weaning. It has also been claimed that leaves picked on a Thursday morning and thrown over the right shoulder brings good luck (Anon 1986).

1.3.0 GENERAL REVIEW OF ANTIOXIDANT AND HEPATOPROTECTIVE STUDIES

1.3.1 OXIDATIVE STRESS (OS)

Oxidative stress (OS) is a general term used to describe the steady state level of oxidative damage in a cell, tissue, or organ, caused by the reactive oxygen species (ROS). This damage can affect a specific organ or the entire organism. ROS such as free radicals and peroxides, represent a class of molecules that are derived from the metabolism of oxygen and exist inherently in all aerobic organisms.

OS is caused by an imbalance between the production of reactive oxygen species and detoxifier (antioxidants). All forms of life in normal state maintain an equilibrium redox reaction. Distortion of this normal redox state can cause toxic effects through the production of peroxides and free radicals that can damage components of the cell, including proteins, lipids, and DNA (Aroma 1993).

The level of oxidative stress is determined by the imbalance between the rate at which oxidative damage is induced and the rate at which it is efficiently repaired and removed. The rate at which damage is caused is determined by how fast the reactive oxygen species are generated and then inactivated by endogenous defense agents called antioxidants. The rate at which damage is removed is dependent on the level of repair enzymes. The determinants of oxidative stress are regulated by an individualôs unique heredity factors, as well as his/her environment and characteristic lifestyle. Unfortunately, under the present day life-style conditions many people

run an abnormally high level of oxidative stress that could increase their probability of early incidence of decline in optimum body functions (Aroma 1993).

In humans, oxidative stress is involved in many diseases, such as atherosclerosis, Parkinsonôs disease and Alzheimerôs disease and it may also be important in ageing. However, reactive oxygen species can be beneficial, as they are used by the immune system as a way to attack and kill pathogens and as a form of cell signaling (Rice-Evans, et al., 1995).

In chemical terms, oxidative stress is a large increase in the cellular reduction potential, or a large decrease in the reducing capacity of the cellular redox couples, such as glutathione. 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 (Seis, 1997).

A particularly destructive aspect of oxidative stress is the production of reactive oxygen species, which include free radicals and peroxides. Some of the less reactive of these species (such as superoxide) can be converted by redox reactions with transition metals or other redox cycling compounds including Quinone into more aggressive radical species that can cause extensive cellular damage (Valko, et al., 2005). Most of these oxygen- derived species are produced at a low level by normal aerobic metabolism and the damage they cause 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).

1.3.2 REACTIVE OXYGEN SPECIES (ROS)

Reactive oxygen species are chemical species which are responsible for toxic effects in the body through various tissue damages. They are formed either by the loss of a single electron from a non-radical or by the gain of a single electron by a non-radical.

Examples of ROS are listed in table 1

Table 1: Description of oxidants

Oxidant	Description
$^{\cdot}O_2^-$ superoxide anion	One-electron reduction state of O_2 , formed in many autoxidation reactions and by the electron transport chain. Rather unreactive but can release Fe^{2+} from iron-sulphur proteins and ferritin. Undergoes dismutation to form H_2O_2 spontaneously or by enzymatic catalysis and is a precursor for metal-catalyzed $^{\cdot}OH$ formation.
H_2O_2, hydrogen peroxide	Two-electron reduction state, formed by dismutation of $^{\cdot}O_2^-$ or by direct reduction of O_2. Lipid soluble and thus able to diffuse across membranes.
$^{\cdot}OH$, Hydroxyl radical	Three-electron reduction state formed by Fenton reaction and decomposition of peroxynitrite. Extremely reactive, will attack most cellular components.
ROOH, organic hydro peroxide	Formed by radical reactions with cellular components such as lipids and nucleobases.
$RO^{\cdot}$, alkoxy and $ROO^{\cdot}$, Peroxy radicals.	Oxygen centered organic radicals. Lipid forms precipitates in lipid peroxidation reactions. Produced in the presence of oxygen by radical addition to double bonds or hydrogen abstraction.
HOCL, hypochlorous acid.	Formed from H_2O_2 by myeloperoxidase. Lipid soluble and highly reactive. Will readily oxidize protein constituents, including thiol groups, amino groups and methionine.
$OONO^-$, peroxynitrite	Formed in a rapid reaction between $^{\cdot}O_2^-$ and $NO^{\cdot}$ Lipid soluble and similar in reactivity to hypochlorous acid. Protonation forms peroxynitrous acid, which can undergo hemolytic cleavage to form hydroxyl radical and nitrogen dioxide.

The most important source of reactive oxygen species under normal conditions in aerobic organisms is probably the leakage of activated oxygen from mitochondria during normal oxidative respiration. Other enzymes capable of producing superoxide are xanthine oxidase, reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidases and cytochromes

P450. Hydrogen peroxide is produced by a wide variety of enzymes including monoxygenases and oxidases. Reactive oxygen species play important roles in cell signaling, a process termed redox signaling. Thus, to maintain proper cellular homeostasis, a balance must be struck between reactive oxygen production and consumption (Aroma 1993).

Cell damage is induced by reactive oxygen species (ROS). ROS are either free radicals, reactive anions containing oxygen atoms, or molecules containing oxygen atoms that can either produce free radicals or are chemically activated by them. Under normal conditions, ROS are cleared from the cell by the action of superoxide dismutase (SOD), catalase, or glutathione (GSH) peroxide. The main damage to cells results from the ROS-induced alteration of macromolecules such as polyunsaturated fatty acids in membrane lipids, essential proteins, and DNA (Nishikimi, *et al* 1972).

Exogenous sources of ROS include exposure to cigarette smoke, environmental pollutants such as emission from automobiles and industries, consumption of alcohol in excess, asbestos, exposure to ionizing radiation, and bacteria, fungi or viral infections.

Poor nutrition in general contributes to OS. When the body is fed poorly, it slowly starves and all of its systems suffer. Weak organ systems are prime targets for free radical attack.

Even psychological and emotional stress can contribute to OS. When the body is under stress, it produces certain hormones that generate free radicals. Moreover, the liver must eventually detoxify them and that process also generates free radicals.

Heightened OS has also been observed in athletes after intensive workouts due to the physical stress placed on the body. Both physical and emotional stress also prompts the release of

endogenous cortisol, an adrenal hormone that reduces inflammation, but also suppresses the immune system (Seis, 1997).

Endurance exercise can increase oxygen utilization from 10 to 20 times over the resting state. This greatly increases the generation of free radicals, prompting concern about enhanced damage to muscles and other tissues (Rice ï Evans *et al* 1995).

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 metal ion or metal. 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 (www.en.wikipedia.org/oxidative-stress). 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 oxidize 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 can significantly increase the level of oxidative stress (Valko, *et al*2005).

Certain organic compounds in addition to metal redox catalyst can also produce reactive oxygen species. One of the most important classes of these is the quinones. Quinones can redox cycle with their conjugate semiquinones and hydroquinones, in some cases catalyzing the production of superoxide from dioxide or hydrogen peroxide from superoxide (Valko *et al*2005).

1.3.3 LIPID PEROXIDATION AND FREE RADICALS

Lipid peroxidation refers to the oxidation degradation of lipids. It is the process whereby free radicals ñstealò electrons from the lipids in cell membranes, resulting in cell damage. Lipid

hydroperoxides are non-radical intermediates derived from unsaturated fatty acids, phospholipids, glycolipids, cholesterol esters and cholesterol itself. Their formation occurs in enzymatic or non ï enzymatic reactions involving activated chemical species known as ñreactive oxygen speciesò (ROS) which are responsible for toxic effects in the body via various tissue damages. They are formed either by the loss of a single electron from a non-radical or by the gain of a single electron by a non-radical. They can easily be formed when a covalent bond is broken if one electron from each of the pair shared remains with each atom, this mechanism is known as hemolytic fission. In water, this process generates the most reactive species, hydroxyl radicals OH. Chemists know well that combustion which is able at high temperature to rupture C ï C, C ï H or C ï O bonds is a free radical process. The opposite of this mechanism is the heterolytic fission in which, after a covalent break, one atom receives both electrons (this gives a negative charge) while the other remains with a positive charge. This process proceeds by a free radical chain reaction mechanism. It most often affects polyunsaturated fatty acids, because they contain multiple double bonds in between which lies methylene ï CH2- groups that possess especially reactive hydrogen (McCay *et al.,* 1984).

As with any radical reaction, the reaction consists of three major steps: initiation, propagation and termination. Initiation is the step whereby a radical is produced. The initiators in living cells are most notably reactive oxygen species (or ROS), such as OH, which combines with a hydrogen atom to make water and a fatty acid radical(www.wikipedia.org/wiki/lipid-peroxidation).

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 species thatôs reacts with another free fatty acid producing a different fatty acid radical and a hydrogen peroxide or cyclic

peroxide if it had reacted with itself. This cycle continues as the new fatty acid radical reacts in the same way. This is the propagation stage(www.wikipedia.org/wiki/lipid-peroxidation).

In termination stage a radical reacts with 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 species. This happens only when the concentration of radical species is high enough for there to be a high probability of two radicals actually colliding. Living organisms have evolved different molecules to catch free radicals and protect the cell membrane. One important such antioxidant is alpha-tocopherol, also known as vitamin E (www.wikipedia.org/wiki/lipid-peroxidation).

Free radicals are highly unstable molecules that interact quickly and aggressively with other molecules in our bodies to create abnormal cells. Free radicals are atoms or groups of atoms with an odd (unpaired) number of electrons and can be formed when oxygen interacts with certain molecules. Their instability causes them to react almost instantly with any substance in their vicinity. Oxygen, or oxyl, free radicals are especially dangerous. Once formed, these highly reactive radicals can start a chain reaction, like dominoes. Their chief danger comes from the damage they can do when they react with important cellular components such as DNA, or the cell membrane; enzymes. Cells may function poorly or die if this occurs. They are capable of penetrating into the DNA of a cell and damaging its ñblueprintò so that the cell will produce mutated cells that can the replicate without normal controls. They accelerate aging and contribute to the development of many diseases, including cancer and heart disease (Zhang, *et al*, 1993).

Surprisingly, however, free radicals are involved in many cellular functions and are a normal part of living. When, for example, mitochondrion within cell burns glucose for fuel, the mitochondria

oxidize the glucose and in so doing generates free radicals. White blood cells also use free radicals to attack and destroy bacteria, viruses and virus-infected cells. The detoxifying actions of the liver also require free radicals (Lennon, *et al*., 1991).

It is important to note that free radicals are also released in the body from the breaking down or detoxification of various chemical compounds; drugs, artificial food colorings and flavorings, smog, preservatives in processed foods, alcohol, cigarette smoke, chlorinated drinking water, pesticides, radiation, cleaning fluids, heavy metals such as cadmium and lead, and assorted chemicals such as solvent traces found in processed foods and aromatic hydrocarbons such as benzene and naphthalene (found in moth balls). Additionally, certain foods contain free radicals which when eaten, enter the body and damage it. The major sources of dietary free radicals are chemically altered fats from commercial vegetable oils, vegetable shortening and all oils heated to very high temperatures (Buege, and Aust, 1978).

Some free radicals arise normally during metabolism. Sometimes the bodyôs immune systemôs cells purposefully create them to neutralize viruses and bacteria. However, environmental factors such as pollution, radiation, cigarette smoke and herbicides can also spawn free radicals (Nathan *et al*., 2000).

Because it is not possible to directly measure free radicals in the body, scientists have approached the questions of how effectively can athletes defend against the increased free radicals from exercise by measuring the by-products that result from free radical reactions. If the generation of free radicals exceeds the antioxidant defenses then one would expect to see more of these by-products. These measurements have been performed in athletes under a variety of conditions (Ellman, 1959).

Several interesting concepts have emerged from these types of experimental studies. Regular physical exercise enhances the antioxidant defense system and protects against exercise induced free radical damage. This is an important finding because it shows how smart the body is about adapting to the demands of exercise. These changes occur slowly over time and appear to parallel other adaptations to exercise.

On the other hand, intense exercise in untrained individuals overwhelms defenses resulting in increased free radical damage. Thus, the ñweekend warriorò who is predominantly sedentary during the week but engages in vigorous bouts of exercise during the weekend may be doing more harm than good. To this end there are many factors that may determine whether exercise induced free radical damage occurs, including degree of conditioning of the athlete, intensity of exercise and diet (Sies, 1997).

Normally, the body can handle free radicals, but if antioxidants are unavailable, or if the free-radical production becomes excessive, damage can occur. Of particular importance is that free radical damage accumulates with age.

1.3.4 Biological uses of Reactive Oxygen species

The immune system uses the lethal effects of oxidants as a central part of its mechanism of killing pathogens; with activated phagocytes producing both ROS and reactive nitrogen species (Nathan, *et al*., 2000). Although the use of these highly reactive compounds in the cytotoxic response of phagocytes causes damage to host tissue, the non-specificity of these oxidants is an advantage since they will damage almost every part of their target cell (Rice-Evans, *et al*., 1995).this prevents a pathogen from escaping this part of immune response by mutation of a single molecular target.

More recently, it has become apparent that ROS also have important roles as signaling molecules. A complex network of enzymatic and small molecule antioxidants controls the concentration of ROS and repairs oxidative damage, and research is revealing the complex and subtle interplay between ROS and antioxidants in controlling plant growth, development and response to the environment.

1.3.5 Consequences of Oxidative Stress.

Oxidative stress contributes to tissue injury following irradiation and hyperoxia. It has been implicated in disease states, such as neurodegenerative diseases including Lou Gehrigôs disease (aka MND or ALS), Parkinsonôs disease, Alzheimerôs disease, Huntingtonôs disease and aging. Oxidative stress is thought to be linked to certain cardiovascular disease, since oxidation of low density lipoprotein (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.

Other disease conditions associated with oxidative stress include: Diabetes, pancreatitis, liver damage, and leaky gut syndrome, hypertension and multiple sclerosis, atherosclerosis (Steinberg, *et al*., 1989), coronary thrombosis, asthma, emphysema, chronic pulmonary disease, cataracts, retinopathy, macular degeneration, rheumatoid arthritis (Aroma, 1993), glomerulonephritis, vitiligo, wrinkles (Pryor W.A., 1991), cancer, autoimmune diseases, inflammatory states (Symons and Dowling, 1987), AIDS and Lupus (Montagnier, Oliveier, and Pasquier, 1998).

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).

1.3.6 ANTIOXIDANTS

To prevent free radical damage the body has a defense system of antioxidants. Antioxidants are intimately involved in the prevention of cellular damage the common pathway for cancer, aging, and a variety of diseases. Fortunately, the body maintains a sophisticated system of chemical and biochemical antioxidants scavenge free radicals, that is, they stabilize the unstable free radicals by giving them the electron they need to ñcalm downò. The antioxidants are usually consumed or used up in this process, i.e., they sacrifice themselves.

Antioxidants are molecules that can safely interact with free radicals and terminate the chain reaction before vital molecules are damaged. Although there are several enzyme systems within the body that scavenge free radicals, the principle micronutrient (vitamins) antioxidants are vitamin E, beta-carotene, and vitamin C. Additionally, Selenium, a trace metal that is required for proper function of one of the bodyôs antioxidant enzyme systems, is sometimes included in this category. The body cannot manufacture these micronutrients so they must be supplied in the diet. Therefore the main antioxidants are vitamins A, E, and C, beta-carotene, glutathione, bioflavonoids, selenium, Zinc, CoQ10 (ubiquinone), and various phyto-chemicals from herbs and foods. Green tea, for example, is rich in polyphenols-powerful antioxidants that help fight cancer.

The best studied cellular antioxidants are the enzymes superoxide dismutase (SOD), catalase, and glutathione peroxidase. Less well studied (but probably just as important) enzymatic antioxidants are the peroxiredoxins and the recently discovered sulfiredoxin. Other enzymes that have antioxidant properties (though this is not their primary role) include paraoxonase, gluthione-S transferases, and aldehyde dehydrogenases.

Biochemical antioxidants not only scavenge free radicals, but also inhibit their formation inside the body. These include lipoic acid, and repair enzymes such as catalase, superoxide dismutase (SOD), glutathione peroxidase. Melatonin, a hormone produced by the pineal gland, is also a potent antioxidant. Cholesterol, produced by the liver, is another major antioxidant, which the body uses to repair damaged blood vessels. It is probably for this reason that serum cholesterol levels rise as people age. With age comes more free radical activity and in response the body produces more cholesterol to help contain and control the damage (Seis, 1997).

Of all the antioxidants, glutathione appears to be pivotal. Made up of three amino acids (cysteine, glycine, and glutamic acid), glutathione is part of the antioxidant enzyme glutathione peroxidase and is the major liver antioxidant. It is a basic tenet of natural medicine that health cannot exist if the liver is intoxicated. Not surprisingly, extremely low levels of glutathione are found in people suffering from severe OS. People with AIDS, cancer and Parkinsonôs disease, for example, typically have low glutathione levels.

As noted earlier, oxidative stress occurs when the amount of free radicals in the body exceeds its pool of available antioxidants. Obviously, knowing the varied sources of free radicals and avoiding them in an important part of minimizing their harmful effects.

Diet can be a major source of free radical stressors with processed or highly heated oils being the main offenders. Replace these harmful fats with natural, cold pressed oils such as olive oil (which can be used for cooking) and small amounts of flax oil or walnut oil (which should never be heated). Food grade, unrefined coconut oil and organic butter are also excellent choices, especially for cooking. Both of these naturally saturated fats are rich in certain fatty acids that have proven activity against bacteria, harmful yeasts, fungi and tumor cells.

Additionally, since saturated fats (from animal foods and the tropical oils) and monounsaturated oils (from olive oil and cold-pressed nut oils) are more chemically stable, they are much less susceptible to oxidation and rancidity than their polyunsaturated analogues, which are mostly found in vegetable oils. As a general rule, then, although the body does require a small amount of naturally occurring polyunsaturated oils in the diet each day, it is best not to consume too much of them as they are more prone to free radical attack in the body. As Linus Pauling, noted: ñA diet high in unsaturated fatty acids, especially the polyunsaturated ones, can destroy the bodyôs supply of vitamin E and cause muscular lesions, brain lesions, and degeneration of blood vessels. Care must be taken not to include a large amount of polyunsaturated oil in the dietò (Linus Pauling, 1998).

The best food sources for polyunsaturated are fish, flax oil, sesame oil, walnut oil and dark green, leafy vegetables. One caveat: canola oil is not recommended due to its chemical instability and its content of trans-fatty acids (TFAs), formed during processing. TFAs are increasingly being linked with cancer, immune system dysfunction and heart disease.

A. VITAMIN C

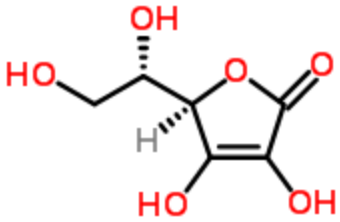


fig.2

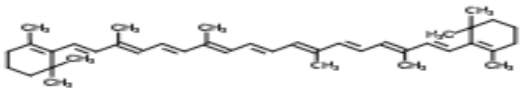
L ï Ascorbic acid

Ascorbic acid is a water- soluble vitamin present in citrus fruits and juices, green peppers, cabbage, spinach, broccoli, kale, cantaloupe, kiwi, and strawberries. The RDA is 60mg per day. Intake above 2000 mg may be associated with adverse side effects in some individuals. Vitamin C is the most abundant water-soluble antioxidant in the body and acts primarily in cellular fluid. It is of particular note in combating free-radical formation caused by pollution and cigarette smoke. Also helps return Vitamin E to its active form (Hickey, and Roberts, 2004).

The vitamins C and E are thought to protect the body against the destructive effects of free radicals. Antioxidants neutralize free radicals by donating one of their own electrons, ending the electron- ñstealingò reaction. The antioxidant nutrients themselves donôt become free radicals by donating an electron because they are stable in either form. They act as scavengers, helping to prevent cell and tissue damage that could lead to cellular damage and disease (Padayatty,*et al*., 2003).

B. BETA-CAROTENE

fig.3



Carotene is a terpene, synthesized biochemically from eight isoprene units. It comes in two primary forms designated by characters from the Greek alphabet: alpha- carotene (-carotene) and beta-carotene (-carotene). Gamma, delta and epsilon (- carotene) also exist. Beta-carotene is composed of two retinyl groups, and is broken down in the mucosa of the small intestine by Beta-carotene dioxygenase to retinol, a form of vitamin A. carotene can be stored in the liver and converted to vitamin A as needed, thus making it a provitamin.

Beta-carotene is a precursor to vitamin A (retinol) and is present in liver, egg yolk, milk, butter, spinach, carrots, squash, broccoli, yams, tomato, cantaloupe, peaches, and grains. Because beta-carotene is converted to vitamin A by the body, there is no set requirement. Instead the RDA is expressed as retinol equivalents (RE), to clarify the relationship. (NOTE: Vitamin A has no antioxidant properties and can be quite toxic when taken in excess). In people who smoke, beta-carotene may increase cardiovascular mortality (Todd, et al., 1999, Omenn, et al., 1998).in men who smoke and have had a prior myocardial infarction (MI), the risk of fatal coronary heart

disease increases by as much as 43% with low doses of beta-carotene. There are some evidence that beta-carotene in combination with selenium, vitamin C and vitamin E might lower high-density lipoprotein 2 (HDL2) cholesterol levels. HDL levels are protective so this considered being a negative effect. Dizziness, reversible yellowing of palms, hands, or soles of feet and to a lesser extent the face (called carotenoderma) can occur with high doses of beta-carotene. Loose stools, diarrhea, unusual bleeding or bruising and joint pain have been reported.

C. GLUTATHIONES

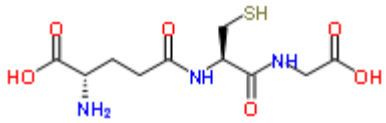


fig. 4

Glutathione (gamma-glutamyl-cysteinyl-glycine; GSH) is the most abundant low-molecular-weight thiol within cells. Two cytosolic enzymes, gamma-glutamylcysteine synthetase and glutathione synthetase catalyze the synthesis of glutathione from glutamate, cysteine, and glycine. Compelling evidence shows that glutathione synthesis is regulated primarily by gamma-glutamylcysteine synthetase activity, cysteine availability, and glutathione feedback inhibition. Animal and human studies demonstrate that adequate protein nutrition is crucial for the maintenance of *glutathione* homeostasis.

In aerobic cells, free radicals are constantly produced mostly as reactive oxygen species. Once produced, free radicals are removed by antioxidant defenses including the enzymes catalase, glutathione peroxidase, and superoxide dismutase. Reactive oxygen species, including nitric oxide and related species, commonly exert a series of useful physiological effects. Imbalance between prooxidant and antioxidant defenses in favor of prooxidants results in oxidative stress, this results in damage to lipids, proteins, and nucleic acids. Alone or in combination with primary factors, free radicals are involved in the cause of hundreds of diseases.

Glutathione ï or L Glutathione ï is a powerful antioxidant found within every cell. Glutathione plays a role in nutrient metabolism, and regulation of cellular events including gene expression, DNA and protein synthesis, cell growth, and immune response. Glutathione taken as a supplement may not be able to cross the cell membrane and thus may not be effective. Consider acetylcysteine instead because it is the N-acetyl derivative of the amino acid, L-cysteine, and is a precursor in the formation of the antioxidant glutathione in the body. The thiol (sulfhydryl) group confers antioxidant effects and is able to reduce free radicals and also acetylcysteine is a good alternative since it can help produce more glutathione.

This antioxidant, made from the combination of three amino acids cysteine, glutamate, and glycine, forms part of the powerful natural antioxidant glutathione peroxidase that is found in our cells. Glutathione peroxidase plays a variety of roles in cells, including DNA synthesis and repair, metabolism of toxins and carcinogens, enhancement of the immune system, and prevention of fat oxidation. However, glutathione is predominantly known as an antioxidant protecting our cells from damage caused by the free radical hydrogen peroxide. Glutathione also helps the other antioxidants in cells stay in their active form. Brain glutathione levels have been found to be lower in patients with Parkinsonôs disease (Zhang, 1993).

Glutathione is found in foods, particularly fruits, vegetables and meats. Cyanohydroxybutene, a chemical found in broccoli, cauliflower, Brussels sprouts and cabbage, is also thought to increase glutathione levels. Various herbs for instance cinnamon and cardamom have compounds that can restore healthy levels of glutathione. Although glutathione is available in pill form over the counter, its utilization by the body is questionable since we donôt know if it can easily enter cells, even after it is absorbed in the bloodstream. Certain nutrients help raise tissue levels of glutathione including acetylcysteine, methyl donors, alpha lipoic acid, polyphenols such as pycnogenol, and vitamin B12 (Silva, *et al.,* 1995*)*.

An excellent review article in the April 1998 issue of Alternative Medicine Review summarizes the known effects of acetylcysteine. The author writes, ñN- acetylcysteine is an excellent source of sulfhydryl groups, and is converted in the body into metabolites capable of stimulating glutathione synthesis, promoting detoxification, and acting directly as a free radical scavenger. Acetylcysteine has historically been as a mucolytic [mucus dissolving] agent in a variety of respiratory illness; however, it appears to also have beneficial effects in conditions characterized by decreased glutathione or oxidative stress, such as HIV infection, cancer, heart disease, and cigarette smokingò. The frequent use of acetaminophen (paracetamol) depletes glutathione peroxidase levels. There appear to be feedback inhibition in glutathione synthesis. This means that if glutathione levels are excessively increased with the help of the nutrients, the body may decrease its natural production (Kelly, 1998).

Glutathione is solid in pills with dosages ranging from 50 to 250mg. Glutathione is a promising antioxidant. However, due to the inconsistence in the medical literature on the ability of glutathione to enter tissues and cells when ingested orally, its beneficial effect to oral dosing may be questionable. Oral administration is poorly tolerated, owing to high doses required (due to low

oral bioavailability), very unpleasant taste and odor, and adverse effects (particularly nausea and vomiting). In a research conducted by Baker, it was concluded that oral N-acetylcysteine was identical in bioavailability to cysteine precursors. Glutathione deficiency contributes to oxidative stress, which plays a key role in aging and the worsening of many diseases including Alzheimerôs disease, Parkinsonôs disease, liver disease, cystic fibrosis, sickle cell anemia, HIV, AIDS, cancer, heart attack, and diabetes. The concentration of glutathione declines with age and in some age-related diseases (Liu, *et al.,* 2004).

Staying on top of oxidative stress is a necessity in our increasingly toxic world. Taking care to avoid those toxins as much as possible and to enrich our diets with life-giving antioxidants is a wise step to take in our endless quest for wellness.

FOOD SOURSES OF ANTIOXIDANTS

CoQ10 (ubiquinone): Beef heart, beef liver, sardines, spinach, peanuts.

Beta carotene: All orange and yellow fruits and vegetables; dark green vegetables.

Zinc: Oysters, herring, lamb, whole grains.

Selenium: Butter, meats, seafood, whole grain.

Vitamin A: Cold liver oil, butter, liver, all oily fish.

Vitamin E: Cold-pressed, unrefined nut and seed oils; wheat germ oil.

Vitamin C: Berries, greens, broccoli, kale, kiwi, parsley, guava.

Glutathione (GSH): Fresh fruits and vegetables, fresh meats, low-heat dried whey.

Bioflavonoids: Most fruits and vegetables, buckwheat.

Polyphenols: Greentea,berries.

Herbal sources: Milk thistle, Ginkgo biloba, turmeric, curry (Padma 28, a packaged Ayurvedic herbal formula, is a special blend of herbal antioxidants).

1.3.7 BIOTRANSFORMATION OF CARBON TETRACHLORIDE

Metabolism of carbon tetrachloride is initiated by cytochrome P-450 mediated transfer of an electron to the C ï Cl bond forming an anion radical that eliminates chloride, trichloromethyl radical (Pohl *et al*., 1981). This radical may undergo both oxidative and reductive biotransformation. The isoenzymes implicated in this process are the cytochrome P2E1, cytochrome P2B1 and cytochrome P2B2 (Gruebele*et al*., 1996). Some isoforms may preferentially be susceptible to degradation of carbon tetrachloride. Evidence that carbon tetrachloride inactivates CYP2E, and reduces total CYP2E protein has been obtained by Dai and Cederbaum (1995). When protein synthesis is blocked, inactivation and degradation of CYP2E1 by carbon tetrachloride are more pronounced.

The formation of carbon tetrachloride ï cytochrome P-450 complexes has been demonstrated. The most important pathway in the elimination of trichloromethyl radicals is the reaction with molecular oxygen, resulting in the formation of trichloromethyl peroxyl radicals as proposed by McCay*et al.,* (1984).

Carbon tetrachloride has been reported to be metabolized to CO_2 in the liver homogenates. The biotransformation of carbon tetrachloride to carbon IV oxide in vivo has been reported by Reynolds *et al*., (1984).

1.3.8 SERUM ENZYME DETERMINATIONS AS A DIAGNOSTIC TOOL

Normally most enzymes reside within cells, where they function in various phases of intermediary metabolism and only small quantities are present in the serum. During certain acute physiologic insults such as myocardial infarction or acute hepatitis, cellular content escapes with extra cellular fluid and eventually reaches the serum in high concentration.

1.3.9 ENZYMES IN THE DIAGNOSTIC PATHOLOGY: ALT, AST AND ALP

I. Alanine Transaminase (ALT)

Alanine Transaminase (ALT) formerly called Glutamate-Pyruvate Transaminase (GPT) is an enzyme present in hepatocytes (liver cells), and in less amount in kidney, heart and skeletal muscle. When a cell is damaged, it leaks this enzyme into the blood, where it is measured. ALT rises dramatically in acute liver damage, such as viral hepatitis than AST (Song, *et al*., 2010).

II. Aspartate Transaminase (AST)

Aspartate Transaminase (AST) formerly called Glutamate-Oxaloacetate Transaminase (GOT) is similar to ALT in that it is another enzyme associated with liver parenchymal cells. It is raised in acute liver damage, myocardial infarction, myopathies muscular disease (muscular dystrophy, rhabdomyolisis) or trauma but is also present in red cells, brain, cardiac and skeletal muscles. It is therefore less specific to liver disease.

III. Alkaline phosphatase (ALP)

Alkaline phosphatase (ALP) is an enzyme in the cells lining the biliary ducts of the liver. ALP levels in plasma will rise with bile duct obstruction, intra-hepatic cholestasis or infiltration disease of the liver. ALP is also present in bone and placental tissue, so it is higher in growing children.

IV. Bilirubin

Increased total bilirubin causes jaundice and its increased production causes hemolytic anemia and internal hemorrhage. Deficiencies in bilirubin metabolism can cause cirrhosis and viral hepatitis, while its deficiencies in excretion can bring about obstruction of the bile duct (Schmidt and Schmidt, 1963).

1.4.0 Rationale of study

Most of the health benefits observed in people that use the extracts of *Annonasenegalensis*Pers. (Annonaceae) stem bark for the management of many ailments are attributed to its pharmacological and medicinal properties. This study is aimed at understanding the baseline pharmacological and toxicological effects of the extracts of A. Senegalensis stem bark in hepatotoxic and normal rats. The rationale of this work is linked to the hepatoprotective effect of *A.senegalensis*stem bark extract on the liver (Dalziel, 1995).

1.4.1 Aim of study

- To determine pharmacognostic standards of *Annona Senegalensis* Pers.
- To determine phytochemical constituents of pulverized bark of *Annonasenegalensis*.
- To evaluate the antioxidant activities of the extract and fractions of *Annona senegalensis.*
- To evaluate the hepatoprotective activities of the extract and fractions of *Annona senegalensis.*

CHAPTER TWO

2.0 MATERIALS AND METHODS

2.1 Plant Collection

Stem bark of *Annona senegalensis* was collected on March 2014 from Enugu Ezike in Igbo-Eze North Local Government Area, Enugu State, Nigeria. It was identified and authenticated by Mr. Alfred Ozioko of the International Centre for Ethno medicine and Drug Development (InterCEDD) Nsukka, Enugu State. The voucher specimen (INTERCEDD 0314) was deposited at InterCEDD.

2.2 Preparation of Extract

The pulverized stem bark (2kg) was extracted with Dichloromethane - Methanol (1:1) for 48hours using cold maceration method. The choice of using this solvent combination for extraction was to make sure that both lipid and non-lipid materials from the crude stem bark were extracted. The mixture was filtered and the filtrate concentrated using rotary evaporator under a reduced pressure to obtain the extract (400g).

2.3 Experimental animals

Thirty five (35) white albino Wistar rats (86 - 100 g) of either sex were procured from the Laboratory Animal Unit of the Faculty of Veterinary Medicine, University of Nigeria, Nsukka. They were kept in stainless steel cages and were fed *ad-libitum* with standard laboratory animal feed (Guinea Feed®), except in situations, where fasting was required. They were also provided with clean tap water. They were maintained in accordance with the recommendation in the Guide for the Care and Use of Laboratory Animals (DHHS, NIH Publication No. 85-23, 1985). They were allowed 2 weeks to acclimatize before the start of the experiments

2.4 Fractionation Procedures

400g of the crude extract was fixed on Silica gel (60-200mesh) and subjected to column chromatography using n-hexane, ethyl acetate and methanol as eluent.

2.5 Phytochemical screening

The preliminary phytochemical test was carried out using methods described by Trease and Evans, (1989).

a.TEST FOR CARBOHYDRATE

Molisch test

0.1g of the extract was boiled with 2ml of distilled water and filtered. To the filtrate, few drops of naphthol solution in ethanol (molischôs reagent) were added. Concentrated sulphuric acid was then gently poured down the side of the test tube to form a lower layer. A purple interfacial ring indicates the presence of carbohydrate.

b. TEST FOR ALKALOIDS

20ml of 3% sulphuric acid in 5% ethanol was added to 2g of the extract and heated on boiling water both for 10minutes, cooled and filtered. 2ml of the filtrate was tested with few drops of Mayerôs reagent (Potassium Mercuric Iodide solution), Dragendorffôs reagent (bismuth potassium iodide solution), Wagnerôs reagent (iodine in potassium iodide solution), and picric acid solution (1%).

The remaining filtrate was placed in 100ml separator funnel and made alkaline with dilute ammonia solution. The aqueous alkaline solution was separated and extracted with two 5ml

portions of dilute sulphuric acid. The extract was tested with a few drops of Mayerôs Wagnerôs, Dragendorffôs reagent and picric acid solution. Alkaloids give milky precipitate with few drops of Mayerôs reagent; reddish brown precipitate with few drops Wagnerôs reagent; yellowish precipitate with few drops of picric acid and brick red precipitate with few drops of Dragendorffôs reagent.

c. TEST FOR REDUCING SUGAR

5ml of a mixture of equal parts of Fehlingôs solution I and II were added to 5ml of aqueous extract and then heated on a water bath for 5 minutes. A brick red precipitate shows the presence of reducing sugar.

d. TEST FOR GLYCOSIDES

5ml of dilute sulphuric acid was added to 0.1g of the extract in a test tube and boiled for 15 minutes on a water bath, then cooled and neutralized with 20% potassium hydroxide solution. 10ml of a mixture of equal parts of Fehlingôs solution I and II was added and boiled for 5 minutes. A brick red precipitate indicates the presence of glycoside.

e. TEST FOR SAPONINS

20ml of distilled water was added to 0.25g of the extract and boiled on a hot water bath for 2 minutes. The mixture was filtered, while hot and allowed to cool and filtrate was used for the following tests;

Frothingtest: 5ml of the filtrate was diluted with 15ml of distilled water and shaken vigorously. A stable froth (form) upon standing indicates the presence of saponins.

Emulsion test: To the fronth solution was added 2 drops of olive oil and the contents shaken vigorously. The formation of emulsion indicates the presence of saponins.

Fehling's test: To 5ml of the filtrate was added 5ml of Fehlingôs solution (equal parts of I and II) and the contents were heated on a water bath.

f. TEST FOR TANNINS

1g of the powdered material was boiled with 20ml of water, filtered and used for the following test;

Ferric chloride test: To 3ml of the filtrate, few drops of ferric chloride were added. A greenish, black precipitate indicates the presence of tannins.

Lead acetate test: To a little of the filtrate was added lead acetate solution. A reddish colour indicates the presence of tannins.

g. TEST FOR FLAVONOIDS

10ml of ethyl acetate was added to 0.2g of the extract and heated on a water bath for 3 minutes. The mixture was cooled, filtered and the filtrate was used for the following tests;

Ammonium test: 4ml of filtrate was shaken with 1ml of dilute ammonia solution. The layers were allowed to separate and the yellow colour in the ammoniacal layer indicates the presence of flavonoids.

1% Aluminum chloride solution test: Another 4ml portion of the filtrate was shaken with 1ml of 1% Aluminum chloride solution. The layers were allowed to separate. A yellow colour in the Aluminum chloride layer indicates the presence of flavonoids.

h. TEST FOR RESINS

Precipitating test: 0.2g of the extract was extracted with 15ml of 96% ethanol. The alcoholic extract was then poured into 20ml of distilled water in a beaker. A precipitating occurring indicates the presence of resins.

Colour test: 0.2g of the extract was extracted with chloroform and the extract was concentrated to dryness. The residue was re-dissolved in 3ml of acetone and another 3ml concentrated hydrochloric acid was added. This mixture was heated in a water bath for 30 minutes. A pink colour which changes to magenta red indicates the presence of resins.

i. TEST FOR FATS AND OIL

0.1g of the extract was pressed between filter paper and the paper was observed. A control was also prepared by placing 2 drops of olive oil on filter paper. Translucency of the filter paper indicates the presence of fats and oil.

j. TEST FOR STEROIDS AND TERPENOIDS

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

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

k. TEST FOR ACIDIC COMPOUNDS

About 0.1g of the powder was shaken vigorously with 5ml of distilled water and filtered. The filtrate was used in the following tests:

Fehling's test: To 1ml portion of the filtrate was an added equal volume of Fehlingôs solution I and II and boiled on water bath for few minutes. A brick red precipitate indicates the presence of reducing sugar.

Benedict's test: To 1ml portion of the filtrate was added 2ml of Benedictôs reagent. The mixture was shaken, heated on a water bath for 5 minutes. A rusty brown precipitate indicates the presence of reducing sugar.

2.6 Microscopic Examinations

The microscopic examinations were carried out using method described by Trease and Evans, (1989). Microscopic slide and cover slip were properly washed and air dried. A tip of a needle was used to apply the powder on the microscopic slide. To the microscopic slide, 2 drops of chloral hydrate was placed to cover the powder, and then the cover slip was placed over the preparation on the microscopic slide. Slide was passed over the flame for 5 seconds, in order to allow the chloral hydrate dissolve and clear the colouring pigment surrounding the structures. The microscopic slide was allowed to cool completely. The slide was viewed under the microscope.

2.7.0 Determination of Total Flavonoids

Total flavonoids were estimated using the method of Ordonez *et al* (2006)method. To 0.5 ml of sample, 0.5 ml of 2% $AlCl_3$ ethanol solution was added. After one hour at room temperature, the absorbance was measured at 420 nm. A yellow color indicated the presence of flavonoids. Extract samples were evaluated at a final concentration of 0.1 mg/ml. Total flavonoid content were calculated as rutin (mg/g)

2.7.1 Determination of Total Phenolics

Total phenol contents in the extracts were determined by the modified Folin-Ciocalteu (1927) method. An aliquot of the extract was mixed with 5 ml Folin-Ciocalteu reagent (previously diluted with water 1:10 v/v) and 4 ml (75 g/l) of sodium carbonate. The tubes were vortexed for 15 sec and allowed to stand for 30 min at 40 °C for color development. Absorbance was then measured at 765 nm using the Hewlett Packard UV-VS spectrophotometer. Samples of extract were evaluated at a final concentration of 0.1 mg/ml. Total phenolic content were expressed as mg/g gallic acid equivalent.

2.8 Determination of some Pharmacognostic Standards of the Pulverized stem back of *AnnonaSenegalensis* Pers.

The methods adopted for the determination of ash values follow the specification given by Glen *et al*., (1967).

I. Total Ash Value.

A tarred nickel crucible was ignited to a constant weight at a dull red heat, cooled and stored in a desiccator. 2g of the pulverized stem bark was weighed into the nickel crucible and heated gently

until all the moisture has evaporated. The heat was increased until most of the carbon had completely charred, after which the sample was heated at about 450^0C until the residue was free from carbon; the residue was cooled and a constant weight was achieved. This was done for three more times and an average taken.

II. Acid Insoluble Ash Value

The resultant weight for the total ash was used. The ash content was transferred to a beaker containing 25ml of dilute hydrochloric acid, heated in water bath for 5 minutes and filtered through an ash-less filter paper. The residue was washed repeatedly until free from acid. The filter paper was dried in the oven, folded into a narrow cone and inserted into a tarred crucible and heated at 450^0C until it was completely decomposed. The residual ash was then heated more strongly and after cooling in a desiccator, it was reweighed. This was repeated for three more times and an average taken.

III. Sulphated Ash Value

A nickel crucible was ignited to a constant weight at a dull heat in the oven. 2g of the pulverized sample of *A.Senegalensis* stem bark was spread in the bottom of the crucible, which was reweighed. The plant material was moistened with dilute sulphuric acid (5%) and ignited at a low heat initially to burn off the carbon content. The crucible was cooled in a desiccator, more dilute sulphuric acid was added and heating continued to about 800^0C with occasional cooling until a constant weight was obtained. This was repeated for three more times and an average taken.

IV. Alcohol Extractive Yield

5g of the pulverized sample was weighed and placed in a 250ml stopped conical flask. 100ml of 90% ethanol was added and the stopper firmly replaced. The content of the flask was shaken mechanically for 6 hours, then allowed to macerate for another 18 hours and then filtered. 20ml of the filtrate was evaporated to dryness in a 25ml beaker on a water-bath. The residue was dried to constant weight at 105^0C. This was repeated for three more times and an average taken.

V. Water Extractive Yield

5g of the pulverized sample was weighed and placed in a 250ml stopped conical flask. 100ml of chloroform water (1:400) was added and the stopper firmly replaced. The content of the flask was shaken mechanically for 6 hours, then allowed to macerate for another 18 hours and then filtered. 20ml of the filtrate was evaporated to dryness in a 25ml beaker on a water-bath. The residue was dried to constant weight at 105^0C. This was repeated for three more times and an average taken.

VI. Determination of Moisture Content

A tarred evaporating dish was heated to a constant weight and stored in a desiccator. 3g of the pulverized sample was added to the evaporating dish and dried in an oven maintained at a temperature of 105^0C to a constant weight. This was repeated for three more times and an average taken. The formula used was: Percentage of moisture in the sample (%w) =

$$\frac{\text{weight of sample before drying (g)}}{\text{weight of sample after drying (g)}} \times 100$$

2.9.0 In vitro anti-oxidant analysis

Extract/fractions and positive standards (ascorbic acid, butylated hydroxytoluene, catechin and gallic acid) were assay and for different *in vitro* anti-oxidant capacities. Of each sample 800 µg was dissolved in 1 ml analytical methanol. These solutions were further serially diluted to 400, 200, 100, 50 and 25 µg/ml. In all the different antioxidant assays, same dilutions of sample and standards were used while standard altered as per assay requirement. The sample at different concentrations were prepared in triplicates.

A. Evaluation of antioxidant capacity using the 1, 1-diphenyl-2-picrylhydrazyl radical (DPPH) spectrophotometric assay

The free radical scavenging activity of extract/fraction was analyzed by the DPPH assay following a standard method (Mensor,*et al.,* 2001). A given volume (2 ml) of the extract at varying concentrations ranging from 800-25 µg/ml each was mixed with 1 ml of 0.5 mM DPPH (in methanol) in a cuvette. The absorbance at 517 nm was taken after 30 min of incubation in the dark at room temperature. The experiment was done in triplicate. The percentage antioxidant activity was calculated as follows:

% Antioxidant Activity [AA] = 100 ï [{(Abs sample ï Abs blank) X 100}/Abs control].

Methanol (1.0 ml) plus 2.0 ml of sample was used as the blank while 1.0 ml of the 0.5 mM DPPH solution plus 2.0 ml of methanol was used as the negative control. Ascorbic acid was used as reference standard.

B. Ferric reducing/antioxidant power (FRAP) assay

The total antioxidant potential of sample was determined using a ferric reducing ability of plasma (FRAP) assay of Benzie and Strain (1996) as a measure of ñantioxidant powerò. FRAP assay measures the change in absorbance at 593 nm owing to the formation of a blue colored Fe(II)-tripyridyltriazine compound from colorless oxidized Fe(III) form by the action of electron donating antioxidants. Standard curve was prepared using different concentrations (100-1000 µmol/L) of $FeSO_4$ x $7H_2O$. All solutions were used on the day of preparation. In the FRAP assay, the antioxidant efficiency of the extracts under the test was calculated with reference to the reaction signal given by an Fe^{2+} solution of known concentration, this representing a one-electron exchange reaction. Ascorbic acid was measured within 1 h after preparation. Extract/fractionswere first adequately diluted to fit within the linearity range. All determinations were performed in triplicate.

Calculations were made by a calibration curve.

FRAP value of sample (µM) =

$$\frac{\text{Changes in absorbance from 0-4 min}}{\text{Changes in absorbance of std 0 min-4 min}} \times \text{FRAP value of std (1000 µM)}$$

C. Hydrogen peroxide scavenging assay

The method of Bokhari *et al*. (2013) was followed to investigate hydrogen peroxide scavenging capacity of samples. Hydrogen peroxide (2 mM) solution was prepared in phosphate buffer (50 mM, pH 7.4). Samples (100 µl) were pipetted into flasks and their volume made up to 400 µl with 50 mM phosphate buffer (pH 7.4). H_2O_2 solution (600 µl) was added and absorbance at 230

nm was taken 10 min after vortexing the flasks. Percent scavenging activity was determined by following formula;

H_2O_2 % scavenging activity = (1- absorbance of sample) x 100

Absorbance of control

Ascorbic acid served as standard.

D. Hydroxyl radical scavenging assay

The antioxidant activity was evaluated by method reported by Halliwell *et al* (1981). The reaction mixture comprised of 2-deoxyribose (2.8 mM, 500 µl) in 50 mM of phosphate buffer, 100 µl of 0.2 M hydrogen peroxide solution, 200 µl of 0.1M ferric chloride, 0.1M EDTA and 100 µl of test sample. The reaction was initiated by the addition of 100 µl of ascorbate (0.3M). The mixture was incubated at 37 °C for 60 min. TCA (2.8% w/v, 1 ml) and 1 ml of thiobarbituric acid (TBA) solution in 50 mM of sodium hydroxide (1%; w/v) was added. This reaction mixture was heated for 15 min in boiling water bath and then allowed to cool. Absorbance was recorded at 532 nm.

Hydroxyl scavenging activity (%) =1-(Absorbance of sample ×100)

Absorbance of control

E. ABTS radical cation scavenging activity

Re *et al*. (1999) methodology with slight modification was followed for ABTS (2, 2 azobis, 3 ethylbenzothiozoline-6-sulphonic acid) radical cation scavenging activity. ABTS (7 mM) solution was reacted with 2.45 mM potassium persulfate and kept overnight in dark for generation of dark colored ABTS radicals. For the assay, the solution was diluted with 50 % ethanol for an initial absorbance of 0.7 at 745 nm. Activity was determined by adding 100 µl

sample of different dilution with 1 ml of ABTS solution in glass cuvette. Decrease in absorbance was measured after one min and 6 min of mixing. The difference was calculated and compared with control. Percent inhibition was calculated by formula:

$$\% \text{ ABTS scavenging effect} = \frac{\text{control absorbance - sample absorbance}}{\text{Control absorbance}} \times 100$$

F. β -Carotene bleaching assay

Elzaawely *et al.* (2007) modified method was used for ɓ-carotene bleaching assay. ɓ-Carotene (2 mg) was dissolved in 10 ml of chloroform and blended with 20 mg of linoleic acid and 200 mg of Tween 20 followed by removal of chloroform under nitrogen with subsequent addition of 50 ml of distilled water and vigorous shacking to prepare ɓ-carotene linoleic acid emulsion. An aliquot of each sample (50 µl) was mixed with 1ml of the emulsion, vortexed and absorbance was determined at 470 nm immediately against the blank solution. Capped tube was then kept in a water bath at 45 °C for 2 h and the difference between the initial readings was calculated by measuring the reading after 2 h. ɓ-Carotene bleaching inhibition was estimated by the following equation:

$$\% \text{ bleaching inhibition} = \frac{(A_{ot} - A_{120t})}{A_{oc} - A_{120}} \times 100$$

G. Superoxide anion radical scavenging assay

Riboflavin light NBT system assay was followed for superoxide radical scavenging activity as described by Nishikimi (1972). The reaction mixture containing 0.5 ml of phosphate buffer (50 mM, pH 7.6), 0.3 ml riboflavin (50 mM), 0.25 ml PMS (20 mM), and 0.1 ml NBT (0.5 mM), prior to the addition of 1 ml sample in methanol. Florescent lamp was used for starting the

reaction. Absorbance was recorded at 560 nm after incubation for 20 min under light. The percent inhibition of superoxide anion generation was calculated using the following formula:

% Percent scavenging activity = (1- Absorbance of sample /Absorbance of control) ×100

2.9.1In vivo anti-oxidant analysis

Animals

Albino mice weighing 28-35 g of both sexes were used for the experiments. They were housed at 25 ± 5 °C under a 12-h light/12-h night conditions with free access to standard pelleted feed and clean drinking water. All experiments carried out in this study were approved by the Animal Ethics Committee, University of Nigeria, Nsukka.

A. TOTAL PROTEINS DETERMINATION. (DIRECT BIURET METHOD)

The method used for the determination of total protein was that described by Wiechselbaum (1946), and Tietz (1995).

PRINCIPLE:

Cupric ions, in an alkaline medium interact with protein peptide bonds resulting in the formation of coloured complex, which is photometrically measured.

PROCEDURE:

Three test ï tubes were labeled blank (BL), standard (ST) and sample (SA) respectively before the set of the experiment. To the test-tube labeled SA, 0.02ml of serum was added. To the test ï tube labeled ST, 0.02ml of standard (CAL) was added and 0.02ml was added to the test tube labeled BL.

Then, 1.00ml of the biuret reagent(Is made of sodium hydroxide (NaOH) and hydrated copper(II) sulfate, together with potassium sodium tartrate) was added to all the three test tubes.

After the addition, it was mixed well and incubated at 30minutes at room temperature (20-25^{0}C). The absorbance of the sample and standard was measured against the reagent blank at 546nm.

The absorbance of the standard is 0.365.

B. Lipid peroxidation assay

Lipid peroxidation in the serum from animals on Day 28 was estimated colorimetrically as thiobarbituric acid reactive substances (TBARS) using the method of Buege and Aust (1978). A principal component of TBARS is malondialdehyde (MDA), a product of lipid peroxidation. In brief, 0.1 ml of tissue homogenate (Tris-HCl buffer, pH 7.5) was treated with 2 ml (1:1:1 ratio) of TBA-TCA-HCl reagent (thiobarbituric acid 0.37 %, 0.25 N HCl and 15 % TCA). The mixture was placed in a water bath for 15 min; it was then allowed to cool. The absorbance of clear supernatant was measured against reference blank at 535 nm. Concentration was expressed as nmol/ml.

C. Assay of catalase (CAT) activity

Catalase activity was measured according to the method of Aebi (1984). A given volume (0.1 ml) of the serum was pipetted into cuvette containing 1.9 ml of 50 mM phosphate buffer of pH 7.0. Reaction was started by the addition of 1.0 ml of freshly prepared 30 % (v/v) hydrogen peroxide (H_2O_2). The rate of decomposition of H_2O_2 was measured spectrophotometrically from changes in absorbance at 240 nm. The enzyme activity was expressed as units/ml protein.

D. Assay of Superoxide Dismutase

Superoxide Dismutase in the serum from animals on day 28 was estimated using the procedure of Mclord,, and Fridovich, (1969). A given volume (0.1ml) of the serum was pipetted into cuvette containing 1 M KOH buffer of pH 7.8 at 25^0C. Reaction was started by the addition of 1.0 ml of freshly prepared 30 % (v/v) superoxide Dismutase (SOD). The rate of decomposition of SOD was measured spectrophotometrically from changes in absorbance at 550nm. The enzyme activity was expressed as units/ml protein.

E. Assay of reduced glutathione (GSH) concentration

Reduced glutathione was determined by the method of Ellman, (1959). A volume (1.0 ml) of serum was treated with 0.5 ml of Ellmanôs reagent (19.8 mg of 5, 5- dithiobisnitrobenzoic acid (DTNB) in 100 ml of 0.1 % sodium nitrate) and 3.0 ml of phosphate buffer (0.2 M, pH 8.0). Then 0.4 ml of distilled water was added. The mixture was thoroughly mixed and absorbance was read at 412 nm and expressed as units/ml.

2.9.2 Acute Toxicity Study

The lethal dose (LD_{50}) is the dose that produces death in 50% of the animals. Lethal dose (LD_{50}) determination is essential because it gives room for safe dosing in subsequent whole animal experiments which will be carried out in the course of the study.

The Lorke, D. (1983) procedure of LD_{50} determination was used.

The experiment involved first, a preliminary test in which three group of mice (n=3) where orally administered 10, 100 and 1000 mg/kg of *Annona Senegalensis* extract. The animals were constantly observed for first 2 hours, intermittently for the next 4 hours and then overnight. No death of animal per group was recorded at the end of 24 hours. From the result obtained above, the second stage of the acute toxicity test was performed using doses of 1000, 1600, 2900 and 5000 mg/kg administered orally to (n = 3).

2.9.3 Treatment of animals

Rats were divided into five groups (n=5). Group 1served as the control and received 3 % tween 80, group 2 constituted the hepatotoxic group and was treated similarly to group 1, group 3 received the reference drug, Silymarin (100 mg/kg body weight), and groups 4 and 5 received the methanol-methylene chloride (1:1) extract (200 and 400 mg/kg body weight respectively). These were given to the animals once daily for 3 days. On day 3, one hour after administration of the appropriate drug for each group, carbon tetrachloride was given to the different groups apart from the group one (control). 48 hours After carbon tetrachloride administration, blood was collected from the retro-orbital plexus to be used for the assessment of biochemical parameters.

2.9.4 BIOCHEMICAL TESTS

I. Liver function tests

Blood was collected from all the groups via the retro-orbital plexus at room temperature and the serum was separated by centrifuging at 2500 rpm for 10 minutes. The serum was used for estimation of the biochemical parameters to determine the functional state of the liver. Serum aminotransferase activities including AST and ALT, Alkaline phosphatase, total bilirubin values were assayed using the ôRANDOXô commercial Enzyme kit according to the method of Reitman and Frankel (1957) and Schmidt (1963) was used.

2.9.5 Statistical analysis

All data were expressed as Mean ± S.E.M. or % mean. Data were analyzed using one way analysis of variance (ANOVA) at 5% level of significance.

CHAPTER THREE

3.0 RESULTS

3.1.0 Extract and fractions

The 2kg of the plant material yielded 400g of the extract after maceration procedures. This implies 20% yield. Partitioning with n ï hexane, EtOAc produced 20.1 and 120.66g respectively, while the residual of methanol was 180.20g. Table 2 shows the summary of the results.

Table2. Percentage yield of the Extract and fractions

Plant materials	2 Kg	Yield in %
Extract	400 g	20%
N - hexane fraction	20.1 g	5.01%
EtOAc fraction	120.66 g	30.17%
MeOH fraction	180.20 g	45.05%

3.2 Acute Toxicity Test (LD_{50})

No death was recorded even at 5000 mg/kg. This shows that the *A. senegalensis* extract/fractions was safe at higher dose.

3.3.0 Phytochemical analysis

Table 3 shows the phytochemical constituents of *A. senegalensis* stem bark. It indicates the presence of the following; carbohydrate, alkaloids, tannins, flavonoids, terpenoids, proteins, reducing sugar, glycosides, saponins, resins, oil, and steroids in the crude extract; presence of protein, terpenoids, glycosides, oil and resins in n – Hexane fraction; presence of alkaloids, carbohydrate, flavonoids, terpenoids, reducing sugar, glycosides, resins, and steroids in EtOAc; and presence of alkaloids, carbohydrates, flavonoids, tannins, terpenoids, reducing sugar, glycosides, resins and saponins in MeOH respectively.
However, crude extract and EtOAc indicates the degree of abundance of these metabolites with alkaloids, carbohydrates, flavonoids, terpenoids, resins and steroids.

Table 3: Phytochemical Constituents of *A. senegalensis*

		EXTRACT	**N-Hexane**	**EtOAc**	**MeOH**
S/N	**PHYTOCHEMICAL CONSTITUENTS**				
1	Alkaloids	+++	-	+++	+
2	Carbohydrates	++	-	++	+
3	Flavonoids	+++	-	+++	+
4	Proteins	++	++	-	-
5	Tannins	++	-	-	++
6	Terpenoids	+++	+	+++	+
7	Reducing sugar	++	-	+	+
8	Glycosides	+	+	+	+
9	Oil	+++	+++	-	-
10	Resins	++	++	++	++
11	Saponins	+	-	-	+
12	Steroids	++	-	++	-

Key:

+++ = high in concentration

++ = medium in concentration

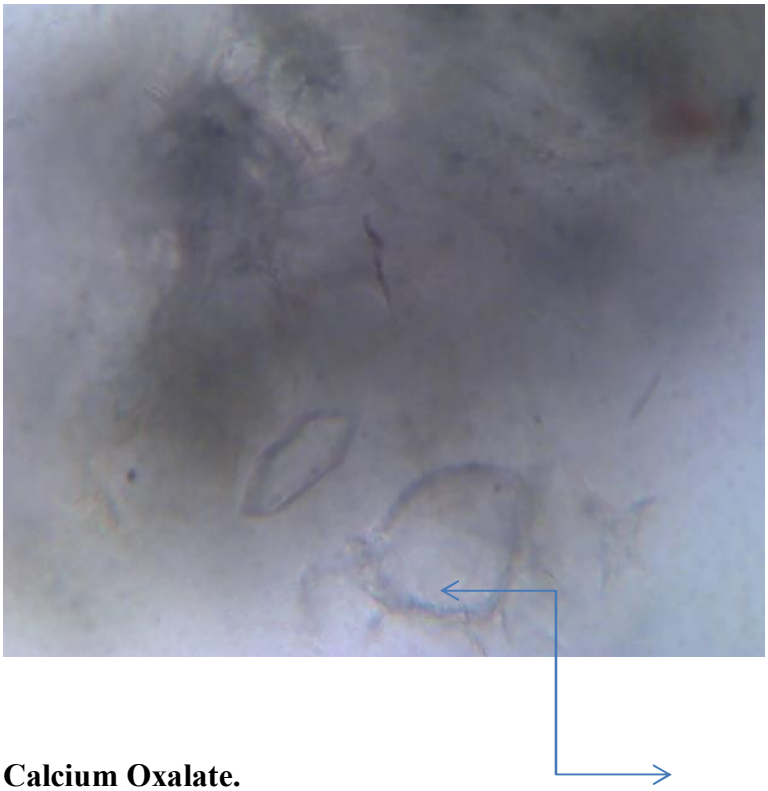
\+ = low in concentration

\- = absence

3.4,0 Microscopic examinations.

Fig. 5 shows the microscopic examination of the stem bark powder of *A. senegalensis.* It indicates the presence of calcium oxalate, stone cells/sclereids, fibres, cork cells, and phloem parenchyma cells as obtained in a standard stem bark of a plant.

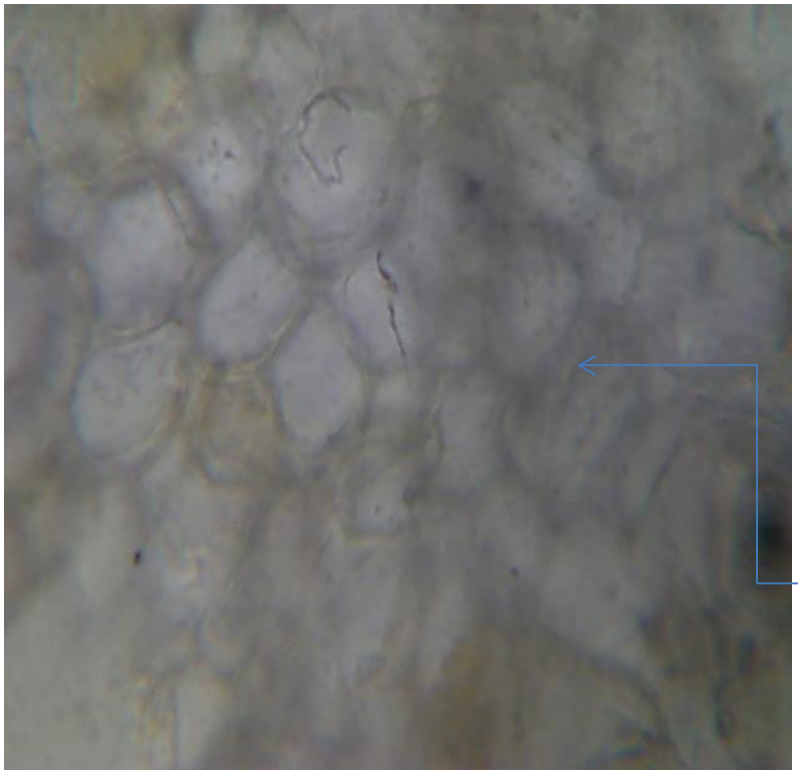
Fig 5. All magnifications are in ×400.



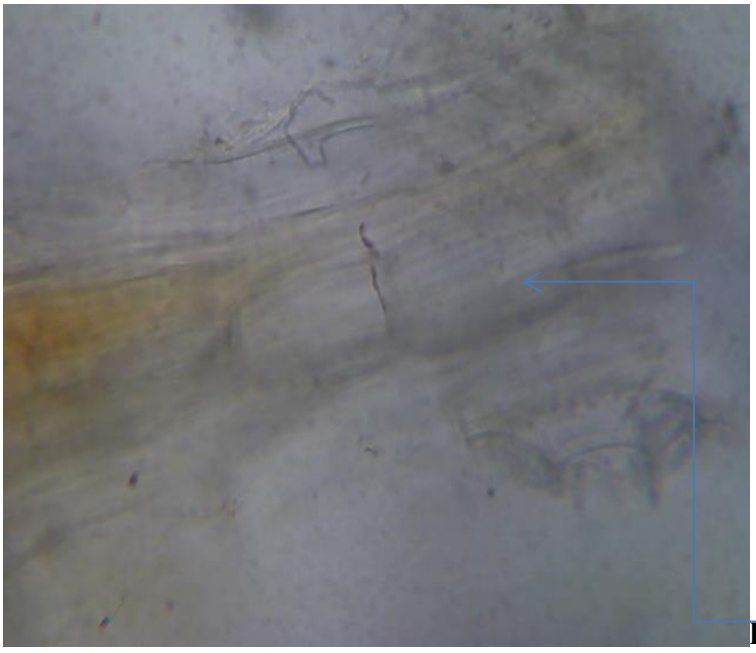
Calcium Oxalate.

Sclereids/stone cells

Fibre



Cork cells



Phloem Parenchyma cells

3.5.0 Total Flavonoid and Total Phenolic Contents

The initial qualitative tests on each fraction, informed the choice of the subsequent quantitative phytochemical test. Total flavonoid and total phenolic contents were determined.

Table 4 shows the total flavonoid and total phenolic contents of *A. senegalensis* stem bark. It indicates the presence of total flavonoids and total phenolic contents of crude extract, ethyl acetate fraction, and methanol fraction of the plant, while absence in n ï hexane.

Table 4: Total Flavonoid and Total Phenolic Contents

Extract/Fraction	Total flavonoid content of *A. senegalensis* (mg rutin equivalent/g extract or fraction)	Total Phenolic content of *A. senegalensis* (mg Gallic acid equivalent/g extract or fraction)
Crude Extract	845.67±93.62	866.67±8.41
n-hexane fraction	-	-
Ethyl Acetate fraction	587.33±50.83	582.00±1.73
MeOH fraction	113.67±9.28	115.33±3.84

*****P < 0.05 compared to respective negative control

3.6.0 Pharmacognostic Standards of Pulverized Stem bark of *A. senegalensis.*

Physicochemical constants like moisture content, total ash, insoluble ash, sulphated, water soluble extractive and alcohol soluble extractive determined and summarized in Table 5. This would help in substantiate standardization of *A. senegalensis.*

Table 5: Pharmacognostic Standards of Pulverized Stem bark of *A. senegalensis.*

Total Ash	7.250(%)
Acid insoluble Ash	5.50(%)
Sulphated Ash	6.90(%)
Water insoluble Ash	7.80(%)
H_20 extractive value	3.750(%)
Alcohol extractive value	3.820(%)
Moisture content	4.643(%)

3.7.0Spectrophotometric reading for anti-oxidant of the leaf and bark of *A. senegalensis* when compared withVit. C.

Table 5 shows spectrophotometric investigation carried out on the leaf and stem bark of *A. senegalensis* for antioxidant as compared to Vitamin C. it indicates that the stem bark of the plant at various concentrations has higher values than the leaf and Vit. C, the standard. This was why the work was done on only the stem bark of the plant.

Table 6: Spectrophotometric reading for anti-oxidant of the leaf and bark of *A. senegalensis* when compared withVit. C

Leaf	Bark	Vit. C (Std.)	Concentration (ug)
0.027	0.03	0.002	25
0.033	0.046	0.011	50
0.041	0.061	0.015	100
0.048	0.069	0.020	200
0.051	0.078	0.025	400

3.8.0 IC_{50} Values of different antioxidant Assays of *A. senegalensis* (IC_{50}, µg/ml)

Table 7 shows the IC_{50} values of different antioxidant assays of *A. senegalensis.* 1,1-diphenyl-2-picylhydrazyl radical (DPPH) indicates that the crude extract and the fractions from n-hexane, Ethyl Acetate, MeOH had better antioxidant activities when compared to Vitamin C, the standard. Ethyl acetate fraction exhibited greater activity than other extract/fractions with IC_{50} value of 121.67µg/ml. The crude extract from FRAP assay indicated more inhibition than other fractions. Hydrogen radical scavenging capacity of Ethyl acetate fraction with IC_{50} value of 75.67±6.33µg/ml indicates higher activity than others when compared with the standard with Ic_{50} of 62.00±1.73µg/ml. Ethyl acetate indicates higher activity than others in beta-carotene bleaching activity, hydrogen peroxide, superoxide radical and ABTS scavenging activities.

Table 7: IC_{50} Values of different antioxidant Assays of *A. senegalensis* (IC_{50}, µg/ml)

Test Method Activity	Crude Extract	n-hexane fraction	Ethyl Acetate fraction	MeOH fraction	Standard (Vitamin C)
DPPH Scavenging activity	752.67±15.60	355.67±4.49	121.67±2.40	293.67±16.91	69.67±1.86
FRAP activity	63.00±12.22	281.67±15.34	81.00±1.00	275.67±6.33	71.66±0.33
Hydrogen peroxide scavenging activity	390.33±0.88	437.00±29.05	256.33±0.88	404.00±37.70	107.00±2.52
Hydroxyl radical scavenging activity	418.00±5.29	371.67±36.25	75.67±6.33	370.67±7.80	62.00±1.73
Superoxide radical scavenging activity	333.67±2.40	140.67±7.97	79.33±1.45	235.33±0.88	68.00±0.58
ɓ-Carotene Bleaching activity	351.67±4.33	358.33±7.22	145.67±5.55	254.33±4.98	57.00±1.15
ABTS Scavenging activity	372.00±3.46	391.00±14.57	126.67±1.20	217.00±4.04	78.33±1.76

*$p<0.05$ significantly different from reference standard compound (Vitamin C).

3.9.0 Anti-oxidant *in vivo* study: The effect of extract/fractions on anti-oxidant parameters

Table 8 shows the anti-oxidant *in vivo* study of the extract/fractions of *A. senegalensis.* Crude extract indicates that at dose of 100mg/kg, superoxide dismutase, lipid peroxide, and total protein has no activity, but as dose increases from 200mg/kg to 400mg/kg the activity increased. The fractions of ethyl acetate, n ï Hexane of the plant at doses of 400mg/kg indicates good antioxidant activities. It is dose dependent.

Table 8: Anti-oxidant *in vivo* study: The effect of extract/fractions on anti-oxidant parameters

Ext./fraction	Dose (Mg/kg)	SOD (Mg/dl)	L.H_2O_2 (mmol/L)	Vit. C (Mg/dl)	T. Protein (Mg/dl)
Ext.	100	38.00±4.56	0.32±0.04	14.40±2.25	7.66±0.54
ñ	200	30.40±3.66**	0.26±0.02	18.60±1.57**	8.42±0.43*
ñ	400	23.00±2.00**	0.20±0.012*	20.00±2.24**	10.46±0.53**
3% Tween	5ml/kg	45.80±2.35	0.31±0.03	10.00±0.71	6.38±1.12
FRACTIONS					
n-Hex	400	24.80±2.71**	0.18±0.02**	18.40±2.01**	9.60±0.58**
EtOAc	400	19.80±0.80**	0.17±0.014**	20.40±1.12**	10.84±0.56**
MeOH	400	34.20±3.57	0.27±0.03**	17.60±1.57**	8.54±0.38
Crude Ext.	400	23.00±2.00**	0.20±0.02**	20.00±2.24**	10.46±0.53**
3% Tween 80	5ml/kg	40.00±2.30	0.37±0.02	10.40±0.51	16.94±0.81

Value in mean ±S.E. (standard error) n = 5, *p < 0.05, **p < 0.01, ***p < 0.001 when compared with the control.

NB: SOD= superoxide dismutase, L.H_2O_2 = lipid peroxide, T. protein = total protein.

3.9.1Biochemical tests (Liver function tests).

Table 9 shows the liver function tests of *A. senegalensis.* It indicates that higher activities in concentrations of the parameters signify liver damage. 400mg/kg extract produces reductions greater than the control followed by 200mg/kg while 100mg/kg the least. Fraction of 400mg/kg Ethyl acetate of*A. senegalensis* reduced the ALT, AST and bilirubin levels significantly followed by n-Hexane fraction and methanol fraction produced the least effect.

Table 9: Biochemical tests (Liver function tests).

		AST	**ALP**	**ALT**	**BIL**
EXTRACT	**DOSES(mg/kg)**	**Mean±SEM**	**Mean±SEM**	**Mean±SEM**	**Mean±SEM**
	100	1.11±14.01ھھر	1.60±12.82	46.80±2.31ھھھر	1.80±0.45
	200	1.21±18.90ھھر	1.17±8.19ھھھر	51.80±4.43ھھھر	1.94±0.15ھھر
	400	1.41±13.18صر	1.16±8.77ھھھر	67.00±3.33ھھھر	2.08±0.11صر
Control	5ml/kg	1.96±11.32	1.93±11.72	99.20±4.51	1.56±0.09
FRACTIONS					
n ï Hex.	400	1.05±2.56ھھھر	1.58±6.55	59.80±7.47صر	2.08±0.16صر
EtOAc	400	1.46±1.56ھھر	1.65±18.63	63.40±10.30صھر صر	2.78±0.14ھھھر
MeOH	400	1.56±11.43ھھر	1.87±10.63ھھر	74.40±3.84صر	2.10±0.14صر
Control	5ml/kg	2.04±10.80	1.54±8.80	1.10±5.42	1.56±0.01

Value in mean ±S.E. (standard error) n = 5, *p < 0.05, **p < 0.01, ***p < 0.001 when compared with the control.

CHAPTER FOUR

4.0 DISCUSSION AND CONCLUSION

4.1 Discussion

Several techniques have been used to determine the antioxidant activity *in vitro* in order to allow rapid screening of substances since substances that have low antioxidant activity *in vitro*, will probably show little activity *in vivo.* Free radicals are known to play a definite role in a wide variety of pathological manifestations. Antioxidants fight against free radicals and protect us from various diseases. They exert their action either by scavenging the reactive oxygen species or protecting the antioxidant defense mechanisms(Sies, 1997).

The information obtained from preliminary phytochemical screening will be useful in finding out the genuity of the drug. Ash values, extractive values can be used as a reliable aid for detecting adulteration. The simple but reliable standards will be useful to a lay person in using the drug as a home remedy. Also manufacturers can utilize them for identification and selection of raw material for drug production. A number of scientific reports indicated that certain flavonoids, triterpenoids and steroids have protective effect on liver due to its antioxidant properties. The presence of high concentration of flavonoids,terpenoids, and steroids in the *A. senegalensis* may be responsible for the hepatoprotective effect.However, crude extract/EtOAcfraction had more phenolic contents of 866.67±8.41 and 582.00±1.73 mg Gallic acid equivalent/g respectively.

In this study, a spectrophotometric investigation was carried out on the leaf and stem bark of *A. senegalensis* for antioxidant as compared to Vitamin C. Result showed that the stem bark of the

plant at concentration of 100µg has a very high value of 0.061 the leaf has 0.041and vitamin C the least with 0.015 (Table 6). However, the stem bark of *A. senegalensis* seems to be more active than its leaf, which was why I worked only on the stem bark of the plant.

Microscopic examination of the stem bark powder of *A. senegalensiss* showed a clear features of a standard stem bark of a plant, which includes: calcium oxalate, stone cells/sclereids, fibres, cork cells and phloem parenchyma cells as shown in Fig. 5.

The acute toxicity method employs 15 albino mice with the following assumption: substances more toxic than 1 mg/kg are so highly toxic that it is not important to calculate the LD_{50} exactly, LD_{50} value greater than 5000 mg/kg are of no practical interest and an approximate figure for the LD_{50} is usually adequate to estimate the risk of acute intoxication. The acute toxicity result showed that stem bark of *A. senegalensis* is safe at high doses of 5000mg/kg.

The electron donation ability of natural products can be measured by 1, 1-diphenyl-2-picrylhydrazyl radical (DPPH) purple-coloured solution bleaching. The method is based on scavenging of DPPH through the addition of a radical species or antioxidants that decolourize the DPPH solution. The degree of colour change is proportional to the concentration and potency of the antioxidants (Mensor, *et al*., 2001). A large decrease in the absorbance of the reaction mixture indicates significant free radical scavenging activity of the compound under test. In the present study the result by 1, 1-diphenyl-2-picrylhydrazyl radical (DPPH) showed that crude extract and the fractions from n-hexane, Ethyl Acetate, MeOH had better antioxidant activities when compared to the standard, vitamin C (Table 5). However, Ethyl Acetate fraction exhibited greater activity than other extract/fractions with IC_{50} value of 121.67µg/ml which is significant to the standard value of 69.67µg/ml as shown on Table 7.

The ability of plant extracts to reduce $Fe^{3\div}/Fe^{2\div}$ was determined by FRAP assay which measures the reducing capacity by increased sample absorbance based on the formed ferrous ions.The assay may not be complete even several hours after the reaction starts, such that a single end-point of the reaction cannot be determined. The change in absorbance at 593 nm owing to the formation of blue coloured $Fe^{2\div}$ - TPTZ complex from the colourless oxidized $Fe^{3\div}$ form by the action of electron donating antioxidants (Benzie, I.F. and Strain, J.J.1996). The FRAP results were similar to the DPPH with Ethyl Acetate fractions exhibiting the highest activity of FRAP value of 81.00±1.00µg/ml which is slightly higher than the standard value of 71.66±0.33µg/ml. The crude extract from FRAP assay exhibited more inhibition than the standard and other fractions with 63.00±12.22µg/ml. Since FRAP assay is easily reproducible and linearly related to molar concentration of the antioxidants present, it can be reported that extract/fractions of *A. senegalensis* may act as free radical scavenger, capable of transforming reactive free radical species into stable non radical products.

Hydroxyl radical is regarded as a detrimental species in pathophysiological processes and capable of damaging almost every molecule of biological system and contributes to carcinogenesis, mutagenesis and cytotoxicity. Hydroxyl radicals were produced by the reaction of H_2O_2 and the ferrous that would react with 2-deoxyribose. The reaction was stopped by adding TBA reagent that would give a red colour if the malonaldehyde was formed as the result of the reaction between the radical and 2-deoxyribose. Hydroxyl radical scavenging capacity of an extract or compound is directly proportional to its antioxidant activity which is depicted by the low intensity of red colour. The sample of *A. senegalensis* when added to the reaction mixture actively scavenged the hydroxyl radicals and prevented the degradation of 2-deoxyribose (Halliwell, and Gutteridge, 1981). In this present study, Ethyl Acetate fractions with

Ic_{50} value of 75.67±6.33µg/ml showed higher activity than others when compared with the standard with Ic_{50} of 62.00±1.73µg/ml.

ABTS radical scavenging assay involves a method that generates a blue/green ABTS+ chromophore via the reaction of ABTS and potassium persulfate (Re *el al.,* 1999). The ABTS radical cation is generated by the oxidation of ABTS with potassium persulfate, its reduction in the presence of hydrogen-donating antioxidants is measured spectrophotometrically at 745 nm. The extract of *A. senegalensis* and its fractions possessed strong ABTS scavenging activity as noticed in this study when compared to the standard.

The ɓ-carotene bleaching assay is a commonly used model to analyze the antioxidant activity of the plant extracts because ɓ-carotene is extremely sensitive to free radical mediated oxidation of linoleic acid.ɓ-carotene in this model system undergoes rapid discoloration in the absence of an antioxidant. This is because of the coupled oxidation of ɓ-carotene and linoleic acid, which generates free radicals. The linoleic acid free radical formed upon the abstraction of a hydrogen atom from one of its diallylic methylene groups attacks the highly unsaturated ɓ-carotene molecules (Elzaawely *et al.* 2007). As a result, ɓ-carotene will be oxidized and broken down in part; subsequently the system loses its chromophore and characteristic orange colour, which can be monitored spectrophotometrically. The tested compound inhibited ɓ-carotene oxidation, suggesting that the antioxidant activity could be related to free hydroxyl groups in the compound. Free radical chain reaction is widely accepted as a common mechanism of lipid peroxidation and it is generally thought that the inhibition of lipid peroxidation by antioxidants may be due to their free radical-scavenging activities. Radical scavengers may directly react and quench peroxide radicals to terminate the peroxidation chain reaction and improve the quality

and stability of food products. This study demonstrated that the extract and its different fractions possess a lipid peroxidation inhibitory activity.

Superoxide and hydroxyl radicals are important mediators of oxidative stress that play vital role in some clinical disorders. Any compound, natural or synthetic with antioxidant activities might contribute towards the total/partial alleviation of such damage. Therefore, removing superoxide and hydroxyl radical could contribute to defense of a living body against disease (Mclord, and Fridovich, 1969).

The antioxidant *in vivo* study of the extract/fractions of *A. senegalensis* (Table 8) showed that at dose of 100mg/kg, superoxide dismutase, lipid peroxide and total protein had no activity, but as doses increased from 200mg/kg to 400mg/kg the activity increased. This showed that it has dose dependent effect. However, at doses of 400mg/kg, fractions of EtoAc, n-Hexane of A. senegalensis exhibited good antioxidant activities.

The hepatoprotective study was undertaken to demonstrate the protective ability of the methanol-methylene chloride (1:1) extract of *A. senegalensis* on 'liver damage induced by Carbon tetrachloride(CCl_4) and the toxic effects of the similar doses in rats.The formation of carbon tetrachloride ï cytochrome P-450 complexes has been demonstrated. The most important pathway in the elimination of trichloromethyl radicals is the reaction with molecular oxygen, resulting in the formation of trichloromethyl peroxyl radicals as proposed by McCay*et al.,* (1984). Carbon tetrachloride has been reported to be metabolized to CO_2 in the liver homogenates. The biotransformation of carbon tetrachloride to carbon IV oxide in vivo has been reported by Reynolds *et al*., (1984). The damage of the liver caused by CCl_4 was evident by the alteration in serum marker enzymes concentration beside the clinical signs. Carbon tetrachloride

is reported to produce free radicals, which affect the cellular permeability of hepatocytes leading to elevated levels of serum biochemical parameters like ALT, AST and ALP. The levels of serum Asparate aminotransaminase (AST), Alanine aminotransaminase (ALT) and Alkaline Phosphate (ALP) were taken as an index for oxidative stress induced by CCl_4. The serum activities in concentrations of these enzymes decreased significantly in the extract ï treated animals when compared to the untreated group and this suggests that extract *A. senegalensis* has a hepatoprotective activity. This is in agreement with the commonly accepted view that serum levels of AST, ALT and ALP return to normal with the healing of hepatic parenchyma and the regeneration of hepatocytes (Schmidt, and Schmidt, 1963). The studied plant extract contains antioxidants and hepatoprotective activity through regulatory action on cellular permeability, stability and suppressing oxidative stress.

Table 9 shows the different concentrations of the liver enzymes used in the biochemical parameters for different treatment groups after induction of hepatotoxicity. Higher activities in concentrations of the parameters signify liver damage. The doses 200 and 400mg/kg of the extract reduced the AST, ALP, ALT and bilirubin levels significantly and implied significant restoration of the liver function. 400mg/kg extract produced reductions greater than the control followed by 200mg/kg while 100mg/kg the least. It showed that the hepatoprotective effect was dose dependent. The study on table 9 showed that at doses of 400mg/kg, the Ethyl acetate fraction of*A. senegalensis* reduced the ALT, AST and bilirubin levels significantly followed by n-Hexane fraction and methanol fraction produced the least effect. Moreover, Methanol fraction of *A. senegalensis* was the only fraction that has effect on ALP level. The plant may be said to have protective effects on the liver.

4.2 Conclusion

The results of the present study demonstrated that *A. senegalensis* stem barkhas antioxidant properties by scavenging free radicals, decreasing lipid peroxidation and increasing the endogenous blood antioxidant enzymes levels. Also the extract/fractions (40Omg/kg) have potent hepatoprotective activity against Carbon tetrachloride induced liver damage and the effects are dose dependent. The antioxidant and hepatoprotective activities are due to the presence of bioactive compounds like flavonoids and tannins. Since the *A. senegalensis*is a very popular drug in traditional medicine, it is a promising candidate for use as an antioxidant and hepatoprotective agent. Further investigation is necessary to determine the phytoconstituents responsible for the hepatoprotective effect.

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