IN VITRO FREE RADICAL SCAVENGING AND DNA PROTECTING ACTIVITIES OF DIFFERENT SOLVENT EXTRACTS OF LEAF AND STEM BARK OF *Gossypium barbadense*

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NOVEMBER, 2016

DECLARATION

I hereby declare that the thesis entitled "**IN VITRO FREE RADICAL SCAVENGING AND DNA PROTECTING ACTIVITIES OF DIFFERENT SOLVENT EXTRACTS OF LEAF AND STEM BARK OF *Gossypium barbadense***" is based on the original research work carried out by me in the department of Biochemistry under supervision of Professor A. J. Nok (*OON, FAS, NNOM*) and Professor H.M. Inuwa. The information derived from the literature has been duly acknowledged in the text and a list of references provided. To the best of my knowledge and belief, no part of this project work has previously formed the basis for award of any degree in any other university or institution.

ADEWUSI, ADEOLA MARY _____ _____

Signature **Date**

CERTIFICATION

This is to certify that the thesis entitled "IN VITRO FREE RADICAL SCAVENGING AND DNA PROTECTING ACTIVITIES OF DIFFERENT SOLVENT EXTRACTS OF LEAF AND STEM BARK OF *Gossypium barbadense*" by Adeola Mary ADEWUSI meets the regulations governing the award of degree of Master of Science (M.Sc.) of Ahmadu Bello University, Zaria, and is approved for its contributions to knowledge and literary presentation

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DEDICATION

To the Invisible, Immortal, the only wise God, be glory and honour forever (Amen).

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ABSTRACT

Prevention and treatment of various degenerative diseases using traditional medicines is increasingly generating interest, especially in clinical research. This study was aimed to evaluate the invitro free-radical scavenging properties and potential to prevent DNA damage (from oxidative stress) of leaf and stem bark extracts of *Gossypium barbadense*. Quantitative and Qualitative phytochemical test of different solvent extracts (hexane extract, ethyl acetate extract and aqueous extract) was carried out and result showed that hexane leaf extract had the highest amount of flavonoids (1734.54 ± 6.36 µg equivalent Quercertin/g dry material) and ethyl acetate leaf extract has the highest phenolic content (86.04 ± 0.85 µg Gallic acid/g of dry sample). Scavenging of DPPH radical by all the extracts was evaluated to estimate their antioxidant potential. All the extracts possess free radical scavenging property with hexane leaf extract having the lowest IC_{50} value of 0.192 ± 0.076 mg/ml; as compared to the standard, ascorbic acid with IC_{50} value of 0.173 ± 0.098 µg/ml. The IC_{50} value is in the increasing order of HL > EL > ES > HS > AqL > AqS. Protection of DNA against free radicals-induced oxidative damage (free radicals from Fentons reagent and photolyzed H_2O_2) by all extracts was studied using Pmax Green Florescence Protein (GFP) plasmid. The result showed that only the aqueous leaf extract had DNA protecting activity. Membrane stabilization test – a measure of anti-inflammatory activity was also carried out. It was observed that all the extracts protected the RBC from hemolysis as compared with a standard, aspirin.

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ABBREVIATIONS, DEFINITIONS, GLOSSARY AND SYMBOLS

AqL	Aqueous leaf
AqS	Aqueous stem
ASC	Ascorbic acid
ATP	Adenosine triphosphate
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
CAT	Catalase
CPFA	Cyclopropenoid fatty acids
DNA	Deoxyribo Nucleic acid
DPPH	1, 1-diphenyl-2-picrylhydrazyl
EL	Ethyl acetate leaf
ELS	Extra long staple
ES	Ethyl acetate stem
GC	Gas chromatography
GFP	Green Flourescent Protein
GSH	glutathione
HL	Hexane leaf
HRBC	Human Red Blood Cells
HS	Hexane stem
MS	Mass Spectroscopy
NADH	Reduced Nicotinamide Adenine Dinucleotide
NADPH	Reduced Nicotinamide Adenine Dinucleotide Phosphate
RBC	Red Blood Cells
ROS	Reactive Oxygen Species
SOD	Superoxide dismutase
UV	Ultraviolet

CHAPTER ONE

1.0 INTRODUCTION

1.1 Free Radicals

Free radical is a chemical compound which contains an unpaired electron spinning on the peripheral electron orbital layer around the nucleus. The family of free radicals generated from oxygen during energy production or oxidation (a normal physiological and metabolic process in the cell) is called reactive oxygen species (ROS) which cause damage to other molecules by extracting hydrogen ion from them or donating electrons to them in order to attain stability (Singh *et al.*, 2009).

However, these free radicals have been claimed to affect human health by causing several diseases including cancer, hypertension, heart attack and diabetes due to over-production of reactive species, induced by exposure to external oxidants or a failure in the defense mechanisms of the body to protect against oxidative damage to cell structures (Amin *et al.*, 2010).

Despite the fact that the body possesses antioxidant defense mechanisms such as enzymatic antioxidant systems – [superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT)] - and non enzymatic antioxidant systems – [ascorbic acid (vitamin C), α-tocopherol (vitamin E), glutathione (GSH), carotenoids and flavonoids] - normal levels of antioxidant system in the body are not sufficient for the eradication of the free radical injury. These injuries occur when natural defenses of the organism are overwhelmed by an excessive generation of Reactive Oxygen Species (ROS), leading to a situation of 'oxidative stress', in which cellular and extra cellular macromolecules (proteins, lipids, and nucleic acids) suffer oxidative damage, thereby causing tissue injury (Adedapo *et al.*, 2009a). These free radicals attack the unsaturated fatty acids in the biomembranes resulting in membrane lipid peroxidation, a decrease in

membrane fluidity, loss of enzymes, loss of receptor activity and damage to membrane proteins leading to cell inactivation. As a result of this, much attention has been focused on the use of antioxidants (compounds that inhibit or delay the oxidation process by blocking the initiation or propagation of oxidizing chain reactions), especially natural antioxidants to inhibit lipid peroxidation and to protect biomolecules from damage and many kinds of cellular degeneration caused by free radicals (Adedapo *et al.,* 2009a). Synthetic antioxidants like butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) that are commonly used have side effects and are also carcinogenic (Ali *et al.,* 2008), thus there is a need for more effective, less toxic and cost effective antioxidants.

1.2 Medicinal Plants

Medicinal plants are now in greater demand due to their increased popularity and it is being suggested by a large number of conservation groups, that wild medicinal plants should be brought into cultivation because numerous medicinal plants as well as their purified components have shown beneficial therapeutic potentials.

Consumption of medicinal herbs is increasing immensely over past decade as an alternative approach to improve the quality of life and maintain good health. Medicinal plants have been used for centuries as remedies for human diseases (Arokiyaraj *et al.,* 2008). It has long been recognized that naturally occurring substances in higher plants have antioxidant activity. The harmful action of the free radicals can be blocked by antioxidant substances, which scavenge the free radicals and detoxify the organism. Current research into free radicals has confirmed that foods rich in antioxidants play an essential role in the prevention of cardiovascular diseases (Adedapo *et al.,* 2009a, Abbas *et al.,* 2013), cancers and neurodegenerative diseases, including Parkinson's and Alzheimer's diseases (Adedapo *et al.,* 2009a), as well as inflammation and

problems caused by cell and cutaneous aging (Kumaran and Karunakaran, 2006; Adedapo *et al.*, 2009a).

A great number of aromatic and other medicinal plants contain chemical compounds that exhibit antioxidant properties. Sources of natural antioxidants are primarily, plant phenolics that may occur in all parts of plants such as fruits, vegetables, nuts, seeds, leaves, roots and barks (Adedapo *et al.,* 2009b). Majority of the antioxidant potential is due to the presence of flavones, flavonoids, isoflavones, anthocyanin, coumarin, catechins and isocatechins in plants (Muhammad *et al.*, 2012).

Phenolic compounds contribute to the quality and nutritional value of food, in terms of the modification of color, aroma, taste, and flavour, and also contribute to the beneficial effects for health. Phenolic compounds have also been reported to serve in defense mechanisms of plants to combat reactive oxygen species (ROS), in order to preserve total antioxidant activity (Suganya *et al.,* 2012). Polyphenolic compounds play an important role in the lipid-oxidation, and are closely associated with antioxidant activity. Therefore, phenolic compounds are considered to contribute directly to oxidative capacity (Abbasi *et al.*, 2012).

1.3 *Gossypium barbadense*

Gossypium barbadense is a species of cotton plant. It is also known as extra long staple (ELS) cotton as it generally has a staple of at least 36mm or longer. It is a tropical, frost-sensitive perennial plant that produces yellow flowers and has black seeds. It grows as a small, bushy tree and yields cotton with unusually long, silky fibers.

Cotton originated from dry regions and requires consistently hot temperatures for best yield; dry conditions during boll maturation contribute to fibre quality. *G. barbadense* is more tolerant to cold temperatures and early planting than *G. hirsutum* (OGTR, 2008).

G. barbadense seedling development in the first two weeks is generally insensitive to temperatures between 15°C and 40°C, although once the seedling has established the height, yield and rate of development can all be affected by temperature the optimum range for *G. barbadense* is between 25–30°C with only 30% yield at 35°C (OGTR, 2008)

Plants are known medicinally as a mean for treatment of diseases and infections and they are an alternative strategy in search for new drugs. *Gossypium barbadense* is a plant well known for the cotton it produces. It also has some medicinal applications in emetics, veneral diseases, tumors, paralysis, epilepsy, convulsions, spasm, and cutaneous and subcutaneous parasitic infection. It has antifungal properties and contains the chemical gossypol, making it less susceptible to insect damage (Turco *et al.*, 2004; Stipanovic, *et al*., 2006). It is also sometimes used as a male anti-fertility drug (Coyle *et al.*, 1994). In Suriname's traditional medicine, the leaves of *G. barbadense* are used to treat hypertension and delayed or irregular menstruation (Hasrat *et al.*, 2004). It is known to have antimalarial effect. In a preliminary report, gossypol which is an active constituent of *G. barbadense*, was reported to have an *in vitro* antimalarial activity against the human pathogen *Plasmodium falciparum.* Also, the plant has been said to have antibacterial activity (Roberta *et al.*, 2009) and anticancer activity (Deshmukh and Sakarkar, 2011).

1.4 Statement of Research Problem / Justification

DNA damage is very frequent especially DNA oxidative damage from reactive oxygen species and it appears to be a fundamental problem for life. DNA damage can trigger different mutations

ranging from lesions on DNA and breaks in DNA strands to faulty links and base gaps in sequences, development of cancer, and accelerate aging. Most DNA damage is removed by DNA repair enzymes, but these repair processes are not completely efficient. DNA oxidative damage can be prevented especially by plants which are known to have free radical scavenging properties.

The beneficial effect of antioxidants on promoting health is believed to be achieved through several possible mechanisms, such as directly reacting with and quenching free radicals, chelating transition metals, reducing peroxides, and stimulating the antioxidative defense enzyme system (Zhou *et al.,* 2004). It has also been proposed that antioxidant activity of plant origin components can be mainly ascribed to the presence of phenolic compounds (Heim *et al.,* 2002). Many polyphenolic compounds as flavonoids and phenolic acid from plant materials such as herb extract have shown the antioxidant activity against ROS. Phenolic compounds are not evenly distributed in plant parts; they are present at elevated amounts in the outer parts of the fruits, leaf and bark (Pieta, 2000).

Very little work has been done about the detailed antioxidant and DNA protecting activities of leaf and stem bark of this plant hence, there is need for study with the main objective of evaluating the antioxidant and DNA protecting activities of this plant, *Gossypiun barbadense.*

1.5 Aims and Objectives

The general aim of this research is to evaluate the free radical scavenging and DNA protecting activities of *Gossypium barbadense in vitro.*

The specific objectives are:

(i) To determine the qualitative and quantitative profile of the total phenols and the total flavonoids in the leaf and stem of *Gossypium barbadense*

(ii) To determine the free radical scavenging activity of the plant, *Gossypium barbadense in vitro*

(iii) To determine the DNA protecting property of the extracts of *Gossypium barbadense in vitro*

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 *Gossypium barbadense* Habitat

Gossypium barbadense L. was named after its assumed habitat of Barbados. The geographical centre for *G. barbadense* is South America (Jenkins 2003) from where it was dispersed to West Indies and the Galapagoes, and from Senegal, it entered into Nigeria. It is a tropical perennial plant with yellow flowers and black seeds. It contains the chemical compound Gossypol which confers insect resistance to the plant.

2.1.1 Classification of *Gossypium barbadense*

Its classification by United States Department of Agriculture, Natural Resources Conservation Service is as follows:

Kingdom	*Plantae* – Plants
Subkingdom	*Tracheobionta* – Vascular plants
Superdivision	*Spermatophyta* – Seed plants
Division	*Magnoliophyta* – Flowering plants
Class	*Magnoliopsida* – Dicotyledons
Subclass	*Dilleniidae*
Order	*Malvales*
Family	*Malvaceae* – Mallow family
Genus	*Gossypium* L. – cotton
Species	*Gossypium barbadense* L. – Creole cotton

2.2 Uses of cotton plant

2.2.1 Commercial uses

Cotton is currently the leading plant fibre crop worldwide and is grown commercially in the temperate and tropical regions of more than 50 countries (Zhu *et al.*, 2011). It is estimated that cotton is cultivated on approximately 2.4% of the world's arable land (Blaise 2006). It is produced in areas where climatic conditions suit natural growth requirements, including periods of hot and dry weather and where adequate moisture is available. *G. barbadense* is grown for its fibre quality as it has longer staple length (44 – 46 staple length) and higher fibre strength than other species of cotton.

Cotton is primarily grown as a fibre crop but it is harvested as seed cotton which is then ginned to separate the seed and the lint. The long lint fibres are further processed by spinning to produce yarn that is knitted or woven into fabrics. Cotton fabrics made from cotton lint are used in clothing, upholstery, towels and other household products (OECD, 2008).

Cotton seeds are also processed into oil, meal and hulls. The oil from cotton seed is used in a variety of products including edible vegetable oils and margarine, soap and plastics (Agarwal *et al.*, 2003). Cotton seed or meal, flour or hull is used in food products and for animal feed, but is limited by presence of natural toxicants in the seeds. Cotton trash can be used as a bulking agent to improve the efficacy of animal manure composting (Brampton 2001). In USA, cotton trash has been investigated as an industrial fuel for power plant or combined with pecan shells to produce BBQ briquettes (Coates, 2000, OGTR, 2008). In addition, there are some interests in using cotton waste to produce ethanol through fermentation (Joel and Agblevor 2001).

In West Africa the fibre of *G. barbadense* is locally used for weaving articles such as waistbands, neckerchiefs and armlets. In southern Nigeria it forms the basis of a local spinning and weaving industry of some importance. In Cameroon the fibre is made into cloth used for dowries and in burials. In Gabon the fibre is made into bags. In Congo it is used for making coarse textiles, cords, string, fishing nets and straps. *G. barbadense* is sometimes grown as an ornamental. It is bee forage, giving higher honey yields than *Gossypium hirsutum* (OECD, 2008).

2.2.2 Nutritional and medicinal uses of cotton plant

In traditional medicine, *G. barbadense* leaves have been used as a treatment for swollen tissue around a wound and for nausea during pregnancy (Essien *et al.,* 2011). Currently, *G. barbadense* extracts are sold for use in alternative medicine for treatment of hypertension, fungal infections, and as menstruation stimulant (Tropilab inc. 2007). Extracts from *G. barbadense* have been shown to have anti-hypotensive effects in rats (Hasrat *et al.,* 2004) and to increase smooth muscle contraction in guinea pigs (Mans *et al.,* 2004). Gossypol has also been studied for its use as a treatment for cancer. Human melanoma cells show cytotoxicity to gossypol, with a 5-fold greater cytotoxic sensitivity to the (–)-gossypol enatiomer than the (+)-enatiomer, suggesting that the (–)-gossypol enatiomer may have some potential therapeutic benefits in melanoma patients. Gossypol has also been investigated as a human contraceptive, and shown to be highly effective, although it has irreversible effects in approximately 20% of men (Coutinho 2002). It has also been investigated as an antiparasitic agent. *In vitro* experiments showed that gossypol reduced the growth of both *Trypanosoma cruzi*, the causal agent of chagas disease, and *Entamoeba histolytica*, which causes amoebiasis (Wang *et al.,* 2008).

Gossypium herbaceum Linn, one of the members of the cotton family, was reported as traditional medicine plant with the unique properties like antifertility, antispermatogenic, antitumor, contraceptive, antidibetic, antiviral, galactagogue, and antibacterial activity**.** It was also reported in the treatment of tooth pain (Sharma *et al.,* 2011; Sultana *et al.,* 2012).

Agarwal *et al.,* (2011) reported that the extract of *Gossypium herbaceum* has antimicrobial property. One of the study showed that aqueous extract o*f Gossypium herbaceum* showed significant antidepressant-like effect due to activation of adenyl cyclase- cAMP pathway in signal transduction system and hence protecting the neurons from the lesion (Dhamija *et al.,* 2012). The aqueous and ethanolic extracts of flowers of *G. herbaceum* L. was reported to increase healing of gastric ulcer and possess potential antiulcer activity (Baiter *et al.,* 2005).

Velmurugan *et al.,* (2012) in their study confirmed that methanolic extract of the leaf of *G. herbaceum* have wound healing activity that may be attributed to presence of different phyto-constituents like flavonoids and tannins. A study reported that Hydro-alcoholic extract of *G. herbaceum* possess antioxidant activity which was attributed to its phenolic content. *G. herbaceum* reduces the free radicals to corresponding hydrazine when it reacted with hydrogen donors from DPPH antioxidant principle (Sharma *et al.,* 2011).

A phyto-therapeutic preparation containing *Gossypium herbaceum* was tested by Mello (Feitosa *et al.,* 2012) for preclinical toxicity, and the results revealed the absence of systemic toxicity at a therapeutic dose.

2.3 Chemical Constituents of Cotton Plant

Cotton is not a pathogen and not capable of causing disease in humans, animals or plants. However, it does contain a number of compounds which may have adverse effects on human and

animal health. The most studied of these is gossypol [1,1',6,6',7,7'- hexahydroxy-5,5'-diisopropyl-3,3'-dimethyl-(2,2-binaphthalene)-8,8'-dicarboxaldehyde]. This is a yellow polyphenolic compound found primarily in the pigment glands of the cotton seeds, leaves and roots (Coutinho 2002) and is generally removed before cotton seed can be eaten. Cotton plants also contain cyclopropenoid fatty acids (CPFA) (including dihydrosterculic, sterculic and malvalic acids) in the seed and tannins in the leaves (OECD, 2008) and flower buds which are both thought to act as deterrents to insect herbivory and may affect utilization as animal feed.

The chemical composition of the essential oil of the Nigerian-grown cotton leaf, *G. barbadense* L., analyzed by GC and GC/MS techniques was reported to have about nineteen components, accounting for 92.6% of the total oil fraction. The major constituents were the monoterpenes comprising of tricyclene (29.6%), bornyl acetate (18.6%), α-pinene (12.8%), α-terpinene (11.1%), Isoledene (6.0%) and β -pinene (5.4%), r-cymene (2.1%) and terpinolene (2.0%). (Essien *et al.,* 2011)

2.4 Reactions of Free Radicals

Free radicals are routinely referred to as Reactive Oxygen Species (ROS) in biological sciences, since most of the biologically important free radicals are all oxygen-centered, although not all free radicals are ROS.

Free radicals and reactive oxygen species (ROS) contribute to the occurrence of many degenerative diseases such as arthritis, cirrhosis, cancer, Alzheimer and aging which can be prevented by the presence of antioxidants (Amin *et al.,* 2010).

Free radicals undergo four primary types of chemical reactions; these are:

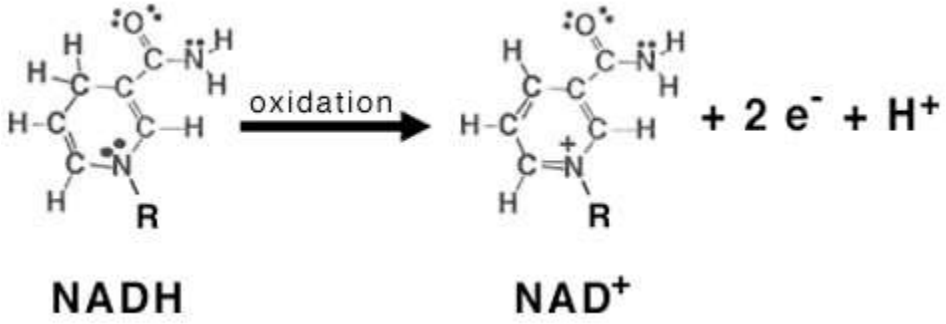
- *Hydrogen abstraction:* in which a radical interacts with another molecule that has a free hydrogen atom (i.e., a hydrogen donor). As a result, the radical binds to the hydrogen atom and becomes stable, whereas the hydrogen donor is converted to a free radical.
- *Addition:* in which the radical binds to another, originally stable molecule, converting the combined molecule into a radical.
- *Termination:* in which two radicals react with each other to form a stable compound.
- *Disproportionation:* in which two identical radicals react with each other, with one of the radicals donating an electron to the other so that two different molecules are formed, each of which is stable

2.4.1 Sources of free radicals in a cellular organism

One chemical element frequently involved in free radical formation in a cellular organism is oxygen. Molecular oxygen (O_2) is essential for cell function because it plays a pivotal role in a series of biochemical reactions occurring in the respiratory chain, which is responsible for most of the production of adenosine triphosphate (ATP), which provides the energy required for a multitude of cellular reactions and functions (Lambeth, 2004).

In the respiratory chain, which takes place in membrane–enclosed cell structures called mitochondria, electrons and a proton (H^+) are removed from a helper molecule (i.e., cofactor) called reduced nicotinamide adenine dinucleotide (NADH). The electron is transferred to the first component of the respiratory chain, and the proton is released into the surrounding fluid. Chemically, NADH is oxidized to NAD^+ in this reaction, whereas the respiratory chain component that accepts the electron is reduced. The NAD^+ subsequently can be used again to

accept new hydrogen atoms that are generated during the metabolism of sugars (e.g., glucose) and other nutrients.



The reduced respiratory chain component, in turn, passes the electron on to other molecules in the respiratory chain until it is finally transferred to O_2, which then interacts with protons in cells to generate water.

$$^1/_2\ O_2 + 2H^+ + 2e^- \text{--> } H_2O$$

These series of electron transfer reactions generate sufficient energy to produce several molecules of ATP for each electron that passes through the respiratory chain.

Molecular oxygen can accept a total of four electrons, one at a time, and the corresponding number of protons to generate two molecules of water. During this process, different oxygen radicals are successively formed as intermediate products, including superoxide ($O_2^{\bullet -}$); peroxide ($O_2^{=}$), which normally exists in cells as hydrogen peroxide (H_2O_2); and the hydroxyl radical ($^{\bullet}OH$). Superoxide, peroxide, and the hydroxyl radicals are considered the primary ROS and have caused major research on the role of free radicals in biology and medicine. (Superoxide can react with itself to produce H_2O_2. Thus, systems producing superoxide also will result in formation of H_2O_2. Technically, H_2O_2 is not a free radical, but it is commonly included among the ROS.)

However, because they are unstable and rapidly react with additional electrons and protons, most of these ROS are converted to water before they can damage cells. It has been estimated that only about 2 to 3 percent of the O_2 consumed by the respiratory chain is converted to ROS (Lambeth, 2004). Nevertheless, the toxic effects of oxygen in biological systems—such as the breakdown (i.e., oxidation) of lipids, inactivation of enzymes, introduction of changes (i.e., mutations) in the DNA, and destruction of cell membranes and, ultimately, cells—are attributable to the reduction of O_2 to ROS (Govindappa *et al.*, 2011).

The major source of ROS production in the cell is the mitochondrial respiratory chain, and utilizes approximately 80 to 90 percent of the O_2 a person consumes. Thus, even though only a small percentage of that oxygen is converted to ROS, the mitochondrial respiratory chain in all cells generates most of the ROS produced in the body (Nathan and Cunningham-Bussel, 2013).

Another major source of ROS, especially in the liver, is a group of enzymes called the cytochrome P450 mixed–function oxidases. Many different variants of these iron–containing enzymes exist, some of which are responsible for removing or detoxifying a variety of compounds present in our environment and ingested (e.g. foods or drugs), including alcohol. Some cytochrome P450 enzymes also are important for metabolizing substances that naturally occur in the body, such as fatty acids, cholesterol, steroids, or bile acids. The biochemical reactions catalyzed by the cytochrome P450 molecules use molecular oxygen, and during these reactions small amounts of ROS are generated. The extent of ROS generation may vary considerably depending on the compound to be degraded and on the cytochrome P450 molecule involved. One type of cytochrome molecule that is especially active in producing ROS is known as CYP2E1 (Wu and Cederbaum, 2003).

Other sources of ROS in the body are two types of immune cells called macrophages and neutrophils, which help defend the body against invading microorganisms. In this case, however, ROS production is beneficial and even essential to the organism because it plays a central role in destroying foreign pathogens (Lambeth *et al.*, 2000). Macrophages and neutrophils contain a group of enzymes called the NADPH oxidase complex, which, when activated, generates superoxide radicals and hydrogen peroxide. Hydrogen peroxide then interacts with chloride ions present in the cells to produce hypochlorite (the active ingredient in bleach), which in turn destroys the pathogen. The NADPH oxidase complex and the resulting ROS production are critical to the body's defense against all kinds of diseases, as is evident in patients with a condition called chronic granulomatous disease, in which ROS production by the NADPH oxidase complex is drastically reduced. Patients with this condition are highly sensitive to infections and usually die at an early age (Lambeth *et al.*, 2000, Park *et al.*, 2006).

Besides the ROS generation that occurs naturally in the body, humans are constantly exposed to environmental free radicals, including ROS, in the form of radiation, UV light, smog, tobacco smoke, and certain compounds referred to as redox cycling agents, which include some pesticides, but also certain medications used for cancer treatment. The toxicity of these medications against tumor cells (as well as normal body cells) results from the fact that the compounds are modified by cellular enzymes to an unstable intermediate, which then reacts with molecular oxygen to produce the original product plus a superoxide radical. Thus, a vicious cycle of chemical reactions involving these compounds continually produces ROS **(**Wu and Cederbaum, 2003**).**

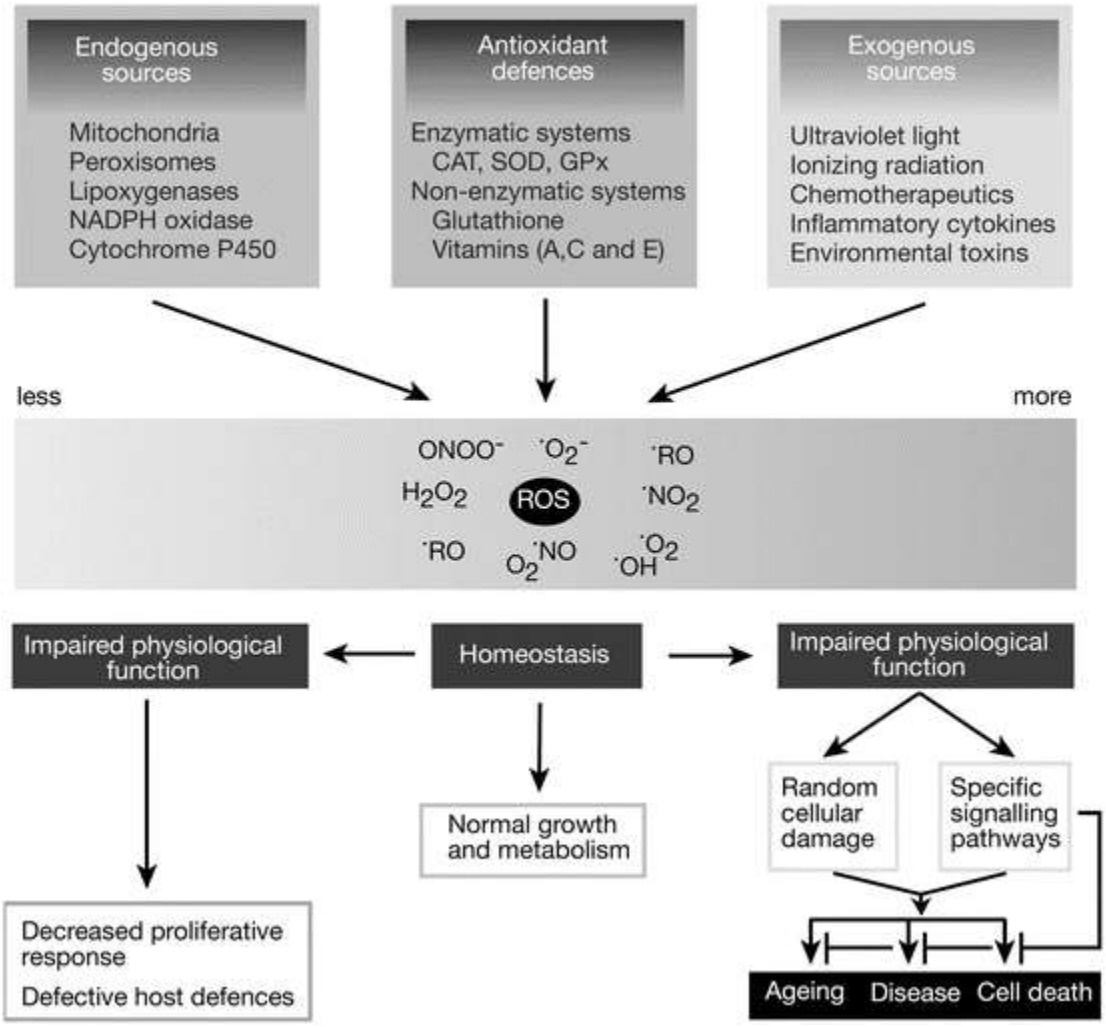


Figure 2.1 Sources of ROS, antioxidant defences, and subsequent biological effects depending on the level of ROS production (Finkel and Holbrook, 2000)

2.4.2 Free Radicals and Antioxidant Systems

There is a well maintained balance which exists between production of free radicals and antioxidant defense mechanisms in a healthy individual (Poljsak *et al.,* 2013). In pathophysiological conditions, this balance shifts towards overproduction of free radicals or lowering of antioxidant defenses results in oxidative stress. Oxidative stress is excess formation and/or incomplete removal of highly reactive molecules such as ROS, including superoxide radical, hydroxyl radical and so on, as well as non-radical species such as hydrogen peroxide. Free radicals are implicated in the etiology of several degenerative diseases such as stroke, coronary artery diseases, rheumatoid arthritis, diabetes and cancer. These radicals are capable of damaging essential biomolecules such as proteins, DNA and lipids (Kekuda *et al.,* 2012; Poljsak *et al.,* 2013). In case of aerobic organisms, cell membrane is the major target for ROS where lipid peroxidation is induced. Membrane structure and function are affected and some oxidation products, such as malondialdehyde, are produced which react with biomolecules and exert cytotoxic and genotoxic effects. Endogenous antioxidants such as vitamin C, vitamin E, and so on, act as primary defense systems. However, in diseased states, there is an extra requirement for antioxidants from exogenous sources.

Substances termed antioxidants can influence the oxidation process through simple or complex mechanisms including the prevention of chain termination, binding to transitional metal ion catalyst, decomposition of peroxides, prevention of continued hydrogen abstraction and radical scavenging. They reduce oxidative damage and prevent or alleviate chronic diseases. In recent years, there is an increasing interest in the search for antioxidants from natural origin. The most effective antioxidants are flavonoids and other phenolic compounds present in plants particularly in herbs, seeds and fruits (Kekuda *et al.,* 2012).

Components of the antioxidant system may be classified into antioxidative enzymes, free radical scavenging dietary factors, endogenous non-enzymatic antioxidants, and compounds with indirect antioxidative properties. Antioxidative enzymes (superoxide dismutase, catalase and glutathione peroxidase) are directly involved in the detoxification of free radicals via catalytic action (Birben *et al*., 2011). These enzymes are synthesized by the organism, but the trace elements needed as cofactors must be supplied by the diet. For example selenium which is a cofactor for glutathione peroxidase is supplied in the diet. These enzymes also contain elements at their binding sites. The superoxide dismutase present in the cytosol contains zinc and copper, while the mitochondrial form of superoxide dismutase contains manganese (Birben *et al*., 2011).

In addition to trace elements that serve as cofactors of antioxidative enzymes, the diet also provides nutrients that react directly with free radicals in a non catalytic manner. Of these, vitamin E, vitamin C and carotenoids have attracted the widest scientific attention (Cordero *et al.*, 2010).

Vitamin C is a water-soluble antioxidant, which is present in the organism at high concentrations. It is able to scavenge superoxide anion radical, hydrogen peroxide, hydroxyl radical, singlet oxygen, and nitrogen oxide species. Vitamin C also regenerates tocopheroxyl radicals back to the reduced tocopherol form, and may react with air pollutants in the respiratory tract. Hence, its usage as standard for some of the antioxidant assays in vitro (Traber and Stevens, 2011).

Diet also contains non-nutrient components that may have antioxidative effects. For instance, phenolic and polyphenolic substances like flavonoids and catechin are potent antioxidants (Fang

et al., 2002). Flavonoids are able to scavenge superoxide, hydroxyl radical and peroxyl radicals (Halliwell *et al.,* 2005).

Accordingly it is evident that the antioxidative defense of the organism consists of a complex network of nutrients and endogenously synthesized components. The antioxidative action of a single substance is dependent on its interplay with other components of the system. Furthermore, antioxidants may also have pro-oxidant effects depending on their chemical and physical environment. For instance, β-carotene may act as a pro-oxidant in high oxygen tension (Clark, 2002), and vitamin E if the concentration of vitamin C, reduced glutathione or NADPH is inadequate. Vitamin C acts as a pro-oxidant in the presence of transition metals iron and copper (Clark, 2002).

Synthetic and natural food antioxidants are used routinely in foods and medicine especially those containing oils and fats to protect the food against oxidation. There are a number of synthetic phenolic antioxidants, butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) being prominent examples. Strong antioxidants activities have been found in berries, cherries, citrus, prunes, and olives. Green and black teas have been extensively studied in the recent past for antioxidant properties since they contain up to 30% of the dry weight as phenolic compounds. Apart from the dietary sources, a lot of medicinal plants also provide antioxidants and these include (with common names in brackets) *Acacia catechu* (kair), *Allium cepa* (Onion), *A. sativum* (Garlic,), *Aleo vera, Azadirachta indica* (Neem), *Bacopa monniera*), *Butea monosperma* (Palas,), *Camellia sinensis* (Green tea), *Cinnamomum verum* (Cinnamon), *Curcma longa* (Turmeric,), *Mangifera indica* (Mango), *Momordica charantia* (Bitter gourd), *Murraya koenigii* (Curry leaf), *Nigella sativa* (Black cumin), *Ocimum sanctum* (Holy basil), *Spirulina fusiformis*

(Alga), *Tinospora cordifolia* (Heart leaved moonseed), *Withania somifera* (Winter cherry), and *Zingiber officinalis* (Ginger) (Devasagayam *et al.,* 2004)

There are two main types of antioxidants (free radical scavengers) namely primary and secondary antioxidants which differ in their mechanisms of action (Lim *et al.,* 2007). Primary antioxidants scavenge free radicals and donate a hydrogen atoms or electrons to make the free radicals more stable. On the other hand, secondary antioxidants act by suppressing the formation of radicals thus preventing oxidative damage. One of the known free radical scavenging activity that occurs exogenously in human body is the inhibition mechanism of lipid oxidation (Barros *et al.,* 2007).

DPPH (1,1-diphenyl-2-picryl hydrazyl) is a free radical generating compound and is used to determine the radical scavenging activity of plant extracts. The DPPH radical scavenging assay has been used widely to evaluate the radical scavenging activity of the different type of antioxidant substances (Seow-Mun *et al.,* 2012).

2.4.3 Oxidative DNA damage

DNA is the cell's genetic material, and any permanent damage to the DNA can result in changes (i.e., mutations) in the proteins encoded in the DNA, which may lead to malfunctions or complete inactivation of the affected proteins. Thus, it is essential for the viability of individual cells or even the entire organism that the DNA remain intact. The building blocks of DNA molecules are called nucleotides; they consist of a sugar component and an organic base. Each DNA molecule consists of two strands of nucleotides held together by weak chemical bonds in a supercoiled form. Changes in the nucleotides in one strand can result in mismatch with the nucleotides in the other strand, yielding subsequent mutations (Wu and Cederbaum, 2003).

Different types of DNA damage have been identified that result from

(i) alkylating agents (essential for a number of biosynthetic processes), that can turn a legitimate base into either a mutagenic, miscoding deviant, or a lethal, noncoding lesion,

(ii) hydrolytic deamination that can directly change one base into another and

(iii) free radicals and reactive oxygen species formed by various photochemical processes (for example radiation) (Donat and Rajeshwar 2002)

Free radicals are a major source of DNA damage, causing strand breaks, removal of nucleotides, and a variety of modifications of the organic bases of the nucleotides. Although cells have developed repair mechanisms to correct naturally occurring changes in the DNA, additional or excessive changes caused by ROS or other agents can lead to permanent changes or damage to the DNA, with potentially detrimental effects for the cell.

The Fe^{2+}-dependent decomposition of hydrogen peroxide is called the Fenton reaction. Exposure of plasmid DNA to Fenton's reagent ultimately results in strand breaks formation, mainly due to the generation of reactive species - hydroxyl radical - and the subsequent free radical-induced reaction on plasmid DNA. The vast majority of DNA damage affects the primary structure of the double helix; that is, the bases themselves are chemically modified. These modifications can in turn disrupt the molecules' regular helical structure by introducing non-native chemical bonds that do not fit in the standard double helix. Hydroxyl radical react with nitrogenous bases of DNA producing base radicals and sugar radicals. The base radicals produced as a result of this, react with the sugar moiety causing breakage of sugar phosphate backbone of nucleic acid, resulting in strand break formation (Yumrutas and Saygideger, 2010).

Free radicals may also be generated by ionizing or ultraviolet radiation. Planned radiation exposure during radiotherapy and radiodiagnosis as well as unplanned exposures (occupational, accidental and natural background exposure) are increasing the risk for man and will continue to increase as the whole world is in the race of nuclear weapons testing. Exposure of a biological system to ionizing radiation results in a rapid burst of reactive oxygen species (ROS) such as $O_2^{\bullet-}$, $H^{\bullet}$, $HO_2^{\bullet}$ and $OH^{\bullet}$ (Saini *et al.,* 2007), which are highly damaging for cellular macromolecules like DNA, proteins, lipids and up to some extent for carbohydrates. Exposure of plasmid DNA to UV radiation in the presence of H_2O_2 produces hydroxyl radical which in turn reacts with the bases of DNA.

2.4.3.1 Mechanisms of oxidative damage to DNA

Of the reactive oxygen species, the highly reactive hydroxyl radical ($^{\bullet}OH$) reacts with DNA by addition to double bonds of DNA bases and by abstraction of an H atom from the methyl group of thymine and each of the C-H bonds of 2-deoxyribose (Cooke *et al.,* 2003; Dizdaroglu and Pawel, 2012). The non - radical reactive oxygen species most commonly responsible for oxidative damage is hydrogen peroxide, the precursor to the hydroxyl radical.

Hydroxyl radical is highly reactive with a half-life in aqueous solution being less than 1 ns. Thus when produced *in vivo* it reacts close to its site of formation. It can be generated through a variety of mechanisms. Ionizing radiation causes decomposition of H_2O, resulting in formation of $^{\bullet}OH$ and hydrogen atoms. $^{\bullet}OH$ is also generated by photolytic decomposition of alkyl hydroperoxides. Production of $^{\bullet}OH$ close to DNA could lead to this radical reacting with DNA bases or deoxyribosyl backbone of DNA to produce damaged bases or strand breaks. It has been proposed that the extent of DNA strand breaking by •OH is governed by the accessible surface

areas of the hydrogen atoms of the DNA backbone. Hydroxyl radical also causes addition to DNA bases leading to generation of a variety of oxidative products (Valko *et al.,* 2004).

The reaction of hydroxyl radical with a biomolecule will produce another radical, usually of lower reactivity. As a result of the high reactivity of $^{\bullet}OH$, it often abstracts carbon-bound hydrogen atoms more or less non-selectively, e.g. from glucose. Production of $^{\bullet}OH$ close to an enzyme molecule present in excess in the cell, such as lactate dehydrogenase, might have no biological consequences. However, attack by $^{\bullet}OH$ on a membrane lipid can cause a series of radical reactions that can severely damage the membranes (Valko *et al.*, 2004)

Addition to the C_5-C_6 double bond of pyrimidines leads to C_5-OH and C_6-OH adduct radicals and H atom abstraction from thymine results in the alkyl radical. Adduct radicals differ in terms of their redox properties, with C_5-OH adduct radicals being reducing and C_6-OH adduct radicals oxidizing. Radicals are reduced or oxidized depending on their redox properties, redox environment, and reaction partners (Cooke *et al.,* 2003). Product types and yields depend on absence and presence of oxygen and on other conditions. In the absence of oxygen, the oxidation of C_5-OH adduct radicals, followed by addition of OH^- (or addition of water followed by deprotonation), leads to cytosine glycol and thymine glycol (Cooke *et al.,* 2003; Dizdaroglu and Pawel, 2012). The alkyl radical yields 5-hydroxymethyluracil. C_5-OH-6-peroxyl radicals are formed by addition of oxygen to C_5-OH adduct radicals at diffusion-controlled rates. C_5-OH-6-peroxyl radicals eliminate $O_2^{\bullet-}$, followed by reaction with water (addition of OH^-) to yield thymine and cytosine glycols. Oxygen reacts with the alkyl radical, leading to 5-hydroxymethyluracil and 5-formyluracil. Thymine peroxyl radicals are reduced, followed by protonation to give hydroxyl hydroperoxides, which decompose and yield thymine glycol, 5-hydroxymethyluracil, 5-formyluracil, and 5-hydroxy-5- methylhydantoin (Cooke *et al.,* 2003).

Products of cytosine may deaminate and dehydrate. Cytosine glycol deaminates to give uracil glycol, 5-hydroxycytosine, and 5-hydroxyuracil. However, cytosine glycol, uracil glycol, 5-hydroxycytosine, and 5-hydroxyuracil were all detected in irradiated cytosine, indicating that all these compounds may simultaneously be present in damaged DNA. In the absence of oxygen, C_5-OH adduct radicals may be reduced, followed by protonation to give 5-hydroxy-6-hydropyrimidines. 5-Hydroxy-6-hydrocytosine readily deaminates into 5-hydroxy-6-hydrouracil. Similarly, C_6-OH adduct radicals of pyrimidines may lead to 6-hydroxy-5 hydropyrimidines. These products are typical of anoxic conditions because oxygen inhibits their formation by reacting with OH adduct radicals (Dizdaroglu and Pawel, 2012).

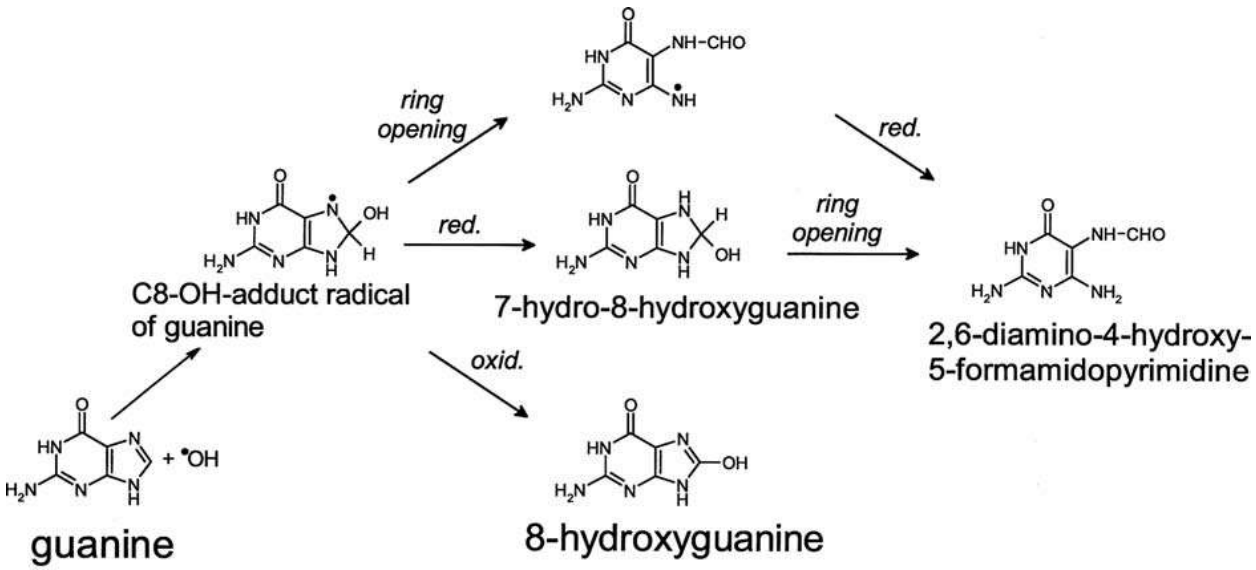


Figure 2.2 Structures of various products formed from the C8-OH–adduct radical of guanine, which itself is formed by attack of •OH on the C8-position of guanine (Valko *et al.*, 2004)

Further reactions of C_5-OH-6-peroxyl and C_6-OH-5- peroxyl radicals of cytosine result in formation of 4-amino- 5-hydroxy-2, 6(1H,5H)-pyrimidinedione and 4-amino-6- hydroxy-2,5(1H,6H)-pyrimidinedione, respectively, which may deaminate to give dialuric acid and isodialuric acid, respectively. The detection of 4-amino-6-hydroxy- 2,5 (1H,6H)-pyrimidinedione and isodialuric acid in DNA suggested that both compounds may simultaneously exist in DNA. Oxygen oxidizes dialuric acid to alloxan (9, 11). Decarboxylation of alloxan yields 5-hydroxyhydantoin upon acidic treatment. Intramolecular cyclization of cytosine C_5-OH-6-hydroperoxide gives rise to *trans*-1-carbamoyl- 2-oxo-4, 5-dihydroxyimidazolidine as a major product in cytosine (Teoule, 1987; Cooke *et al.,* 2003). Hydroxyl radical add to the C_4, C_5, and C_8 positions of purines generating OH adduct radicals. In the case of adenine, at least two OH adducts are formed: C_4-OH and C_8-OH adduct radicals. C_4-OH and C_5-OH adduct radicals of purines dehydrate and are converted to an oxidizing purine $(-H)^{\bullet}$ radical, which may be reduced and protonated to reconstitute the purine. C_4-OH adduct radicals possess oxidizing properties, whereas C_5-OH and C_8-OH adduct radicals are primarily reductants. The guanine radical cation ($guanine^{\bullet -}$) is formed by elimination of OH^- from the C_4-OH adduct radical of guanine and may deprotonate depending on pH to give guanine $(-H)^{\bullet}$. The radical cation does not hydrate to lead to the C_8-OH adduct radical and then to 8-hydroxyguanine by oxidation; however, it may react with 2-deoxyribose in DNA by H abstraction causing DNA strand breaks (Cooke *et al.,* 2003; Dizdaroglu and Pawel, 2012).

Reactions of pyrimidines and purines result in multiple products in DNA. Most of these modified bases were identified in DNA in vitro and in mammalian cells upon exposure to free radical generating systems. Another reaction of base radicals is the addition to an aromatic amino acid of proteins or combination with an amino acid radical, leading to DNA–protein cross-linking

(Cooke *et al.,* 2003). Reactions of $^{\bullet}OH$ with the sugar moiety of DNA by H abstraction give rise to sugar modifications and strand breaks. These compounds represent a concomitant damage to both base and sugar moieties and are considered tandem lesions. Oxygen inhibits their formation by reacting with the C_5-centered sugar radical before cyclization. Were it not for cellular defenses such as low molecular weight antioxidants, enzymic antioxidants, and DNA repair, levels of such oxidatively modified bases would rapidly represent the majority of bases in DNA (Cooke *et al.,* 2003; Dizdaroglu and Pawel, 2012).

2.4.3.2 Mechanisms of self DNA repair

Accumulation of damaged DNA has been considered to contribute to some of the features of aging. It is not surprising that a complex set of cellular surveillance and repair mechanisms has evolved to reverse the potentially deleterious damage that would otherwise destroy the precious blueprint for life. Some of these DNA repair systems are so important that life cannot be sustained without them. An increasing number of human hereditary diseases that are characterized by severe developmental problems and/or a predisposition to cancer have been found to be linked to deficiencies in DNA repair.

DNA Repair DNA repair can be grouped into two major functional categories: A) Direct Damage reversal B) Excision of DNA damage (Sancar *et al.,* 2004).

The direct reversal of DNA damage is by far the simplest repair mechanism that involves a single polypeptide chain, with enzymatic properties which binds to the damage and restores the DNA genome to its normal state in a single-reaction step. The major polypeptides involved in this pathway are DNA photolyases, the enzymes responsible for removing cyclobutane pyrimidine dimers from DNA in a light-dependent process called as photo reactivation and 6-methylguanine-DNA methyltransferase I and II (MGMT), also called DNA alkyltransferases

which remove the modified bases like 6-alkylguanine and 4-alkylthymine. The photolyase protein is not found in all living cells however, the DNA alkyltransferases are widespread in nature.

The excision repair mechanisms is the most ubiquitous and versatile modes of DNA repair and are targeted at the removal of bulky DNA adducts and UV-induced photoproducts, base-pair alterations and purine loss, DNA mismatch, single- and double- strand DNA breaks. In addition, DNA replication, recombination and transcription are all involved in DNA repair pathways (Lisa *et al.*, 2002).

The excision repair include i) Base excision repair (BER) ii) Nucleotide excision repair (NER), iii) Mismatch repair (MMR) and iv) Strand break repairs.

i) Base Excision Repair (BER) is initiated by DNA glycosylases (e.g. enzyme uracil DNA glycosylase), which catalyze the hydrolysis of the N-glycosidic bonds, linking particular types of chemically altered bases to the deoxyribose-phosphate backbone. DNA damage is excised as free bases, generating sites of base loss called apurinic or apyrimidinic (AP) sites. The AP sites are substrates for AP endonucleases which produce incisions in duplex DNA as a result of the hydrolysis of a phosphodiester bond immediately 5' or 3' to each AP site. The ribose-phosphate backbone is then removed from the DNA through the action of a specific exonuclease called deoxy ribophosphodiesterase or dRpase. Finally, the DNA polymerase and a ligase catalyze the incorporation of a specific deoxyribonucleotide into the repaired site, enabling correct base pairing (Lisa *et al.*, 2002).

ii) Nucleotide excision repair (NER) is a much more complex biochemical process than BER, especially in eukaryotic cells. This mechanism is used to replace regions of damaged DNA up to

30 bases in length. Common causes of such DNA damage include ultraviolet (UV) light, which induces the formation of cyclobutane pyrimidine-pyrimidine dimers, and smoking, which causes formation of benzo[a]pyrene-guanine adducts. Several gene products are required in a multiple step process, during which the ordered assembly of DNA proteins provides an enzymatic complex that discriminates damaged from undamaged DNA. In eukaryotic cells, the enzymes cut between the third to fifth phosphodiester bond (3' from the lesion and on the 5' side) the cut is somewhere between the twenty-first and twenty-fifth bonds thus, a fragment of DNA 27–29 nucleotides long is excised. After the strand is removed, it is replaced again by exact base pairing, through the action of yet another polymerase, and the ends are joined to the existing strands by DNA ligase.

iii) Mismatch repair (MMR): This mechanism corrects errors made in a single mismatch base pair or a short region of unpaired DNA when DNA is copied. (For example, a Cytosine could be inserted opposite an Adenine rather than a thymine to Adenine, or the polymerase could slip or stutter and insert two to five extra unpaired bases). Specific proteins scan the newly synthesized DNA, using adenine methylation within a GATC sequence as the point of reference. The template strand is methylated, and the newly synthesized strand is not. This allows the repair enzymes to identify the strand that contains the errant nucleotide which requires replacement. If a mismatch or small loop is found, a GATC endonuclease cuts the strand bearing the mutation at a site corresponding to the GATC. An exonuclease then digests this strand from the GATC through the mutation, thus removing the faulty DNA. This can occur from either end if the defect is bracketed by two GATC sites. The defect is then filled in by normal cellular enzymes according to base pairing rules.

iv) Strand Breaks Repairs: Single-strand breaks (SSBs) and double-strand breaks (DSBs) in the DNA backbone are produced by ionizing radiation and certain chemicals. Breaks in a single strand of the DNA molecule are repaired using the same enzyme systems that are used in Base-Excision Repair (BER). There are two mechanisms by which the cell attempts to repair a complete break in a DNA molecule (i.e. Double-Strand Break). The first mechanism include direct joining of the broken ends and this requires proteins that recognize and bind to the exposed ends and bring them together for ligating. This type of joining is also called Non Homologous End-Joining (NHEJ). The second mechanism involves a homologous Recombination where the broken ends are repaired using the information on the intact sister chromatid, or on the homologous chromosome (Lisa *et al.,* 2002).

2.5 Stabilization of RBC and Inflammation

Hemolysis is the breakage of the red blood cells (RBC's) membrane, causing the release of the hemoglobin and other internal components into the surrounding fluid.

Inflammation is the reaction of living tissues to injury, infection or irritation. Lysosomal enzymes released during inflammation produce a variety of disorders which leads to the tissue injury by damaging the macromolecules and lipid peroxidation of membranes which are assumed to be responsible for certain pathological conditions as heart attacks, septic shocks and rheumatoid arthritis etc. The extra cellular activity of these enzymes is said to be related to acute or chronic inflammation (Chippada *et al.,* 2011). Stabilization of lysosomal membrane is important in limiting the inflammatory response by inhibiting the release of lysosomal constituents of activated neutrophil such as bactericidal enzymes and proteases, which cause further tissue inflammation and damage upon extra cellular release or by stabilizing the lysosomal membrane (Rajendran and Lakshmi, 2008). HRBC or erythrocyte membrane is

analogous to the lysosomal membrane and its stabilization implies that the extract may as well stabilize lysosomal membranes. Stabilization of human red blood cell membrane (HRBC) by hypo tonicity induced membrane lysis can be taken as an in vitro measure of anti inflammatory activity of the drugs or plant extracts (Chippada *et al.*, 2011).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals and Reagents

Pmax GFP plasmid (DNA labs, Kaduna) Folin-Ciocalteu reagent, 2, 2-diphenyl-1-picrylhydrazyl (DPPH), 50mM Phosphate buffer, 10% H_2O_2, 1mM $FeSO_4$, potassium ferricyanide [$(K_3Fe(CN)_6$, hexane (Sigma-Aldrich), ethyl acetate (Sigma-Aldrich),, 10% ammonia solution, benzene, 10% hydrochloric acid, 0.1% ferric chloride, Mayer's reagent, Draggendorff's reagent, 2N Folin-Ciocalteu reagent, 10% Na_2CO_3, Gallic acid, Quercertin, 95% alcohol, 10% aluminium chloride hexahydrate ($AlCl_3 . 6H_2O$) , 1M potassium acetate (CH_3COO^-), 3% H_2O_2.

3.1.2 Equipment

Jenway 6305 Spectrophotometer, Grant JB Series Water Bath, Heraus Labofuge 300 Centrifuge, Gallenkamp Incubator, RS-232C Weighing Balance, Gel Doc 2000 (BioRad), **3.1.3 Plant sample, source and classification**

The leaf and bark of the plant *Gossypium barbadense* were obtained from Ilorin, Kwara State, Nigeria, in December, 2012. It was then identified and authenticated by Chief Botanist at Plant Biology; Department, University of Ilorin, Ilorin, Kwara State with number UIH977.

3.2 Methods

3.2.1 Preparation of Plant Extract

The plant parts of *Gossypium barbadense* were rinsed in clean water and then dried at room temperature in the laboratory for two weeks. After which it was ground to coarse powder with a mechanical grinder and kept in a clean air-tight glass container.

The dried sample was then extracted by three different solvents separately (hexane, ethyl acetate and distilled water) with a volume of solvent at ratio 1:10 each for 3 days to allow total extraction process. The extraction done in aqueous medium was left for 24hrs to avoid fermentation. The extract was concentrated on a water bath and reconstituted for the different analysis carried out.

3.2.2 Phytochemical Screening

The extracts were qualitatively tested for the presence of chemical constituents using the following methods.

3.2.2.1 Test for reducing sugars (Fehling's test)

The different solvent extracts were added to boiling Fehling's solution (A and B) in a test tube. The solution was observed for a colour reaction (Ayoola *et al.,* 2008).

3.2.2.2 Test for anthraquinones

Test for Free Anthraquinones (Borntrager's Test): Small portion (0.1g) of the extract was shaken with 10ml of benzene and then filtered. Exactly 5ml of a 10% of ammonia solution was then added to the filtrate and stirred using a stirrer. The production of a Pink-red or Violet colour indicates the presence of free anthraquinones.

Test for Combined Anthracene (Modified Borntrager's Test): Sample (0.1g) was boiled with 5ml of 10% hydrochloric acid for 3 mins. This process hydrolyzed the glycosides to yield aglycones which are soluble in hot water only. The solution obtained was then filtered hot; the filtrate was cooled and extracted with 5ml of benzene. The benzene layer was filtered off and shaken gently with half its volume of 10% ammonia solution. A rose-pink or cherry red colour indicates combined anthracene (Ayoola *et al.,* 2008)

3.2.2.3 Test for terpenoids (Salkowski test)

To 0.1g each of the powdered extract, 2 ml of chloroform was added. A 3 ml portion of concentrated H_2S0_4 was then carefully added to form a layer, and the mixture was then observed for colour (Ayoola *et al.*, 2008).

3.2.2.4 Test for flavonoids

Two methods were used to test for flavonoids as described by Ayoola, 2008. First, dilute ammonia (5 ml) was added to 0.1g of the extract of *Gossypium barbadense*. Concentrated sulphuric acid (1 ml) was then added. A yellow colouration that disappears on standing indicated the presence of flavonoids.

Secondly, a portion of 0.1g of the extract of *Gossypium barbadense* was heated with 10 ml of ethyl acetate over a steam bath for 3 min. The mixture was then filtered and 4 ml of the filtrate shaken with 1 ml of dilute ammonia solution (Ayoola *et al.*, 2008).

3.4.2.5 Test for saponins

To 0.1g of the powdered extracts of *Gossypium barbadense*, 5 ml of distilled water was added in a test tube. The solution was shaken vigorously and observed for a stable persistent froth. The frothing was then mixed with 3 drops of olive oil and shaken vigorously after which it was observed for the formation of an emulsion (Ayoola *et al.*, 2008).

3.2.2.6 Test for tannins

To 0.1g of the extract of *Gossypium barbadense* boiled in 10 ml of water in a test tube and then filtered, a few drops of 0.1% ferric chloride was added and observed for brownish green or a blue-black colouration which indicates the presence of tannins (Ayoola *et al.*, 2008).

3.2.2.7 Test for alkaloids

To 0.1g of the extract, 10 ml acid alcohol was added, boiled and filtered. To 5 ml of the filtrate obtained, 2 ml of dilute ammonia was added followed by 5ml of chloroform and shaken gently to extract the alkaloidal base. The chloroform layer was extracted with 10 ml of acetic acid. This was then divided into two portions. To one portion, Mayer's reagent was added and Draggendorff's reagent was added to the other, these were then observed for colour change (Ayoola *et al.,* 2008).

3.2.2.8 Test for cardiac glycosides (Keller-Killiani test)

To 0.1g of the different plant extract of *Gossypium barbadense*, 5ml of water was added followed by 2ml of glacial acetic acid containing one drop of ferric chloride solution. This was underlayed with 1ml of concentrated sulphuric acid. A brown ring at the interface which indicates the presence of a deoxysugar characteristic of cardenolides was looked out for (Ayoola *et al.,* 2008).

3.2.2.9 Phenols test:

This was carried out by treating the different solvent extracts of *Gossypium barbadense* with 5 ml of $FeCl_3$ solution**.** Appearance of bluish-black coloration indicates the presence of phenols.

3.2.3 Quantitative Determination of Total Phenols

Principle of reaction: The reaction is based on the reduction of phosphor-wolframate-phosphomolybdate complex by phenolics to a blue reaction product with a λ_{max} of 765nm which can be measured spectrophotometrically (Amin *et al.,* 2004).

Procedure: Total phenolics of various solvent extracts of plant studied were determined by the method of Makkar *et al.,* (1993). This was conducted with 0.1 ml of different solvent extracts combined with 2.8 ml of 10% Na_2CO_3 and 0.1 ml of 2N Folin-Ciocalteu reagent. After 40 minutes absorbance at 765 nm was measured by UV-visible spectrophotometer. Gallic acid was

used as a standard to get a seven-point standard curve (0–200 mg/L). The levels of total phenolic contents in various fractions obtained were determined using the standard curve. The data obtained was expressed as milligram of gallic acid equivalents (GAEs)/g powder and converted to microgram gallic acid equivalents (GAEs)/ g of the sample of *Gossypium barbadense*.

3.2.4 Quantitative Determination of Total Flavonoids

The total flavonoid content of *Gossypium barbadense* was quantified by using aluminum chloride colorimetric method described by Chang *et al.,* 2002. Aliquots of 0.001 g of different solvents extracts were dissolved in 1ml of deionized water. This solution (0.5 ml) was then mixed with 1.5ml of 95% alcohol, 0.1ml of 10% aluminium chloride hexahydrate ($AlCl_3 . 6H_2O$), 0.1ml of 1M potassium acetate (CH_3COO^-), and 2.8ml of deionised water.

After incubation at room temperature for 40min, the reaction mixture absorbance was measured using spectrophotometer (Jenway 6305 Spectrophotometer) at 415 nm. The deionized water was used as blank. Quercetin was used as standard to get a seven-point standard curve (0–50 mg/L). The levels of total flavonoid contents in the extracts of *Gossypium barbadense* were determined in triplicate, respectively. The data obtained was expressed as milligram of quercetin equivalents /g powder and converted to microgram quercetin equivalents /g of *Gossypium barbadense* (Suganya *et al.,* 2012).

3.2.5 Free Radical scavenging activity

3.2.5.1 Scavenging effect on 1,1-diphenyl-2-picrylhydrazyl (DPPH)

Principle of reaction: 1, 1-Diphenyl-2-picrylhydrazyl (DPPH) contains an odd electron in its structure. Its purple colour is reduced to yellow-coloured diphenylpicryhydrazine when it reacts

with an antioxidant, which can donate a hydrogen atom or a proton to it. The change in colour was measured spectrophotometrically at 520 nm using a UV/Visible light spectrophotometer

Procedure: The DPPH radical scavenging activities of various solvent extract of *Gossypium barbadense* was examined by comparison with that of known antioxidant, Ascorbic acid using the method of Lee and Shibamoto with slight modifications (2001). This was conducted with 0.1ml of extracts mixed with 2.9ml of solutions of DPPH (0.1mM in Methanol). The mixture was then shaken vigorously in a mechanical shaker and allowed to stand at room temperature for an hour. Then absorbance was measured at 517nm against blank. Lower absorbance indicated higher free radical scavenging activity.

The percent of DPPH decoloration of the samples were calculated according to the formula:

$$\% \text{ inhibition} = \left[1 - \frac{A_1 - A_2}{A_o}\right] X \ 100$$

Where A_0 = absorbance control with DPPH only

A_1 = absorbance of extract + DPPH

A_2 = absorbance control with extract only

Each sample was assayed in triplicate and mean values were calculated. (Lee and Shibamoto 2001)

3.2.6 DNA protection assay

3.2.6.1 Using Fenton's reagent

Principle of reaction: This is the iron-salt-dependent decomposition of dihydrogen peroxide, generating the highly reactive hydroxyl radical, possibly via an oxoiron (IV) intermediate, which can cause both single-strand and double-strand DNA breaks

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^{\cdot} + OH^{-}$$

Procedure: DNA protection assay was performed using plasmid DNA (Pmax GFP) by the method of Lee *et al.,* 2002 with slight modifications. A mixture containing 5μl of the various solvent extract of *Gossypium barbadense* and 1.5μl of plasmid Pmax GFP (476ng) was incubated for 10min at room temperature followed by the addition of 10 μl of Fenton's reagent (10% H_2O_2 and 1mM $FeSO_4$). The final volume of the mixture was made up to 20μl with 50mM Phosphate buffer (pH 7.4) and incubated for 30min at 37°C. The DNA was analyzed on 1% agarose gel using G stain (Suganya *et al.,* 2012).

3.2.6.2 Using UV radiation and H_2O_2

The potential of each extract of *Gossypium barbadense* to prevent DNA damage was tested by photolyzing plasmid DNA via UV radiation in the presence of H_2O_2 and performing agarose gel electrophoresis with the irradiated DNA using the method of Guha *et al.,* 2011 with slight modifications. Into polyethylene microcentrifuge tubes, 1.5µL aliquots of Pmax GFP (476 ng/mL) were placed, and then 1 mg/ml of each extracts was separately added to individual tubes. One tube did not have any extract added to it, which served as the irradiated control. Into all the tubes, 5 µL of 3% H_2O_2 was added, and then they were placed directly on the surface of a UV transilluminator (100%). The samples were irradiated for 10 min at room temperature; 1 µL aliquot of stock plasmid DNA was placed in a separate tube and served as the non-irradiated control. The final volume of the mixture was made up to 20μl with 50mM Phosphate buffer (pH 7.4). The DNA was analyzed on 1% agarose gel using G stain (Guha *et al.*, 2011).

3.2.7 Membrane stability test (Sakat *et al.,* 2010)

3.2.7.1 Preparation of red blood cells (RBCs) suspension

Fresh whole human blood (10ml) was collected and transferred to the centrifuge tubes and centrifuged at 3000 rpm for 10 min, then washed three times with equal volume of normal saline.

The volume of blood was measured and re-constituted as 10% v/v suspension with normal saline (Sakat *et al.,* 2010).

3.2.7.2 Heat induced hemolysis

Principle of reaction: The principle involved here is stabilization of human red blood cell membrane by hypotonicity induced membrane lysis.

Procedure: The reaction mixture (2ml) consisted of 1 ml of plant extract and 1ml of 10% RBCs suspension, instead of the extract, only saline was added to the control test tube. Aspirin was taken as a standard drug. All the centrifuge tubes containing reaction mixture was incubated in water bath at 56°C for 30 min. At the end of the incubation the tubes were cooled under running tap water. The reaction mixture was centrifuged at 2500 rpm for 5 min and the absorbance of the supernatants was taken at 560 nm. The experiment was performed in triplicates for all the plant extracts.

The percentage of hemolysis of HRBC membrane was calculated as follows:

% Hemolysis = (Absorbance of Test sample / absorbance of Control) X 100

The percentage of HRBC membrane stabilisation was calculated as follows:

% Protection = 100 – [(Absorbance of Test sample / absorbance of Control) X 100]

This method is as described by Sakat *et al.,* 2010.

3.3 Statistical analysis

The data was analyzed by the analysis of variance (ANOVA). The results were expressed as mean ± standard deviation (SD) except where otherwise stated. P value less than 0.05 was taken as significant ($P< 0.05$)

CHAPTER FOUR

4.0 RESULTS

4.1. Yield Value Determination

The percentage yield of the solvent extracts (hexane, ethyl acetate and aqueous) of the stem and leaf of *G. barbadense* is represented in Table 4.1. The percentage yield follows the trend AqL > AqS > EL > HL > ES > HS. Aqueous extract of leaf has the highest yield (21.38%).

Table 4.1 Yield values of different solvent extracts

Solvent used for extraction	% yield
Hexane leaf	2.37
Hexane stem	0.93
Aqueous leaf	21.38
Aqueous stem	8.1
Ethyl acetate leaf	4.03
Ethyl acetate stem	1.07

4.2. The Qualitative Determination of Phytochemicals in *Gossypium barbadense*

The qualitative determination of some phytochemicals present in the hexane extracts, aqueous extracts and ethylacetate extracts of stem bark and leaf of *Gossypium barbadense* is presented in table 4.2. The result shows that carbohydrates, cardiac glycosides, flavonoids, phenols, alkaloids, triterpenes, steroids and tannins are present in all the solvent extracts. Saponin is present only in the aqueous extracts while anthraquinone is absent in all the solvents extracts.

Phytochemical test	Aqueous Leaf	Aqueous Stem	Hexane Leaf	Hexane Stem	Ethyl acetate Leaf	Ethyl acetate Stem
Saponin	+	+	-	-	-	-
Carbohydrate	+	+	+	+	+	+
Cardiac Glycosides	+	+	+	+	+	+
Phenols	+	+	+	+	+	+
Flavonoids	+	+	+	+	+	+
Tannins	+	+	+	+	+	+
Alkaloids	+	+	+	+	+	+
Anthraquinone	-	-	-	-	-	-
Terpenoids	+	+	+	+	+	+
Triterpenes	+	+	+	+	+	+
Steroids	+	+	+	+	+	+

Table 4.2 Results of phytochemical screening

+ Present **-** Absent

4.3 Quantitative Profile of PolyPhenols

The total polyphenol of different solvent extracts of stem and leaf of *G. barbadense* is presented in Table 4.3. The result shows that ethyl acetate leaf extract had the highest amount of the totalphenol (86.04 ± 0.85 µg gallic acid/g of dry sample) followed by hexane leaf extract (34.62 ± 0.19 µg gallic acid/g of dry sample) and aqueous stem extract had the least total phenol (6.79 ± 0.19 µg gallic acid/g of dry sample).

Table 4.3 Total PolyPhenols content of extracts from leaf and stem of *G. barbadense*

Extracts	Polyphenols (µg equivalent gallic acid/g dry material)
Hexane Leaf (HL)	34.62 ± 0.19
Hexane Stem (HS)	20.91 ± 0.89
Aqueous Leaf (AQL)	13.43 ± 0.14
Aqueous Stem (AQS)	6.79 ± 0.19
Ethyl acetate Leaf (EL)	86.04 ± 0.85
Ethyl acetate Stem (ES)	24.21 ± 0.30

4.4 Quantitative profile of Flavonoids

The total flavonoids of different solvent extracts of stem and leaf of *Gossypium barbadense* is represented in Table 4.4. The result shows that hexane leaf extract had the highest total flavonoids (1734.54 ± 6.36 µg quercetin/g of dry sample) followed by ethyl acetate stem extract (1134.24 ± 5.92 µg quercetin/g of dry sample) and aqueous stem extract had the least total flavonoids (115.46 ± 6.30 µg quercetin/g of dry sample).

Table 4.4 Total Flavonoid content of extracts from leaf and stem of *G. barbadense*

Extracts	Flavonoids (µg equivalent Quercertin/g dry material)
Hexane Leaf (HL)	1734.54 ± 6.36
Hexane Stem (HS)	1033.18 ± 8.36
Aqueous Leaf (AqL)	199.09 ± 19.43
Aqueous Stem (AqS)	115.46 ± 6.30
Ethyl acetate Leaf (EL)	1051.51 ± 5.92
Ethyl acetate Stem (ES)	1134.24 ± 5.92

4.5 In vitro Free Radical scavenging effect on DPPH

A plot of % scavenging inhibition of free radicals versus extracts concentrations representing the antioxidant activity of the different solvent extracts of stem and leaf of *G. barbadense* is shown in Figure 4.1. This is based on their scavenging ability on DPPH (a stable purple-coloured radical which is reduced to yellow-coloured diphenylhyrazine during the reaction). The scavenging inhibition of the standard (ascorbic acid) decreased with increasing concentration. The % inhibition of the aqueous leaf extract increased with increasing concentration while the inhibition of other extracts fluctuated with concentration.

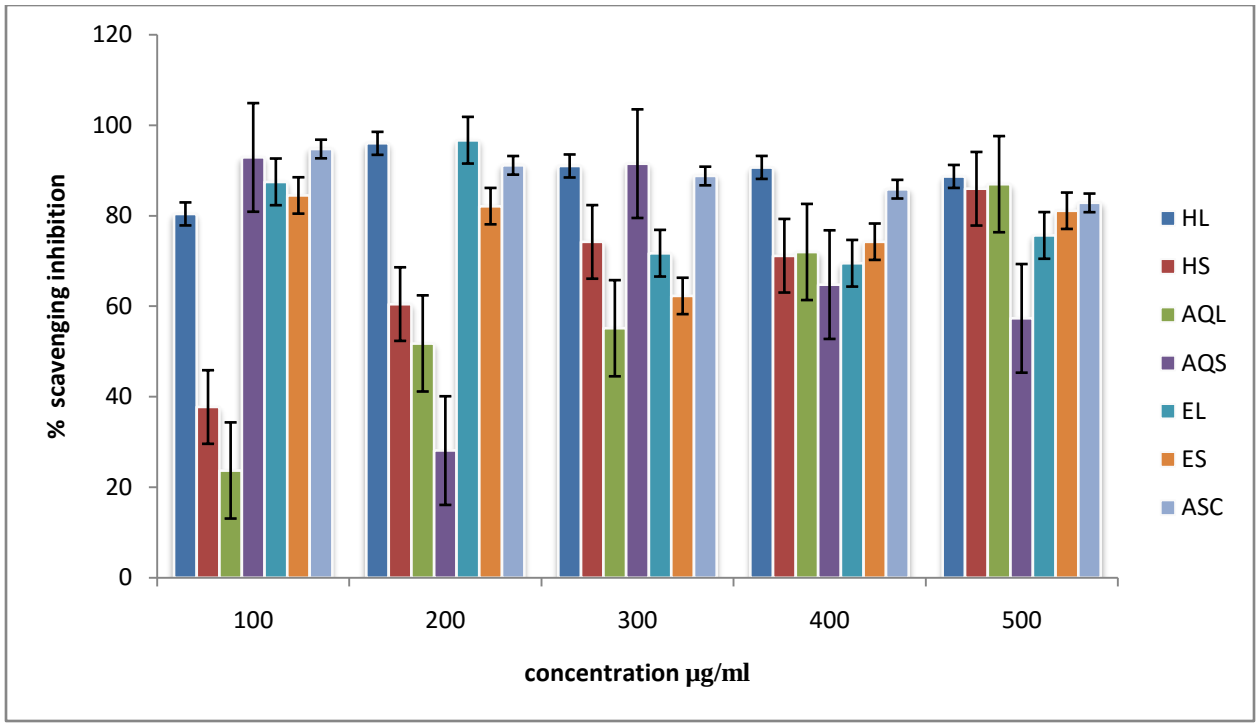


Figure 4.1 Percentage DPPH scavenging inhibition of the different solvent extracts of *G. barbadense* and ascorbic acid

HL=Hexane Leaf, extract, HS = Hexane stem extract, AQL = Aqueous leaf extract, AQS = Aqueous stem extract, EL = Ethyl acetate leaf extract, ES = Ethyl acetate stem extract, ASC = Ascorbic acid

Table 4.5 presents the IC_{50} values of the different solvent extracts of stem and leaf of *G. barbadense* and Ascorbic acid. The result shows that ascorbic acid (standard used) had the lowest IC_{50} value (0.173 ± 0.098 mg/ml), followed by hexane leaf extract with IC_{50} value of 0.192 ± 0.076 mg/ml and that of ethyl acetate leaf extract (0.197 ± 0.116 mg/ml).

Table 4.5 IC_{50} values of stem and leaf extracts of *Gossypium barbadense* and Ascorbic acid

Extracts	IC_{50} values (mg/ml)
Hexane Leaf (HL)	$0.192 \pm 0.076^{a,b}$
Hexane Stem (HS)	$0.214 \pm 0.069^{a,b}$
Aqueous Leaf (AqL)	$0.248 \pm 0.043^{a,b}$
Aqueous Stem (AqS)	0.263 ± 0.153^{b}
Ethyl acetate Leaf (EL)	$0.197 \pm 0.116^{a,b}$
Ethyl acetate Stem (ES)	$0.200 \pm 0.105^{a,b}$
Ascorbic acid (ASC)	0.173 ± 0.098^{a}

Values are means ± SD n=3 determinations with different superscripts are significantly different ($p<0.05$)

4.7 DNA protecting assay

Figure 4.2 presents the DNA protecting activity of the different solvent extracts of stem and leaf of *G. barbadense.* From the result obtained, it was shown that only the leaf aqueous extract was able to prevent the DNA damage induced by Fentons reagent.

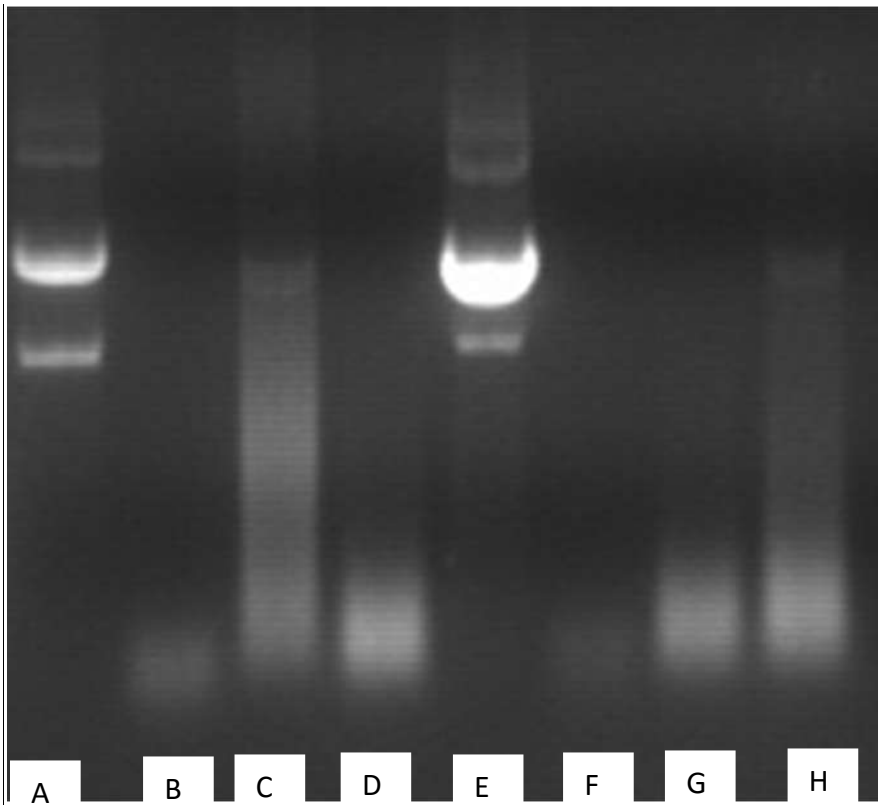
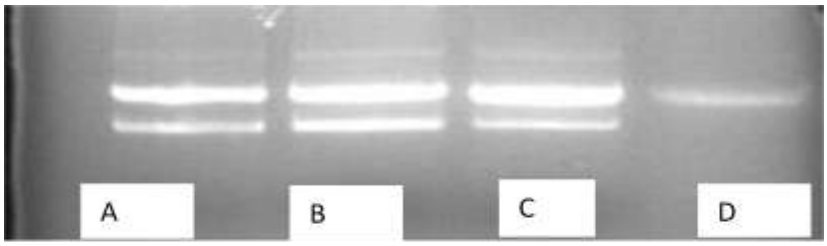


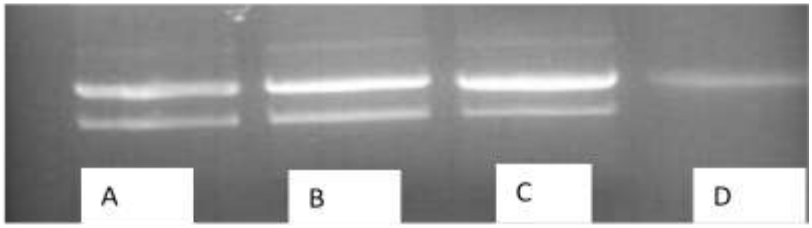
Plate 4.1 Electrophoregram showing the DNA protecting activity

A – untreated DNA, B – DNA + Fenton's reagent, C – DNA + Fenton's reagent + hexane leaf extract, D – DNA + Fenton's reagent + hexane stem extract, E – DNA + Fenton's reagent + aqueous leaf extract, F – DNA + Fenton's reagent + aqueous stem extract, G – DNA +Fenton's reagent + ethyl acetate leaf extract, H – DNA + Fenton's reagent + ethyl acetate stem extract

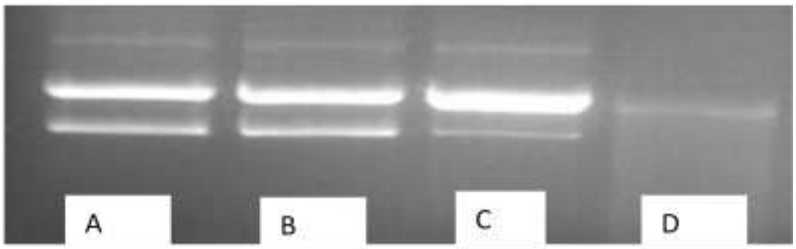
Figure 4.3 (A – C) below present the DNA protecting activity of aqueous leaf extracts of *G. barbadense* using Fentons reagent with varying incubation time of 10 minutes, 30 minutes and 50 minutes. From the result obtained, the leaf aqueous extract was able to prevent the plasmid DNA from damage induced by free radicals.



(A) INCUBATION TIME: 10mins



(B) INCUBATION TIME: 30mins



(C) INCUBATION TIME: 50mins

Plate 4.2: Electrophoregram showing the DNA protecting activity of aqueous leaves extract incubated with Fentons reagent for various minutes

A – plasmid only, B – plasmid + aqueous leaves extract,
C – plasmid + aqueous leaves extract + Fenton's reagent , D – plasmid + Fenton's reagent

Figure 4.4 (A – C) below present the DNA protecting activity of aqueous leaf extracts of *G. barbadense* using H_2O_2-induced UV radiation with varying exposure time of 10 minutes, 30 minutes and 50 minutes. From the result obtained, the leaf aqueous extract was able to prevent the plasmid DNA damage on exposure to H_2O_2-induced UV radiation.

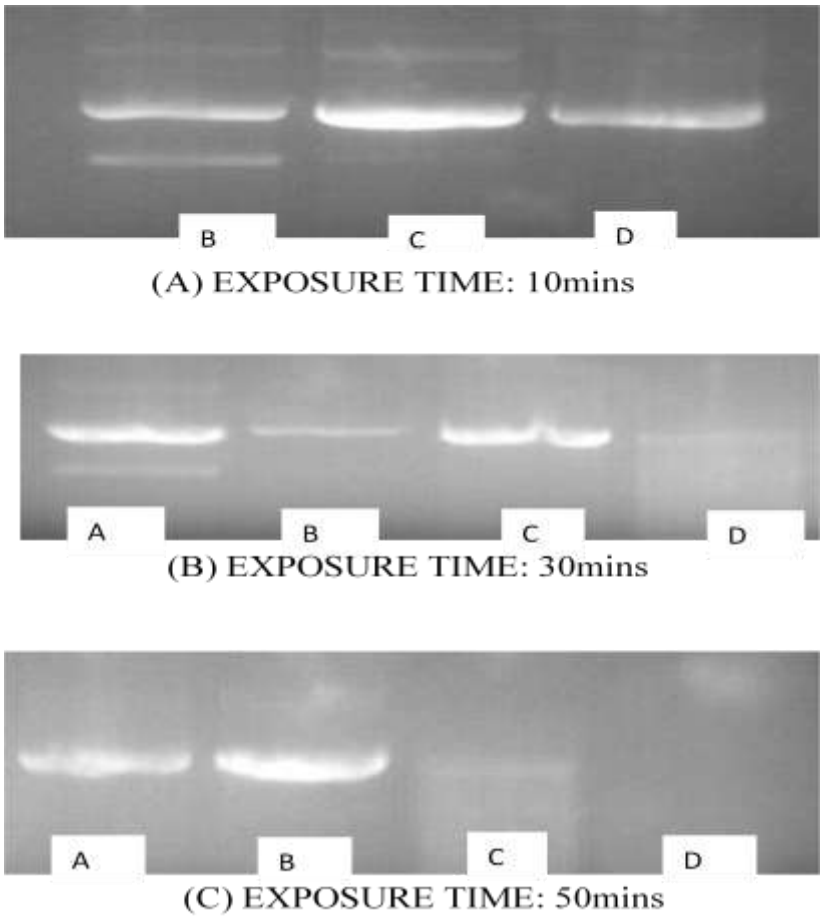


Plate 4.3: Electrophoregram showing the DNA protecting activity of aqueous leaves extract exposed to UV radiation for various minutes

A – plasmid + UV only, B – plasmid + aqueous leaves extract + UV,
C – plasmid +aqueous leaves extract + 3% H_2O_2 + UV, D – plasmid + 3% H_2O_2 + UV

4.7 Membrane stability test

Table 4.7 presents the percentage hemolysis and percentage protection from hemolysis of hexane extracts, aqueous extracts and ethyl acetate extracts of stem and leaf of *G. barbadense* and aspirin (standard drug). The increasing order of protection as shown by the result is EL>AqL>ES>HS>AqS>ASPIRIN>HL.

Table 4.7 Percentage hemolysis and protection from hemolysis of stem and leaf extracts of *Gossypium barbadense* and standard drug

Plants extracts	% Hemolysis	% Protection
HL	9.92	90.08
HS	4.76	95.24
AQL	16.77	83.23
AQS	6.91	93.09
EL	37.03	62.97
ES	12.36	87.64
Aspirin	5.34	94.66

CHAPTER FIVE

5.0 DISCUSSION

Phytochemicals in plants have long been studied in the prevention of certain chronic diseases besides maintaining freshness in fruits and prolonging food storage. The presence of phytochemicals such as phenolics, anthocyanins and other flavonoid contents has contributed to the antioxidant properties in plants (Seow-Mun *et al.,* 2012). The phytochemicals which might be responsible for the scavenging activity in this species are phenolic and flavonoid constituent (Mazandarani *et al.,* 2011). Phenols are very important plant constituents because of their scavenging ability due to their hydroxyl groups. The phenolic compounds have inhibitory effects on mutagenesis and carcinogenesis in humans, when ingested up to 1g daily from a diet rich in fruits and vegetables. Therefore, it was reasonable to determine the total phenolic content in the plant extracts. Phenolic compounds are the key phytochemicals with high free radical scavenging activity. It has generated a great interest among the scientists for the development of natural antioxidant compounds from plants (Abass *et al.,* 2012).

Phenols may not be solely responsible for the antioxidant activity. Plant extracts with high phenolic contents also show high flavonoid content as reported for other plant species. The study of flavonoids has also been intensive due to their antioxidant properties that contribute to good health of human kind. The action of flavonoids can be divided into two different mechanisms: scavenging or the chelating process. This may be explained due to the fact that flavonoids are one of the classes of plant polyphenol and polyphenols have structural requirements of free radical scavengers. That is to say that the polyphenols scavenging potential and metal chelating

ability are dependent upon their unique structure, the number and position of the hydroxyl groups which may vary from one part of the plant to another(Jayathilakan *et al.,* 2007).

1,1-diphenyl 2-picryl hydrazyl (DPPH) radical Scavenging activity assay is considered to be primary antioxidant assay because it is one of the most effective methods for evaluating the concentration of radical-scavenging materials actively by a chain-breaking mechanism and is a model for a lipophilic radical. Antioxidant potential is inversely proportional to Inhibitory concentration value (IC_{50} - the amount of antioxidant required to inhibit 50% of DPPH free radicals under the experimental conditions) which was calculated from the linear regression. In the present study the antioxidant activity test was found to be positive for each extract as compared with the standard and this can be attributed to the presence of phenols and flavonoids as shown in the phytochemical screening test. The extracts with high phenolic and flavonoids contents recorded high DPPH scavenging effect but were not comparable statistically to the DPPH activity of Ascorbic acid (standard). The results suggest that the presence of these components present in the extracts synergises their actions responsible for the DPPH activity.

A cell depends on its DNA for coding information to synthesize various types of proteins that includes enzymes, certain hormones, transport proteins and structural proteins, which support life. When the genetic information containing the "blueprint" for these substances is disturbed, cell homeostasis is disrupted resulting in a wide range of alterations that can lead mutations in genetic coding and lastly to cancer. DNA strand scission was induced by hydroxyl radicals. Hydroxyl radical is the most reactive radical found in biological systems, it has the shortest half life compared with other radicals and is considered to be responsible for much of the biological damage in free radical pathology. It can attract hydrogen atoms from biological molecules, including thiols, leading to the formation of sulphur radicals capable to combine with oxygen to

generate oxysulfur radicals, which damage biological molecules (Wu and Cederbaum, 2003). The radical has the capacity to cause strand breakage in DNA, which contributes to carcinogenesis, mutagenesis and cytotoxicity (Poorna *et al.,* 2012). This is first report to date on the protective effect of aqueous leaf extract of *G. barbadense* on DNA damage. The DNA protecting activity of *G. barbadense* was carried out using plasmid DNA (Pmax GFP). All the extracts were incubated with the plasmid before addition of Fentons reagent (mixture of $FeSO_4$ and H_2O_2). Only the aqueous leaf extract gave a positive result (protecting activity) (Fig. 4.2). Three bands were observed in the control (Plasmid only). The same bands were also observed for the aqueous leaf extract treated with plasmid, samples that did not show any bands contained extracts that did not have the ability to inhibit oxidative DNA damage. DNA plus Fentons reagent and other extracts except the aqueous leaf extracts exhibited complete degradation of DNA. This result was also obtained for *E. agallocha* Linn in which only the aqueous fraction of *E. agallocha* Linn has shown very good protective activity and has retained all three forms of the plasmid used (Poorna *et al.,* 2012).

Aqueous leaf extracts of *G. barbadense* was able to confer protection on DNA and prevented damage caused by hydroxyl radical. The high DNA protecting ability of aqueous leaf extract is likely due to the presence of saponinis. This ability of the extract might be due to their chelating activity on iron or hydroxyl radical scavenging or both. A study reported the protective effects of saponin-emulsified vitamin A and E against oxidative stress by demonstrating that the reactions leading to lipid peroxidation, protein carbonylation, and DNA damage were more efficiently suppressed by saponin-emulsified vitamins E than by non-emulsified vitamin (Kim *et al.,* 2016). Increase in ultraviolet radiation at the Earth's surface due to the depletion of the stratospheric ozone layer has recently fuelled interest in the mechanisms of various effects it might have on

organisms especially the release of free radicals from molecules (Birben *et al*., 2011). DNA is first vital target molecule for radiation injury in biological systems. Therefore, efforts was made in the present study to see whether incubation of plasmid DNA with *G. barbadense* aqueous extract prior to exposure to H_2O_2-induced UV radiation provides protection to DNA or not. It was seen that the aqueous extract was able to confer protection on the plasmid DNA when incubated for more than 30 minutes. It is known that there is a synergistic effect of both H_2O_2 and UV radiation on DNA molecules which lead to excessive damage of DNA. Only UV radiation could cause damage to cellular molecules. H_2O_2 alone also is known to cause DNA breaks in intact cells and purified DNA. 1mMol H_2O_2 produced significant toxicity and caused DNA damage in the form of SSBs and DSBs, chromosomal aberrations (Saini *et al.,* 2007). Kadam *et al.,* 2008) reported that sugarcane juice has protective effect against radiation induced DNA damage.

Shetty *et al.,* (2005) reported that *Hemidesmus indicus* root extract protected DNA *in vitro* against gamma radiation induced strand breaks, which was observed as reduction in quantity of supercoiled form of plasmid DNA. Diverse plant resources were observed to protect DNA from oxidative stress due to UV-induced photolysis of H_2O_2. UV-photolysis of H_2O_2 generates OH radicals, which cause colossal oxidative damage. OH bound to DNA leads to strand breakage, deoxysugar fragmentation, and base modification. Moreover, oxidation of lipids induced by OH and other ROS can generate end products, such as malondialdehyde and unsaturated aldehydes, that can attach to DNA and produce mutagenic adducts.

Stabilization of human red blood cell membrane (HRBC) by hypo tonicity induced membrane lysis can be taken as an in vitro measure of anti inflammatory activity of the drugs or plant extracts (Chippada *et al.,* 2011).

Stabilization of the RBCs membrane by *G. barbadense* was studied to further establish the mechanism of anti-inflammatory action of *G. barbadense.* Exposure of red blood cells (RBCs) to injurious substances such as hypotonic medium, heat, methyl salicylate or phenylhydrazine may result in the lysis of the membranes, accompanied by haemolysis and oxidation of haemoglobin. Since human red blood cell (HRBC) membranes are similar to lysosomal membrane components (Mounnissamy *et al.,* 2008), the inhibition of hypotonicity and heat induced red blood cell membrane lysis was taken as a measure of the mechanism of anti-inflammatory activity of *G. barbadense* extract. The haemolytic effect of hypotonic solution is related to excessive accumulation of fluid within the cell resulting in the rupturing of its membrane. Injury to red cell membrane will render the cell more susceptible to secondary damage through free radical induced lipid peroxidation (Halliwell and Whiteman, 2004). Membrane stabilization leads to the prevention of leakage of serum protein and fluids into the tissues during a period of increased permeability caused by inflammatory mediators (Chaitanya *et al.,* 2011). *G. barbadense* extract perhaps stabilized the red blood cell membrane by preventing the release of lytic enzymes and active mediators of inflammation.

Many anti-inflammatory plants and agents modify inflammatory responses by accelerating the destruction or antagonizing the action of the mediators of inflammatory reaction. Foods and fruits rich in flavonoids and other phenolic compounds have been associated with decreased risk of developing inflammatory and other related diseases (Sadik *et al.,* 2003; Sies *et al.,* 2005). Therefore, our present in vitro studies on *G. barbadense* extracts demonstrate the depression of inflammation. The presence of active principles such as flavonoids and tritrepenoids and related polyphenols may be responsible for this activity. Hence, *G. barbadense* can be used as a potent anti inflammatory agent.

CHAPTER 6

CONCLUSIONS AND RECOMMENATIONS

6.1 CONCLUSIONS

The results of this study has shown that leaf and stem bark extracts of *G. barbadense* possess DPPH scavenging property (antioxidant activity) with highest activity observed in the hexane leaf extract; all with respect to a known antioxidant – Ascorbic acid. This activity may be due to the presence of flavonoids and polyphenols in the plant.

Also, the study showed that the aqueous leaf extract of *Gossypium barbadense* has the ability to protect plasmid DNA against free radical-induced damage.

It was observed from the results obtained that *G. barbadense* extract has anti-inflammatory activity.

6.2 RECOMMENDATIONS

On the basis of the conclusion drawn from this study, it can be recommended that *Gossypium barbadense* could be a very good source of medicinal uses as alternative therapy.

Further research on *in vivo* studies of DNA protection and the toxicity of the plant *Gossypium barbadense* should be evaluated.

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APPENDICES
APPENDIX 1.0

Yield value of each extracts was calculated as:

$$\frac{\text{Extracts Obtained}}{\text{Total amount of crude}} \times 100\%$$

Yield Value Determination

For hexane extraction;

Yield Value of leaf (HL): (1.42g/60 g) × 100% = 2.37%

Yield Value of Stem: (HS) (0.28g/30 g) × 100% = 0.93%

For aqueous extraction;

Yield Value of leaf (AQL): (12.83g/60 g) × 100% = 21.38%

Yield Value of stem: (AQS) (2.43g/30 g) × 100% = 8.1%

For ethyl acetate extraction;

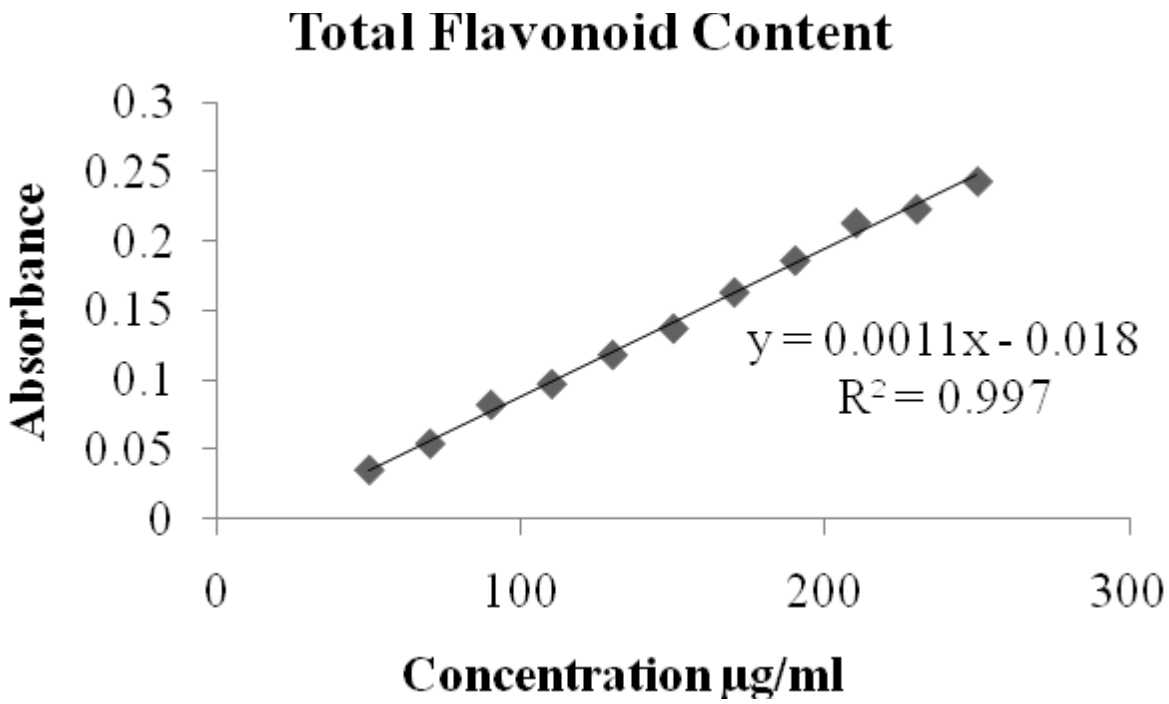
Yield Value of leaf (EL): (2.42g/60 g) × 100% = 4.03%

Yield Value of stem: (ES) (0.32g/30 g) × 100% = 1.07%

Table 4.6 percentage scavenging activity of stem and leaf extracts of *Gossypium barbadense* and Ascorbic acid

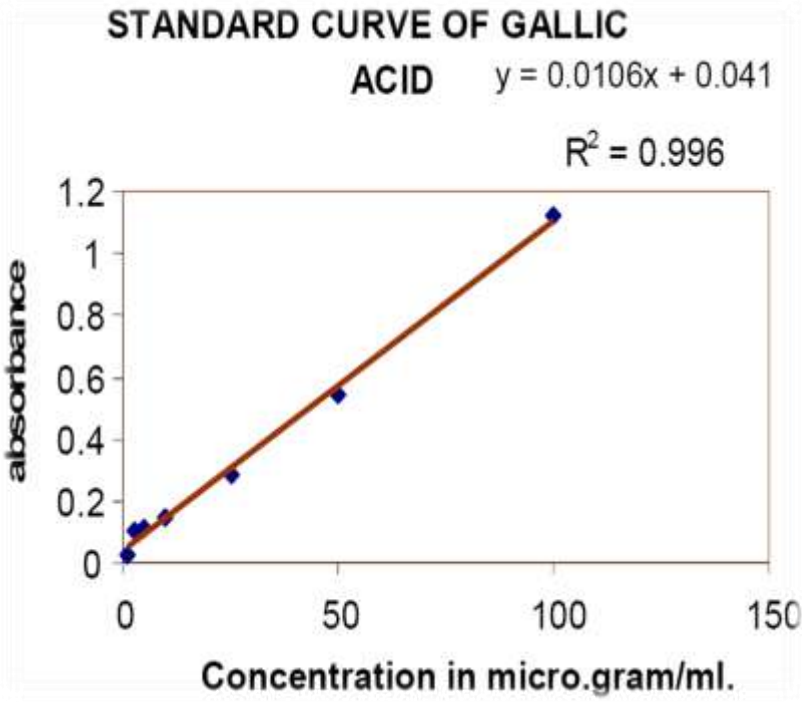
Extracts	% activity at different concentrations (µg/ml)				
	100	200	300	400	500
HL	80.40±0.12	95.98±0.00	90.98±0.23	90.66±0.12	88.67±0.12
HS	37.73±0.16	60.47±0.81	74.25±0.32	71.15±0.16	85.95±0.20
AQL	23.72±0.29	51.78±0.46	55.14±1.00	71.98±0.16	86.96±0.08
AQS	92.88±0.05	28.11±0.32	91.49±0.12	64.77±0.35	57.33±0.12
EL	87.42±0.05	96.67±0.00	71.71±0.40	69.49±0.16	75.64±0.05
ES	84.47±2.15	82.11±0.00	62.27±0.00	74.25±0.12	81.09±0.17
ASC	94.73±0.00	91.12±0.00	88.77±0.00	85.85±0.00	82.83±0.00

APPENDIX 2.0



Calibration curve of Quercertin

APPENDIX 3.0



Calibration curve of Gallic acid