IN VITRO ANTIOXIDANT ACTIVITIES OF METHANOLIC EXTRACTS OF *Caesalpinia volkensii* Harms, *Acacia hockii* De Wild AND *Vernonia lasiopus* O. Hoffm

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DECLARATION

I, Beatrice Muthoni Guchu, declare that the work presented in this thesis is my original work and have not been submitted for a degree or any other awarding any other university or any other institution.

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We hereby confirm that the candidate carried out the work reported in this thesis under our supervision.

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DEDICATION

I dedicate this thesis to my husband David Biaru, my son Caleb Kamau, my daughter Janet Muthoni, my father Nelson Guchu and my mother Pricilla Wambui. You have been my great source of love and inspiration.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS AND ACRONYMS

AIDS	Acquired immunodeficiency syndrome
ATP	Adenosine triphosphate
ANOVA	Analysis of variance
BHA	Butylated Hydroxyl Anisole
BHT	Butylated Hydroxyl Toluene
COX	Cyclooxygenase
DM	Diabetes mellitus
DNA	Deoxyribonucleic acid
DPPH	2, 2-diphenyl-1-picrylhydrazyl
Gpx	Glutathione peroxidase
GSH	Reduced glutathione
GSSG	Oxidized glutathione
H_2O_2	Hydrogen peroxide
HOCl	Hipochloric acid
LDL	Low density lipoproteins
LOX	Lipoxygenase
NADPH	Dihydronicotinamide-adenine dinucleotide phosphate
NF-kB	Nuclear factor kappa-B
$^{\cdot}O_2^-$	Superoxide anion
$^{\cdot}OH$	Hydroxyl radical
O_3	Ozone
PUFA	Polyunsaturated fatty acids
RBC	Red blood cell
ROS	Reactive oxygen species
SOD	Superoxide dismutase
UV	Ultraviolet
WHO	World health organization

ABSTRACT

Antioxidants are chemical substances that inhibit oxidation (oxidation is a chemical reaction that can produce free radicals, leading to a chain reaction that may damage cells or other molecules) Free radicals are reactive oxygen species and reactive nitrogen species generated by our bodies naturally during normal metabolic functions. If free radicals overwhelm the body ability to regulate them, a condition known as oxidative stress ensues. Oxidative stress leads to the destruction of biomolecules such as lipid membranes leading to lipid peroxidation which is responsible for the pathogenesis of various disease conditions such as cancer, hypertension, and cardiovascular diseases. Hence the application of an external source of antioxidant can assist in coping with the treatment of this oxidative stress. Research on natural antioxidant has gained interest in the recent past as demand for safer and readily available antioxidant by food industries and preventive medicine has gained momentum. This study was carried out to determine the *in vitro* antioxidant potential of Methanolic extracts of *Caesalpinia volkensii, Acacia hockii* and *Vernonia lasiopus*. The plant samples were collected with the help of local herbalists in Embu County, Kenya and transported to Kenyatta University Biochemistry and Biotechnology laboratories for cleaning, air drying, milling, and extraction using methanol as a solvent. The antioxidant activities of the extract were evaluated through the scavenging effect of 2, 2-diphenyl-1-picrylhydrazyl (DPPH), hydrogen peroxide (H_2O_2) and total ferric reducing power. The antioxidant data obtained was assayed against that of the standard (Ascorbic acid). All the extracts demonstrated significant DPPH scavenging activities with the highest percentage of 87.22% for ascorbic acid and 82.73%, 76.86%, 67.36% for *A. hockii, V. lasiopus* and *C. volkensii* respectively. Ascorbic acid which was used as a standard demonstrated lowest IC_{50} of 0.198. The IC_{50} for the plant extracts were 0.601, 0.40 and 0.47 for *C. volkensii, A. hockii* and *V. lasiopus* respectively. They also demonstrated reducing the power that increased with increase in extracts concentration. In hydrogen peroxide assay increase in extracts, the concentration decreased the scavenging activity for all the extracts. There was a significant difference between the extracts and the standard. The methanolic extracts of *Caesalpinia volkensii, Acacia hockii* and *Vernonia lasiopus* demonstrated significant antioxidant activity. Qualitative phytochemical screening indicated the presence of secondary metabolites associated with antioxidant activities. The present study therefore validates and supports the traditional use of *Caesalpinia volkensii, Acacia hockii* and *Vernonia lasiopus* in management of oxidative stress and recommends further research for the development of phytomedicine with antioxidant properties.

CHAPTER ONE

INTRODUCTION

1.1 Background information

Reactive species including oxygen and nitrogen radicals are highly unstable molecules generated in aerobic organisms in the course of respiration. These free radicals have unpaired electrons which pair with other radicals making them highly reactive. Intracellular and environmental factors are some of the sources of these reactive species. Endogenous reactive species are formed in the metabolic processes, for instance, the destruction of microbes using oxidants such as superoxide, during inflammation, mental stress, excessive exercise and ischemia (Ahmed, 2014).

Exogenous reactive oxygen species (ROS) are produced in the environment mostly by human activities and when they penetrate the body they are decomposed into free radicals. Their sources include air and water pollution, waste from industries, active and passive smoke from cigarettes, radiations from industry and cosmic rays, the metallic chemical element such as copper, lead and mercury, alcohol, unsaturated fat and certain drugs such cyclosporine and tacrolimus (Mwihia, 2017).

Oxidative damage is induced by the accumulation of free radicals in the body that results in tissues injuries due to oxidation of body's macromolecules (Ahmed *et al*., 2013). However, living organisms are endowed with antioxidant and defence mechanism that stabilizes radicals thereby preventing their harmful effects.

These reducing agents act as electron donors, radical scavengers, chelating agents, peroxide breakers, dioxygen molecule suppressors, synergists and blockers of catalytic processes involving free radicals. The antioxidant mechanisms collectively defend the body from oxidative damage (Bhattacharya, 2015). The oxidants reducing system comprises both catalytic and non-catalytic antioxidants. Metallic ions such as iron, copper, zinc as well as magnesium that facilitate catalytic reactions in enzymatic antioxidant alter harmful oxidative products into harmless products in a sequential manner. On the other hand, biologics such vitamins and phytochemicals comprise non-enzymatic antioxidant that disrupts free radical chain reactions (Birben *et al*., 2012).

Inadequate antioxidant mechanisms are the main source of various metabolic disorders such as chronic hyperglycemia as well as different types of cancers, cardiovascular diseases, rheumatoid arthritis inflammation and ageing (Esmaeili *et al*., 2009). Once the manifestation of free radicals surpasses natural mechanisms ability to counteract them, subsequent damage is reflected by oxidative stress (Mwihia, 2017). Globally, diseases associated with oxidative stress have huge economic burdens to individuals, families public and private sectors in terms of their treatment and management. These diseases, for example, diabetes have complications like diabetic neuropathy which increase the cost of treatment. They also cause premature death which reduces the workforce in industries and therefore cut productive lives (Cavallo *et al*., 2018).

Conventional antioxidants that are commercially available include Propyl gallate (PG) Butylated hydroxyl anisole (BHA) and Butylated hydroxyl toluene (BHT). These

synthetic antioxidants have proven to be harmful, costly, and have associated side effects such as hypoglycemia, damage to the liver and are carcinogenic (Virdi *et al*., 2003). The unintended pharmacologic effects with consumption of conventional antioxidants in the management of oxidative damage and related disorders necessitate the need for alternative therapy for oxidative stress using herbal remedies that are safe, inexpensive, and biodegradable besides having fewer side effects (Lobo *et al*., 2010)

Different herbs have been used as a source of antioxidants. Extracts of S*trychno henningsii* have shown to possess antioxidant potential dependent on the concentration, this shows the ability of *Strychnos henningsii* to minimize oxidative stress in the body (Oyedemei *et al.,* 2010). The methanol extracts of *T. chebula* inhibits lipid peroxidation as well as enhancing superoxide and hydroxyl radicals scavenging (Bag *et al.,* 2013), therefore preventing oxidative damage.

The major groups of phytochemicals that contribute to the total antioxidant capacity of plants include polyphenols and vitamins (A, C and E). Phenolic compounds of plants are hydroxylated derivatives of benzoic acid and cinnamic acids, which possess antioxidant and anticarcinogenic effects. They include phenols, flavonoids, coumarins, tannins, and anthocyanidins. These phytoactive complexes are important in plant defence mechanisms against biotic and abiotic stresses (Rajashekar *et al*., 2009). When plants or plant products rich in these phytoactive principles are consumed, they are deemed to confer the same beneficial effects to humans. For instance, flavonoids have for long been recognized to

possess anti-inflammatory, anti-allergic, antiviral, immunomodulatory, anti-ageing and antiproliferative properties (Panche *et al*., 2016*)*.

For centuries, medicinal plants have had a great contribution in health care provision. This research aimed to explore and provide information on *in vitro* antioxidant activities of *Caesalpinia volkensii, Acacia hockii* and *Vernonia lasiopus*as well as determining their phytochemical composition. The finding of the study will be effective in the generation of herbal formulation which is affordable, readily available and a remedy for complementary management of oxidative damage and associated disorders.

1.2 Statement of the problem

Oxidative stress is the result of the disparity between prooxidants and antioxidants in an organism and it is important in the pathogenesis of several degenerative disorders, such as arthritis, Alzheimer's, cancer and cardiovascular diseases. The use of antioxidants is effective in delaying the oxidation of biomolecules. However, the conventional methods in the prevention and management of oxidative damage are not readily available to the patients due to factors such as cost and inaccessibility.

1.3 Justification of the Study

Exploration of antioxidant properties in indigenous plants is significant since the consumption of plant-based food is favourable for reduction of oxidative-stress related diseases. The risk associated with oxidative damage such as cardiovascular diseases, hypertension and drawbacks of conventional management such as toxicity and adverse

side effects necessitates the need to look for alternative ways of managing oxidative stress. Traditional use of *Caesalpinia volkensii, Acacia hockii* and *Vernonia lasiopus* should be scientifically validated to confirm their use. The study aimed to know the qualitative phytochemical associated with them. It is important to explore alternative oxidative damage management options. Herbal medicine has been found to provide such alternatives. It is, therefore, important to scientifically evaluate the antioxidant potential of *C. volkensii, A. hockii* and *V. lasiopus* to support their traditional use as antioxidants. It is also important to determine the phytochemical composition of the extract to understand their antioxidant capacity.

Medicinal plants have been used for centuries by man to manage diseases, and have a host of antioxidant complexes. Traditionally, *Caesalpinia volkensii, Vernonia lasiopus* and *Acacia hockii* have folkloric remedies against associated oxidative stress-mediated complications. However, the upsurge in its use has not been accompanied by scientific validations to support these claims. Oxidative damage is associated with health risk, which is attributed to the development of various diseases.

1.4 Hypotheses

i. Methanolic extracts of *C. volkensii*, *V. lasiopus* and *A. hockii* have *in vitro* ferric reducing activities.
ii. Methanolic extracts of *C. volkensii*, *V. lasiopus* and *A. hockii* have *in vitro* DPPH radical scavenging activities.
iii. Methanolic extracts of *C. volkensii*, *V. lasiopus* and *A. hockii* have *in vitro* hydrogen peroxide scavenging activities.

iv. Methanolic extracts of *Caesalpinia volkensii, Acacia hockii* and *Vernonia lasiopus* have total phenolic and flavonoid content associated with antioxidant activities.

v. Methanolic extracts of *C. volkensii*, *V. lasiopus* and *A. hockii* have phytochemicals associated with antioxidant activities.

1.5 Objectives

1.5.1 General objective

To determine *in vitro* antioxidant effects and qualitative phytochemical composition of methanolic extracts of *C. volkensii, A. hockii* and *V. lasiopus*.

1.5.2 Specific objectives

i) To determine *in vitro* reducing the power of methanolic extracts of *Caesalpinia volkensii, Acacia hockii* and *Vernonia lasiopus.*

ii) To determine *in vitro* DPPH scavenging activities of methanolic extracts of *Caesalpinia volkensii, Acacia hockii* and *Vernonia lasiopus*.

iii) To determin*e in vitro* hydrogen peroxide scavenging activities of methanolic extracts of *Caesalpinia volkensii, Acacia hockii* and *Vernonia lasiopus*.

iv) To determine the total phenolic and flavonoid content of methanolic extracts of *Caesalpinia volkensii, Acacia hockii* and *Vernonia lasiopus*.

v) To determine the qualitative phytochemical composition of methanolic extracts of *Caesalpinia volkensii***,** *Acacia hockii* and *Vernonia lasiopus*.

CHAPTER TWO

LITERATURE REVIEW

2.1 Oxidative stress

The inabilities of the body to neutralize reactive oxygen/nitrogen species (ROS and RNS) and eliminate them at the rate which they are generated in the system have the consequence of oxidative stress. The ROS are generated when cells are exposed to the environment with a lot of oxygen. ROS and RNS comprise of reactive unpaired electrons, for instance, hydroxyl radical and superoxide anion in addition to non-radical species that include hypochloric acid, singlet oxygen, hydrogen peroxide as well as ozone (Nabavi *et al.*, 2013).

Aerobic organisms possess antioxidant defence system which includes the dietary antioxidant system which relies on the diet and antioxidant enzyme system provided by different catalytic complexes such as superoxide, dismutase, catalase and peroxides. The defence system reduces the harmful effects caused by free radicals (Ahmad *et al*., 2013). Failure of the antioxidant defence system results in a build-up of free radicals at the cellular membranes causing lipid peroxidation and also exposes them to oxidative damage (Ansari *et al*., 2015). Subsequently, long term pathological conditions, for instance, rheumatoid arthritis, vascular dysfunction, diabetics, chronic inflammation, stroke and septic shock, premature ageing as well as cancer may develop (Bruce *et al*., 1991).

Low antioxidant diet intake has been linked to the development of some cancers. Besides, a review by Valko *et al*. (2004), established that carcinogenesis is associated with oxidative damage on DNA molecules. In most cases, initiation of cancer is associated with chromosomal defects and mutation of oncogenes, due to cellular interaction with high levels of reactive species. The mutagenic damage on DNA bases leads to chemical carcinogenesis (Kryston *et al*., 2011). However, free radicals in their right amount help the body in performing different physiological functions such as health ageing (Lagouge and Larsson, 2013).

2.2 Free radicals

Molecules or atoms with unstable electrons form free radicals once a covalent linkage is broken forming unpaired electrons. Reactive oxygen and nitrogen species are formed by unpaired electrons of oxygen and nitrogen respectively. These unstable species can either give or receive an electron from other atoms (Halliwell, 2007). In the majority of the pathological condition, the oxygen-containing unstable molecule is important in the progression of terminal illnesses. Some of the prominent free radicals include hydrogen peroxide, hypochlorite, hydroxyl and superoxide anion radicals as well as peroxynitrite and nitric oxide radicals (Fang *et al*., 2002). These reactive species are destructive to biological molecules thereby disrupting homeostatic processes in the tissues.

2.3 Sources of free radicals

2.3.1 Endogenous sources

The body produces free radicals by different mechanisms ranging from the normal metabolic processes, elimination of parasites and pathogens through oxidizing systems such as peroxides, superoxide and nitric oxide. Moreover, fatty acids degradation by peroxisome produces hydrogen peroxide as a byproduct as well as in the inflammatory response, immune cell activation, ischemia, infection and during excessive mental and physical stress (Ahmed, 2014).

One of the mechanisms that the body's immune system responds to a stimulus or foreign particle is the process of inflammation (Bala and Haldar, 2013). During these process, different immune cells such as neutrophils and macrophages are activated to undergo oxidative burst, which is a chain of biochemical responses that phagocytes undergo when exposed to a foreign particle leading to the production of unstable reactive molecules like nitric oxide and superoxides that aid in the destruction of the foreign particle or organism. During oxidative burst free radicals are produced in large amounts, hence there is a possibility that there will be excess free radicals that will create an imbalance unless neutralized by antioxidants (Valko *et al*., 2007; Bala and Haldar, 2013).

Similarly, during the phagocytic processes, the body's leukocytes produce a high amount of superoxide to help in providing antibacterial activity, but in the process, superoxide is transformed enzymatically to hydrogen peroxide by superoxide dismutase. The generated

hydrogen peroxide may partially dissociate to form a more potent oxidant or a hydroxyl radical (Knight, 2000; Wu and Cederbaum, 2003).

2.3.2 Exogenous sources

Free radicals can be obtained from external sources including polluted air and water, cigarette smoke (primary or secondary), medical radiations, industrial waste, sunbeams, as well as heavy metals, alcohol, unsaturated fat and certain drugs (Arika *et al*., 2019). Once they gain entry into the tissues, the particular extrinsic complexes are converted to radicals which serve as an exogenous supply of oxidants to the body. Exposure to different pollutants in the environment such as air and water pollution leads to stirring up of the immune cells to fight off the foreign particles. The cells themselves produce free radicals to eliminate the foreign particles and in the process, contribute to their accumulation in the body.

Foods that we use contain chemicals some of which were used as pesticides and fertilizers. These chemicals some of them contain free radicals and some when ingested they produce free radicals. These radicals will either react with others producing more destructive radicals or can directly react with different cellular components leading to damage (Wu and Cederbaum, 2003). Tobacco smoke contains a lot of oxidants that cause oxidative damage to the lungs during smoking. These oxidants deplete antioxidants in the lungs thus leading to a loss of balance between oxidants and antioxidants. Besides, smokers have high levels of neutrophils in their respiratory tract that fights foreign particles. The high levels of neutrophils can themselves lead to increased levels of free

radicals through the activation of processes such as inflammation and respiratory burst (Lobo *et al*., 2010).

2.4 Types of reactive Oxygen and Nitrogen Species

2.4.1 Superoxide radical

Superoxide radicals are dioxide with distinctive reactivity; it is generated in the reduction reaction of oxygen by various oxidases that include xanthin oxidases, cyclooxygenase and NADPH (Dihydronicotinamide-adenine dinucleotide phosphate) oxidase. The electron transport chain of mitochondria during the progression of normal oxidative phosphorylation in energy production in the form of ATP (Adenosine triphosphate) is also involved. Superoxide generation takes place in the mitochondria (Dröse, and Brandt, 2012). The major source of ATP in animal cells is the electron transport chain mitochondrial. There's premature leakage of electrons to oxygen forming superoxide, during energy transduction and this advances to various disorders in the body.

2.4.2 Hydroxyl Radical ($^{\bullet}$OH)

Fenton reaction from hydrogen peroxide, when reacted with ferrous ions, is responsible for the formation of Hydroxyl radical: $Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + {}^{\bullet}OH + OH^-$

Likewise, when hydrogen peroxide and superoxide radicals are reacted in Heber-Weiss reaction: $O_2^{\bullet-} + H_2O_2 \longrightarrow O_2 + {}^{\bullet}OH + OH^-$

The hydroxyl radical formed is extremely unstable hence it destroys many tissue elements (Valko *et al*., 2006).

2.4.3 Hydrogen peroxide (H_2O_2)

It is formed from dismutation reactions by superoxide dismutase or during the respiratory burst. It has two-electron reduction states and readily permeates across the cellular membranes because it's lipophilic. It mainly causes modification in the DNA molecule and protein cross-linking. In oxidation reactions like those involving biosynthesis of biological complexes, it acts as the substrate (Kumar, 2011). In the body, liver cells are also associated with the generation of hydrogen peroxide.

2.4.4 Hydroperoxides (ROOH)

This molecule is formed when lipids, proteins and nucleic acids are reacted with radicals. Hydroperoxides are good oxidants and readily reacts with other molecules (Sudha, 2016).

2.4.5 Nitric oxide

In tissues, nitric oxide is produced by a particular nitric oxide synthase, which converts amino acid arginine to amino acid citrulline eventually forming nitric oxide radical (Ghafourifar and Cadenas, 2005). This reactive species can also be produced by the immune cell during oxidative burst triggered by inflammation.

$$\text{L-Arg} + O_2 + \text{NADPH} \xrightarrow{\text{nitric oxide synthase}} NO^{\bullet} + \text{citrulline}$$

2.4.6 Peroxynitrite ($ONOO^-$)

Nitric oxide can react with superoxide radical during inflammation consequently forming Peroxynitrite ($ONOO^-$), a very strong oxidizer that causes lipid peroxidation and DNA fragmentation (Nimse and Pal, 2015).

$NO^{\cdot} + O_2^{\cdot -} \longrightarrow ONOO^{-}$ **(**Carr *et al*., 2000).

2.5 Beneficial roles of free radicals in the body

Majority of physiological biochemical reactions produces unstable reactive species in a tightly controlled manner. In the human body, these reactions are part of the sequence of events in phagocytic antimicrobial action via NADPH-oxidase, using leukocytes in the defence system. The mechanism is key to the defence system of human against microbial agents and is intended to destroy cellular components of the invading pathogens (Pasupuleti *et al*., 2012). For example, superoxide and nitric oxide are generated in large volumes by macrophages for destroying microbes.

In biochemical processes, free radicals play a regulatory role, for instance, lymphocytes and fibroblasts continually produce superoxide radical in small doses as growth regulators (Asgher e*t al*., 2017). Besides, during an infection phagocytes such as endothelial cells and arterial smooth muscle cells are triggered to release superoxides. Moreover, in their work, Sektioglu *et al*., (2016) reported that macrophage-derived nitric oxide is implicated in the elimination of bacterial and tumour cells.

Furthermore, some of the enzymes' mechanism of action involves free radicals, for instance, prostaglandin synthase, cytochrome P-450 as well as ribonucleoside diphosphate reductase. Additionally, tissue injury accompanying ischemia/reperfusion and production of reactive oxygen species are sources of free radicals. Valko *et al*.,

2007observed that normal and cancerous cell liberates superoxide and hydrogen peroxide suggesting their likely role as cellular "messengers".

2.6 Harmful effects of free radicals

2.6.1 Oxidative damage to protein

Free radicals damage proteins through alteration of individual amino acid, peptide splitting and protein cross-linkages formation attributed to reaction with lipid peroxidation products. Oxidative damage to proteins affects the receptors, membrane transport as well as enzymes activity (Zhang and Zhang, 2014). Proteinous molecules that are damaged through oxidation may contain reactive complexes that contribute to the altered membrane and cellular functions. Proteins are usually oxidized and destroyed by peroxyl radical (Chapple and Matthews, 2007). Similarly, reactive oxygen species can impair amino acids and produce carbonyls which may result to further protein changes comprising of creation of methionine sulfoxide and protein carbonyls along with protein peroxide (Moskovitz and Oien, 2010).

Free radicals may influence polypeptide chains disintegration, the variation of electrons in amino acids, chemical bonding and modification of signal transduction in addition to the oxidation of specific amino acid (Chatgilialoglu *et al*., 2011). These alterations bring about an increase in susceptibility to proteolysis by degradative protease, with a consequence of premature ageing. Unfolding and degradation of proteins result from structural modifications from the oxidation of methionine residues or sulfhydryl groups. Catalysts having metallic ions on their interaction sites are mainly sensitive to metal-catalyzed oxidation (Torreggiani, 2009).In certain instances, amino acid oxidation may

take place like in case of methionine being converted to methionine sulfoxide and phenylalanine being changed to O-tyrosine (Wehr and Levine 2013).

2.6.2 Lipid peroxidation

Biomolecules may be modified oxidatively and this is involved in several physiological and disease progressions in cases like senescence, atherosclerosis, inflammation and tumorigenesis as well as intoxication by medication. Phaniendra *et al* 2015). Peroxidation of lipids is a biochemical mechanism that generates secondary free radical. Lipid peroxidation may be initiated by ROS thereby upsetting phospholipids layers of the plasma membrane consequently inactivating enzymes and membrane-bound receptors thereby increasing permeation of cells (Gęgotek and Skrzydlewska, 2019). Unsaturated aldehydes, which are products of lipid peroxidation, are capable of modifying the majority of the cellular molecule such as amino acids by forming bonds with other molecules. Liu and Pravia 2010 stated that 4-Hydroxy-2-nonenal depletes cellular GSH (Reduced glutathione) and stimulates the production of peroxides alongside activation of receptors of growth factor and induction of fibronectin production on the epidermis.

Isoprostanes and thiobarbituric acid which are reactive elements from lipid peroxidation are utilized as secondary biological indicators of oxidative stress where they are found in plenty in the respired air, secretions of bronchioles and alveoli as well as in the lungs of cigarette smokers(Moriasi *et al*., 2020). Biological membranes and intracellular structures are typically sensitive to oxidative attack attributable to the presence of conjugated fatty acids in their phospholipids bilayers (Catalá, 2009).

2.6.2 Carbohydrates oxidation

Oxidation of carbohydrates comprising of mannitol and glucose is mainly attributed to the free carbon and hydrogen atoms of deoxy sugars (Zhang and Huber, 2018). Reactive unpaired elements interact with carbohydrates molecules to form carbon-centred radicals. These radicals react with another carbohydrate, and the series of autocatalytic chain reaction is initiated in destroying the cells subsequently (Sharma *et al*., 2018). The most common oxidative products of carbohydrates are Ketoamines and ketoaldehydes (Hopps *et al*., 2010).

2.7 Oxidative stress and human diseases

Oxidative damage is attributed to the progressions of different pathological states including atherosclerosis, inflammatory condition, tumours as well as senescence (Bansal and Kaushal, 2014). Oxidative stress is reported to be involved in inflammatory disorders such as arthritis, adult lung diseases. Moreover, ischemic disorders, hemochromatosis, acquired immunodeficiency syndrome, emphysema, gastric ulcers, hypertension in pregnancy, a neurological disorder in addition to musculoskeletal disorders (ASIF,2018).

2.7.1 Cardiovascular diseases

A study by Molavi and Mehta, 2004 demonstrated that oxidative stress is connected with the raised generation of ROS that alters lipids and amino acids thus stimulating peroxidation and oxidation of thiol groups. This prompts alteration in membrane permeability, membrane lipid bilayer disruption and functional modification of various cellular proteins. ROS-induced oxidative stress in hypertensive patients is accompanied by decreased levels of antioxidants such as Vitamin E, GSH, and superoxide dismutase

(SOD), all good scavengers of free radicals. Lipid peroxidation mainly affects polyunsaturated fatty acids (low-density lipoproteins composed of lipids and proteins (Catalá, 2009). Free radical reacts with the lipid component forming oxidized LDL which initiates the inflammatory reaction. This oxidized LDL that is taken up by white blood cells such as macrophages, which collect in the artery wall leading to foam cell formation and atherosclerotic plaques (Lobo *et al*., 2010). These macrophages attract other immune cells to the same point causing a growth inside the artery which blocks or slows the flow of blood to the heart.

2.7.2 Cancer

Cancerous cells, as opposed to healthy cells, bring about cellular oxidative stress as a result of cellular redox imbalance. ROS reacts with nucleotides in the chromosomes thus triggering DNA strands promoting transformation that may lead to carcinogenesis and mutation (Valko *et al*., 2006). ROS interferes with the gene expression as well as some signal transduction cycle that are influential in the progression of carcinogenesis (Poli *et al*., 2004).

One of the major genes in the suppression of cancer is the Tumour protein (Tp53) which codes for amino acids that regulate the cell cycle. The protein that suppresses tumour in the tissues is crucial in inhibiting the initiation of tumorigenesis by stopping impaired cells from undergoing division till chromosomes are repaired or the irreparable cell undergoes programmed cellular death (Rossiello *et al*., 2014).ROS production is heightened by the p53-mediated transcription of apoptotic genes. Moreover, p53 also

promotes the expression of various antioxidant genes that thwart programmed cellular death (Kaiser and Attardi, 2018).

2.7.3 Rheumatoid arthritis

One of the autoimmune disorders that bring about prolonged soreness of the joints and tissue around the joints is Rheumatoid arthritis. It is characterized by permeation of macrophages and activated T cells resulting in inflammation (Mateen *et al*., 2016).

The disease progression is connected with the creation of free radicals at the site of inflammation. Oxidative damage in synovial tissue is also linked with a high rate of p53 mutation. The migration of monocytes and lymphocytes into the rheumatoid arthritis synovium is mediated by the abnormal expression of several adhesion molecules (ELAM-1, VCAM-1, ICAM-1, ICAM-2) (Cunnane *et al*., 2001) and induction of redox-sensitive signalling pathways. Increased ROS levels in RA result in a pro-oxidation environment, which in turn could result in decreased antioxidant activity and increased malondialdehyde (MDA) levels (an oxidative stress parameter) oxidative stress and reductions in the levels of an antioxidant such as sulfhydryl is associated with chronic inflammation associated with the disease (Pedersen-Lane, 2007). Persistent inflammation results in the destruction of cartilage and bone. This occurs through several mechanisms, including the oxidative and proteolytic breakdown of collagen and proteoglycans (Valentino, 2010). They may also undergo a respiratory burst and generate several reactive oxygen species, including superoxide, hydrogen peroxide and possibly hydroxyl radical (Kärkönen and Kuchitsu, 2015).

2.7.4 Aging

Ageing is a continuous decrease in normal biological activities in an organism (Valko *et al*., 2007). This can arise from different effects caused by free radicals (Vilchez *et al*., 2014). The current theories associated with ageing are free radical and mitochondrial theories. The proposed theory suggests that a chain of reaction is generated in the mitochondria in which unstable molecules are formed in large amounts, in so doing enhancing potential damage (Marnett, 2000). Oxidative stress which is indicated in the ageing process is generally exhibited as a continual buildup of various harmful alterations in the body. Besides, ageing escalates the threat of ailments and mortality (Rahal *et al*., 2014).

Studies that have been done recently have indicated that with age, ROS levels accumulate in the liver, heart, brain, and muscular-skeletal regions this is due to their amplified production or minimized elimination(McCormick and Goljanek-Whysall 2017). Therefore, ageing can be described as a progressive weakening of tissues functions about time as well as a decline in the flexibility to stress or a general rise in vulnerability to ailments (Kregel and Zhang, 2007). At the moment oxidative stress theory is the most established description of ageing which holds that upsurges in ROS are a pointer to alteration of bodily functions, disease state and other noticeable signs of ageing and ultimately death (Hagen, 2003).

2.7.5 Neurological disorders

Neurodegenerative disorders are suspected to be as a result of oxidative stress. These disorders include Parkinson's disease, Huntington's disease, Multiple sclerosis, Lou Gehrig's disease, Depression and Alzheimer's disease among others (Patel and Chu, 2011). Reactive nitrogen and oxygen molecules generation, together with antioxidant defence can be monitored as biomarkers indicative of oxidative damage that is associated with the progression of neurodegenerative disorders (Nunomura *et al*., 2006). Interference with mitochondrial aerobic metabolism as well as mitochondrial damage results in increased oxidative stress. This accumulation is associated with the progression of Alzheimer's disease, Parkinson's disease, and other neurodegenerative diseases (Ramalingam and Kim, 2012).

Oxidative stress due to free radicals is thought to be one of the contributing factors that lead to Parkinson’s disease (Kumar *et al*., 2012). Neurons rely on aerobic respiration that occurs during oxidative phosphorylation in the mitochondria. Any mitochondria alterations or dysfunction leads to low levels of ATP production, incomplete reduction of oxygen leading to the production of radicals such as superoxide radicals, hydroxyl and peroxynitrite. Besides, this induces mitochondrial DNA damage. Accumulation of mitochondrial DNA damage initiates ageing and cell death (Bjelakovic *et al*., 2012; Kumar *et al*., 2012).

Microglias are immune cells that occur in the central nervous system, they are involved in the elimination of foreign particles from the brain (Kumar *et al*., 2012). When

activated, they produce free radicals that aid them in the elimination of free radicals. These radicals, in excess, cause oxidative damage to different cells in the central nervous system. They also deprive the system the antioxidants that offer the defence (Koutsilieri *et al*., 2002). The neurotransmitter dopamine is susceptible to auto-oxidation when it is excess in the cytosol leading to the formation of products such as dopamine-quinone capable of covalently modifying different cell molecules. In the process of auto-oxidation different free radicals such as superoxide and hydrogen peroxide are produced (Koutsilieri *et al*., 2002**;** Bjelakovic *et al*., 2012).

2.7.6 Cataract

The current proposition takes oxidative stress as a vital aspect of age-related complications in the tissues, cataract is one of them (Vinson, 2006). The generation of unstable reactive species and the decline of intracellular antioxidants molecules together play a role in cataract development. Oxidative damage brought about by ultraviolet rays also appears to take part in several complications of the eye including deterioration of the retina and cataract formation. Oxidative stress constantly subjects the eye lens to damage through destruction of the crystalline proteins and other components of the eye (Vinson, 2006).

Nevertheless, the eye employs various systems to shield its constituents from oxidative damage and to sustain its redox state. The mechanisms involve catalytic chain reactions along with elevated amounts of ascorbic acid and reduced glutathione (Zorić, 2003).

Though, with advanced age, the buildup of oxidized lens constituents and inefficiency of repair channels may be attributed to the progression of cataract (Zorić, 2003).

Continual exposure to ultraviolet ray encourages the production of reactive oxygen species which are attributed to cataract formation. Peroxidation of lipids has also been suggested as an element in the development of cataract (Berthoud and Beyer, 2009). On the other hand, Sawada *et al*. (2009) established a noteworthy rise in activities of superoxide dismutase and protein concentration in nuclear cataracts, suggestive of oxidative stress involvement.

2.8 Antioxidants

Elements that can bond with and stabilize free radicals to prevent their harmful effects on the tissues are regarded as antioxidants (Nimse and Pal, 2015). These elements are also found in diet and may safeguard the cells from the detrimental effects of free radicals, molecules that can damage cells. Examples of antioxidants include glutathione, ubiquinol, and uric acid, are produced during normal metabolism in the body (Arika *et al*., 2019). They also terminate free oxygen species through enzyme-like superoxide dismutase (SOD), glutathione peroxidase (Gpx), and catalase and thioredoxin reductase. These molecules deter the process of oxidation by giving out an electron to a free radical making it neutral and harmless (Moriasi *et al*., 2020). This defence systems work by hindering the initial generation of free radicals, scavenging the oxidants and transforming them into harmless substances also by stalling the production of harmful secondary metabolites, inflammatory mediators (Mwihia, 2017). Another mechanism of

antioxidants is the obstruction of the production cycle of the secondary oxidants, repairing the molecular damage brought by free radicals or gene expression regulation as well as improving the cellular antioxidant defence mechanisms.

2.8.1 Natural sources of antioxidant

Natural antioxidants either are synthesized in the human body through the metabolic process or are supplemented from other natural sources, and their activity very much depends upon their physical and chemical properties and mechanism of action. This can be further divided into two categories, i.e., enzymatic antioxidants and nonenzymatic antioxidants (Haida and Hakiman, 2019).

Antioxidants act as the radical scavenger, hydrogen donor, electron donor, peroxide decomposer, singlet oxygen quencher, an enzyme inhibitor, synergist, and metal chelating agents. These defence mechanisms work together in protecting the tissues from oxidative damage (Mehla et al., 2017). The antioxidant defence system consists of powerful enzymatic and non-enzymatic antioxidants. Antioxidants can be classified differently depending on their effects; they can be grouped as enzymatic and non-enzymatic (Arika *et al*., 2019). The enzymatic ones act by breaking and eliminating free radicals. For instance, catalyst transforms hazardous oxidative products to hydrogen peroxide (H_2O_2) subsequently into the water, in a process that utilizes inorganic copper, zinc, manganese, and iron (Mwihia, 2017). On the other hand, non-catalytic counterparts work by interfering with free radical formation pathways. Vitamin C and E, a plant

polyphenol, carotenoids as well as glutathione are some of the naturally occurring non-enzymatic antioxidants (Moriasi *et al*., 2020).

2.8.1.1 Superoxide dismutase

They are a class of closely related enzymes that catalyze the breakdown of the superoxide anion into oxygen and hydrogen peroxide (Zelko *et al*., 2002). They are present in almost all aerobic cells and the extracellular fluids. They contain metal ions that can be copper, zinc, manganese or iron. In humans, the copper/zinc superoxide dismutase is present in the cytosol, while manganese superoxide dismutase is present in the mitochondria. There also exists a third form of superoxide dismutase in extracellular fluids, which contains copper and zinc in its active sites (Johnson and Giulivi, 2005) Superoxide dismutase removes superoxide anion by catalyzing a dismutation reaction. In the absence of superoxide dismutase, this reaction occurs non-enzymatically but at a very slow rate (Nozik-Grayck *et al*., 2005).

2.8.1.2 Catalase

Catalase is an enzyme found in all living organism which is exposed to oxygen. It's found in the cell organelle called the peroxisome. It exists as a tetramer composed of 4 identical monomers, each of which contains a heme group at the active site. The function of catalase is to catalyze the decomposition of hydrogen peroxide to water and oxygen. All organs have catalase with the higher concentration being at the liver (Kirkman *et al*., 1999). Degradation of H_2O_2 is accomplished via the conversion between 2 conformations of catalase-ferricatalase (iron coordinated to water) and compound I (iron complexed with an oxygen atom). Catalase also binds NADPH as a reducing equivalent preventing

oxidative inactivation of the enzyme (formation of compound II) by H_2O_2 as it is reduced to water(Goyal and Basak, 2010).

2.8.1.3 Glutathione systems

The glutathione system includes glutathione, glutathione reductase, glutathione peroxidases, and glutathione S-transferases. Glutathione peroxidase is an enzyme-containing four selenium-cofactors that catalyze the breakdown of hydrogen peroxide and organic hydroperoxides. Glutathione peroxidase 1 is the most abundant and is a very efficient scavenger of hydrogen peroxide, while glutathione peroxidase 4 is most active with lipid hydroperoxides. The glutathione S-transferases show high activity with lipid peroxides. These enzymes are at particularly high levels in the liver and also serve in detoxification metabolism (Sharma *et al*., 2004).

Glutathione reductase (GR) is a crucial enzyme that reduces glutathione disulfide (GSSG) to the sulfhydryl form (GSH) by the NADPH-dependent mechanism, an important cellular antioxidant system. GR is a flavoured protein that contains two FAD molecules as a prosthetic group, which is reducible by NADPH. GR is one of the thermostable enzymes. GR belongs to the defence system protecting the organism against chemical and oxidative stress (Hayes *et al*., 2005).

2.8.1.4 Glutathione

In almost all cellular compartments there's the abundance of glutathione the major soluble antioxidant. GSH/GSSG ratio is a major determinant of oxidative stress (Birben

et al., 2012). Glutathione exhibits its antioxidant potential in several ways through detoxification of hydrogen peroxide and lipid peroxides via the action of Glutathione peroxidase (GSH-Px). GSH contributes its electron to H_2O_2 to be reduced into H_2O and O_2 (Kumar and Jain 2015). GSSG is again reduced into GSH by GSH reductase that uses NADPH as the electron donor. GSH-Pxs are also essential for shielding of plasma membrane from lipid peroxidation and protect them from oxidant attacks (Birben *et al*., 2012). GSH is a cofactor for several detoxifying enzymes, such as GSH-Px and transferase. It has a role in converting vitamin C and E back to their active forms. GSH protects cells against apoptosis by cooperating with proapoptotic and antiapoptotic signalling cycle (Ulusu and Tandoğan 2007).

2.8.1.5 Vitamin E

Lipophilic tocopherol is saturated in the aquaphobicinner regions of the cytomembrane and is the primary protection against oxidant-induced injury to the plasma membrane. Alpha-tocopherol is the most active form of vitamin E and the major membrane-bound antioxidant in the cell. Vitamin E donates the electron to peroxyl radical, which is produced during lipid peroxidation (Howard *et al.,* 2011).

Vitamin E stimulates programmed cellular death of malignant cells and obstructs generation of free radical. It is promoted for a range of purposes-from delaying ageing to healing sunburn and powerful antioxidant which shields cellular membranes from oxidants by neutralizing radicals generated in the lipid peroxidation cycle (Dong *et al*., 2011). This removes the free radical intermediates and prevents their propagation. It also

plays a role in immunocompetence by increasing humoral antibody protection, resistance to bacterial infections, cell-mediated immunity, the T-lymphocytes tumour necrosis factor production, inhibition of mutagen formation, repair of membranes in DNA, and blocking micro cell line formation (Sen *et al*., 2006). Therefore, vitamin E may be useful in cancer prevention and inhibit carcinogenesis by the stimulation of the immune system.

2.8.1.6 Vitamin C/Ascorbic acid

Ascorbic acid is soluble in water therefore it offers cellular aqueous-phase antioxidant capacity primarily by scavenging oxygen free radicals (Niki, 2010). These vitamins are present abundantly in fruits and vegetables and because they can't be synthesized in humans they must be obtained from the diet. In some animals, ascorbic acid is synthesized in the tissues therefore not required in the diet. It is preserved in its reduced form in cells by reacting with glutathione, mediated by disulfide isomerase and glutaredoxins (Hamid *et al*., 2010).

Vitamin C is a reducing agent and it neutralizes ROS such as hydrogen peroxide. In addition to its direct antioxidant effects, ascorbic acid is also acted upon by the antioxidant enzyme ascorbate peroxidase, a function that is particularly important in stress resistance in plants (Caverzan *et al*., 2012). Ascorbic acid may be useful in averting cancer where the mode of action may include antioxidant effects, hindering generation of nitrosamines, improvement of the immune response, and hastening of cleansing enzymes of the liver (Linster and Schaftingen, 2007).

2.8.1.7 Carotenoid

Lycopene, β-carotene, lutein, and zeaxanthin are all complexes contained in carotenoid. These are coloured compounds originating from fruits and vegetables and are lipid-soluble. Lutein is commonly found in plenty amount in green vegetables and its renowned for it's protecting the optic nerve against the deleterious properties of frees radicals as well as antiatherosclerotic effects (Sikora *et al*., 2008; Hamid *et al*., 2010). On the other hand, lycopene is sourced from tomatoes while spinach is a source of zeaxanthin. Studies have shown that lycopene is a powerful antioxidant and is an efficient molecule in scavenging singlet oxygen in fruits.

Vitamin A (retinol) originates from β-carotene and it is present in animal proteins, vegetables and grains. Oxidative products can cause genetic damage hence β-carotene protects the tissues against malignancy through its antioxidant properties (Linster and Schaftingen, 2007). Moreover, the photoprotective effects of β-carotene may guard against ultraviolet light-induced carcinogenesis. Immune improvement properties of β-carotene may contribute to cancer protection. β-carotene may also have an anticarcinogenic effect by altering the hepatic metabolism effects of carcinogens (Tanaka *et al*., 2012)

2.8.1.8 Minerals

For the proper functioning of the biocatalysts, minerals are required in the body and biosynthesis of macromolecules can be adversely affected by their absence. This inorganic salt comprises manganese, zinc, copper, iron, and selenium that act as cofactors

for the enzymatic antioxidants (Illanes, 2008). In the biological system iron is the most abundant mineral that is conjugated with proteins. Generally, the amount of circulating iron is low and these concentrations encourage the generation of ROS, lipid peroxidation, subsequently oxidative damage (Barrera, 2012). Therefore, supplementation with iron aids in decreasing oxidative damage.

Magnesium, on the other hand, is a cofactor in pentose pathway which produces NADPH from NADP by glucose-6-phosphate dehydrogenase (G6PD) and 6-phosphogluconate dehydrogenase (6PGD) glucose metabolism preserving the normal ratio of GSH to GSSG and a normal redox state in cells. Insufficiency of magnesium decreases GR effects and GSSG is not changed to GSH, with the consequence of oxidative stress in the cells (Fang *et al.,* 2002).

Conversely, selenium is an essential element of enzymatic antioxidant and in its presence, glutathione peroxidase (GPx) protects the cell membrane against lipid oxidation as well as taking part in H_2O_2 and lipids' hydroxyl peroxide metabolism. Therefore, these mineral acts like tocopherol and can be substituted with vitamin E to avert the predisposition of malignancy and cardiovascular disorders (Sikora *et al*., 2008).

2.8.1.9 Polyphenols

Phenols are a family of secondary metabolites that are potent antioxidants. The antioxidant effects rely on their physicochemical character that controls biochemical reactions determined by their atomic organizations (Ajila *et al*., 2011). Polyphenols comprise of gingerol, curcumin, flavonoids and phenolic acids (Kunwar and Priyadarsini, 2011).

Phytochemicals are bioactive chemicals that are found in plant-based foods and they give them their characteristic colour, smell and taste. They protect plants from diseases and ecological threats like pollutants, oxidants, drought, radiations and herbivores (Rai, 2016). Phytochemicals neutralize carcinogens by inhibiting chemicals that stimulate their production and at the same time stabilize free radicals. For instance, genistein inhibits angiogenesis in tumours and metastasis (Xiao *et al*., 2015)

One of the principal nutrient groups acknowledged by the scientists is the flavonoids. Kaempferol, catechins, quercetin and anthocyanidins comprise some of the best-known flavonoids. This dietary family is popular for its health benefits as a radical stabilizer as well as anti-inflammatory activity in addition to food colourant agent (Holt *et al.,* 2009). Flavonoids are antioxidants that are involved in reducing the effects of free radicals in the tissues. These molecules block the yield of messenger molecules involved in inflammation in addition to the repression of nuclear factor kappa-B (NF-kB) cyclepro-inflammatory signals (Das, 2020). In the circulatory system, they aid in protecting lipoproteins from oxidative damage, for instance, rutin and hesperid which are indicated in the maintenance of the integrity of the blood vessel walls. On the other hand, quercetin and rutin have been shown to have the antiaggregatory property that helps inhibit excessive clumping of platelets that may lead to unwanted clogging of blood vessels which helps support the cardiovascular system (Harborne and Baxter, 1999).

Essential oils, resins and oleoresins comprise of terpenoids amongst the common and chemically diverse family of organic materials that are highly combustible branched chains carbon compounds occurring as liquids (Firn, 2010). These hydrocarbons of plant origin are presented as $(C_5H_8)_n$ and are categorized as mono-, di-, tri- or sesquiterpenoids based on number carbon atoms. Diterpenes (C_{20}) are typically regarded as resins and taxol which is an antineoplastic agent. On the other hand, triterpenes (C_{30}) comprises of cardiac glycosides, sterols, and steroids with anti-inflammatory, sedative and cytotoxic activity (Harborne, 1991).

2.8.2 Synthetic antioxidants

Several manmade phenolic compounds act as antioxidants and they include Nordihydro-guaretic acid, the metal chelating agent, tertiary butyl hydroquinone and butylated hydroxyanisole butylated hydroxytoluene (BHT), propyl gallate(PG) in addition to butylated hydroxyanisole (BHA) (Hamid *et al*., 2010). In cells, the concentration of free radicals together with other unstable molecules is controlled by antioxidant defences, which minimize oxidative damage to cellular components (Halliwell, 2007). Butylated hydroxytoluene (BHT) is extensively used as an antioxidant where free radicals must be controlled. It acts as an artificial equivalent of tocopherol acting as an agent that prevents autoxidation, where oxygen attacks unsaturated organic molecules. This autocatalytic oxidation is stopped by BHT through donating a hydrogen atom by transforming peroxy radicals to hydroperoxides (Wang *et al*., 2016).

Butylated hydroxyanisole (BHA) is another reducing agent that is widely used. It consists of a blend of 3-tert-butyl-4-hydroxyanisole and 2-tert-butyl-4-hydroxyanisole, made from 4-methoxyphenol and isobutylene. BHA has been chiefly used as a preservative in food an antioxidant, cosmetics, rubber and petroleum products industries (Wells *et al*., 2018). As Butylated hydroxytoluene (BHT), the coupled aromatic ring of BHA is capable of neutralizing free radicals, by segregating the unstable molecules thus preventing further reactions.

These molecules have been extensively utilized as antioxidants in the food industry, cosmetics and for therapeutic purposes. Though, physicochemical properties of the synthetic antioxidants such as their instability at high temperatures, strict legislation on the use as food additives carcinogenic nature and consumer preferences have refocused the attention of manufacturers from synthetic to natural antioxidants.

2.9 Management of oxidative stress

2.9.1 Conventional management of oxidative stress

There are several synthetic antioxidants classified as phenolic compounds that capture free radicals preventing them from initiating a series of chemical reactions. They comprise of, metal chelating agent (MCA), Tertiary butyl hydroquinone (TBHQ), Butylated hydroxytoluene (BHT), N-acetylcysteine, alpha-lipoic acid and Propyl gallate (PG), Butylated hydroxyl anisole (BHA) and Nordihydro-guaretic acid (NDGA) (Hamid *et al*., 2010). These have been analyzed to reduce free radical production to amounts that are not harmful to the body. These conventional antioxidants are invaluable but limited

by their restricted activity, cost, and failure rates and associated adverse effects such as low blood glucose, liver damage and carcinogenesis (Virdi *et al*., 2003).

2.9.2 Alternative management of oxidative stress

Fruits, vegetables, nuts as well as organic green tea, provides a source of mineral antioxidants, (Selenium, Copper, Zinc and Iron, which act as co-factors of antioxidants) and vitamins needed for most metabolic processes (vitamin B, C and E). Vitamins A, C, E, lycopene, beta-carotene in addition to resveratrol are some of the antioxidants from dietary sources (Hamid *et al*., 2010; Asif, 2015). Reduction of stress and having sufficient sleep helps to reduce the risk of the increase in oxidation activities that will produce more oxidants (Rahal *et al*., 2014).

Phenols, which are extensively distributed among plants, are regarded as an essential source of dietary antioxidant elements in living systems for the obstructing oxidative stress (Matus-Cádiz *et al*., 2008**)**. Phenolics comprise compounds such as flavonoids which are found in plant pigments and regarded as nature's biological stabilizers since experiments have shown their capacity to regulate the body`s reaction to chemicals, microorganisms and cancer-causing agents (Krifa *et al*., 2013). They demonstrate the ability to resist immune responses towards allergic reactions, inflammation as well as microbial and cancer invasion (Yamamoto and Gaynor, 2001) this is associated with the phenolics hydroxyl moiety linked to the flavonoid (Silva *et al*., 2013).

The scavenging effects of lipid peroxyl radicals and interruption of the free radical chain reaction by vitamin E gives its antioxidant properties (Demain and Sanchez, 2009). A hydrogen atom is removed from the lipid peroxyl radical by the phenyl group of tocopherol thereby stabilizing the molecule that has accepted a hydrogen atom. Sequentially, tocopherol is altered into a molecule that is stable and less reactive. As a result, this vitamin E derived radical is not likely to attack lipids and continue the chain reaction. Rather, it is supposed to react with another peroxyl radical thus becoming steady. This reaction shields tissues from harm caused by free radicals and lipid peroxides.

Saraca indica bark is among the most widely used herbal medicines in India. Traditionally it is used to manage skin infections, and problems of the genital-urinary and central nervous system (Panchawat and Sisodia, 2010). According to studies by Panchawat and Sisodia (2010), has been shown to possess antioxidant potential associated with its high levels of phenolic compounds which inhibit oxidation of different cellular components such lipids and proteins by donation of an electron to the radical molecule (Rahal *et al*., 2013).

In Sub-Himalayan tracts of India *Terminalia chebula* Retz (Combretaceae) has been extensively used in the Ayurveda similarly in Unani and Homeopathic therapy. It has a valuable outcome on digestive, urinary, skin, heart diseases as well as pyrexia constipation, ulcers, vomiting, colic pain and haemorrhoids management (Juang and Sheu, 2004). Phyto-constituents present in this plants is hydrolysable tannins like gallic

acid, chebulagic acid, punicalagin, chebulamin, corilagin, neochebulini acid, ellagic acid, casuarinas and terchebulin. The methanolic extracts from the plant inhibit lipid peroxide formation and scavenge hydroxyl and superoxide radicals (Bag *et al.*, 2013). The methanolic extract of *T. chebula* has antioxidant, anti-inflammatory and anti-cancer ability and the phenolic derivatives, hydrolysable tannins and oleanane-type triterpenoids are the active components (Manosroi *et al*., 2013).

Essentially, the plant-based drugs are biodegradable as well as being regarded as safe, inexpensive and with less adverse effects. With challenges involving the management and treatment of oxidative-stress related disorders through conventional medicine, the use of plant-derived therapy is essential (Jamkhande *et al*., 2014). This involves the use of products that are naturally available hence not very expensive compared to the synthetic antioxidants.

2.10 Study Plants

2.10.1 *Caesalpinia volkensii* Harms

2.10.1.1 Description and distribution

Caesalpinia volkensii (figure 2.10.1) is a perennial wild bush that belonging to Caesalpiniaceae family and it's found throughout East Africa. The plant is commonly found in forested areas in altitude of up to 2100. Occasionally planted near homesteads (Kokwaro, 1993). This climber has a straggling stem supplied with curved and straight spikes, leaves are alternate and compound with 3 to 6 pairs of pinnae. Have opposite leaflets and unbranched inflorescence, bisexual flowers, free sepals and petals, ten free

stamens, superior ovary and a slender style. Fruits are broadly oblong with a flattened pod covered with prickles. Seeds are globose and hard.

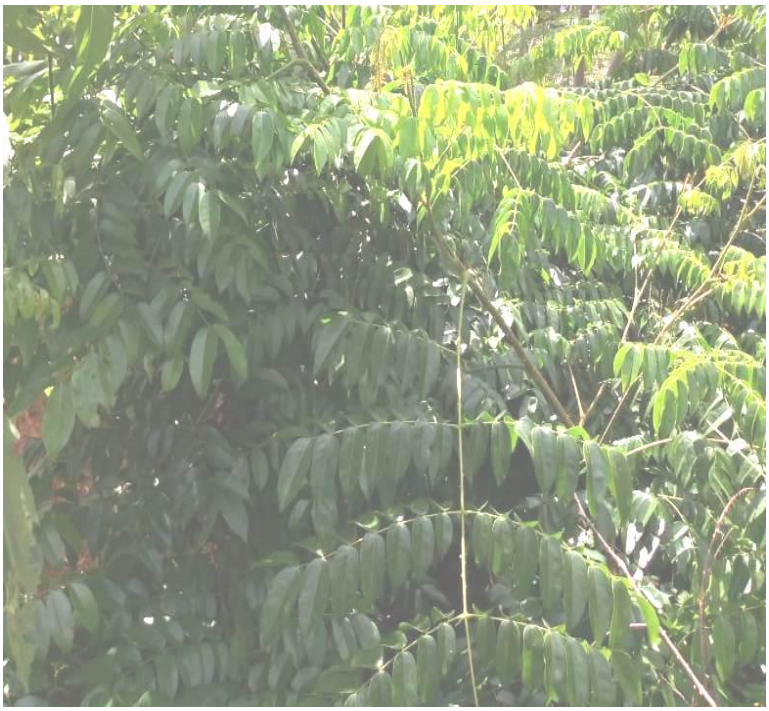

Figure 2.10.1: *Caesalpinia volkensii*

2.10.1.2 Medicinal uses

In Kenya, the leaves are traditionally used for malaria treatment. *In vitro* tests of the leaf extracts have demonstrated activity against plasmodium species that are chloroquine-sensitive and chloroquine-resistant strains (Kuria *et al*., 2001). The water leaves extracts are used for their analgesic effects by expectant mothers who take powdered pods to relieve stomach-ache besides, in Tanzania, the whole plant is used for managing seizure, gastrointestinal ulcers and as an aid in childbirth (Mworia *et al.,* 2015).In Kenya, the seeds are renowned stomach antiulcer while flower buds are ground and used for the

treatment of eye sty as well as retinoblastoma (Ndile *et al*., 2018). In various parts of the world, *C. volkensii* is used for the treatment of malaria, gonorrhoea and bilharzia (Njoroge and Bussmann, 2006).On the other hand, *C. volkensii* leaf extracts have shown hypoglycemic effects on alloxan-induced diabetic mice (Murugi *et al*., 2012).

2.10.2 *Vernonia lasiopus* O. Hoffm.

2.10.2.1 Description and distribution

Vernonia lasiopus (figure 2.10.2) are important traditional medicinal plants in Embu community. It is an erect sparsely branched subshrub that belongs to the Asteraceae family and grows to a height of three meters. The stem is woody at the base and reaches three centimetres in diameter. The upper part of the stem has branches with varying colour shade of green and purple. It has hairs each consisting of along oblique terminal cell on a few many-celled stalks (Burkill, 1985)

Figure 2.10.2: *Vernonia lasiopus*

2.10.2.2 Medicinal uses

Vernonia lasiopus is reputed to have several health benefits traditionally where an infusion of powdered leaves is used to cure indigestion, stomach-ache, malaria and also as a purgative by Kikuyu of Central Kenya. A root decoction is said to be a very effective treatment for the stomach ache (Dharani *et al.,* 2010). Its use in treating other diseases varies among communities in Kenya, for example, malaria (Kikuyu), scabies (Kamba), and venereal diseases (Luo) in addition to sores by the Maasai (Erasto, 2001). The organic fraction extracts of the plant were shown to possess sedative, analgesic (leave and seeds), antiulcerogenic (leaves and seeds), and membrane-stabilizing activity (leaves and roots) as shown by reduced RBC lysis (Erasto, 2001). Tanzanians prepare a bitter decoction from the whole plant and use in the treatment of epilepsy, indigestion and in

childbirth, the root extracts are used to facilitate parturition (Kokwaro, 1976). It is also used by Western Kenya (Bungoma) to control ticks by applying the whole plant to the animal's body (Wanzala *et al*., 2012).

2.10.3 *Acacia hockii* De Wild

2.10.3.1 Description and distribution

Acacia hockii (Figure 2.10.3) is a native plant Fabaceae family found in many dry areas in tropical Africa south of the Sahel, to eastern and southern Africa, and also Yemen. It is *a* multi-stemmed plant 2-4 m tall with an open crown. The bark is green-brown, peeling off in papery layers. The thorns are spinescent stipules. Leaves often have a gland on the petiole and between the top 1(3) pairs of pinnae. Flowers are bright yellow or orange, in axillary. The pods are reddish-brown, narrow, straight or crescent-shaped; Seeds are olive-brown, smooth, and elliptic and compressed (Wickens *et al*., 1995).

Figure 2.10.3: ***Acacia hockii***

2.10.3.2 Medicinal uses

The species has a wide range of uses throughout its native range as a source of fuelwood, resins, bee forage, bark fibre and fodder (Burkill, 1985). In Kenya, it is used by the Mbeere community as glue. Many medicinal uses are reported including as a painkiller, vermifuge and to treat stomach troubles, dropsy, swellings, oedema and gout (Burkill, 1985). In Kenya, it has been used against malaria, abdominal pains and applied to abscesses while in Tanzania, boiled bark is administered to babies to manage pyrexia while the root extract is used against hookworm alongside management of tuberculosis and associated ailments in Uganda (Tabuti *et al*., 2010).

However, mixtures of extracts from different native *Acacia* species resulted in protein precipitating profiles similar to that of wattle extract (Mugedo and Waterman, 1992). The pods and leaves are foraged by herbivores while primates feed on seeds and flowers moreover, bee forage for nectar from the flowers (Wickens *et al*., 1995). The inside of the bark is edible as a famine food. Inner bark fibre is also chewed for its juice, which has a sweet taste, and the Maasai chew the white inner bark to slake thirst. Its chemical composition was reported by Anderson *et al*. (1984).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Collection and preparation of plant materials

Fresh leaves of *Caesalpinia volkensii, Vernonia lasiopus* and stem bark of *Acacia hockii* were collected from their natural habitats in Mbeere North sub-county Embu County. The plants were chosen based on extensive ethnomedical survey and folklore reports from the local practising herbalists. The plant samples were provided to an acknowledged taxonomist for botanical authentication. The voucher specimens were deposited in plant science Herbarium at Kenyatta University for future reference. Samples were sorted out properly and transported to the Department of Biochemistry, Microbiology and Biotechnology laboratories at Kenyatta University and shade dried to complete drying for a period of two weeks. After drying, the plant materials were grounded well using electrical mill into a fine powder, packaged in well-labelled airtight containers at room temperature awaiting extraction.

3.2 Extraction of active components

A weight of 400g of each powdered plant material was soaked in 1L of methanol in a conical flask. The flask containing each plant material was shaken regularly, corked and left to stand for 48 hours at room temperature. The extracts were then filtered using Whatman No.1 filter paper and the filtrate concentrated under rotary evaporator to dryness at 65°C to recover the solid extract. The concentrates were put in airtight containers and stored at 4°C awaiting use *in vitro* bioassay.

3.3 Determination of *in vitro* antioxidant activities

3.3.1 Determination of ferric reducing power

The reducing power of the extracts was determined according to the method described by Oyaizu (1986) with some modifications. Briefly, five different concentrations of methanolic extracts (0.2, 0.4, 0.6, 0.8 and 1 mg/ml) was mixed with 2 ml phosphate buffer (0.2M, pH 6.6) and 2 ml of 1% potassium ferricyanide [$K_3Fe(CN)_6$]. The mixture was incubated at 50°C for 20 minutes. A volume of 2 ml of 10% trichloroacetic acid (TCA) was then added and the mixture was centrifuged at 1000 rates per minute (rpm) for 10 minutes. The upper layer (2 ml) was mixed with 2 ml of distilled water and 1 ml of 0.1% of Ferric chloride ($FeCl_{3)}$ and the absorbance was measured spectrophotometrically at 700 nm. The assays were carried out in triplicates. The extract concentration providing 50% of absorbance (IC_{50}) was calculated from the graph of absorbance at 700 nm against extract concentrations. The same procedure was done for ascorbic acid, which was used as a standard. Distilled water was used as the blank.

3.3.2 Determination of DPPH radical scavenging activities

The DPPH radical scavenging assay was done using 1, 1 diphenyl-2-picrylhydrazyl (DPPH) according to the method described by Ayoola *et al.* (2008) with some modifications. Briefly, five different concentrations of the extracts (0.0625, 0.125, 0.25, 0.5 and 1 mg/ml) were prepared in methanol. The same concentrations were also prepared for ascorbic acid, which was used as a standard. The extract (1 ml) was placed in a test tube, 1 ml of methanol added followed by 0.5ml of 0.1 M DPPH in methanol.

The mixture was shaken vigorously and left to stand in the dark at room temperature for 30 minutes. A blank solution was prepared to contain the same amount of methanol and DPPH. The absorbance of the resulting solution was measured at 517 nm using a spectrophotometer. The experiment was repeated thrice and measurements were taken. The DPPH radical scavenging activity was estimated based on the percentage of DPPH radical scavenged using the following equation:

$$\text{DPPH scavenging activity (\%)} = \frac{\text{Ac} - \text{As}}{\text{Ac}} \times 100$$

Where:

A_S = absorbance of the sample

A_C = absorbance of the control

The half-maximal inhibitory concentration (IC_{50}) of the extracts was computed from a plot of percentage DPPH free radical inhibition versus the extract concentration.

3.3.3 Determination of hydrogen peroxide scavenging activities

The hydrogen peroxide scavenging activities of the extracts were estimated according to the method described by Ruch *et al.* (1989). Briefly, the plant extract was dissolved in distilled water at various concentrations (0.00625, 0.0125, 0.025, 0.05, and 1mg/ml), then mixed with 1 ml of 40mM H_2O_2 solution prepared in phosphate buffer (0.1 M pH 7.4) and left to stand for 10 minutes at room temperature. This procedure was also done for ascorbic acid, which was used as a standard. The experiment was repeated thrice and the absorbance of the solution was taken at 560 nm against the blank solution containing

phosphate buffer without H_2O_2. The hydrogen peroxide scavenging activity of the extracts was calculated using the following formula:

$$\% \text{ Hydrogen peroxide scavenging activity } = \frac{Ac - As}{Ac} \times 100$$

Where:

A_S = absorbance of the sample

A_C = absorbance of the control

3.4 Determination of total phenolic contents

The total phenolic content of the extracts was done according to the Folin-Ciocalteu method with some modifications (Do *et al*., 2014). Briefly, the crude extract (1 ml) was mixed with 2 ml of Folin-Ciocalteu reagent which was prepared by dilution with distilled water in a ratio of 1:10 v/v, after which 1 ml of 20% Sodium Carbonate (Na_2CO_3) was added. The mixture was shaken for 20 seconds and incubated at 40°C for 30 minutes. Absorbance was measured at 765 nm. Gallic acid was used for the generation of the standard curve. The total phenolic content was expressed as mg of gallic acid equivalents (GAE) per gram (g) of the dried sample. The experiment was repeated thrice.

3.5 Determination of total flavonoid contents

The total flavonoid content of the extracts was evaluated through a technique described by Park *et al*. (2008). In a 10 ml test tube, 0.3 ml of the crude extracts, 3.4 ml of 30% methanol, 0.15 ml of $NaNO_2$ (0.5 M) and 0.15 ml of $AlCl_3$.6H2O (0.3 M) were blend. This was followed by addition of 1 ml of NaOH (1 M) after 5 minutes. The solution was mixed well and the absorbance was measured against the reagent blank at 510 nm. The

standard curve for total flavonoids was made using quercetin standard solution (0 to 100 mg/L). The total flavonoids were expressed as milligrams of quercetin equivalents per g of the dried fraction. The experiment was repeated thrice.

3.6 Qualitative phytochemical screening

Qualitative phytochemical screening was executed to detect the existence of secondary metabolites such as sterols, flavonoids, alkaloids, steroids, tannins as well as saponins in the plant extracts as described by Harbone (1998) and Katoke (2000). This is because some of these secondary metabolites have been associated with antioxidant activities.

3.6.1 Test for saponins

Two millilitres of each plant extract solution and five drops of sodium bicarbonate solution were added and stirred briskly for about 15 seconds. The extract was then allowed to settle for about 15-20 minutes. The experiment finding was taken as negative if there was no frothing after this length of time, and positive if there was foaming after 15-20 minutes (Katoke, 2000)

3.6.2 Test for alkaloids

A measure of 2 g of each plant extract was added to 2 ml of 1 molar aqueous concentrated hydrochloric acid to assay for alkaloids presence. The mixture was continually agitated while being heated in a water bath for 5 minutes, followed by cooling. Afterwards, the solution was filtered with Whatman's filter paper number 1 then

two drops of Dragendorff's reagent were added. After addition of Dragendorff's reagent, colour change to orange showed the presence of alkaloids (Katoke, 2000).

3.6.3 Test for terpenoids

A volume of 2 ml of chloroform was added to 0.5 g of plant extract. This was followed by carefully layering 3 ml of concentrated sulfuric acid. A reddish-brown colouration at the border revealed the presence of terpenoids (Ayoola *et al*., 2008).

3.6.4 Test for flavonoids

Two approaches were utilized to assay for the existence of flavonoids in the plant extract. To 5 ml aqueous filtrate of each plant extract, 5 ml of dilute ammonium solution was mixed then of 1 ml sulphuric acid was added. A yellow colouration that faded out on settling indicated the existence of flavonoids. In the other technique, 2 ml of each extract blended with 1 ml of 1% sodium hydroxide. An intense/golden yellow pigments pointed out to the presence of flavonoids (Katoke, 2000; Ayoola *et al*., 2008).

3.6.5 Cardiac glycosides

To test for cardiac glycosides presence, 0.5 g of the extract was dissolved in 2 ml glacial acetic acid containing 2 drops of 10% ferric chloride solution. One millilitre of concentrated H_2SO_4 was then slowly introduced into the underlying mixture. The appearance of either a violet, brown or greenish band at the boundary was observed as positive for the deoxysugars indicative of cardenolides (Ayoola *et al*., 2008).

3.6.6 Steroid test

The existence of steroids in the extract was established by mixing 0.5 g of the extract with 2 ml of chloroform. This was followed by addition of 3ml of 2M sulphuric acid carefully to the sides of the test tube creating a layer. Presence of the steroids was indicated by the reddish-brown colour at the interphase (Katoke, 2000).

3.6.7 Phenols test

To assay for the number of phenols in the extract, 1 ml of ferric chloride solution was mixed with 2 ml of the extract. The appearance of blue to the green colour was indicative of the existence of phenolics (Katoke, 2000).

3.7 Data management and statistical analysis

The scavenging activities of DPPH and hydrogen peroxide together with ferrous reducing powers were measured and documented on a spreadsheet using Microsoft Excel program. The data was then exported into Minitab statistical software version 17.0 for analysis. The data were exposed to descriptive statistics and stated as mean ± standard error of the mean (SEM). The one-way analysis of variance (ANOVA) was used to analyze whether there was any significant difference between the means of different groups. This was preceded by Tukey's tests to separate means and obtain the definite significance discrepancy in the various groups. The values of $p \leq 0.05$ were considered significant. The findings were presented in tables and graphs. Data on qualitative phytochemical analysis

was presented on a table, where the presence of a phytochemical was denoted with a plus (+) sign and the absence with a minus (-) sign.

CHAPTER FOUR

RESULTS

4.1 *In vitro* ferric reducing activities of methanolic extracts of *C. volkensii, V. lasiopus* and *A. hockii*

The methanolic extracts of *A. hockii*, *C. volkensii* and *V. lasiopus* were investigated for their ability to reduce Fe^{3+} to Fe^{2+}. This was shown by a change of colour from yellow to green and eventually blue, subject to the concentration of the extracts.

Generally, the methanolic extracts of three studied medicinal plants exhibited remarkable ferric reducing power as shown in Table 4.1. The reducing power was revealed by an intensified absorbance with an increase in extract concentrations at the 700nm wavelength (Table 4.1). At all extracts concentrations, the methanolic extracts of *A. hockii* had significantly higher ferric reducing power than methanolic extracts of *C. volkensii* and *V. lasiopus* ($p<0.05$; Table 4.1). Notably, at all extracts concentrations, there was significantly higher reducing power for methanolic extracts of *V. lasiopus* in comparison to the *C. volkensii* extracts ($p<0.05$; Table 4.1). However, the control (Ascorbic acid) had a reducing power activity that was significantly higher than the three studied methanolic extracts ($p<0.05$; Table 4.1).

Table 4.1 *In vitro* ferric reducing activities of methanolic extracts of *C. volkensii, V. lasiopus* and *A. hockii*

Absorbance at 700nm					
Treatments	**Concentration in mg/ml**				
	0.2	**0.4**	**0.6**	**0.8**	**1**
Ascorbic acid	1.72±0.01[a]	1.92±0.00 [a]	2.25±0.01 [a]	2.56±0.02 [a]	2.75±0.00 [a]
A. hockii	1.63±0.02[b]	1.82±0.00 [b]	1.88±0.01 [b]	2.18±0.02 [b]	2.54±0.04 [b]
V. lasiopus	1.35±0.01[c]	1.47±0.01 [c]	1.72±0.01 [c]	1.87±0.02 [c]	1.93±0.02[c]
C. volkensii	1.05±0.02[d]	1.23±0.01 [d]	1.42±0.02 [d]	1.65±0.01 [d]	1.81±0.01 [d]

Values expressed as Mean ± SEM of the experiment triplicates in each concentration. Values with the same superscript letters are not significantly different by one way ANOVA followed by Tukey's test ($p>0.05$).

4.2 *In vitro* DPPH scavenging activities of methanolic extracts of *C. volkensii, V. lasiopus* and *A. hockii*

The methanolic extracts of *C. volkensii*, *V. lasiopus* and *A. hockii* also demonstrated remarkable *in vitro* DPPH scavenging potential in a dose-dependent trend (Table 4.2). This was exhibited by the rise in the percentage of DPPH scavenging capacity with the rise in extract concentration. However, the control (Ascorbic acid) exhibited a significantly higher DPPH scavenging capacity than all the studied methanolic plant extracts ($p<0.05$; Table 4.2). At all tested extracts concentrations, *A. hockii* demonstrated significantly higher DPPH scavenging activity compared to those of *C. volkensii* and *V. lasiopus* extracts ($p<0.05$; Table 4.2). There was also a significantly higher DPPH

scavenging activity of *V. lasiopus* compared to that of *C. volkensii* extracts in all the tested concentration ($p<0.05$; Table 4.2). Respectively, the half-maximal inhibitory concentration (IC_{50}) for the *C. volkensii, V. lasiopus* and *A. hockii* extracts were 0.601, 0.47, 0.40 while that of the standard (Ascorbic acid) which was 0.198 (Table 4.2).

Table 4.2 *In vitro* DPPH scavenging activities of methanolic extracts of *C.volkensii, V.lasiopus* and *A. hockii*

	DPPH scavenging activity (% inhibition)					
Treatment	**Concentration in mg/ml**					
	0.0625	**0.125**	**0.25**	**0.5**	**1**	**IC_{50}**
Ascorbic acid	46.81±0.46[a]	62.18±0.18[a]	73.06±1.03[a]	81.35±0.30[a]	87.22±0.75[a]	0.20
C. volkensii	33.16±0.60[b]	36.10±0.75[b]	51.64±0.46[b]	61.31±0.75[b]	67.36±0.30[b]	0.60
V. lasiopus	36.44±0.75[c]	44.73±1.73[c]	50.60±0.17[c]	70.47±1.79[c]	76.86±0.96[c]	0.47
A. hockii	39.38±0.30[d]	53.37±1.30[d]	66.67±1.05[d]	76.34±0.62[d]	82.73±0.62[d]	0.40

Values expressed as Mean ± SEM of the experiment triplicates in each concentration. Values with the same superscript letters are not significantly different by one way ANOVA followed by Tukey's test ($p>0.05$).

4.3 *In vitro* hydrogen peroxide scavenging activities of methanolic of *C. volkensii, V. lasiopus* and *A. hockii*

The methanolic extracts of *C. volkensii*, *V. lasiopus* and *A. hockii* exhibited remarkable *in vitro* hydrogen scavenging activities (Table 4.3). At all the tested extracts concentrations, the methanolic extracts of *A. hockii* demonstrated significantly higher hydrogen peroxide scavenging capacity than those of the *C. volkensii* and *V. lasiopus* extracts ($p<0.05$; Table 4.3). Moreover, at all tested concentrations, the methanolic extracts of *V. lasiopus* demonstrated significantly higher hydrogen peroxide scavenging potential compared to the effects of the *C. volkensii* extracts. However, the *in vitro* H_2O_2 radical scavenging capacity of ascorbic acid was considerably greater than those of *C. volkensii*, *V. lasiopus* and *A. hockii* extracts at all concentrations tested ($p<0.05$; Table 4.3). The half-maximal inhibitory concentration (IC_{50}) values for *C. volkensii, V. lasiopus* and *A. hockii* extracts were 0.56, 0.72 and 0.85, respectively, while that of the standard (Ascorbic acid) was 1.06 (Table 4.3). However, the higher extracts concentration had lower hydrogen peroxide scavenging activities than the lower extracts concentration (Table 4.3).

Table 4.3: ***In vitro*** **hydrogen peroxide scavenging activities of methanolic extracts of C.** ***volkensii, V. lasiopus*** **and** ***A. hockii***

	Hydrogen peroxide scavenging activity (% inhibition)					
Treatment	**Concentration in mg/ml**					
	0.0625	**0.125**	**0.25**	**0.5**	**1**	**IC_{50}**
Ascorbic acid	87.50±1.30[a]	80.56±0.65 [a]	73.29±2.03 [a]	62.39±0.39 [a]	50.64±0.68 [a]	1.05
C. volkensii	66.99±0.32[d]	56.41±0.37 [d]	46.69±0.36[d]	40.71±0.56 [d]	30.98±0.28 [d]	0.56
V. lasiopus	73.93±0.91[c]	61.75±0.83 [c]	53.74±0.65 [c]	47.22±0.21 [c]	38.46±0.56 [c]	0.71
A. hockii	81.52±0.28[b]	73.56±0.28 [b]	63.14±0.56 [b]	50.86±0.39 [b]	43.38±0.28 [b]	0.85

Values expressed as the mean ±SME of the experiment triplicates in each concentration. Values with the same the superscript letter is not significantly different along the row by one way ANOVA followed by Tukey's test ($p>0.05$).

4.4 Total phenolic and flavonoid contents of methanolic extracts of *C. volkensii, V. lasiopus* and *A. hockii*

Determination of the quantity of total phenolic of the methanolic extracts of *C. volkensii*, *V. lasiopus* and *A. hockii* showed that *A. hockii* composed of 41.78±0.93 mg of GAE/g total phenolic content (Table 4.4.a), which was significantly highest among all the three tested plant extracts. On the other hand, it was observed that *V. lasiopus* had a content of 36.04±0.032mg of GAE/g total phenolic (Table 4.4.a), which was significantly higher than that of *C. volkensii* which was 28.51±0.061mg of GAE/g (Table 4.4.a).

Table 4.4(a): Total phenolic contents of methanolic extracts of *C. Volkensii, V. lasiopus* and *A. hockii*

Sample	TPC(mg GAE/g)
Acacia hockii	41.78 ± 0.09^{a}
Vernonia lasiopus	36.04 ± 0.03^{b}
Caesalpinia volkensii	28.51 ± 0.06^{c}

Values expressed as mean ± SEM of the experiment triplicates. Values with different superscript letters are significantly different by one way ANOVA followed by Tukey's test ($p>0.05$)

On the other hand, analysis of the total flavonoids of the of methanolic extracts of *C. volkensii*, *V. lasiopus* and *A. hockii* showed that *A. hockii* contained total phenolic of 39.89±0.04 mg of QE/g (Table 4.4.b), which was significantly highest among all the three tested plant extracts. On the other hand, it was observed that *V. lasiopus* had a total phenolic content of 32.89±0.01mg of QE/g (Table 4.4.b) which was significantly higher than that of *C. volkensii* which was 22.52±0.09 mg of QE/g (Table 4.4.b).

Table 4.4(b) Total flavonoids contents of methanolic extracts of *C. volkensii, V.lasiopus* and *A. hockii*

Sample	TFC(mg QE/g)
Acacia hockii	39.89±0.04[a]
Vernonia lasiopus	32.89±0.01[b]
Caesalpinia volkensii	22.52±0.09[c]

Values expressed as mean ± SEM of the experiment triplicates. Values with different superscript letters are significantly different by one way ANOVA followed by Tukey's test ($p>0.05$)

4.5 Qualitative phytochemical screening

The qualitative phytochemical assay of the stem bark extracts of *A.hockii* contained flavonoids, phenols, steroids, saponins, alkaloids, cardiac glycosides and terpenoids. (Table 4.6). On the other hand, the leaf extracts of *C. volkensii* and *V. lasiopus* exhibited the presence of saponins, terpenoids, flavonoids, alkaloids and phenols though they lacked cardiac glycosides and steroids (Table 4.6).

Table 4.6: Qualitative phytochemical composition of methanolic leaf extracts of *C. volkensii, V. lasiopus* and extracts the stem bark of *A. hockii*

Phytochemicals	*A. hockii*	*C. volkensii*	*V. lasiopus*
Flavonoids	+	+	+
Phenols	+	+	+
Steroids	+	-	-
Saponins	+	+	+
Alkaloids	+	+	+
Cardiac glycosides	+	-	-
Terpenoids	+	+	+

The sign (+) indicates presence while (-) indicates the absence of phytochemical.

CHAPTER FIVE

DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1 Discussion

There have been attempts to treat and manage oxidative stress using conventional methods but still, more research needs to be done on alternative approaches, because they are safer and more affordable in comparison to conventional approaches. According to a report by Verma and Singh, (2008) over 80% of the people in the third world countries depend on herbs alongside traditional remedies for their basic health care as per records of World Health Organization. One of the leading sources of disease state in cells is oxidative damage, which can be controlled by antioxidants use, which protects the tissues from detrimental effects of free radicals (Sreeramulu *et al*., 2013). They act by stabilizing the unstable species or enhancing the protective mechanisms of antioxidant (Umamaheswari and Chatterjee, 2008).

Conventional antioxidants including BHA and BHT are used in the management of oxidative stress (Kadhum *et al*., 2011). They are added as supplements in the food industry. They work by reducing oxygen radicals as well as interfering with the propagation step of oxidation, thereby reducing the levels of free radicals that arise due to oxidation (Shebis *et al*., 2013). However, synthetic drugs used to manage these conditions are associated with unaffordability, unavailability and varied side effects (Moulisha *et al*., 2010). Compounded with all these challenges, attention has been shifted to naturally occurring antioxidants (Yao *et al*., 2004).

The goal of this research was to investigate the *in vitro* activities of methanolic extracts of stem bark of *A. hockii* and leaf extracts of *V. lasiopus* and *C. volkensii*.
The standard in the antioxidant assay was ascorbic acid a naturally occurring antioxidant. On the other hand, the solvent that was used for the separation of active components was methanol which has a polarity index of 5.1 thus; extraction and concentration of bioactive molecules were easy through soxhlet extraction and Rotavapor respectively. It also dissolves many polar molecules.

In the tissues, the defence mechanisms against oxidants and the antioxidant capacity of the antioxidant activities are governed to a large extent by the type of oxidant generated (Choi *et al*., 2002; Roesler *et al*., 2008). Accordingly, different approaches utilized in the analysis of *in vitro* antioxidant potential of different elements display variable outcomes dependent on the selectivity of the compound used as the radical stimulus (Frankel and Meyer, 2001). The mode of action of these methods also differs. Some of the *in vitro* antioxidant tests used include hydrogen peroxide scavenging activity, Lipid peroxidation inhibition capacity (LPIC), Oxygen radical absorbance capacity (ORAC) method, Nitric Oxide scavenging Assay, Ferric reducing antioxidant power (FRAP) as well as DPPH scavenging effects (Akinmoladun *et al*., 2010).

In this study, Ferric Reducing Antioxidant Power (FRAP) technique was employed. The existence of reducing agents in the mixture brings about change of the Fe^{3+}/ferricyanide complex to the ferrous form. Hence, the Fe^{2+} formation can be examined by absorbance capacity at 700 nm (MacDonald-Wicks *et al*., 2006). In the FRAP assay, the mechanism of action is electron transfer (Gülçin *et al*., 2010).

Increases in absorbance indicate an increase in reducing power. This assay may be used as the quantitative pointer of the antioxidant capability of a compound.

In this study, the ferric reducing power properties of methanolic extracts of *A. hockii*, *V. lasiopus* and *C. volkensii* demonstrated that the effects increased with increasing extract concentrations (Table 4.1). This implies that as the extract concentration increases, the number of antioxidant compounds present in the extract increases, thus the amplification in ferric reducing power, which shows an increase in antioxidant potential. The findings were comparable with other *in vitro* antioxidant studies that have been done. A study by Gülçin *et al*. (2003) and Noriham *et al*. (2004) demonstrated the antioxidative activities of *Pimpinella anisum* seeds extract and four types of Malaysian plants respectively. A study by Adesanoye and Farombi (2014) demonstrated dose-dependent reducing activity of methanolic leaf extracts of *Vernonia amygdalina*. Besides, Rahman *et al*. (2015) demonstrated dose-dependent ferric reducing antioxidant activities of methanolic extract of *Tabebuia pallid* in Bangladesh.

Another method used in this study is the use of diphenyl picryl hydrazyl (DPPH) which is a stable radical. A fresh solution DPPH displays a deep violet colour which absorbs polarized light at 517 nm. Molecules are considered to be antioxidant if they can reduce DPPH free radicals by donating hydrogen atoms then change it to a (2, 2-diphenyl-1-hydrazine, or an exchange equivalent hydrazine), consequentially decreasing in absorbance at 517 nm. This technique is among the most commonly used assay in the evaluation of *in vitro* antioxidant effects because it is stable, simple,

fast, and reliable and only requires a spectrophotometer to work (Huang and Prior, 2005).

From this study DPPH scavenging capacity of the methanolic extracts of *A. hockii, C. volkensii* and *V. lasiopus* demonstrated the dose-dependent relationship (Table 4.2). The outcome was corroborated with work by Ravishanker *et al*. (2002) and Mary *et al*. (2003) who reported a DPPH scavenging activity of root bark extracts of *H. indicus* in a dose-dependent manner. Additionally, an assay by Kanwal *et al*. (2015) indicated that the methanolic leaf extracts of *Zanthoxylum armatum* demonstrated a dose-dependent increase in DPPH scavenging activity. This implies that higher concentrations are associated with stronger free radical scavenging potential.

The half-maximal (IC_{50}) DPPH scavenging activities of values of *A. hockii, C. volkensii* and *V. lasiopus* and the standard (ascorbic acid) was 0.4, 0.6, 0.47 and 0.2, respectively (Table 4.2). The methanolic extracts of *A. hockii* showed a slightly lower IC_{50} value than the extracts of *C. volkensii* and *V. lasiopus*. The IC_{50} values of methanolic extracts of *V. lasiopus* were slightly lower than that of the *C. volkensii* extracts. This means that a lower concentration of *A. hockii* was needed to cause DPPH scavenging activity than *C. volkensii* and *V. lasiopus* which would require a slightly higher concentration.

Hydrogen peroxide free radical scavenging assay was the other technique employed in the study to assess the antioxidant capacity of the extracts. Hydrogen peroxide has oxidizing properties and can directly denature some enzymes via oxidation of important thiol (-SH) groups (Nagulendran *et al*., 2007). The effect of hydrogen

peroxide as an active oxygen element has been attributed to its likelihood to generate unstable hydroxyl species through the Fenton reaction: $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^{\bullet}$ (Namiki, 1990; Koksal *et al*., 2009; Jeong *et al*., 2010; Ogasawara *et al*., 2014). Consequently, obstruction of hydrogen peroxide production prevents additional yield of unstable species.

The plant extracts of the study demonstrated a dose-dependent decrease in hydrogen peroxide scavenging activity that was also demonstrated in both acetone and aqueous whole plant extracts of *Bulbine abyssinica* (Kibiti and Afolayan, 2015). However, in an experiment performed by Abimbade *et al*. (2014), hexane extracts of *Cyperus esculentus* exhibited a dose-dependent rise in hydrogen peroxide scavenging activities, while its methanolic extract produced a dose-dependent drop in scavenging activity in hydrogen peroxide. The dose-dependent decrease in hydrogen scavenging activity implies that low concentrations had high scavenging activities hence high antioxidant abilities. This trend in the hydrogen peroxide scavenging activity could have been brought about by saturation of reactive centres of hydrogen peroxide by the high extract concentrations leading to low activities, compared to dilute concentrations that ensure an easier and rapid reaction, leading to high activity.

The half-maximal (IC_{50}) value for *A. hockii*, *C. volkensii* and *V. lasiopus* and standard (ascorbic acid) was 0.85, 0.56, 0.71 and 1.05, respectively (Table 4.3). This means that there was a negative correlation in the concentration of extracts and the probable neutralizing capacity of the hydrogen peroxide.

The Folin-Ciolcalteu method was used in this study to assay for the total phenol content of the extracts. Folin-Ciocalteu’s phenol reagent is a colourless compound

that consists of phosphomolybdic and phosphotungstic acids. It is used for phenol and polyphenol assays. This method is simple, precise and sensitive (Huang and Prior, 2005). When it reacts with a solution containing reductants, it forms molybdenum and tungsten blue, which are responsible for the colour change to blue (Agbor *et al*., 2014).

The methanolic extracts of *A. hockii*, *V. lasiopus* and *C. volkensii* demonstrated significant total phenolic content (Table 4.4, a). The over-all phenolic composition of *A. hockii* was significantly exceeding those of *V. lasiopus* and *C. volkensii*. However, the total phenolic composition of *V.lasiopus* was than those of *C. volkensii*. This explains why the antioxidant activity of *A. hockii* was significantly higher than *V. lasiopus* and *C. volkensii*. The hydroxyl moieties of phenolic compounds are significant because they are involved in rendering radical scavenging capacities of the plant extracts. (Elzaawely and Tawata, 2012).

The aluminium chloride colourimetric technique was utilized to determine the total flavonoid composition of the three plants extracts. This technique depends on the addition of the nitro group on an aromatic ring having a catechol moiety with its positions 3 or 4 unsubstituted or not sterically blocked. When Al (III) is added, a yellow solution of the mixture was formed, that turned into red colour immediately once NaOH was introduced then the absorbance was taken at 510 nm (Park *et al*., 2008).

The methanolic extracts of *A. hockii*, *V. lasiopus* and *C. volkensii* demonstrated significant total flavonoids contents (Table 4.4.b). The total flavonoids content of *A. hockii* were significantly higher than those of *V. lasiopus* and *C. volkensii*. However,

the total flavonoids contents of *V. lasiopus* were significantly higher than that of *C. volkensii*. This explains why the antioxidant activity of *A. hockii* was significantly higher than those of *V. lasiopus* and *C. volkensii*.

The observed *in vitro* antioxidant properties of the three plants extracts in the study could be attributed to the existence of several phytochemicals. The qualitative phytochemical assay of the methanolic extracts of *A. hockii* revealed the existence of phenols, alkaloids, flavonoids, tannins, steroids, terpenoids, cardiac glycosides and saponins. On the other hand, the methanolic extracts of leaf extracts of *C. volkensii* and *V. lasiopus* showed the existence of phenols, saponins, alkaloids, terpenoids and flavonoids but lacked steroids as well as cardiac glycosides.

Phenols are the major secondary metabolites that occur in many different plant species. Phenolic is a renowned antioxidant and the effects of the extracts in the study were probably due to these components (Tepe *et al*., 2006). The antioxidant potential of these molecules is thought to be through the reductive and oxidative capacity that allows absorption as well as counteracting effects of free radicals (Zheng, and Wang, 2001). Many of these secondary metabolites are endowed with significant reductive abilities that are attributed to lesser incidences of death and suffering due to oxidative stress-related disorders (Soobrattee *et al*., 2005). The findings highly propose that phenolics are significant components of these plants and this is attributed to their biological effects.

Flavonoids are one of the phenolics compounds found in plants and they are attributed to various pharmacological activities comprising of anti-inflammatory, antitumor

properties and they are capable of acting as antioxidants that shield the cells from destructive effects of free radicals (Nimse and Pal, 2015). The flavonoids structure and its hydroxyl atom location, as well as its other properties, are important for antioxidant and reactive species neutralizing capacity (Banjarnahor and Artanti, 2014). These molecules demonstrated potent scavenging effects of numerous oxidants and various reactive species that are associated with several disorders (Bravo, 1998). Saeed *et al*. (2012) demonstrated the antioxidant of *T. leptophylla* was due to the presence of flavonoid. Braca *et al*. (*2002*) separated various flavonoids from the leaves of *Licania licaniaeflora* and stated that quercetin products possess strong antioxidant properties

Most flowering plants contain alkaloids as their secondary metabolites these compound act as defence complexes in plants, being efficient against pathogens and predators due to their toxicity. A study by Chung *et al*. (2001), reported that alkaloids have *in vitro* antioxidant activities. On the other hand, terpenoids are natural products divided into different classes based on their carbon skeletons. These classes include monoterpenes, sesquiterpenes, diterpenes, triterpenes, tetraterpenes and polyterpene (Grassmann, 2005). A study by Bourgou *et al*. (2012) revealed that terpenoids from *Nigella sativa* are antioxidant agents in nature. For instance, tetraterpenes, mainly carotenoids provide antioxidant activity by quenching of singlet oxygen and scavenging of peroxyl radical (Grassmann, 2005).

The finding of this work reveals that the extracts from the three plants are potential candidates for further exploitation in the development of plant-derived

pharmacologically active agents. Among all the three plant extracts, *A. hockii* exhibited higher antioxidant activities.

The disparity in the higher half-maximal inhibitory concentration (IC_{50}) in extracts as compared with that of reference (Ascorbic acid) suggested that the crude extracts were not as efficient radical scavengers as the standard which was a pure compound. Nevertheless, this could be attributed to the fact that ascorbic acid is a pure and refined product, whereas the extracts were crude products. Thus, preliminary investigation of antioxidant activities of methanolic leaf extracts of *C. volkensii, V. lasiopus* and stem bark extracts of *A. hockii* may be valuable as a source of biologically active elements and consequently lead to discovery and development of drugs. This confirms the use of *C. volkensii, V. lasiopus* and *A. hockii in* the management of oxidative stress-related disorders in traditional medicine.

5.2 Conclusions

From this study, it is concluded that;

i. The methanolic leaf extracts of *V. lasiopus, C. volkensii* and stem bark extract of *A. hockii* demonstrated *in vitro* ferric reducing antioxidant power (FRAP).
ii. The methanolic leaf extracts of *V. lasiopus, C. volkensii* and stem bark extract of *A. hockii* demonstrated *in vitro* DPPH scavenging activities.
iii. The methanolic leaf extracts of *V. lasiopus, C. volkensii* and stem bark extract of *A. hockii* demonstrated *in vitro* hydrogen scavenging activities.

iv. The methanolic leaf extracts of *V. lasiopus, C. volkensii* and stem bark extract of *A. hockii* contains phytochemicals which are associated with antioxidant activities.

5.3 Recommendation from the study

i) The methanolic leaf extracts of *C. volkensii V.lasiopus* and stems bark extracts of *A. hockii* may be used to develop remedies for oxidative stress-related disorders.

5.4 Suggestions for further studies

i) It is necessary to determine *in vivo* activity of the extracts to provide more information which can be compared with the *in vitro* studies.

ii) Further work is needed to separate and ascertain actual antioxidant components in the extracts.

iii) It is necessary to determine toxicology studies to provide more information on their safety.

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APPENDICES

Appendix I: Analysis of *In vitro* ferric reducing activities of methanolic extractsof *C. volkensii V.lasiopus* and *A. hockii*

One-way ANOVA: AH 0.2, VL 0.2, CV 0.2, AS 0.2

Method
Null hypothesis all means are equal
Alternative hypothesis At least one mean is different
Significance level $\alpha = 0.05$

Equal variances were assumed for the analysis.

Factor Information

Factor Levels Values

Factor 4 AH 0.2, VL 0.2, CV 0.2, AS 0.2

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	3	0.814567	0.271522	350.35	0.000
Error	8	0.006200	0.000775		
Total	11	0.820767			

Model Summary

S	R-sq	R-sq (adj)	R-sq (pred)
0.0278388	99.24%	98.96%	98.30%

Means

Factor	N	Mean	StDev	95% CI
AH 0.2	3	1.6333	0.0252	(1.5963, 1.6704)
VL 0.2	3	1.3500	0.0200	(1.3129, 1.3871)
CV 0.2	3	1.0533	0.0404	(1.0163, 1.0904)
AS 0.2	3	1.7167	0.0208	(1.6796, 1.7537)

Pooled StDev = 0.0278388

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Factor	N	Mean	Grouping
AS 0.2	3	1.7167	A
AH 0.2	3	1.6333	B
VL 0.2	3	1.3500	C
CV 0.2	3	1.0533	D

Means that do not share a letter are significantly different.

One-way ANOVA: AH 0.4, VL 0.4, CV 0.4, AS 0.4

Method

Null hypothesis	all means are equal
Alternative hypothesis	At least one mean is different
Significance level	$\alpha = 0.05$

Equal variances were assumed for the analysis.

Factor Information

Factor	Levels	Values
Factor	4	AH 0.4, VL 0.4, CV 0.4, AS 0.4

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	3	0.910795	0.303598	1318.56	0.000
Error	8	0.001842	0.000230		
Total	11	0.912637			

Model Summary

S	R-sq	R-sq (adj)	R-sq (pred)
0.0151740	99.80%	99.72%	99.55%

Means

Factor	N	Mean	StDev	95% CI
AH 0.4	3	1.82367	0.00651	(1.80346, 1.84387)
VL 0.4	3	1.46867	0.01206	(1.44846, 1.48887)

CV 0.4 3 1.2333 0.0252 (1.2131, 1.2535)
AS 0.4 3 1.92000 0.01000 (1.89980, 1.94020)

Pooled StDev = 0.0151740

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Factor N Mean Grouping
AS 0.4 3 1.92000 A
AH 0.4 3 1.82367 B
VL 0.4 3 1.46867 C
CV 0.4 3 1.2333 D

Means that do not share a letter are significantly different.

One-way ANOVA: AH 0.6, VL 0.6, CV 0.6, AS 0.6

Method

Null hypothesis all means are equal
Alternative hypothesis At least one mean is different
Significance level $\alpha = 0.05$

Equal variances were assumed for the analysis.

Factor Information

Factor Levels Values
Factor 4 AH 0.6, VL 0.6, CV 0.6, AS 0.6

Analysis of Variance

Source DF Adj SS Adj MS F-Value P-Value
Factor 3 1.06731 0.355770 1057.53 0.000
Error 8 0.00269 0.000336
Total 11 1.07000

Model Summary

S R-sq R-sq (adj) R-sq (pred)
0.0183417 99.75% 99.65% 99.43%

Means

Factor	N	Mean	StDev	95% CI
AH 0.6	3	1.88133	0.01060	(1.85691, 1.90575)
VL 0.6	3	1.72000	0.01000	(1.69558, 1.74442)
CV 0.6	3	1.4200	0.0300	(1.3956, 1.4444)
AS 0.6	3	2.24667	0.01528	(2.22225, 2.27109)

Pooled StDev = 0.0183417

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Factor	N	Mean	Grouping
AS 0.6	3	2.24667	A
AH 0.6	3	1.88133	B
VL 0.6	3	1.72000	C
CV 0.6	3	1.4200	D

Means that do not share a letter are significantly different.

One-way ANOVA: AH 0.8, VL 0.8, CV 0.8, AS 0.8

Method

Null hypothesis	all means are equal
Alternative hypothesis	At least one mean is different
Significance level	$\alpha = 0.05$

Equal variances were assumed for the analysis.

Factor Information

Factor	Levels	Values
Factor	4	AH 0.8, VL 0.8, CV 0.8, AS 0.8

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	3	1.43589	0.478631	617.59	0.000
Error	8	0.00620	0.000775		
Total	11	1.44209			

Model Summary

S	R-sq	R-sq (adj)	R-sq (pred)
0.0278388	99.57%	99.41%	99.03%

Means

Factor	N	Mean	StDev	95% CI
AH 0.8	3	2.1800	0.0265	(2.1429, 2.2171)
VL 0.8	3	1.8467	0.0252	(1.8096, 1.8837)
CV 0.8	3	1.6533	0.0231	(1.6163, 1.6904)
AS 0.8	3	2.5633	0.0351	(2.5263, 2.6004)

Pooled StDev = 0.0278388

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Factor	N	Mean	Grouping
AS 0.8	3	2.5633	A
AH 0.8	3	2.1800	B
VL 0.8	3	1.8467	C
CV 0.8	3	1.6533	D

Means that do not share a letter are significantly different.

One-way ANOVA: AH 1, VL 1, CV 1, AS 1

Method

Null hypothesis	all means are equal
Alternative hypothesis	At least one mean is different
Significance level	$\alpha = 0.05$

Equal variances were assumed for the analysis.

Factor Information

Factor	Levels	Values
Factor	4	AH 1, VL 1, CV 1, AS 1

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	3	1.88815	0.629383	384.77	0.000

Error 8 0.01309 0.001636
Total 11 1.90123

Model Summary

S	R-sq	R-sq (adj)	R-sq (pred)
0.0404444	99.31%	99.05%	98.45%

Means

Factor	N	Mean	StDev	95% CI
AH 1	3	2.5377	0.0740	(2.4838, 2.5915)
VL 1	3	1.93333	0.01528	(1.87949, 1.98718)
CV 1	3	1.8067	0.0208	(1.7528, 1.8605)
AS 1	3	2.7500	0.0200	(2.6962, 2.8038)

Pooled StDev = 0.0404444

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Factor	N	Mean	Grouping
AS 1	3	2.7500	A
AH 1	3	2.5377	B
VL 1	3	1.93333	C
CV 1	3	1.8067	D

Means that do not share a letter are significantly different.

Appendix II: Analysis of *In vitro* DPPH scavenging activities of methanolic extracts of *C. volkensii*, *V. lasiopus* and *A. hockii*

One-way ANOVA: AH 0.2, VL 0.2, CV 0.2, AS 0.2

Method

Null hypothesis all means are equal

Alternative hypothesis At least one mean is different

Significance level $\alpha = 0.05$

Equal variances were assumed for the analysis.

Factor Information

Factor Levels Values
Factor 4 AH 0.2, VL 0.2, CV 0.2, AS 0.2

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	3	305.064	101.688	110.86	0.000
Error	8	7.338	0.917		
Total	11	312.402			

Model Summary

S	R-sq	R-sq (adj)	R-sq (pred)
0.957732	97.65%	96.77%	94.71%

Means

Factor	N	Mean	StDev	95% CI
AH 0.2	3	39.378	0.518	(38.103, 40.653)
VL 0.2	3	36.442	1.304	(35.167, 37.717)
CV 0.2	3	33.161	1.036	(31.886, 34.436)
AS 0.2	3	46.805	0.791	(45.530, 48.080)

Pooled StDev = 0.957732

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Factor	N	Mean	Grouping
AS 0.2	3	46.805	A
AH 0.2	3	39.378	B
VL 0.2	3	36.442	C
CV 0.2	3	33.161	D

Means that do not share a letter are significantly different.

One-way ANOVA: AH 0.4, VL 0.4, CV 0.4, AS 0.4

Method

Null hypothesis	all means are equal
Alternative hypothesis	At least one mean is different
Significance level	$\alpha = 0.05$

Equal variances were assumed for the analysis.

Factor Information

Factor	Levels	Values
Factor	4	AH 0.4, VL 0.4, CV 0.4, AS 0.4

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	3	1132.09	377.363	89.72	0.000
Error	8	33.65	4.206		
Total	11	1165.74			

Model Summary

S	R-sq	R-sq (adj)	R-sq (pred)
2.05084	97.11%	96.03%	93.51%

Means

Factor	N	Mean	StDev	95% CI
AH 0.4	3	53.37	2.26	(50.64, 56.10)
VL 0.4	3	44.73	2.99	(42.00, 47.46)

CV 0.4 3 36.097 1.304 (33.366, 38.827)
AS 0.4 3 62.176 1.036 (59.446, 64.907)

Pooled StDev = 2.05084

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Factor N Mean Grouping
AS 0.4 3 62.176 A
AH 0.4 3 53.37 B
VL 0.4 3 44.73 C
CV 0.4 3 36.097 D

Means that do not share a letter are significantly different.

One-way ANOVA: AH 0.6, VL 0.6, CV 0.6, AS 0.6

Method

Null hypothesis all means are equal
Alternative hypothesis At least one mean is different
Significance level $\alpha = 0.05$

Equal variances were assumed for the analysis.

Factor Information

Factor Levels Values
Factor 4 AH 0.6, VL 0.6, CV 0.6, AS 0.6

Analysis of Variance

Source DF Adj SS Adj MS F-Value P-Value
Factor 3 857.18 285.727 141.91 0.000
Error 8 16.11 2.013
Total 11 873.29

Model Summary

S R-sq R-sq (adj) R-sq (pred)
1.41897 98.16% 97.46% 95.85%

Means

Factor	N	Mean	StDev	95% CI
AH 0.6	3	66.67	1.82	(64.78, 68.56)
VL 0.6	3	56.131	0.791	(54.242, 58.020)
CV 0.6	3	51.641	0.791	(49.752, 53.530)
AS 0.6	3	73.06	1.87	(71.17, 74.95)

Pooled StDev = 1.41897

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Factor	N	Mean	Grouping
AS 0.6	3	73.06	A
AH 0.6	3	66.67	B
VL 0.6	3	56.131	C
CV 0.6	3	51.641	D

Means that do not share a letter are significantly different.

One-way ANOVA: AH 0.8, VL 0.8, CV 0.8, AS 0.8

Method

Null hypothesis	all means are equal
Alternative hypothesis	At least one mean is different
Significance level	$\alpha = 0.05$

Equal variances were assumed for the analysis.

Factor Information

Factor	Levels	Values
Factor	4	AH 0.8, VL 0.8, CV 0.8, AS 0.8

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	3	666.68	222.228	69.46	0.000
Error	8	25.59	3.199		
Total	11	692.28			

Model Summary

S	R-sq	R-sq (adj)	R-sq (pred)
1.78863	96.30%	94.92%	91.68%

Means

Factor	N	Mean	StDev	95% CI
AH 0.8	3	76.339	1.079	(73.957, 78.720)
VL 0.8	3	70.47	3.11	(68.08, 72.85)
CV 0.8	3	61.313	1.304	(58.931, 63.694)
AS 0.8	3	81.347	0.518	(78.966, 83.728)

Pooled StDev = 1.78863

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Factor	N	Mean	Grouping
AS 0.8	3	81.347	A
AH 0.8	3	76.339	B
VL 0.8	3	70.47	C
CV 0.8	3	61.313	D

Means that do not share a letter are significantly different.

One-way ANOVA: AH 1, VL 1, CV 1, AS 1

Method

Null hypothesis	all means are equal
Alternative hypothesis	At least one mean is different
Significance level	$\alpha = 0.05$

Equal variances were assumed for the analysis.

Factor Information

Factor	Levels	Values
Factor	4	AH 1, VL 1, CV 1, AS 1

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	3	662.28	220.759	149.51	0.000

Error 8 11.81 1.477
Total 11 674.09

Model Summary

S	R-sq	R-sq (adj)	R-sq (pred)
1.21513	98.25%	97.59%	96.06%

Means

Factor	N	Mean	StDev	95% CI
AH	1	3	82.729	1.079 (81.111, 84.347)
VL	1	3	76.857	1.666 (75.239, 78.474)
CV	1	3	67.358	0.518 (65.740, 68.975)
AS	1	3	87.219	1.304 (85.602, 88.837)

Pooled StDev = 1.21513

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Factor	N	Mean	Grouping
AS 1	3	87.219	A
AH 1	3	82.729	B
VL 1	3	76.857	C
CV 1	3	67.358	D

Means that do not share a letter are significantly different.

Appendix III: **Analysis of *In vitro* hydrogen peroxide scavenging activities of methanolic of *C. volkensii*, *V. lasiopus* and *A. hockii***

One-way ANOVA: CV 0.2, VL 0.2, AH 0.2, AS 0.2

Method

Null hypothesis all means are equal
Alternative hypothesis At least one mean is different
Significance level $\alpha = 0.05$

Equal variances were assumed for the analysis.

Factor Information

Factor Levels Values
Factor 4 CV 0.2, VL 0.2, AH 0.2, AS 0.2

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	3	718.17	239.389	118.49	0.000
Error	8	16.16	2.020		
Total	11	734.33			

Model Summary

S	R-sq	R-sq (adj)	R-sq (pred)
1.42138	97.80%	96.97%	95.05%

Means

Factor	N	Mean	StDev	95% CI
CV 0.2	3	66.987	0.555	(65.095, 68.880)
VL 0.2	3	73.932	1.581	(72.039, 75.824)
AH 0.2	3	81.517	0.490	(79.625, 83.409)
AS 0.2	3	87.50	2.24	(85.61, 89.39)

Pooled StDev = 1.42138

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Factor	N	Mean	Grouping
AS 0.2	3	87.50	A
AH 0.2	3	81.517	B
VL 0.2	3	73.932	C
CV 0.2	3	66.987	D

Means that do not share a letter are significantly different.

One-way ANOVA: CV 0.4, VL 0.4, AH 0.4, AS 0.4

Method

Null hypothesis all means are equal
Alternative hypothesis At least one mean is different
Significance level $\alpha = 0.05$

Equal variances were assumed for the analysis.

Factor Information

Factor Levels Values
Factor 4 CV 0.4, VL 0.4, AH 0.4, AS 0.4

Analysis of Variance

Source DF Adj SS Adj MS F-Value P-Value
Factor 3 1083.85 361.285 360.71 0.000
Error 8 8.01 1.002
Total 11 1091.87

Model Summary

S R-sq R-sq (adj) R-sq (pred)
1.00080 99.27% 98.99% 98.35%

Means

Factor N Mean StDev 95% CI
CV 0.4 3 56.410 0.641 (55.078, 57.743)
VL 0.4 3 61.752 1.445 (60.420, 63.085)
AH 0.4 3 73.504 0.490 (72.172, 74.837)
AS 0.4 3 80.556 1.126 (79.223, 81.888)

Pooled StDev = 1.00080

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Factor N Mean Grouping
AS 0.4 3 80.556 A
AH 0.4 3 73.504 B
VL 0.4 3 61.752 C
CV 0.4 3 56.410 D

Means that do not share a letter are significantly different.

One-way ANOVA: CV 0.6, VL 0.6, AH 0.6, AS 0.6

Method

Null hypothesis all means are equal
Alternative hypothesis At least one mean is different
Significance level $\alpha = 0.05$

Equal variances were assumed for the analysis.

Factor Information

Factor Levels Values
Factor 4 CV 0.6, VL 0.6, AH 0.6, AS 0.6

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	3	1201.33	400.444	106.80	0.000
Error	8	30.00	3.750		
Total	11	1231.33			

Model Summary

S	R-sq	R-sq (adj)	R-sq (pred)
1.93639	97.56%	96.65%	94.52%

Means

Factor	N	Mean	StDev	95% CI
CV 0.6	3	46.688	0.667	(44.110, 49.266)
VL 0.6	3	53.739	1.126	(51.161, 56.317)
AH 0.6	3	63.141	0.962	(60.563, 65.719)
AS 0.6	3	73.29	3.52	(70.71, 75.87)

Pooled StDev = 1.93639

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Factor	N	Mean	Grouping
AS 0.6	3	73.29	A
AH 0.6	3	63.141	B
VL 0.6	3	53.739	C
CV 0.6	3	46.688	D

Means that do not share a letter are significantly different.

One-way ANOVA: CV 0.8, VL 0.8, AH 0.8, AS 0.8

Method

Null hypothesis all means are equal
Alternative hypothesis At least one mean is different
Significance level $\alpha = 0.05$

Equal variances were assumed for the analysis.

Factor Information

Factor Levels Values
Factor 4 CV 0.8, VL 0.8, AH 0.8, AS 0.8

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	3	744.259	248.086	508.42	0.000
Error	8	3.904	0.488		
Total	11	748.163			

Model Summary

S	R-sq	R-sq (adj)	R-sq (pred)
0.698541	99.48%	99.28%	98.83%

Means

Factor	N	Mean	StDev	95% CI
CV 0.8	3	40.705	0.962	(39.775, 41.635)
VL 0.8	3	47.222	0.370	(46.292, 48.152)
AH 0.8	3	50.855	0.667	(49.925, 51.785)
AS 0.8	3	62.393	0.667	(61.463, 63.323)

Pooled StDev = 0.698541

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Factor	N	Mean	Grouping
AS 0.8	3	62.393	A
AH 0.8	3	50.855	B
VL 0.8	3	47.222	C

CV 0.8 3 40.705 D

Means that do not share a letter are significantly different.

One-way ANOVA: CV 1, VL 1, AH 1, AS 1

Method

Null hypothesis all means are equal
Alternative hypothesis At least one mean is different
Significance level $\alpha = 0.05$

Equal variances were assumed for the analysis.

Factor Information

Factor Levels Values
Factor 4 CV 1, VL 1, AH 1, AS 1

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	3	615.926	205.309	299.78	0.000
Error	8	5.479	0.685		
Total	11	621.405			

Model Summary

S	R-sq	R-sq (adj)	R-sq (pred)
0.827561	99.12%	98.79%	98.02%

Means

Factor	N	Mean	StDev	95% CI
CV 1	3	30.983	0.490	(29.881, 32.085)
VL 1	3	38.462	0.962	(37.360, 39.563)
AH 1	3	43.376	0.490	(42.274, 44.478)
AS 1	3	50.641	1.156	(49.539, 51.743)

Pooled StDev = 0.827561

Tukey Pairwise Comparisons
Grouping Information Using the Tukey Method and 95% Confidence

Factor	N	Mean	Grouping
AS 1	3	50.641	A
AH 1	3	43.376	B
VL 1	3	38.462	C
CV 1	3	30.983	D

Means that do not share a letter are significantly different.

Appendix IV: Research Authorization form Kenyatta University

KENYATTA UNIVERSITY
GRADUATE SCHOOL

E-mail: deans-graduate@ku.ac.ke
Website: www.ku.ac.ke

P.O. Box 43844, 00100
NAIROBI, KENYA
Tel. 8710901 Ext. 57530

Our Ref: **I56/CE/25204/2014**

DATE: 18th April 2016

Director General,
National Commission for Science, Technology
& Innovation
P.O Box 30623-00100
NAIROBI

Dear Sir/Madam,

RE: RESEARCH AUTHORIZATION FOR GUCHU BEATRICE MUTHONI – REG. NO. I56/CE/25204/2014.

I write to introduce Ms. Guchu Beatrice Muthoni who is a Postgraduate student of this University. She is registered for M.Sc degree programme in the Department of Biochemistry & Biotechnology.

Ms. Guchu intends to conduct research for a M.Sc. Proposal entitled, "**In Vitro Antioxidant Activity of Methanolic Extracts of *Caesalpinia volkensii*, *Acacia hockii* and *vernonia lasiopus***".

Any assistance given will be highly appreciated.

Yours faithfully,

MRS. LUCY N. MBAABU
FOR: DEAN, GRADUATE SCHOOL

Appendix V: Research Authorization from NACOSTI

THIS IS TO CERTIFY THAT:
MS. BEATRICE MUTHONI GUCHU
of KENYATTA UNIVERSITY, 4057-1000
Thika,has been permitted to conduct
research in Embu County

Permit No : NACOSTI/P/19/11985/30593
Date Of Issue : 7th June,2019
Fee Recieved :Ksh 1000

on the topic: IN VITRO ANTIOXIDANT ACTIVITIES OF METHANOLIC EXTRACTS OFCAESALPINIAVOLKENSIN ACACIA MOCKII AND VERNONIA LASIOPUS

for the period ending:
6th June,2020

.....
Applicant's
Signature

.....
Director General
National Commission for Science,
Technology & Innovation

RE: RESEARCH AUTHORIZATION

Following your application for authority to carry out research on ***"In vitro antioxidant activities of methanolic extracts of caesalpini wolkensii acacia hockii and vernonia lasiopus."*** I am pleased to inform you that you have been authorized to undertake research in **Embu County** for the period ending **6th June, 2020.**

You are advised to report to **the County Commissioner and the County Director of Education, Embu County** before embarking on the research project.

Kindly note that, as an applicant who has been licensed under the Science, Technology and Innovation Act, 2013 to conduct research in Kenya, you shall deposit **a copy** of the final research report to the Commission within **one year** of completion. The soft copy of the same should be submitted through the Online Research Information System.

BONFACE WANYAMA
FOR: DIRECTOR-GENERAL/CEO

Copy to:
The County Commissioner
Embu County.

The County Director of Education
Embu County.