CHAPTER ONE

INTRODUCTION

1.1 Background of the study

Throughout history, natural products have continued to play significant role as medicines and serve as repository of numerous bioactive compounds that serve as important leads in drug discovery (Dias *et al*., 2012). The significance of natural product based medicines is demonstrated by the reliance of more than half of the world's population on natural products for their primary health care (Ekeanyanwu, 2011).

The role of natural products in meeting the health needs of the Nigerian population has been stressed in many studies (Sofowora, 1982; Osemene *et al*., 2013). These natural products are rarely used solely for a particular disease condition. In some cases, a particular herbal product may be used for the treatment of related disease conditions or disease conditions with similar pathogenesis. There are situations where single herbal product is used for numerous unrelated disease conditions and for general body healing – this is the category where traditional use of *Millettia aboensis* falls.

The contribution of natural products in disease control has been well acclaimed; however, the use of many plant based medicines for the treatment of disease conditions is yet to be fully accepted due to lack of scientific evidence on their efficacy and safety (Firenzuoli and Gori, 2007). The knowledge and uses of some medicinal plants are still based on cultural or folkloric believes. The full therapeutic potentials of herbal products would optimally be harnessed when their efficacy and toxicity are clearly validated and documented using scientific procedures.

Millettia aboensis is one of the plants considered to be an all-purpose plant in most parts of Africa because of the multiplicity of its use (Banzouzi *et al*., 2008). The leaf is used for the treatment of constipation in children, as laxative, and for treating cold, headaches, dysentery, chicken pox and measles (Borokini and Omotayo, 2012; Harrison *et al*., 2011; Onyegeme-Okerenta and Essien, 2015).

1.2 Statement of the problem

Accumulating evidence derived from specific validated biomarkers revealed that oxidative stress is implicated in the initiation, progression and pathogenesis of over 150 human disease conditions (Halliwell and Gutteridge, 2007). On the other hand, compromised effective immune response results to expression of diverse disease conditions (Cochran *et al*., 2000). The search for substances and leads with antioxidant and immune-enhancing activities has become a field of major interest in health care sectors. Though there is poor understanding on the mechanisms that mediate the general body healing potentials of *M. aboensis*, it is hypothesized that scavenging of free radicals and ability to increase immunity in general might likely be associated with its traditional use.

1.3 Aim of the study

The specific aim of this study was to evaluate the antioxidant and immune-enhancing potentials of ethanol leaf extract, fractions and active constituents of *Millettia aboensis.*

1.4 Objectives of the study

The specific objectives of this study are:

Evaluation of the antioxidant activity of the ethanol leaf extract and different polarity solvent fractions of *M. aboensis*.

Determination of inhibition of lipid peroxidation and oral effective dose of the extract.

In vivo effect of the extract and fractions on site specific and systemic oxidative stress.

The effect of the extract and fraction on humoral immune responses as well as pattern of induction of cytokine expression by the most active fraction.

Isolation and characterization of the major compounds in the active fractions of the extract.

In vitro evaluation of the antioxidant activity of compounds isolated from the fraction with the highest inhibitory effect on oxidative stress.

In vitro immune enhancing activity of compound(s) isolated from the fraction with highest immunostimulatory effect.

CHAPTER TWO

LITERATURE REVIEW

2.1 Role of free radicals in oxidative stress

Free radicals are reactive chemicals (an atom, groups of atom or molecular species) with an unpaired electron in an outer orbital that are capable of independent existence (Poljsak *et al*., 2013). Majority of these radicals are oxygen driven (reactive oxygen species) with reactive nitrogen, iron, copper and sulfur species occurring less frequently. These radicals cause chain reactions in a bit to return to their ground state through obtaining an electron from a surrounding atom or molecule thereby leading to chains of reactive substances (Jones, 2008).

Free radicals may be generated through exogenous as well as endogenous sources. Environmental pollutants like hydrocarbons and gases primarily released in automobile exhausts form major sources of exogenous free radical (Cao *et al*., 2011). Other exogenous sources include cigarette smoke (Valavanidis *et al*., 2009), ionizing radiation (Svobodova *et al*., 2006) and some xenobiotics (Deavall *et al*., 2012). Most of the free radicals produced by these exogenous substances are mediated through metabolic activation by cytochrome P450 (CYP450) (Weber *et al*., 2003).

Enzyme catalyzed reactions constitute a major aspect of *in vivo* radical formation. NADPH oxidase enzyme catalyzed reactions in phagocytic cells leading to deliberate superoxide ($O_2^{\bullet}$) radical production. Several other enzymes including peroxidases, oxidases and dehydrogenases are capable of reducing O_2 to $O_2^{\bullet}$ (Petrulea *et al*., 2012). Auto-oxidation of many biological molecules in the presence of transition metal ions also contribute to endogenous free radical production (Guttmann and Powell, 2012).

Naturally, production of free radicals in the biological system is maintained at equilibrium by antioxidant defense system. Oxidative stress manifests when there is an imbalance in the homeostatic production and scavenging of free radicals leading to more free radical production and scavenging inefficiency. A major consequence of oxidative stress is damage to nuclei acids, lipids and proteins which can severely compromise cell function and viability or induce a variety of cellular responses through generation of secondary reactive species, ultimately leading to cell death (Stocker and Keaney, 2004).

2.2 Antioxidants

Antioxidants are molecules stable enough to neutralize free radicals through donation of an electron thus, reducing the ability of free radicals to induce oxidative damage (Lobo *et al*., 2010). Antioxidants therefore are the first line of defense against free radical damage and as such are critical for maintaining optimum health and well-being. The biological system is equipped with endogenous antioxidants that regulate systemic free radical levels however, due to increasing exposure to exogenous sources of free radicals and subsequent reduced effiency of endogenous antioxidant defence system due to defective production pathway, age and diseases, the need for exogenous complementary antioxidants becomes critical in maintaining optimal oxidative state of biological system (Poljsak *et al*., 2013). To protect biological system of the body against reactive species and free radicals, the endogenous and exogenous antioxidants function interactively to neutralize free radicals and their damaging consequences (Shukla *et al*., 2014). These antioxidants include:

2.2.1 Enzymes

Antioxidant enzymes are endogenous proteins that catalyze direct scavenging of free radicals. These enzymes include superoxide dismutase (SOD), catalase (CAT) and the Glutathion family of enzymes which include glutathione reductase (GR), glutathione peroxidase (GPx) and Glutathione transferase (GST).

Metalloenzyme superoxide dismutase is one of the most effective intracellular enzymatic antioxidants which is ubiquitous in all aerobic organisms and in all subcellular compartments prone to ROS mediated oxidative stress (Gill and Tuteja, 2010). SOD catalyzes the conversion of superoxide anion to dioxygen and hydrogen peroxide. SOD exists in several isoforms which differ in nature of active metal centre, amino acid composition, co-factors and other features. SOD neutralizes superoxide ions by going through successive oxidation and reduction cycles of transition metal ions at its active site (Chaudiere and Ferrari-Iliou, 1999). The copper ions in SOD catalyze dismutation by undergoing alternate oxidation and reduction while Zn^{2+} does not function in the catalytic cycle but help stabilize the enzyme (Chaudiere and Ferrari-Iliou, 1999). The specificity of SOD for $O_2^{\bullet}$ is often used to establish the involvement of $O_2^{\bullet}$ in biological processes (Eboh, 2014).

Dismutation of $O_2^{\bullet}$ generates H_2O_2 in a reaction catalyzed by SOD and several oxidase enzyme. Hydrogen peroxide can be removed by two types of enzymes. The catalase catalyzes direct decomposition of H_2O_2 to ground-state O_2 while peroxidases remove H_2O_2 by using it to oxidize another substrate. In mammals, catalase is present in all organs, but especially concentrated in

the liver. Catalase in erythrocytes help protect them against H_2O_2 generated by dismutation of $O_2^{\bullet}$ from haemoglobin autoxidation (Mates *et al*., 1999).

Glutathione reductase is a flavor-protein oxidoreductase found in both prokaryotes and eukaryotes. It plays an essential role in defense system against ROS by sustaining the reduced status of GSH (Gill and Tuteja, 2010). While glutathione peroxidase uses GSH to reduce H_2O_2 and lipid hydroperoxides, glutathione transferase catalyse the conjugation of reduced glutathione with compounds that contain an electrophilic reactive centre through the formation of thioether bond between the sulphur atom of GSH and the substrate (Noctor *et al*., 2002). Classically, GSTs play a major role in phase II drug metabolism where they contribute to cell survival by detoxification of phase I (particularly CYP 450) metabolized reactive xenobiotics (Jancova *et al*., 2010). Epoxide and alkyl containing compounds are examples of compounds that undergo GST-catalyzed conjugation reaction. In addition to conjugation reactions, a number of GST isoenzymes exhibit other GSH-dependent catalytic activities including the reduction of organic hydroperoxides.

2.2.2 Vitamins

In humans, α-tocopherol (vitamin E) is the most active form and the major powerful membrane bound antioxidant employed by the cell (Hensley *et al*., 2004). The main function of vitamin E is to protect against lipid peroxidation (Pryor, 2000). During antioxidant reaction, α-tocopherol is converted to an α-tocopherol radical by the donation of a labile hydrogen to a lipid or lipid peroxyl radical, and the α-tocopherol are regenerated in the presence of ascorbic acids. α-tocopherol has higher vitamin E activity and single oxygen-quenching ability whereas γ-

tocopherol has better nitrogen dioxide and peroxynitrite radical-scavenging ability than α-tocopherols (Gregory, 1996). The phytyl chain of tocopherols fit in the membrane bilayer and the active chroman ring is closely positioned to the surface. This unique structure enables α-tocopherol to act as effective antioxidant and to be regenerated through reaction with other antioxidants such as ascorbic acid (Papas, 1999).

Ascorbic acid (Vitamin C) is a 6-carbon lactone ring structure with 2,3-enediol moiety. The antioxidant activity of ascorbic acid resides in its 2,3-enediol (Hacisevki, 2009). Ascorbic acid first changes to semi-dihydroascorbic through donating a hydrogen atom and electron, and then to dihydroascorbic acid by donating a second hydrogen atom and electron. Both ascorbic acid and dihydroascorbic acid retain the vitamin C activity. Ascorbic acid is highly susceptible to oxidation in the presence of metal ions such as Cu^{2+} and Fe^{3+}. Oxidation of ascorbic acid is also influenced by heat, light exposure, pH and oxygen concentration (Gregory, 1996).

The antioxidant mechanism of ascorbic acid is based on hydrogen atom donation to lipid radicals, quenching of singlet oxygen, and removal of molecular oxygen. Scavenging aqueous radicals and regeneration of α-tocopherol from the tocopheroxyl radical species are also well known antioxidant mechanisms of ascorbic acid (Hacisevki, 2009). Ascorbic acid is an excellent electron donor because of the low standard one electron reduction potentials, the generation of relatively stable semi-dehydroascorbic acid and the easy conversion of dehydroascorbic acid to ascorbic acid (Rumsey *et al*., 1999).

2.2.3 Carotenoids

These are group of tetraterpenoids with basic structural backbone consisting of isoprenoid units formed either by head-to-tail or by tail-to-tail biosynthesis. There are primarily two classes of carotenoids: carotenes and xanthophylls. Lycopene and β-carotene are typical carotenes whereas lutein in green leaves and zeaxanthin in corn are typical xanthophylls. Carotenoids contain conjugated double bond and their antioxidant activity arises due to the ability of these to delocalize unpaired electrons (Mortensen *et al*., 2001). The efficacy of carotenoids for physical quenching is related to the number of conjugated double bonds present in the molecules.

2.2.4 Thiol antioxidants

The major thiol antioxidant is the tripeptide glutathione (GSH) which is a multifunctional intracellular antioxidant and is considered to be the major thiol-disulphide redox buffer of cell (Masella *et al*., 2005). It is abundant in cytosol, nuclei and mitochondria, and is the major soluble antioxidant in these cell compartments (Masella *et al*., 2005). The antioxidant capacity of thiol compounds is due to the sulphur atom, which can easily accommodate the loss of a single electron (Karoui *et al*., 1996). The oxidized glutathione (GSSG) is accumulated inside the cells and the ratio of GSH/GSSG is a good measure of oxidative stress of an organism (Droge, 2002). The main protective roles of glutathione against oxidative stress are that it can act as a co-factor for several detoxifying enzymes, scavenge hydroxyl radical and singlet oxygen directly, and regenerate vitamin C and E back to their active forms (Masella *et al*., 2005).

Another thiol antioxidant is the thioredoxin (TRX) system. They are proteins with oxidoreductase activity and are ubiquitous in both mammalian and prokaryotic cells (Eboh,

2014). It also contains a disulphide and possesses two redox-active systems within a conserved active site. Thioredoxin contains two adjacent –SH groups in its reduced form that are converted to a disulphide unit in oxidizd form when it undergoes redox reaction with multiple proteins. Both TRX and GSH have overlapping as well as compartmentalized functions in the activation and regulation of transcription factors (Valko *et al*., 2006).

2.2.5 Bilirubin

The pigment bilirubin is essentially bound by albumin in human plasma. Albumin bound to bilirubin can synergize with lipoprotein associated α-tocopherol and by doing so, effectively inhibit LDL lipid oxidation, particularly if the latter process is caused by lipid soluble radical oxidant (Neuzil and Stocker, 1994). Bilirubin is also an effective inhibitor of protein oxidation (Neuzil and Stocker, 1994).

2.2.6 Coenzyme Q

Coenzyme Q (CoQ) or ubiquinone is a redox-active and lipophilic substance present in most cellular membranes. CoQ functions as a mobile redox agent shuttling electron and protons in the electron transport chain. CoQ is highly effective in preventing lipid, protein and DNA oxidation and it is continuously regenerated by intracellular reduction system. CoQ in its reduced form as the hydroquinone (ubiquinol) is a potent lipophilic antioxidant and is capable of recycling and regenerating other antioxidants such as tocopherol and ascorbate (Crane, 2001). In some pathologic processes when tissue CoQ content is decreased, it may be advantageous to supplement CoQ by dietary administration.

2.2.7 Polyphenols

Polyphenols are naturally occurring compounds found largely in the fruits, vegetables, cereals and beverages. All plant phenolic compounds arise from a common intermediate phenylalanine, or a close precursor, shikimic acid (Watson, 2014). Primarily they occur in conjugated forms with one or more sugar residue linked to hydroxyl groups, although direct linkages to aromatic carbon also exist. Association with other compounds like carboxylic and organic acids, amines, lipids and linkage with other phenol is also common (Kondratyuk and Pezzuto, 2004).

Flavonoids are the most common type of phenolic compounds found in plants. Each flavonoid group is differentiated by the number of hydroxyl and other substituents on its two benzene rings. Hydrogen donation and chelation of metal ions are the main mechanism of action of polyphenolic compounds (Bravo, 1998). After donating a hydrogen atom, phenolic compounds become resonance-stabilized radicals, which do not easily participate in other radical reactions.

2.2.8 Synthetic antioxidants

Butylated hydroxyl anisole (BHA), butylated hydroxyl toluene (BHT), propyl gallate (PG) and tertiary butyl hydroquinone (TBHQ) all fall into this class of antioxidants. Butylated hydroxyanisole is perhaps the most extensively used antioxidant in the food industry – for preservation of low fat food, fish products, packaging materials and mineral oils (Shalaby and Shanab, 2013). BHT is also widely used in combination with other antioxidants such as BHA, propyl gallate and citric acid for the stabilization of oil and high fat foods (Shanab *et al*., 2010).

The same chemical properties which make BHA and BHT excellent preservatives have been implicated in their adverse health effects. The oxidative characteristics and/or metabolites of BHA and BHT contribute to carcinogenicity or tumorigenicity. Repeated studies have also shown that these substances increases the risk of cancer which has led some health organizations to enlist these substance as carcinogen and as such barred it for use in food industries (Shalaby and Shanab, 2013).

2.3 The Immune system as biological defense machinery

The immune system consists of a network of cells, tissues, organs and molecules that work together to defend the body against attacks by foreign invaders which may be microbes or toxins (Nworu *et al*., 2007). The system also keeps surveillance on threatening endogenous substances within the body. Immune response involves a coordinated reaction of this defense network against the foreign invaders through an elaborate and dynamic communication. The physiological function of the immune system is therefore to protect the body from diseases through searching and destruction of health-damaging agents (Mills, 2004).

The importance of the immune system in health is demonstrated by the frequent observation that individuals with defective immune responses are susceptible to serious, often life-threatening infections. The emergence of acquired immune deficiency syndrome (AIDS) also emphasizes the importance of the immune system in defending individuals against infections. Nevertheless, for effective response, the immune system must be well coordinated as abnormal immune response may predispose to disease conditions with serious morbidity and mortality.

In animals, defense mechanisms can be passive or active. Passive defense comes in the form of natural barriers that hinder infection. Examples are skin, which prevents access of microbes to the underlying tissue and gastric acid in the stomach which can kill many microbes that might be ingested with food. Their existence is quite independent of the presence of infections. Active defense is brought about by immune responses that involve a diversity of different effector mechanisms that are induced by the presence of infection and which may eliminate the microbes. The immune system though highly complex, consists of two categories of defense mechanisms- the innate (non-specific) and the adaptive (specific) system (Janeway *et al*., 2005). These two mechanisms could be modified by substances to either enhance or suppress their ability to resist invasion by pathogens (Williams, 2001).

2.3.1 Innate immunity

This is the first line of defense against pathogens and it represents a rapid and stereotyped response to a large but limited number of stimuli. It is represented by physical, chemical and biological barriers, specialized cells and soluble molecules present in every normal individual irrespective of previous contact with offending agents or immunogens and does not change qualitatively or quantitatively after contact (Cruvinel *et al*., 2010).

The main effector cells of innate immunity are macrophages, neutrophils, dendritic cells and natural killer cells. Phagocytosis, release of inflammatory mediators, activation of complement system proteins, as well as synthesis of acute phase proteins ant cytokines are some of the mechanisms in innate immunity (Mogensen, 2009). These mechanisms are activated by specific stimuli, represented by molecular structures of ubiquitous occurance in microorganisms, but not

in human species. Molecules commonly found on the surface of microorganism such as lipopolysaccharides, mannose and tricholic acids constitute pathogen-associated molecular patterns (PAMPs) and activate innate immune response by interacting with different receptors known as pattern recognition receptors (PRR) (Thompson *et al*., 2011).

Phagocytosis begins with adhesion of the phagocyte surface receptors to the pathogen, which then is internalized into vesicle called phagosomes. Inside the phagocyte, the phagosome fuses to lysosome, whose content are released with consequent digestion and pathogen elimination. Generation of ROS represent essential mechanism in phagocytosis. Absence of ROS determines serious deficiency in the destructive capacity of phagocytes, being responsible for a significant primary immunodeficiency called chronic granulomatous disease (Kobayashi *et al*., 2001). Many microbes have however evolved strategies to overcome innate defense, and in this situation, the more potent and specialized acquired immune response is required to eliminate the pathogen. Cytokines produced by the innate immune system signals that infectious agents are present and influence the type of acquired immune response that develops.

2.3.2 The acquired/adaptive immune system

This aspect of immune system is inducible and thus develops more slowly than the inflammatory response of the innate immune system. The immune response is also specific and has memory, conferring a permanent or long-term protection against specific microorganism. Many components of innate resistance are necessary for the development of the adaptive immune response. Conversely, products of the adaptive immune response activate components of innate

resistance. Thus both systems are essential for complete protection against infectious disease (Bonilla and Oettgen, 2010).

The products of the adaptive immune response include a type of serum protein, immunoglobulin (Ig) or antibodies and a type of blood cell, lymphocytes. Immunoglobulins are produced by a bone marrow derived cells known as the B lymphocytes (B-cells), while the T-lymphocytes (T-cells) by thymus derived cells. There are five classes of immunoglobulins (IgG, IgA, IgM. IgE and IgD) which are characterized by differences in structure and function. Several distinct subclasses of IgG and IgA also exist. Antibodies circulate in the blood and bind to antigens on infectious agents. The interaction can result in direct inactivation of the microorganism or activation of a variety of inflammatory mediators that will destroy the pathogen. Antibodies are primarily responsible for protecting against many bacteria and viruses. This arm of immune response is termed humoral immunity (Chaplin, 2010).

T cells are a subset of lymphocytes that undergo differentiation during an immune response and develop into several subpopulations of effector T cells that have an effect on many other cells. Some develop into T-cytotoxic (Tc) cells that attack and kill targets directly. Targets for Tc cells include cells infected by viruses, as well as cells that have become cancerous. Others may develop into T cells that can stimulate the activities of other leukocytes through cell to cell contact or through the secretion of cytokines. This arm of immune response is termed cellular, or cell mediated immunity.

The success of an acquired immune response depends on the function of both the humoral and cellular responses, as well as the appropriate interactions between them. The collaboration between the B cells and a subset of T cells (T-helper cells) is essential for almost all antibody response to antigen. Additionally, both arms produce specialized subpopulations of memory cells, which are capable of remembering the specific antigen and responding more readily and efficiently against future infections.

2.4 Cytokines as a mediator of the immune system

Cytokines are a group of low molecular weight regulatory proteins secreted by leucocytes and a variety of other cells, in response to a number of inducing stimuli. Cytokines in general act as immune ―messenger molecules that modulate, stimulate and regulate various aspects of the immune response by acting on specific receptors on the surface of target cells (Coondoo, 2011). Cytokines that are secreted from lymphocytes are termed lymphokines, whereas those secreted by monocytes or macrophages are termed monokines. Many of the lymphokines are also known as interleukins (ILs), since they are not only secreted by leukocytes but also have an effect on leukocytes (Coondoo, 2011).

There is growing evidence to suggest that the systemic immune response is at least partially controlled by a group of cytokines that allow the cellular component of this defense mechanism to communicate with each other and which are also capable of directing the nature of the response. Some of these molecules such as TNF-α is predominantly synthesized and secreted by activated macrophages. On the other hand, IL-2, IL-4, IL-5, IL-10 and IFN-γ are produced by activated T-lymphocytes. These 2nd group of cytokines have now been classified into 2 subsets

(Th1-type and Th2-type) based on the types of cells that produce them. Th1 cells secrete mostly IL-2 and IFN-γ, while Th2 cells secrete IL-3, IL-4, IL-5, IL-6 and IL-10 (Lyu and Park, 2005). Th1 cells are responsible for cell mediated immunity, phagocyte-dependent host response, cytotoxicity, and macrophage activation. IL-2 has effect on the several other immune cells, including natural killer cells, B-cell monocytes/macrophages and neutrophils (Lyu and Park 2005).

The major producers of the cytokines are the activated T-Helper ((Th-1 and Th-2) cells and macrophages. These cells are important in the development of cellular and humoral immune responses, induction of inflammatory reactions, and regulation of hematopoiesis (Chaplin, 2010). IFN-γ is one of the cytokine produced by these cells and whose main biological activity is immunomodulation. Nearly all cells express receptors for IFN-γ and respond to IFN-γ binding by increasing the surface expression of class II MHC proteins, thereby promoting the presentation of antigen to T-helper (CD_4^+) cells (Gattoni *et al*., 2006). IFN-γ has very important role in regulation of the phenotype of subsequent antigen-specific T-cell response (Gattoni *et al*., 2006). It enhances T-helper cell activity, and it is one of the Th1-specific cytokines, which promote Th1 responses and diminish Th2 responses (Kaiko *et al*., 2008).

Interleukin-4 (IL-4) was first described as stimulating or growth factor of B cell (Chaplin, 2010). In addition to regulation of B cell growth it also affects T cells. Their major roles include Th2 cell differentiation, immunoglobulin E (IgE) secretion and eosinophil recruitment. In addition to Th lymphocytes, IL-4 may be derived from cytotoxic T cells, eosinophils, mast cells, and basophils. IL-4 is generally considered to favor humoral immunity (Kapp *et al*., 2010).

TNF-α is a potent cytokine produced by monocytes and macrophages as well as by other cells including T and B lymphocytes and fibroblasts in response to inflammation, infection and injury. It induces the expression of other autocrine growth factors, increases cellular responsiveness to growth factors and induces signaling pathways that lead to proliferation. Like other growth factors, TNF-α induces expression of a number of nuclear proto-oncogene as well as several interleukins (Granet and Miossec, 2004).

Pro inflammatory cytokines such as TNF-α and IL-1β play important roles in inflammatory diseases. Both TNF-α and IL-1β activate the inflammatory cell and induce the production of other inflammatory mediators that in turn modulate the important cellular events including gene expression, DNA damage and cellular proliferation contributing to various inflammatory disorders.

Colony Stimulating Factors (CSFs) are cytokines that stimulate the proliferation of specific pluripotent stem cells of the bone marrow in adults. Granulocyte-CSF (G-CSF) is specific for proliferative effects on cells of the granulocyte lineage. Macrophage-CSF (M-CSF) is specific for cells of the macrophage lineage. Granulocyte-macrophage-CSF (GM-CSF) has proliferative effects on both classes of cells. Erythropoietin (EPO) is also considered a CSF as well as a growth factor, since it stimulates the proliferation of erythrocyte colony-forming units. IL-3 (secreted primarily from T-cells) is also known as multi-CSF, since it stimulates stem cells to produce all forms of hematopoietic cells (Shi *et al*., 2006). The well documented effects of GM-CSF on the immune response include, activation of macrophage, increasing MHC class II

antigen expression, enhancing dendritic cell maturation and migration. Its immunostimulating functions include inducing localized inflammation at the site of injection and exerting immunomodulatory effects by its systemic impact on the hematopoietic cytokine network (Shi *et al*., 2006).

2.5 Immunomodulation

This is a very broad term which refers to any changes in the immune response and may involve induction, expression, amplification or inhibition of any part or phase in the immune response. It may involve strengthening or suppression of the indicators of cellular and humoral immunity and unspecific defense factors. Modulation may be specific and limited to a given antigen/agent or non-specific, with a general effect on immune response. The manifestations of immunomodulation action of biologically active substance are immunosuppression or immunostimulation of their immune reaction (Sagrawat and Khan, 2007). Stimulation of the immune response is desired for certain people such as immunocompromised patient, whereas suppression of the immune response is beneficial for others such as transplant recipient or patients with autoimmune or inflammatory diseases. The ability of immunomodulator to enhance or suppress immune responses can be affected by a number of factors such as dosage, route of administration, timing and frequency of administration (Tzianabos, 2000)

2.5.1 Immunostimulation

This involves the enhancement or reinforcement of the response by immune system against antigens. Stimulation of nonspecific defense mechanisms in man has a long tradition in medicine (Sima *et al*., 2015). Immunostimulation constitutes an attractive alternative to conventional

chemotherapy and prophylaxis of infections, especially when the host defense mechanism has to be activated under conditions of impaired immune responsiveness (Sahu *et al*., 2010). Immunostimulation may reflect as an increase in the rate, intensity and duration of immune response. It may also manifest as induction of immune response to an otherwise non-immunogenic substance. Primary targets of immunostimulants are T or B lymphocytes or the complement system, while an increase in phagocytosis by macrophages and granulocytes also plays central role in immunostimulation (Gertsch *et al*., 2011). The second most important role is the stimulation of T lymphocytes which can be achieved either directly or indirectly through macrophages (Gertsch *et al*., 2011).

2.5.2 Immunosuppression

Immunosuppression involves reduction of the activation or efficacy of any component of the immune system leading to decrease in immune response. Pancytopenia, leukopenia, lymphopenia, decreased globulin levels, hypocellularity of immune system and decreased immune organ weight (such as thymus, spleen, lymph nodes and bone marrow) are all clinical indicators of immunosuppression. Immunosuppression can occur as a result of adverse drug reaction and as an intended pharmacodynamics effect of drugs. Also, some portions of the immune system exhibit immunosuppressive effects on other parts of the immune system (Guenova *et al*., 2013).

Deliberate immunosuppression is induced in the treatment of autoimmune disease or diseases that are most likely of autoimmune origin (eg rheumatoid arthritis, myasthenia gravis, systemic lupus erythematosus, crohn's disease and ulcerative colitis). Immunosuppression is also applied

in the treatment of some other non-autoimmune inflammatory diseases (such as long term allergic asthma) as well as in the prevention of organ and tissue transplant rejection. Some examples of drugs use in immunosuppression include glucocorticoids (methylprednisolone), cytostatic drugs (cyclophosphamide, azathioprine, dactinomycin), antibiotics (alemtuzumab) and other drugs acting on immunophilins (cyclosporin) (Gold *et al*., 2008; Rathee *et al*., 2012).

2.6 Immunodeficiency

Normal host defense require the participation of four major immune systems: T-lymphocytes, B-lymphocytes, phagocytes and complement. Impairment of any of the major components of the immune system may result in clinical immunodeficiency. Immune defects can arise from intrinsic or acquired defects of lymphoid elements, failure of normal cellular differentiation, viral infection or other acquired causes (Fried and Bonilla, 2009). Primary immunodeficiency results from defects in genes for components of the immune system while secondary/acquired deficiency arise due to factors that have adverse impact on the immune system. Though primary immune deficiency is genetic, some of the diseases associated with this deficiency are diagnosed in later stage in life due to compensating immune functions that keep serious problems from developing earlier in life or due to slowly deteriorating immune function, genetically determined that does not become significant until later in life (Medkaikar *et al*., 2013).

Clinical impairment of immunity is expressed as a marked susceptibility to opportunistic and pathogenic organisms which are difficult to control and by an increased risk of malignancy, allergy and autoimmune disease (Jiang *et al*., 2008). Certain immunodeficiency disorders are associated with aberrant immune regulation. The major types of immune deficiency are

characterized by unique patterns of infections depending on the level at which the defect occurs and the pathogenic mechanisms of the parasite.

2.7 Antioxidant medicinal plants

Many medicinal plants have been reported to possess antioxidant effects. Some of these medicinal plants include:

2.7.1 *Gongronema latifolia*

This is commonly called ‘utazi’ and ‘arokeke’ in the South Eastern and South Western parts of Nigeria respectively. *G. latifolia* is a tropical rainforest plant primarily used as spice and vegetable in traditional folk medicine. It belongs to Ascepiadaceae family.

G. latifolium has been used in the treatment of diverse disease conditions. It has been very useful in the management of diabetes and a good supplement in women after child birth. The extracts from *G. latifolium* leaves have been shown to possess both *in vitro* and *in vivo* antioxidant properties. Both the aqueous and ethanol leaf extracts have been shown to increase the activity of superoxide dismutase enzyme and the levels of reduced glutathione (Ugochukwu and Babady, 2002). Decrease in streptozotocin induced lipid peroxidation was found to be part of the mechanisms of antioxidant activity of *G. latifolium* (Ugochukwu and Babady, 2002). The tannin leaf extract of this plant has also been shown to inhibit oxidative effect mediated through lipoxygenase enzyme reaction (Eze and Nwanguma, 2013).

2.7.2 *Vernonia amygdalina*

Vernonia amygdalina, Del. commonly known as bitter leave belongs to the Compositae family. It is one of such plants reported with high medicinal properties. The name bitter leaf originated from the bitter taste impacted by the leaves and stem. It is a major vegetable and occupies significant position in the diet of several Nigerian ethnic groups (Oriakhi *et al*., 2014). They are widely distributed in tropical Africa and Asia and are most common in natural forest. It has long history in traditional medicine and parts including the leaves, stem and roots have been exploited in the treatment of various ailments. Particularly, the leaves are utilized in the treatment of malaria, diabetes mellitus, venereal disease, wounds, hepatitis and cancer (Kambizi and Afolayan, 2001; Hamill *et al*., 2003; Erasto *et al*., 2007). The antihelminthic properties of the plants have been reported (Danquah *et al*., 2012). *In vitro* antioxidant studies of the ethanol extract of the leaves revealed inhibition of diphenyl picryl hydrazyl radical (Ayoola *et al*., 2008). Similarly, *in vivo* antioxidant of *V. amygdalina* has been shown to ameliorate oxidation of linoleic acid and lipid peroxidation induced by Fe/ascorbate in rat (Mbang *et al*., 2008).

2.7.3 *Psidium guajava*

Psidium guajava, L. (guava) found in the Myrtaceae family is another plant with diverse medicinal values. Guava is reported to be rich in tannins and is widely cultivated in the tropics and subtropics for its edible fruits (Chuanoi *et al*., 2009). The leaves contain considerable amount of phenols and have been used as health tea. The phenolic content of the leaves has been considered to be responsible for its antioxidant activity as well its use in the management and treatment of chronic disease conditions such as diabetes, cancer and heart diseases (Chuanoi *et al*. (2009). High total phenolic compounds were found to be obtained using absolute ethanol as

extraction solvent. This solvent extract exhibited good ferric ion reducing ability as well as total antioxidant capacity (Ekaluo *et al*., 2015).

2.7.4 *Azadirachta indica*

Azadirachta indica A. Juss, known as neem, belongs to the family meliaceae and is widely distributed in Asia, Africa and other tropical parts of the world. . Each part of the neem tree has some medicinal property and is thus commercially exploitable. Neem oil, bark and leaf extracts have been therapeutically used in folk medicine to control diseases like leprosy, intestinal helminthiasis, respiratory disorders, constipation and skin infections. The bark and leaf part of the plant contains phenolic compounds and flavonoids which exhibit antioxidant activity by inactivating lipid free radicals and preventing decomposition of hydroperoxides into free radicals. Different systems of assay such as Hydroxyl radical scavenging assay and DPPH assay have been used to demonstrate antioxidant activities of different parts of this plant (Bhutkar and Bhise, 2011; Nahak and Sahu, 2011).

Table 1: Some medicinal plants with antioxidant activity

Plant species	Family	Antioxidant activity¶	References
Ocimum sanctum L (Tulsi, Sacred Basil)	Lamiaceae	1, 2, 6	Kath and Gupta, 2006; Green *et al*., 2013.
Daucus carrota L (Carrot)	Apiaceae	2, 6, 3, 8	Sun *et al*., 2009; Singh *et al*., 2012.
Camellia sinensis (Green tea)	*Theaceae*	3,4, 5, 8, 9, 10	Manian *et al*., 2008; Tariq and Reyaz, 2013.
Withania somnifera (Ashwagandh)	Solanaceae	3, 4, 7, 9, 12	Sumathi*et al*., 2007; Alam *et al*., 2012.
Glycyrrhiza glabra (Licorice, Mulathi)	*Leguminosae*	3, 4, 14, 16	Dong *et al*., 2014; Asan-Ozusaglam and Karakoca *et al*., 2014.
Curcuma longa (Turmeric)	Zingiberaceae	3, 4, 11, 17	Chatterjee *et al*., 1999; Maizura *et al*., 2011.
Zingiber officinale (Ginger)	Zingiberaceae	3,4, 9, 11, 15	Stoilova *et al*., 2007; Maizura *et al*., 2011
Melia azedarach L. (Margosa)	Meliaceae	1, 2, 3, 4, 9, 18, 19	Ahmed *et al*., 2008; Orhan *et al*., 2012.
Crataegi folium (Hawthorn)	Aceraceae	8, 11	Sokol-Letowska *et al*., 2007; Demiray *et al*., 2009.
Alchornia cordifolia	Euphorbiaceae	2, 3, 6	Olaleye *et al*., 2007; Osadebe *et al*., 2012.
Ludwigia octovalvis	Onagraceae	3, 20, 21	Shyur *et al*., 2005
Pseuderanthemum palatiferum	Acanthacea	1, 2, 3, 4, 7, 8, 22	Chayarop *et al*., 2011; Pamok *et al*., 2014.
Premna integrifolia	Lamiaceae	4, 7, 10, 12, 13, 15, 20	Gokani *et al*., 2011
Lycium barbarum	Solanaceae	3, 4, 10, 12, 20, 23	Li *et al*., 2007; Mocan *et al*., 2014.
Doronicum hookeri	Asteraceae	1, 2, 3, 4, 6, 8, 9, 19, 20	Gupta *et al*., 2011; Syed *et al*., 2014.
Morinda citrifolia	Rubiaceae	2, 3, 4, 5, 8, 15, 20	Kumar *et al*., 2014; Srinivashan *et al*., 2014.
Uncaria tomentosa (cat's claw)	Rubiaceae	20, 24	Pilarski *et al*., 2006.

Plant species	**Family**	**Antioxidant activity¶**	**References**
*Sempervivum tectorum*L.	Crassulaceae	1, 2, 11, 19	Sentjurc *et al*., 2003; Florin *et al*., 2014.
Donax grandis	Marantaceae	3	Daud *et al*., 2011.
Cassia fistula Linn	Fabaceae	3, 4, 9, 25	Irshad *et al*., 2012.
Anthocephalus indicus	Rubiacae	1, 2, 4, 13	Kumar *et al*., 2010; Sanadhya *et al*., 2012.
Syzygium cumini (Jambul)	Myrtaceae	3, 4, 13, 15	Ruan *et al*., 2008; Eshwarappa *et al*., 2014.
Xanthium strumarium	Asteraceae	3, 5, 13, 15	Kamboj *et al*., 2014; Kaur *et al*., 2015.
Pueraria tuberose Linn	Leguminosae	7, 8, 9, 15, 20	Pandey *et al*., 2007
Cassia tora Linn	Caesalpiniaceae	3, 5, 13, 15, 20, 21	Sirappuselvi and Chitra, 2012

¶ Legends: inhibition of in vivo lipid peroxidation-1; increase in antioxidant enzymes-2; inhibition of DPPH radical-3; ferric reducing antioxidant ability-4; total antioxidant capacity-5; inhibition of liver oxidative damage-6; inhibition of in vitro lipid peroxidation-7; inhibition of ABTS radicals-8; metal ion chelating potency-9; inhibition of H_2O_2 induced hemolysis-10; in vitro inhibition of lipid peroxidation-11; inhibition of β-carotene bleaching-12; nitrate/nitric oxide scavenging activity-13; protective effect on H_2O_2-injured PC12 cell damage-14; hydroxyl radical scavenging activity-15; cupric ion reducing antioxidant capacity-16; inhibition of γ-irradiation oxidation-17; inhibition of erythrocyte oxidative damage-18; increase in reduced gluthathion-19; superoxide anion scavenging activity-20; inhibition of H_2O_2 induced DNA strand cleavage-21; inhibition of AAPH-induced hemolysis-22; inhibition of hemoglobin ascorbate peroxidase activity-23; peroxyl radical-trapping capacity-24; hydrogen peroxide scavenging activity-25.

2.8 Plants with immunomodulatory activity

Plants have been widely studied as potential sources of immunomodulatory substances. Some of these plants include:

2.8.1 *Morinda citrifolia L. (Rubiaceae)*

Morinda citrifolia (Noni) has been used in folk medicine for many years and has been reported to have a broad range of therapeutic effects, including antibacterial, antiviral, antifungal, antitumor, anthelmintic, analgesic, hypotensive, anti-inflammatory, and immune enhancing effects (Wang *et al*., 2002). The alcoholic extract of *M. citrifolia* was found to contain a polysaccharide-rich substance that inhibited tumor growth through activation of the host immune system (Hirazumi and Furusawa, 1999). The extract was also capable of stimulating the release of several mediators from murine effector cells, including TNF-α, interleukin-1beta (IL-1β), IL-10, IL-12, interferon-gamma (IFN-γ) and nitric oxide (NO) (Mukherjee *et al*., 2014). A number of major components have been identified in the Noni plant such as scopoletin, octoanoic acid, terpenoids, alkaloids, anthroquinones, β-sitosterol, carotene, flavone glycosides, linoleic acid and rutin (Rethinam and Sivaraman, 2007).

2.8.2 *Allium sativum L. (Alliaceae)*

Allium sativum (Garlic) contains high concentration of sulfur compounds which are responsible both for its pungent odor and many of its medicinal effects (Londhe *et al*., 2011). Increased T-lymphocyte blastogenesis and phagocytosis, as well as modulation of cytokine production are some of the reported in vitro and in vivo immunomodulatory effects of Garlic extract (Ebrahimi *et al*., 2013). The extract also has been found to inhibit the antigen specific histamine release and IgE mediated skin reaction. It is also reported that low concentration of garlic extract reduced IL-12, TNF-α, IL-1α, IL-6, IL-8, T-cell interferon-gamma (IFN-γ), IL-2, TNF-α and an up-

regulation of IL-10 production (Keiss *et al*., 2003). These activities demonstrate diverse immunomodulatory effects of Garlic on different immune functions.

2.8.3 *Bidens pilosa L*. (Asteraceae)

The folkloric use of *B. pilosa* has been recorded in America, Africa, Asia, and Oceania (Oliveira *et al*., 2004). Aqueous infusion of *B. pilosa* has been shown to enhance the cytokine production and white blood cells population while hot water extracts and its butanol fraction increased IFN-γ promoter activity (Bartolome *et al*., 2013). Centaurein and its aglycone derivative (centaureidin) isolated from this plant showed augmentation of IFN-γ promoter activity and also act as NFκB enhancers (Chang *et al*., 2007).

Table 2: Immunomodulatory activity of some medicinal plant

Plant species	Family	Immunomodulatory activity¶	References
Garcinia Kola (Heckel)	Clusiaceae	1, 2, 3	Nworu *et al*., 2007; Okoko and Oruambo, 2008.
Curcuma longa L.	Zingiberaceae	4	Gautam *et al*., 2007.
Azadiracta indica A. Juss	Meliaceae	1, 4, 5	Baral *et al*., 2005; Mandal-Ghosh *et al*., 2007.
Eclipta prostrate L.	Asteraceae	6, 7, 8, 9	Christybapita *et al*., 2007.
Glycyrrhiza glabra L.	Leguminosae	8, 10, 11	Hong *et al*., 2009; Pandit *et al*., 2011.
Jatropha curcas L.	Euphorbiaceae	7, 11, 12	Abd-Alla *et al*., 2009
Nelumbo nucifera	Nymphaeceae	13, 14	Mukherjee *et al*., 2010a; Mukherjee *et al*., 2010b
Piper longum L.	Piperaceae	7, 12, 15, 16	Sunila and Kuttan, 2004.
Baliospermum montanum	Euphorbiaceae	17, 18	Patil *et al*., 2009.
Calendula officinalis L.	Asteraceae	19	Jimenez *et al*., 2006.
Chrysanthemum indicum L.	Compositae	1, 6, 7,	Lee *et al*., 2009.
Desmodium gangeticum	Fabaceae	20, 21	Mishra *et al*., 2005
Prunella vulgaris L.	Lamiaceae	13, 22	Harput *et al*., 2006

Plant species	Family	Immunomodulatory activity¶	References
Psoralea corylifolia L.	Fabaceae	23, 24	Lee and Kim, 2008
Tamarindus induca L.	Leguminosae	25, 26	Librandi *et al*., 2007
Terminalia chebula	combretaceae	1, 7	Shivaprasad *et al*., 2006

¶ Legends: inhibition of delayed type hypersensitivity-1; adjuvant activity on specific antiboby production-2; reduction of lipopolysaccharide activation of macrophage-3; anticomplementary activity-4; induction of cytotoxic macrophages-5; increased phagocytic index-6; induction of antibody titer-7; increase in non-specific immune response-8; increase in lysosomal activity of the humoral responses-9; stimulation of immune cells by CD69 expression on CD4 and CD8 T cells-10; stimulation of macrophage function-11; increase in lymphocyte cells-12; reduction of NO production-13; protection of mast cells degranulation-14; increased bone marrow cellularity-15; increased α-esterase positive cells-16; enhanced neutrophil locomotion and chemotaxis-17; stabilization of mast cell degranulation-18; inhibition of tumor cell proliferation-19; enhanced NO production-20; provided resistance against infection established in peritoneal macrophages by the protozoan parasite *Leishmania donovani*-21; stimulation of T-lymphocytes proliferation-22; up-regulation of production of OVA-specific Th1 cytokine-23; down-regulation of OVA-specific Th2 cytokine-24.

2.9 *Millettia aboensis*

2.9.1 Description

Millettia aboensis is a small tree of about 30 – 40 feet high with reddish-brown pubescence on the petioles, branches, inflorescence and fruits. The flowers are purple in erect woody racemes up to 18 inches long. It has conspicuously rusty-hairy leaves.

2.9.2 Taxonomy

Domain: Eukaryota

Kingdom: Plantae

Subkingdom: Viridaeplantae

Phylum: Trachophyta

Subphylum: Euphyllophytina

Class: Angiospermae

Subclass: Eudicots

Superoder: Rosids

Order: Fabales

Family: Fabaceae

Subfamily: Papilionoidae

Tribe: Tephrosieae

Genus: Millettia

Species: aboensis – (Hook. F.) Baker

Botanical name: *Millettia aboensis* (Hook. F.) Baker

Figure 1: *Millettia aboensis* plant

2.9.3 Common Names

West Africa: Nigeria – awo (Yoruba); erurumesi, mkpukpu manya, ogba otolo, Izi ndu ezi (Igbo), ukperumwesi, Kolokuma (Hausa), Odudu (Efik).

2.9.4 Geographical distribution

Millettia aboensis is found commonly in low land rain forest areas. It is widely distributed in Asia, Western and Central African continent. It occurs mostly in the forest zones of Nigeria, Cameroun and Equatorial Guinea (Burkil, 2005).

2.9.5 Uses of *Millettia aboensis* in traditional medicine

The leaf is used by traditional herbalist for general healing. The root is used in treating gastro intestinal disturbances and liver problems. Also the leaf, stem and root decoction is used for the treatment of venereal diseases such as gonorrhea and syphilis.

2.9.6 Previous pharmacological studies on *Millettia aboensis*

The leaf extract of *Millettia aboensis*has been shown to possess anti-diabetic and anti-lipidemic activities in alloxan induced diabetic rats (Onyegeme-okerenta and Essien, 2015). The leaf extract has also been found to possess potent anti-pseudomonal activity against pseudomonal species expressing extended spectrum beta lactamase (Ikegbunam *et al*., 2012). Its anti-inflammatory activity was found to involve inhibition of prostaglandin synthesis, leukocyte migration and membrane stabilization (Ajaghaku *et al*., 2013). Both alcohol and aqueous root extract showed hepato protective effect on CCl_4 induced hepatotoxicity (Ugwueze *et al*., 2013).

2.9.7 Toxicological studies on *Millettia aboensis*

Acute toxicity evaluation of ethanol leaf extract of *M. aboensis* was reported to be relatively safe with estimated LD_{50} value of 4699 mg/kg (Ajaghaku *et al*., 2012). Acute administration of the aqueous extract of the leaves at high concentrations (1000 – 5000 mg/kg) was reported to produce dose dependent hepatotoxicity evidenced by elevated serum liver marker enzymes (Onyegeme-Okerenta and Onyeike, 2015). Sub-chronic toxicity study on the ethanol leaf extract produced hepatotoxic effects on the liver cells with elevated serum liver marker enzymes at high concentrations (1000 – 4000 mg/kg) (Ajaghaku *et al*., 2012). Sub-chronic effect of the ethanol leaf extract on the hematological system revealed marked elevation of the white blood cell count and negligible effects on the haemoglobin, packed cell volume and red blood cell count (Ajaghaku *et al*., 2012).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Plant material

The leaves of *M. aboensis* were collected in November, 2012 from Nsukka, Enugu State, Nigeria and were authenticated by Mr. Alfred Ozioko of Bioresource Development and Conservation Project, Nsukka, Enugu State, Nigeria. A voucher specimen has been deposited at the herbarium of the Department of Pharmacognosy, Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University, Awka, with the herbarium number PCG/474/A/021. The leaves were air dried for 18 days and pulverized with mechanical grinder (Gx160 Delmar 5.5HP, Honda Motor CO., LTD, Japan).

3.2 Chemicals

***General solvents*:**

The following solvents, dichloromethane, ethyl acetate, n-hexane, methanol and ethanol were used. All solvents were of analytical grade and obtained from Sigma-Aldrich, Germany.

***Solvents for HPLC*:**

Methanol was LiChroSolv HPLC (Merck), nano-pure water (distilled metals free water) was obtained by passing distilled water through nano- and ionexchange filter cells (Barnstead, France).

***Solvents for NMR*:**

Deuterated methanol (Uvasol, Merck) was used for NMR measurements.

Chromatography

Pre-coated TLC plates (Silica Gel 60 F254, layer thickness 0.2mm)	Merck
Silica Gel 60, 40−63 μm mesh size	Merck
Sephadex LH 20, 25−100 μm mesh size	Merck

General instruments

Ultra sonicator RK 510H	Bandelin
Rotary evaporator	Büchi Rotavapor R-200
Vacuum pump	CVC 2000 Vacuubrand
Centrifuge Pico	Heraeus
Nitrogen generator UHPN 3001	Nitrox
Air generator ZA 20	WGA
Fraction collector Retriever II	ISCO
Lyvac GT2 (Freeze dryer)	Steris
Vacuum pump Trivag D10E (Freeze dryer)	Leybol
Syringe	Hamilton 1701 RSN
Magnetic stirrer	Variomag Multipoint HP
Behrotest PH 10-Set	

Semipreparative HPLC

Pump: L-7100	Merck/Hitachi
Detector: UV-L7400 (Photodiode array detector)	Merck/Hitachi
Printer: Chromato-Intergartor D-2000	Merck/Hitachi
Column: Eurospher 100-C18, [10 μm; 300 mm × 8 mm]	Knauer
Pre-column: Eurospher 100-C18, [10 μm; 30 mm × 8 mm]	Knauer

Analytical HPLC

Pump: P 580A LPG	Dionex
Autosampler: ASI-100T (injection volume = 20 μL)	Dionex
Detector: UVD 340S (Photodiode array detector)	Dionex
Column oven: STH 585	Dionex
Column: Eurospher 100-C18, [5 μm; 125 mm × 4 mm]	Knauer
Pre-column: Vertex column, Eurospher 100-5 C18 [5-4 mm]	Knauer

Software: Chromeleon (V. 6.30)

HPLC-MS

Analytical HPLC: Agilent 1100 series (Photodiode array detector)	Agilent
MS: Finigan LCQ-DECA	Thermoquest
Ionizer: ESI and APCI	Thermoquest
Vacuum pump:	Edwards 30 BOC
Column: Eurospher 100-C18, [5 μm; 227 mm × 2 mm]	Knauer
Pre-column: Vertex column, Eurospher 100-5 C18 [5−4 mm]	Knauer

NMR

ARX-600	Bruker

Other chemicals

1,1-diphenyl-2-picrylhydrazil (DPPH),epinephrine, glacial acetic acid,2,2-Azino-bis(3-ethylbenzthiazolin-6-sulfonic acid) were product of Sigma Aldrich, Germany.Multi-Analyte ELISArray cytokine kit (Qiagen, Germany), total antioxidant assay kit (Cyaman chemical, Canada), ferric chloride (Griffin & George, England), Folin-Ciocalteu's reagent (Loba Chemie,

India), hydrogen peroxide (Avondale Laboratories, England), thiobarbituric acid (TBA) (Guangdong Guanghua Chemical Factory Co., Ltd, China). HCL, potassium dichromate and potassium ferricyanide were products of Hopkin and Williams Ltd, England.alkaline phosphatase reagent kit (Teco Diagonostics, USA), aspartate aminotransferase and alanine aminotransferase reagent kitwere products of Randox Laboratories Limited, United Kingdom. Freshly prepared distilled water was used when required.

3.3 Animals

Swiss-female albino rats and mice were used. The animals were obtained from the animal house of the Department of Pharmacology and Toxicology, Nnamdi Azikiwe University, Awka. The animals were maintained in standard laboratory animal conditions and fed with rodent feed (Guinea Feeds Nigeria Ltd). They were allowed free access to water *ad libitum*. All animal experiments were conducted in compliance with NIH guide for care and use of laboratory animals (Pub No: 85-23 Revised 1985).

3.4Extraction, fractionation and phytochemical analysis

3.4.1 Extraction

About 2 kg of the pulverized leaves were cold macerated in 10 litres of aqueous ethanol (70%) for 72 h with intermittent shaking of solvent. The resulting solution was filtered. About 2.5 litres of the filtrate were pre-concentrated *in vacuo* using rotary evaporator at 40^{o}C thereafter dried to a constant weight using open water bath at the same 40^{o}C to obtain the ethanol extract. The remaining filtrate was concentrated to one-quarter the volume and was used for fractionation.

3.4.2 Liquid-liquid fractionation

The pre-concentrated filtrate of the ethanol extract was subjected to liquid–liquid partition successively with 2.5 L of n-hexane, ethyl acetate, and then butanol using separating funnel to give the n-hexane, ethyl acetate and butanol soluble fractions, respectively. The fractions were pre-concentrated using rotary evaporator at 40^{o}C thereafter to dryness using open water bath at 40^{o}C.

3.4.3 Phytochemical analysis

Qualitative determination of relative occurance of secondary metabolites in the extract and fraction of *M. aboensis* was determined using standard procedures (Odebiyi and Sofowora, 1978; Trease and Evans, 1983).

3.5 Bioactivity guided isolation and purification of active compounds

The selection of solvent fractions of ethanol extract of *M. aboensis* for further purification and isolation of active compounds was based on *in vitro* and *in vivo* antioxidant screening as well as *in vivo* humoral and cell mediated immunomodulatory activities of the fractions.

3.5.1 Vacuum liquid chromatographic separation

About 2 g of ethyl acetate fraction was subjected to Vacuum Liquid Chromatography (VLC) (Siliga gel 500 g, sintered funnel 5 L) and eluted with 500 mL each of hexane:ethyl acetate (80:20, 60:40, 40:60, 20:80, 0:100) followed with dichloromethane:methanol (90:10, 70:30,

50:50, 30:70, 10:90, 0:100) and these resulted to 11 pooled fractions D1 to D11. The ethyl acetate sub-fractions were screened for *in vitro* antioxidant activity using DPPH test.

3.5.2 Sephadex separation

About 200 mg each ofethyl acetate sub-fraction D5 and butanol fraction were subjected to Sephadex LH-20 (3X60) separation using dichloromethane:methanol 1:1. The flow rate was maintained at 0.4 mL/min. Eluents were collected in 2 mL aliquots and up to 120 fractions were collected. The fractions were bulked using silica gel coated aluminium thin layer chromatographic plates. Each fraction was spotted on the TLC plate 1 cm apart from neighbouring fraction and 2 cm apart from the base. The samples were drawn up the plate (10 cm) via capillary action using dichloromethane:methanol 1:1 as mobile phase. The plate was allowed to develop in the TLC tank till the solvent was about 1 cm below the top of the plate. The fractions were bulked based on their characteristic colour and movement when visualised with a UV lamp.

3.5.3 Semi-preparative high pressure liquid chromatography

Semipreparative HPLC was performed with Merck Hitachi L-7100 (Merck/Hitachi, Germany) coupled to a UV detector (L-7400). The Sephadex fractions subjected to semipreparative HPLC were each dissolved in HPLC grade methanol to final concentration of approximately 40 mg/mL. The eluant used in semi-preparative HPLC comprises a linear gradient of nanopure water and methanol. 50 µL of approximately 40 mg/mL solution of the substance was injected for each time. The flow rate was stabilized at 5 mL/min, and the paper speed of the recorder was 5

mm/min. The eluted peaks were collected respectively by manual work based on the records of a UV-vis detector.

3.6 Structural elucidation

3.6.1 High pressure liquid chromatography (HPLC) analysis

A. Preparation of samples

About 1 mg of the each of the pure compounds were reconstituted with 2 mL of HPLC grade methanol and 50 µL of the dissolved samples were each transferred into HPLC vials containing 500 µL of HPLC grade methanol.

B. HPLC analysis

HPLC analysis was performed on the samples with a Dionex P580 HPLC system coupled to a photodiode array detector (UVD340S, Dionex Softron GmbH, Germany). Detection was at 235, 254, 280 and 340 nm. The separation column (125 × 4 mm; length × internal diameter) was prefilled with Eurospher-10 C18 (Knauer, Germany), and a linear gradient of nanopure water (adjusted to pH 2 by addition of formic acid) and methanol was used as eluent.

3.6.2 Liquid-chromatographic-mass spectroscopy

HPLC-ESI-MS was carried out using a ThermoFinningan LCQ-Deca mass spectrometer connected to an UV detector. The samples used for the HPLC analysis were each injected to the HPLC-ESI-MS set up. A solution of the sample was then sprayed at atmospheric pressure through a 2-5 kV potential. HPLC was run on a Eurospher C-18 (6 x 2 mm, i.d.) reversed phase column. The mobile phase was H_2O 0.1% Formic acid (A), to which MeOH (B) or ACN (C) was added by a linear gradient: initial, 0% of B; 45 min, 80% of B; 55 min, 100% of B. The flow rate

was at 400 µL/min and the absorbance detected at 254 nm. ESI (electrospray ionization) was performed at a capillary temperature of 200^{o}C and drift voltage of 20eV. Since the molecular ion peak is the most abundant ion in ESI spectra, it is also possible to perform MS/MS experiments. In the MS/MS experiment, the molecular ion from an initial fragment ion corresponds to a certain functional group.

3.6.3 Nuclear magnetic resonance spectroscopy

Nuclear magnetic resonance spectroscopy (NMR) is one of the most efficient methods for studying molecular structures. An NMR spectrum provides detailed molecular structure, that would be difficult, if not impossible, to obtain by any other methods. The ^{1}H NMR and ^{13}C NMR spectra were recorded at 300^{o}K on ARX 600 MHz NMR spectrometers (Bruker, Germany). All 1D and 2D spectra were obtained using the standard Bruker software. The pure compounds were freeze-dried for 2 days and dissolved in deuterated methanol (CD_3OD), the choice of which was dependent on the solubility of the samples. Tetramethylsilane (TMS) was used as internal standard reference signal. The observed chemical shifts (δ) were recorded in ppm and the coupling constants (*J*) were recorded in Hz.

3.7 Pharmacological evaluations

3.7.1 *In vitro*DPPH free radical scavenging activity

The DPPH freeradical scavenging activities of the extract and fractions were evaluated with modification of the method described by Patel and Patel (2010). Freshly prepared DPPH solution (25 µl, 0.6 mmol) was added to 25 µl of different concentrations of the extract and fractions (3.125, 6.25, 12.5, 25, 50, 100 and 200 µg/mL). The volume of the solution was adjusted with methanol to a final volume 200 µl. The control tube contains 175 µl methanol and 25 µl of

DPPH. After incubation in the dark for 30 min at room temperature, absorbance of the mixtures was obtained at 490 nm using micro plate reader. All the tests were performed in triplicate and ascorbic acid was used as standard. The DPPH radical percentage scavenging potentials of the extracts, fractions and standard (ascorbic acid) were calculated from the equation below.

$$\% \text{ Inhibition of free radical} = \left[\frac{A_0 - A_t}{A_0}\right] \times 100 \ldots (1)$$

Where A_0 is the absorbance of the control, and A_t is the absorbance of the test/standard. The EC_{50} was determined from a plot of percentage scavenging potentials against concentration.

3.7.2 Hydrogen peroxide scavenging activity

Hydrogen peroxide scavenging activities of the extract and fraction were determined using the method of Kumaran and Karunakaran (2007). A solution of hydrogen peroxide (20 mM) was prepared in phosphate buffer saline (pH 7.4). This solution (2 mL) was added to 1 mL of different concentrations of the extract and fractions (3.125, 6.25, 12.5, 25, 50, 100 and 200 µg/mL) and absorbance of hydrogen peroxide content of the mixture taken at 230 nm after 10 min against a blank solution containing phosphate buffer without hydrogen peroxide. Ascorbic acid served as the standard. The percentage scavenging potentials was calculated using the following equation:

$$\text{Hydrogen peroxide scavenging activity (\%)} = \left\{1 - \frac{Abs\ of\ control\ - Abs\ of sample}{Abs of control}\right\} x\ 100 \ldots (2)$$

3.7.3 Ferric-reducing antioxidant power (FRAP) assay

Different concentrations of each of the extract and fractions (3.125, 6.25, 12.5, 25, 50, 100 and 200 µg/mL) were mixed with phosphate buffer (1.25 mL, 0.2 M pH 6.6) and potassium ferricyanide [K3Fe(CN)6] (1.25 mL, 1%). The mixture was incubated at 50°C for 20 min, followed by addition of 1.25 mL of 10% tricholoroacetic acid and subsequent centrifugation at 3,000 rpm for 10 min at room temperature. The upper layer of solution (1.25 mL) was mixed with distilled water (1.25 mL) and ferric chloride ($FeCl_3$) (0.25 mL, 0.1%) and the absorbance of the reaction mixture was measured at 700 nm (Kumar*et al*., 2005). Percentage reducing potentials was determinedusing the formular:

$$\left[\frac{\mathrm{AS}-\mathrm{AC}}{AC}\right] \times 100 \ldots (3)$$

Where AC = Absorbance of control; AS = Absorbance of sample

3.7.4 Estimation of total phenolic content

The total phenolic content of the extract and fractions were determined using the method described by Kim *et al*., (2003) with modification. The extract/fractions (1 mL, 0.1 mg/mL) were mixed with 0.2 mL of Folin-Ciocalteu's phenol reagent. After 5 min, 1 mL of 7.6% Na_2CO_3 solution was added to the mixture followed by the addition of 2 mL of deionised distilled water. The mixtures (in triplicate) were incubated at 40^{o}C for 30 min, after which the absorbances were read at 760 nm. The total phenolic content was determined from extrapolation of calibration curve which was made from different concentrations of gallic acid solution (0.01 – 0.06 mg/mL). The result was expressed as milligrams of gallic acid equivalents (GAE) per g of the extract/fractions.

3.8 Determination of ED_{50} of the extract

The ability of the extract to inhibit ethanol induced liver lipid peroxidation was used in determining the effective dose of the extract. This study was conducted using 36 albino rats divided into 6 groups of 6 animals each. Oxidative stress was induced by oral administration of 5 mL/kg of 20% ethanol for 21 days. Different oral doses of the extract (100, 200, 400, 800 and 1600 mg/kg) were given 2 h before administration of the ethanol. Control group received 5 mL/kg of 5% Tween 20 (vehicle). After 24 h of last treatment, the rats were sacrificed by cervical dislocation and the livers were removed immediately, washed with ice-cold saline and a 10% of homogenate prepared in 0.15M Tris-HCl buffer. The homogenate was centrifuged at 12,000 g at 4^{o}C for 20 min and the supernatant used for lipid peroxidation assay.

3.8.1 Lipid peroxidation (LPO) assay

Malondialdehyde (MDA) an index of lipid peroxidation was determined using the method of Buege and Aust (1978). The supernatant from liver homogenate (1.0 mL) was added to 2 mL of (1:1:1) TCA-TBA-HCL-reagent (thiobarbituric acid 0.37%, 0.24N HCL and 15% TCA) tricarboxylic acid-thiobarbituric acid-hydrochloric acid reagent boiled at 100°C for 15 min, and allowed to cool. Flocculent materials were removed by centrifuging at 3000 rpm for 10 min. The absorbance of supernatant was measured using spectrophotometer at 532 nm against a blank. MDA was calculated using the formula:

$$\text{MDA (M)} = \text{Absorbance at 532 nm}/1.56 \text{ X } 10^{5} \quad \ldots (4)$$

(Molar extinction coefficient for MDA TBA-complex = $1.56 \times 10^{5}\ M^{-1}CM^{-1}$)

Mean MDA inhibition of the test groups relative to control group was plotted against concentration and the dose corresponding to 50% inhibition was taken to be the ED_{50}

Where percentage inhibition = $\frac{MDA\ conc.of\ control - MDA\ conc.of\ test}{MDA\ conc\ of\ control}$ . . . (5)

3.9 Local/Site specific oxidative damage assay

Fourteen groups of 6 animals each were used. Groups 1 - 3 received the extract, groups 4 - 6 n-hexane fraction, groups 7 – 9 ethyl acetate fraction, groups 10 – 12 butanol fraction at the oral doses of 200, 300 and 400 mg/kg respectively. Group 13 and 14 (controls) received 100 mg/kg silymarin and 5 mL/kg 5% Tween 20 respectively. Blood samples (1 mL) were collected from the animals through retro-orbital plexus into plain tubes for pre-treatment basal serum liver function marker enzyme assay. After the blood collection, the animals were treated with the extract and fractions for 21 days. The animals were then given acute dose of CCl_4 (1.5 mL/kg)in equal ratio (v/v) with extra virgin olive oil intraperitoneally. Blood samples were collected from the animals through retro orbital plexus 8 h after CCl_4 administration. The serum was used for the determination of liver function marker enzymes while the liver was used for determination of antioxidant enzymes. Lipid peroxidation assay (on the liver homogenate) was done as previously described in section 2.8.1. Liver from one animal from each group was preserved in 10% formalin for histological examination.

3.10 Liver marker enzyme function assay.

Blood samples were allowed for 30 min to clot before they were centrifuged at 3000 rpm for 10 minutes. The serum (supernatant) was separated into an Ependorf tubes, and used for the determination of liver function marker enzymes.

3.10.1 Quantitative determination of alanine aminotransferase (ALT) (Reitman and Frankel, 1957).

Serum alanine aminotransferase concentration was monitored using RANDOX kit. The enzyme activity was monitored by the concentration of pyruvate hydrazine formed with 2,4-dinitrophenylhydrazine. Mixture of 200 mmol/l L-alanine, α-oxoglutarate (2.0 mmol/l) and phosphate buffer (100 mmol/l) (0.5 mL) was added to 0.1mL of each sample and blank (containing distled water). They were mixed and incubated at 37°C for exactly 30 min in a water bath. Thereafter, 0.5mL of 2,4-dinitrophenylhydrazine was added into the sample and blank test tubes and incubated again at room temperature for 20 min. Sodium hydroxide (5 ml) was added to all the test tubes and the absorbance of the sample read at 546 nm using a UV spectrophotometer. The ALT concentration was extrapolated from a graph of concentration against absorbance of known ALT concentrations (Appendix 5).

3.10.2 Quantitative determination of aspartate aminotransferase (AST) (Reitman and Frankel, 1957)

Serum aspartate aminotransferase concentration was monitored using RANDOX kit. The enzyme activity was monitored by the concentration of oxaloacetate hydrazone formed with 2,4-dinitrophenylhydrazine. Mixture of L-aspartate (100 mmol/l), α-oxoglutarate (2.0 mmol/l) and phosphate buffer (100 mmol/l) (0.5 mL) was added to 0.1mL of each sample and blank (containing distled water). They were mixed and incubated at 37°C for exactly 30 min in a water bath. Thereafter, 0.5mL of 2,4-dinitrophenylhydrazine was added into the sample and blank test tubes and incubated again at room temperature for 20 min. Sodium hydroxide (5 ml) was added to all the test tubes and the absorbance of the sample read at 546 nm using a UV

spectrophotometer. The AST concentration was extrapolated from a graph of concentration against absorbance of known AST concentrations (Appendix 5).

3.10.3 Quantitative determination of alkaline phosphatase (ALP) (King and Armstrong, 1954)

Serum alkaline phosphatate concentration was monitored using TECO DIAGNOSTIC kit. The principle of the test is based on alkaline solution mediated blue chromogen formation upon reaction of the enzyme with buffered sodium thymolphthalein monophosphate. Alkaline Phosphatase substrate (3.6 mM sodium thymolphthalein monophosphate in 0.2 M 2-amino-2-methyl-1-propanol buffer, 0.5 mL) was dispensed into sample and blank labelled test tubes and equilibrated to 37°C for 3 min. Thereafter, 0.05 mL of standard (thymolphthalein in 0.5 mM/L n-propanol), control (deionized water), and samples were added to their respective test tubes. The mixture was incubated for 10 min at 37°C. Alkaline phosphatise colour developer (0.1 M Sodium hydroxide and 0.1 M sodium carbonate, 2.5 mL) was added and the absorbance of the sample read at 590 nm using a UV spectrophotometer.

Calculation of ALP concentration

$$\frac{Absorbance\ of\ unknown}{Absorbance\ of\ standard} \times standard\ concentration\ \left({}^{IU}/_{L}\right) \ldots (6)$$

3.11 Determination of superoxide dismutase (SOD) activity

Superoxide dismutase activity was determined by its ability to inhibit the auto-oxidation of epinephrine determined by the increase in absorbance at 480 nm as described by Sun and Zigma (1978). The reaction mixture (3 mL) containing 2.95 mL 0.05 M sodium carbonate buffer pH

10.2, 0.02 mL of liver homogenate supernatant and 0.03 mL of epinephrine in 0.005 N HCl was used to initiate the reaction. The reference cuvette contained 2.95 mL buffer, 0.03 mL of substrate (epinephrine) and 0.02 mL of water. Enzyme activity was calculated by measuring the change in absorbance at 480 nm for 5 min. An extinction coefficient for epinephrine at 480 nm of 4020 $M^{-1}cm^{-1}$was used in monitoring the auto-oxidation of epinephrine per minute. Amount of SOD that produced 50% inhibition of auto-oxidation of epinephrine was taken as one unit of enzyme activity.

3.12 Determination of catalase activity (CAT)

Plasma catalase activity was determined according to the method of Beers and Sizer as described by Usoh *et al.*, (2005). The reaction mixture (3 mL) contained 0.1 mL of liver homogenate supernatant and 2.9 mL of 30 mM H_2O_2 in phosphate buffer pH 7.0. Enzyme activity was calculated by measuring the change in absorbance at 240 nm for 5 min. An extinction coefficient for H_2O_2 at 240 nm of 40.0 $M^{-1}cm^{-1}$ as described by Aebi, (Aebi, 1984) was used for monitoring the disappearance of H_2O_2. Amount of catalase enzyme that decomposed 1 μmol H_2O_2 per min was taken as one unit of enzyme activity.

3.13 Histopathological examination

The liver was processed through a series of aqueous ethyl alcohol of ascending strength (70, 80 and 95 %) for a period of 1 h; twice in absolute alcohol and xylene respectively 1 h in order to render the tissue elements transparent. The tissues were then infiltrated with molten paraplast at 58°C. This was done twice for 1 h each time. The transparent tissues, after clearing all elements from it, were embedded in a solid mass of paraplast. The blocks were labelled, allowed to cool

and the metal blocks were removed. The solid mass was then trimmed free of excess paraplast, leaving some free margins around the embedded tissues. Three microns thick longitudinal sections were cut with a rotary microtone. The sections were mounted on thoroughly cleaned gelatinized slides and the slides were then placed on hot plates at 37°C for 24 h for proper fixation. The slides were stained with H & E stain according to the prescribed staining method (Bancroft and Stevens, 1990). The stain was prepared by dissolving hematoxylin in absolute alcohol. The mixture was boiled rapidly and mercuric oxide was then added. The stain was cooled rapidly in a cold water bath; glacial acetic acid was then added and the stain was ready for immediate use. Several slides were prepared accordingly. The stained slides, after drying and labelling, were examined using light microscope at X 100 and 400 maginification for comparative morphological and pathological changes in the liver of the animals.

3.14 Diabetes induced systemic oxidative stress

Albino rats (n = 100) were fasted overnight for 12h and fasting blood glucose levels taken from tail vein using Accu-check Glucometer (Roche, Germany) before the induction of diabetes with nicotinamide adinine dinuclotide-streptozotocin (NAD-STZ). About 1 mL blood sample was collected through retro-orbital plexus for determination of basal antioxidant parameters. STZ was dissolved freshly in 0.05M citrate buffer (pH 4.5) while NAD was dissolved in normal saline and administered 15 minutes before STZ. Oxidative stress was induced by administering 50 mg/kg NAD and 70 mg/kg STZ i.p. After 72 h, animals with fasting blood glucose levels > 160 mg/dl were confirmed diabetic and selected for the study. The animals were grouped in fourteen groups of six animals each. Groups 1 - 3 received the extract, groups 4 - 6 n-hexane fraction, groups 7 – 9 ethyl acetate fraction, groups 10 – 12 butanol fraction at the oral doses of 200, 300 and 400

mg/kg respectively. Group 13 and 14 (controls) received 5 mg/kg glibenclamide and 5 mL/kg 5% Tween 20 respectively. Treatment with the extract and fractions continued for 21 days with fasting blood glucose determined every 7 days. On the 22nd day, blood samples were collected through retro-orbital plexus into a plain tube. The blood was allowed to clot for about 30 min and was centrifuged at 3000 rpm for 10 min to obtain the serum. Serum total antioxidant status was determined. Also, lipid peroxidation andantioxidant enzymes were determined as previously described in sections 2.7.1, and 2.10 - 2.11 respectively .

3.15 Determination of serum total antioxidant capacity (Kampa *et al*., 2002)

This assay relies on the ability of antioxidants in the sample to inhibit the oxidation of 2,2-Azino-di-3-ethylbenzthiazoline sulphonate (ABTS) by metmyoglobin. Trolox standards (0, 0.045, 0.090, 0.135, 0.180, 0.225 and 0.330 mM) and 10 fold diluted samples (10 µl) were added to the standard and sample wells respectively in duplicates. Metmyoglobin (10 µl) and ABTS – chromogen (150 µl) were added to both sample and standard wells. The reaction was initiated by adding 40 µl of 441 µM hydrogen peroxide to all wells being used and the absorbance read after 5 min at 750 nm using microplate reader.

The average absorbances of each standard and sample were calculated. Average absorbance of standards as a function of Trolox concentration (mM) was plotted and antioxidant concentration of the samples (quantified as millimolar Trolox equivalents) were extrapolated from the standard curve using the following equation:

$$\text{Antioxidant mM} = \frac{sampleaverageabsorbance \quad -(y-intercept\ )}{slope} \text{ X Dilution factor . . . (7)}$$

3.16 Immunomodulatory screening of the extract and fractions

3.16.1 Immunization

Swiss albino mice divided into 15 groups of 6 animals each were used for this study. Groups 1 - 3 received the extract, groups 4 - 6 n-hexane fraction, groups 7 – 9 ethyl acetate fraction, groups 10 – 12 butanol fraction at the oral doses of 200, 300 and 400 mg/kg respectively. Group 13 and 14 (controls) received 100 mg/kg Noni capsule and 5 mL/kg 5% Tween 20 respectively. Group 15 served as the naïve group and was maintained on normal animal diet without immunization. Theanimals were treated for 29 days.Primary and secondary immunization with tetanus toxoid at the dose of 50 µl/footpad was done on the 5^{th} and 18^{th} day respectively. Blood samples were collected through retro-orbital plexus on the $19^{th}$and 29^{th} day for the determination of primary and secondary immunoglobulins.

3.16.2 Determination of immunoglobulin levels

Blood samples collected on the 19^{th} and 29^{th} day were allowed for 30 min to coagulate and then centrifuged at 3000 rpm for 20 min to obtain the serum. The levels of IgG1 and IgG2a were determined using enzyme linked immunosorbent assay (ELISA) as described by Duddukuri *et al*., (2001). Serum samples collected on the 19^{th} and 29^{th} day were taken as the primary and secondary antibody titre respectively.

Each well of the ELISA plates was first coated with 100 µl of 10 fold diluted tetanus toxoid in bicarbonate buffer (pH 9.6). The plates were covered and incubated for 12 h at 4^{o}C. The plates were washed thereafter with 0.05% Tween 20 in 0.1M phosphate buffer saline (washing buffer). The unbound sites were blocked with 200 µl/well of 5% w/v solution of fat free milk in phosphate buffer saline and the plates incubated at room temperature for 1h. The plates were

washed 3 times with washing buffer. 100 µl of the sera (1:20 dilution in 2% fat free milk) were added to their respective wells and incubated for 1h at room temperature followed by washing with the washing buffer. Using 2% fat free milk, 1:1000 dilution of the detection antibodies (anti mouse IgG1 HRP and IgG2a HRP) were freshly prepared and 100 µl of these preparations were added to their respective plates, incubated for 1 h at 25°C and the plates washed 4 times with washing buffer. The substrate ABTS (2,2'-azino-bis-3-ethylbenzothiaziolin-6-sulfonic acid) 100 µl per well were added and the plates read after 20 min in ELISA machine at 405 nm.

3.16.3 Cytokine expression assay

The effect of the butanol fraction (BF) (most active humoral immunostimulating fraction) on cytokine expression was monitored using QIAGEN Multi-Analyte ELISArray kit which allows for simultaneous detection of levels of multiple cytokines using the conventional and simple sandwish-based enzyme-linked immunosorbant assay technique.

The 96-well ELISA microplate was precoated with a panel of 12 target-specific capture antibodies for IL1A, IL1B, IL4, IL6, IL10, IL12, IL17A, IFNγ, TNFα, G-CSF and GM-CSF. 50 µl of double fold diluted serum samples (obtained after secondary immunization with tetanus toxoid) and antigen standards were added to their appropriate wells. Serum samples from untreated immunized mice were used as control. The rest of the procedure as contained in the manufacture's instructional manual was followed strictly which involved addition of detection antibody solution, Avidin-HRP, developing solution, stop solution and washing with buffer after specific incubations. Absorbance was taken at 450 nm. Mean absorbance of the samples were compared with the control and cytokine expression quantified in percentage relative to control group.

3.16.4 Cyclophosphamide immunosuppresion assay

Both the preventive and immune boosting effect of the extract and fraction on cyclophosphamide induced leukopenia was determined. For the preventive effect, the method of Gupta *et al*., (2010) was adopted with minor modification. The animals were (mice) divided into 5 groups of 6 animals each. Groups 1-3 received 200, 300 and 400 mg/kg of BF for 21 days. Groups 4 and 5 served as the control and received noni capsule (100 mg/kg) and 5% Tween 20 respectively. On the 22nd day, 30 mg/kg cyclophosphamide was administered intraperitoneally to all the animals. Pre-treatment and 3 days post cyclophosphamide administration blood samples were collected through retro-orbital plexus into EDTA pre-coated tubes. Total leukocyte count was conducted manually using improved neubauer haemocytometer. The blood samples were diluted (20 fold) with white blood cell diluting fluid (Turk solution-Bio-Optica Milano, Italy) before counting with microscope X 10 objetctive lens magnification. Total leukocyte count was calculated thus:

$$\text{WBCs/mm}^3 = \left(\frac{10}{4} x\ 20\right)\ X\ no\ of\ cells\ counted\ from\ 4\ large\ squares\ \ldots (8)$$

Where $\frac{10}{4}$ = volume from which the cells have been counted

20 = dilution factor

For the differential cell count, a drop of blood was thinly spread over a glass slide, air dried, and stained with a Romanofsky stain. One hundred cells were counted and classified based on their morphological differences. The absolute number of each type of WBC was calculated relative to total number of leukocytes.

A follow-up study on the boosting effect of BF (most active fraction) on cyclophosphamide induced leukopenia was evaluated using modification of the method described by Park *et al* (2014). Animal groupings were as in preventive model above. Total and differential leukocyte

counts were performed prior to intraperitoneal administration of 20 mg/kg cyclophosphamide per day for 3 days. Blood samples were analysed again on the 5^{th} day prior to fraction administration from the 6^{th} day. Blood samples were collected every 3 days during the 12 days treatment period.

3.17 Screening of the isolated compounds for antioxidant activity

3.17.1 Preparation of liver microsome

After overnight fasting, 5 female rats were euthanized by pentobarbitone overdose. Liver was perfused with 10 mL of 0.1 M phosphate buffered saline (PBS) and then isolated. The liver microsome was prepared by slight modification of the methods ofSchenkman and Cinti,(1978); and Umathe *et al*., (2008). The liver was minced and homogenised in 50 mL of 0.25 M sucrose containing 10 mM Tris-HCl (pH 7.4), and then centrifuged at 600 x g for 5 min followed by 12, 000 x g for 10 min using ultra refrigerated centrifuge (TGL-20M, China). The post-mitochondrial supernatant was separated, mixed with solid $CaCl_2$ so that its concentration in the given volume of supernatant was 8.0 mM and then centrifuged at 20, 000 x g for 20 min to obtain pinkisk microsomal pellet, which was suspended in 0.5 mL of 0.1 M potassium phosphate buffer containing 20% glycerol and stored at -20^0C until needed. Protein concentration of the sample was also determined by Biuret method using bovine serum albumin as the standard.

3.17.2 Biuret protein assay

This assay is based on the principle that substances containing more than two peptide bonds form a purple complex with copper salts in alkaline solution. Biuret reagent was prepared by dissolving 1.5 g copper (II) sulphate pentahydrate and 6 g sodium potassium tartrate in 500 mL water. NaOH 10% w/v (300 mL) was added and the volume made up to 1 Litre with distilled water. Standard curve was plotted by preparing series of bovine serum albumin from 0.5 – 10

mg/mL such that the final volume for the assay was 0.5 mL. 2.5 mL of biuret reagent was added to each tube, votexed and allowed toreact for 30 min and the absorbance read at 540 nm using spectrophotometer. Protein concentration of the liver microsomal sample was prepared in a similar way as standard such that the final volume was 0.5 mL before the addition of biuret reagent. The protein concentration was extrapolated from the standard curve.

3.17.3 Liver microsome lipid peroxidation assay.

The assay was done as described by Van-de-sluis*et al*., (2000) with little modification. Microsomes were thawed on ice, diluted with Tris-HCl buffer (50 mM, pH 7.4) to a final concentration 0.5 mg/mL protein. The microsomes (aliquots of 240 µl) were preincubated for 5 min at 37 °C. A 30 µl of different concentrations of compounds **1** and **2** (5, 10 20 40 and 80 µg/mL) were added to their respective tubes while Trolox®(water soluble analog of vitamin E) and methanol served as the positive control and blank respectively. Lipid peroxidation was induced by adding 15 µl of ascorbic acid (4 mM) and 15 µl of FeSO4 (0.2 mM). After incubation for 60 min at 37 °C the reaction was stopped by addition of 0.5 mL of 0.83% thiobarbituric acid dissolved in TCA-HCl (16.8% w/v trichloroacetic acid in 0.125 N HCl). Lipid peroxidation was assessed by measuring thiobarbituric acid reactive species (TBARS) after the tubes were heated for 15 min at 80°C and subsequent centrifugation (2500 rpm, 15 min). Absorption was read at 540 nm using spectrophotometer. Percentage inhibition of lipid peroxidation was calculated thus:

$$\frac{A_{blank} - A_{sample}}{A_{blank}} X\ 100 \ldots (9)$$

Where A_{blank} = absorbance of the blank and A_{sample} = absorbance of the sample.

3.17.4 Radical scavenging interacting effect of isolated compounds

The interactive antioxidant activity of compound **1**and **2**isolated from ethyl acetate fraction was evaluated using DPPH test. The individual scavenging activity of the compounds were determined at 5, 10, 20, 40 and 80 µg/mL while equal combination of these compounds at same doses were used to assess their interactive effect. DPPH test was done as previously described. ED_{50} values of the compounds separately and when combinedwere used for the calculation of their interactive effect using the method described by Tallarida, (2011).

$$\frac{\left(\frac{A+B}{2}\right)}{AB} = X \ldots (10)$$

Where A = ED_{50} of compound 1, B = ED_{50} of compound 2, AB = ED_{50} of the equal ratio combination of the compounds.

When X = 1 ---------- additive interaction

X > 1 ---------- synergistic interaction

X < 1 ----------antagonistic interaction

3.18 *In vitro* screening of the isolated compound for immunomodulatory activity

3.18.1 Mice

BALB/c mice obtained fron Janvier (Le Genest-ST-Isle, France) were used in the study. The animals were maintained on standard livestock pellets (Altromin Spezialfutter GmbH & Co. KG, Lage, Germany) and were allowed unrestricted access to drinking water. The use and care of laboratory animals in the study were in accordance with ethical guidelines as contained in the

European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (EEC Directive 86/609/EEC) of 1986.

3.18.2 Preparation of mouse cells

Mice were sacrificed by CO_2asphyxiation and the spleens recovered aseptically into ice-cold Hanks' Balance Salt Solution (HBSS, Gibco). Single cell suspension was prepared by gentle dispersion of the cells and straining through 70-μM nylon strainer. Red blood cells were lysed by the addition of 3 mL of ACK lysing buffer (Lonza, Walkersville) per mouse spleen for 5 min. The cells were washed and suspended in R-10, consisting of RPMI 1640 medium (Gibo, Germany) supplemented with 10% heat-inactivated foetal calf serum (FCS), 50 μM 2-mercaptoethanol (Gibco, Germany), 100 U/mL penicillin, and 100 μg/mL streptomycin.

3.18.3 Intracellular cytokine staining in cultures of splenic T-lymphocytes

The effect of treatment with compound **3** on the expression cytokines (IFNγ, IL2) by cultures of splenic T-lymphocytes was investigated by the intracellular cytokine staining technique (ICS). Mice splenocytes (3 X 10^6 cells/well) were stimulated at 37^oC in CO_2 incubator for 16 h with either graded concentrations of compound **3** (6.25, 12.5 and 25 μg/mL) or with PMA/ionomycin (5/500 ng/mL) and compared to the unstimulated control wells. Each well also received monensin (2 μM), which inhibited cytokine secretion out of the cells (Jung *et al*., 1993). After stimulation, cells were washed with PBS/bovine serum albumin/azide, and Fc (II/III) receptors were blocked with a mixture of anti-CD16 and anti-CD32 antibody (BD Biosciences). Thereafter, cells were labelled with either allophycocyanin (APC)-conjugated anti-CD8 or anti-CD4 antibodies (BD Biosciences). Cells were then washed with PBS and fixed in 2% paraformaldehyde, followed by permeabilization with 0.5% saponins in PBS/bovine serum

albumin/azide buffer. Cytokines expressions within the cells were detected by staining for 30 min with anti-IFNγ-PE and anti-IL-2-FITCS (BD Bioscience). Cells were analysed in a FACSCalibur™ using CellQuestPro software (BD Bioscience) and reanalysed with Flowjo™ (TreeStar Inc. Ashland, Oregon, USA).

3.19 Statistical analysis

Graphs of percentage inhibition against concentrations for the determination of half maximal effective response (EC_{50}) were drawn using Microsoft Excell 2010. Results of grouped experiments were presented as Mean $\pm$ SEM. Comparative significant differences were done between drug treatments and vehicle treated groups using SPSS version 18. Using one way analysis of variance, P values < 0.05 were considered to be significant. Further post hoc analysis using LSD was used for multiple comparisons.

CHAPTER FOUR

RESULTS

4.1 Extraction, fractionation and phytochemical analysis

Phytochemical analysis of the ethanol extract of *Millettia aboensis* revealed abundant phytocompounds. Tannins and resins were found to be abundantly present. Also, very high concentrations of flavonoids, carbohydrates, steroids, terpenoids, glycosides and proteins were detected in the extract (Table 3). These phyto-compounds were found to distribute differentially in solvents of variable polarities. Steroids and resins particularly accumulated in the n-hexane fraction while the ethyl acetate fraction selected the phenolic compounds – flavonoids and tannins with traces of terpenoids. Some phenolic compounds were also detected in the butanol fraction with abundant glycosides. (Table 3). N-hexane fraction produced the highest yield from the extract while ethyl acetate fraction was the least.

Pharmacological guided screening of theextract and fractions for antioxidant and immunomodulatory activities, revealed ethyl acetate and butanol fractions as having distinguished activities which led to further purification of these fractions through several fractionation techniques. Vacuum liquid separation of ethyl acetate fraction yielded 10 sub-fractions (Table 4) that were screened for *in vitro* antioxidant activity. Sub-fraction D5 having shown the highest scavenging activity was further purified through Sephadex LH-20 fractionation. This resulted to 15 bulked fractions (Table 5). D5N bulked from fractions 106-112 was identified through its HPLC spectrum to contain the major compound and as such further processed for structural elucidation. On the other hand, HPLC fingerprint of butanol fraction

showed 5 major peaks (Figure 2) numbered A(Rt=18.38 min), B(19.08 min), C (20.64 min), D(21.61 min) and E (22.01 min). Peak numbers A and B correspond to simple phenolic derivatives – flavonoid glycosides based on their UV curves. Peak number C (the major compound) exhibited a UV curve typical of quercetin glycosides while peaks D and E exhibited UV curves typical of kaempferol glycosides. The characteristic UV curve of the major compound was used in monitoring the compound through Sephadex fractionation. This led to further processing of the Sephadex bulked fraction 8 for structural elucidation.

Table 3: Phytochemical analysis of the extract and fractionsof *Millettia aboensis*

Phytocompounds	Ethanol extract	N-hexane fraction	Ethyl acetate fraction	Butanol fraction
Flavonoids	+++	-	+++	++
Saponins	++	-	-	+++
Fats & oil	++	++	-	-
carbohydrates	+++	-	-	+++
Tannins	++++	-	++	+++
Reducing sugar	++	-	-	++
Steroids	+++	+++	-	-
Terpenoids	+++	+	+	++
Alkaloids	-	-	-	-
Glycosides	+++	-	-	++++
Resins	++++	++++	-	-
Proteins	+++	-	-	+++
Yield (%)	24.53[a]	40.06[b]	23.11[b]	35.27[b]

+ present in little concentration, ++ moderately present in high concentration, +++ present in very high concentration, ++++ abundantly present, - not present. a - calculated from 2kg pulverised leaves, b – calculated from 100 g extract.

Table 4 Vacuum liquid chromatographic sub-fractions of the ethyl acetate fraction

Sample code	solvent	Weight (mg)
D1	H:E 80:20	146
D2	H:E 60:40	58
D3	H:E 40:60	144
D4	H:E 20:80	158
D5	H:E 0:100	233
D6	D:M 90:10	225
D7	D:M 70:30	207
D8	D:M 50:50	107
D9	D:M 30:70	45
D10	D:M 10:90	38
D11	D:M 0:100	36

H:E = n-hexane:ethylacetate and D:M = dichloromethane:methanol

Table 5: Sephadex LH-20 fractions of D5

Sample code	Bulked fractions	Weight (mg)
D5A	1 - 22	22.2
D5B	23 - 27	24.2
D5C	28 - 32	23.6
D5D	33 - 36	11.9
D5E	37 - 40	7.4
D5F	41 - 49	10.3
D5G	50	1.7
D5H	51 - 60	8.8
D5I	61 - 69	7.7
D5J	70 - 74	10.4
D5K	75 - 80	7.8
D5L	81 - 90	9.3
D5M	91 - 105	7.0
D5N	106 - 112	20.8
D5O	113 - 120	19.5

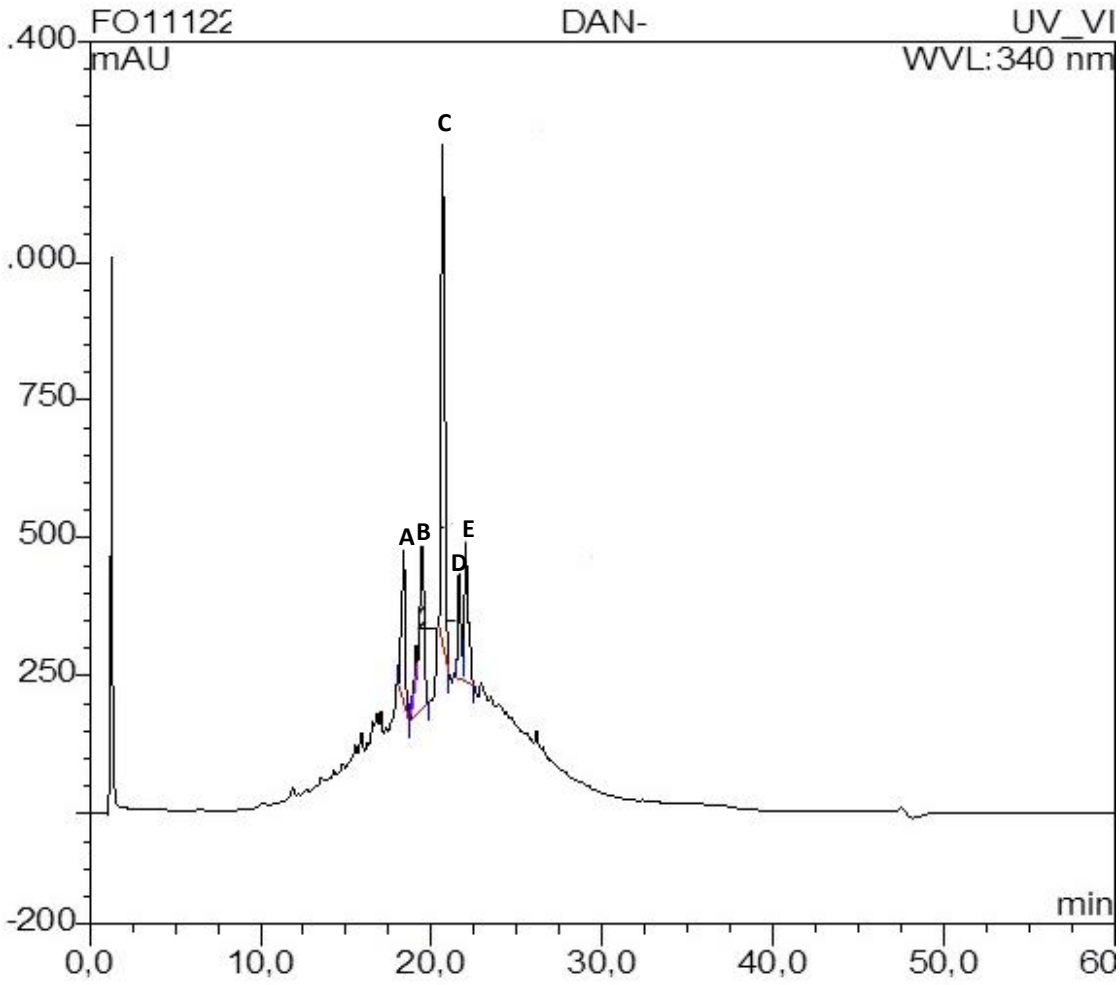


Figure 2: HPLC fingerprint of butanol fraction

A- flavonoid glycosides, B- flavonoid glycosides, C- Quercetin glycosides, D-Kaempferol glycosides, E- Kaempferol glycosides

4.2 Structure elucidation of the isolated compounds

4.2.1 Compound 1

Compound **1** was isolated as light brown amorphous powder. It showed UV absorbance maximum at 278 nm typical of proanthocyanidins (Appendix 1). LC-ESIMS of **1** exhibited strong peaks at *m*/*z* 576.9 $[M+H]^+$ in the positive mode and at *m*/*z* 575.3 $[M-H]^-$ in the negative mode (Appendix 1), which is consistent with the molar mass of 576 g/mol. The molecular formula was established as $C_{30}H_{24}O_{12}$ based on the HR-ESIMS pseudomolecular ion peak at *m/z* 577.1339 $[M+H]^+$. The molecular formula corresponds to biflavonoid of proanthocyanidin A type. The proton NMR showed the presence of isolated AB coupling system at δ_H4.13 d and 4.23 d with $J_{3,4}$ = 3.4 Hz assignable to H-3 and H-4 of the upper unit, which has been described as the diagnostic feature of the C-ring protons of the upper unit of the A-type proanthocyanidin (Lou *et al*., 1999). Further evidence of the A-type proanthocyanidin structure was provided by the absence of H-2 signal and the highly deshielded signal (100.7 ppm) of the ketal carbon (C-2) formed as a result of additional oxygen linkage. There are two sets of ABX coupling systems at 7.14 d (2.1), 6.82 d (8.3) and 7.03 dd (2.1, 8.3) assignable to H-2', H-5' and H-6' of the upper limit, and 6.98 d (2.1) 6.85 d (8.3) and 6.86 dd (2.1, 8.3) assignable to H-2', H-5' and H-6' of the lower limit. These together with the observed high coupling constant value between H-2 and H-3 of the terminal unit ($J_{2,3}$ = 7.9 Hz) (Table 6) support the presence of epicathechin sub-structure in the upper unit and cathechin sub-structure in the terminal unit. All the proton and carbon signals of the compound were assigned by the analysis of the DEPT, HSQC and HMBC (Table 6). Compound **1** was thus unequivocally elucidated as epicathechin-(2β→O→7, 4β→8)-cathechin(Procyanidine A1) (Figure 3). The NMR data are in agreement with those previously reported (De Bruyne *et al*., 1999).

4.2.2 Compound 2

Compound **2** was isolated as light brown amorphous powder. It showed UV absorbance maxima at 242 and 282 nm typical of proanthocyanidins (Appendix 2). LC-ESIMS of **2** exhibited strong peaks at *m*/*z* 577.0 $[M+H]^+$ in the positive mode and at *m*/*z* 575.3 $[M-H]^-$ in the negative mode (Appendix 2), which is consistent with the molar mass of 576 g/mol. The molecular formula was established as $C_{30}H_{24}O_{12}$ based on the HR-ESIMS pseudomolecular ion peak at *m/z* 577.1334 $[M+H]^+$. The NMR spectra of compound **2** are very similar with that of compound **1**. The major difference is the observed small coupling constant value between H-2 and H-3 of the terminal unit ($J_{2,3}$). These two protons appeared as broad singlet thus supporting a cis configuration, which is consistent with epicathechin sub-structure for the terminal unit. The cis configuration also resulted in the deshielded positions of the H-2 and H-3 signals. All the proton and carbon signals of the compound were assigned by the analysis of the DEPT, HSQC and HMBC (Table 7). The compound was thus elucidated as epicathechin-(2β→O→7, 4β→8)-epicathechin(Procyanidine A2) (Figure 4). The NMR data are also in agreement with those previously reported (De Bruyne *et al*., 1999).

4.2.3 Compound 3a/b

Compound **3** was isolated as a yellow powder. It showed UV absorbance maxima at 256 and 352 nm typical of quercetin glycosides (Appendix 3). LC-ESIMS of **3** exhibited strong peaks at *m*/*z* 610.9 $[M+H]^+$ in the positive mode and at *m*/*z* 609.3 $[M-H]^-$ in the negative mode (Appendix 3), which is consistent with the molar mass of 610 g/mol. The molecular formula was established as $C_{27}H_{30}O_{16}$ based on the analysis of the MS and the C-13 NMR data. The LC-ESI-MS also

showed fragment peaks at *m/z* 464.9 $(M-146)^+$ corresponding to a loss of desoxyhexose and 303.2 $[M-146-162]^+$ a subsequent loss of a hexose suggesting that compound **3** is a diglycoside. The fragment 303.2 is also diagnostic of quercetin aglycone and this was supported by the observed UV maxima (λ_{max}) of 256.0 and 352.0 nm. ^{1}H-NMR spectrum of compound **3**showed two sets of ABX aromatic spin systems, one atδ_H 6.88(d, *J*=2.1, 1H) 7.62(dd, *J*=2.1, 8.4, 1H) 7.67(d, *J*=2.1, 1H) and the other at δ_H6.88(d, *J*=2.1, 1H) 7.62(dd, *J*=2.1, 8.4, 1H) 7.87(d, *J*=2.1, 1H) both assignable to H-5', H-6' and H-2' respectively of the of Ring 'B' of the quercetin. There are also two sets meta-coupled aromatic protons at δ_H6.20 (d, *J*=2.0, 1H) and 6.39 (d, *J*=2.0, 1H) both assignable to H-6 and H-8, respectively of the 'Ring A' of quercetin nucleus. The NMR spectrum also showed four anomeric proton signals δ_H 5.11 ppm (d,*J*=7.8 Hz, 1H); 5.07 ppm (d, *J*=7.8 Hz,, 1H); 4.53 ppm (brs, 1H)and 4.52 (brs, 1H). Careful analysis of the HNMR and HHCOSY spectra showed that Compound **3** is an isomeric mixture as shown in Table 3.6. The anomeric proton signal at δ_H 5.11 ppm (d,*J*=7.8 Hz, 1H) corresponds to a β-D-glucosyl unit based on the observed proton and C-13 signals as shown in Table 8. The observed HMBC correlation of the anomeric proton of the rhamnosyl unit (δ_H 4.52) to C-6" of the glucosyl unit suggested a 1→6 interglycosidic linkage in compound **3a.** This was further confirmed by a downfield shift of the C-6 signal. The attachment of the glucosyl moiety at C-3 of the quercetin nucleus was confirmed by the HMBC correlation of the anomeric proton (δ_H5.11) of the glucosyl moiety to C-3 (δ_C) of the quercetin nucleus. Compound **3a**was thus identified as **Rutin** {Quercetin-3-O-[α-L-rhamnopyranosyl(1→6) β-D-glucopyranoside]} (Fig. 3.7). Careful analysis of signals belonging to compound **3b**, the second isomer, showed that **3b** is very similar to **3a**, the major difference being the slightly shielded signals of the anomeric proton at δ_H5.07 compared with that of the glucosy unit. This proton signal belonged to a continuous spin system

as shown in Table 8, which exhibited chemical shifts and coupling constants characteristic of a galactopyranose unit e.g. deshielded position of H-2 signal and H-4 signal appearing as broad singlet (Ohtsuki *et al*., 2008; Gaidi*et al*., 2004). Compound **3b** was thus identified as **Quercetin-3O-robinobioside**{Quercetin-3-O-[α-L-rhamnopyranosyl(1→6)β-D-galactopyranoside]} (Figure 5).

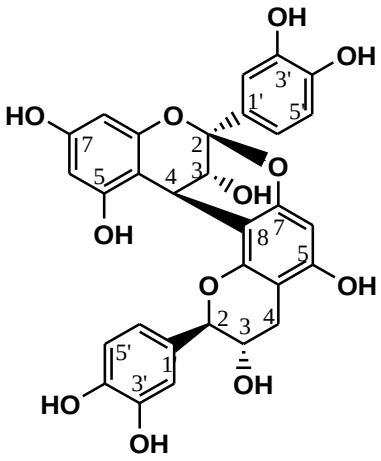


Figure 3: Structure of compound 1 isolated from ethyl acetate fraction

Sample code: D5N2

Sample amount: 1 mg

Physical properties: light brown

Molecular formula: $C_{30}H_{24}O_{12}$

Molecular weight: 576.504 g

Retention time (HPLC): 18.81 min

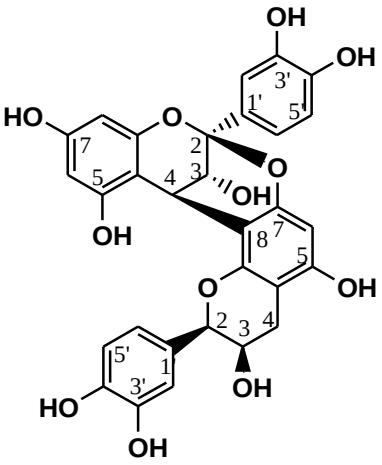


Figure 4:Structure of compound 2 isolated from ethyl acetate fraction

Sample code: D5N3

Sample amount: 1 mg

Physical properties: light brown

Molecular formula: $C_{30}H_{24}O_{12}$

Molecular weight: 576.504 g

Retention time (HPLC): 20.25 min

Table 6: NMR spectra data of compound 1

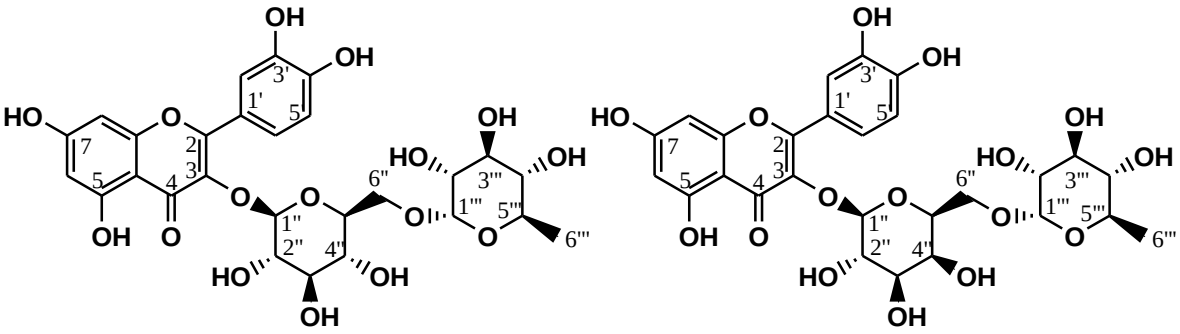
Position	δ_H (u-unit)	δ_C (u-unit)	δ_H (t-unit)	δ_C (t-unit)
2	-	100.7	4.74 (dJ = 7.9)	84.1
3	4.13 d(3.5)	67.9	4.06 m	68.7
4	4.23 d(3.4)	29.4	2.96 HA 2.56 HB	28.9
5	-	157.4	-	156.8
6	6.05 d(2.3)	96.8	6.08 s	96.8
7	-	157.4	-	157.4
8	5.93 d(2.3)	98.3	-	102.9
9	-	156.3	-	151.0
10	-	104.0	-	106.6
1'	-	132.5	-	131.1
2'	7.14 (dJ = 2.1)	115.9	6.96 (dJ = 2.1)	115.6
3'	-	145.9	-	146.6
4'	-	147.0	-	147.0
5'	6.82 (dJ = 8.3)	115.8	6.85 d(8.3)	116.5
6'	7.03 (ddJ = 2.1, 8.3)	120.0	6.86 (ddJ = 2.1, 8.3)	120.5

NB: u-unit = upper unit of the molecule; t-unit = terminal unit of the molecule

Table 7: NMR spectra data of compound 2

Position	δ_H (u-unit)	δ_C (u-unit)	δ_H (t-unit)	δ_C (t-unit)
2	-	100.4	4.93 brs	82.0
3	4.41 d(3.2)	68.2	4.25 brs	67.1
4	4.06 d(3.3)	29.41	2.95 dd HA 2.77 dd HB	30.10
5	-	156.8	-	156.8
6	6.07	96.6	6.09 s	96.6
7	-	156.8	-	158.2
8	6.01	98.5	-	102.6
9	-	152.5	-	152.3
10	-	104.0	-	107.4
1'	-	132.6	-	131.4
2'	7.13 brs	115.8	7.16 brs	116.1
3'	-	145.8	-	146.5
4'	-	146.1	-	146.9
5'	6.82	116.2	6.81	115.8
6'	7.02	119.9	6.99	120.5

NB: u-unit = upper unit of the molecule; t-unit = terminal unit of the molecule



3a **3b**

Figure 5:Structure of compound 3a/bisolated from butanol fraction

Sample code: DBu-8

Sample amount: 2 mg

Physical properties: yellow powder

Molecular formula: $C_{27}H_{30}O_{16}$

Molecular weight: 610.52 g

Retention time (HPLC): 25.36 min

Table 8: NMR spectra data of compound 3a/b

	Compound 3a		Compound 3b	
Position	δ_H	δ_C	δ_H	δ_C
2	-		-	
3	-		-	
4	-		-	
5	-		-	
6	6.22 brs	99.9	6.22 brs	99.9
7	-		-	
8	6.41 brs	94,8	6.41 brs	94,8
9	-		-	
10	-		-	
1'	-		-	
2'	7.67 d	117.5	7.87	117.5
3'	-		-	
4'	-		-	
5'	6.88 d	115.8	6.88	115.8
6'	7.62 dd	123.5	7.62	123.5
1"	5.11 d	104,6	5.07	106.0
2''	3.47 t	75.8	3.83 d	73.2
3''	3.41 t	78.1	3.56	75.1
4''	3.26 d	71.4	3.82 brs	70.0
5''	3.32 d	77.3	3.64	75.2
6''	3.81 m HA	68.5	3.75dd HA	67.4
	3.39 m HB		3.41 m HB	
1'''	4.52 brs	102.5	4.54 brs	101.8
2'''	3.63 dd	72.2	3.58	72.0
3'''	3.42	71.5	3.53	72.0
4'''	3.28	73.8	3.27	73.8
5'''	3.53	69.7	3.44	69.7
6'''	1.19 d (6.2)	18.1	1.12 d (6.2)	18.0

4.3 *In vitro* antioxidant activity of the extract and fractions of *M. aboensis*

In vitro DPPH scavenging activity of the extract and fractions revealed a dose response relationship with progressive decrease in the deep purple colouration of DPPH with increasing concentrations of the extract and fractions (Figure 6). The resulting discolouration is stoichiometric with respect to the number of electrons taken up by the DPPH radical. The extract inhibited the DPPH radical with EC_{50} 116.67 µg/mL higher than that of ethyl acetate and butanol fractions with EC_{50} 35.33 and 79.17 µg/mL respectively (Table 9). Of all the treatment, n-hexane fraction produced weak inhibition of the DPPH radical that did not scavenge up to 50% of the radical at the highest concentration tested.

The scavenging of hydrogen peroxide radical by the extract and fractions was another indicator of the antioxidant potentials of *M. aboensis* (Figure 7). The EC_{50} of the extract against hydrogen peroxide radical was established at 62.5 µg/ml while ethyl acetate and butanol fractions showed EC_{50} at 16.67 and 43.75 µg/ml respectively.The order of potency of the extract and fractions followed the same trend as in DPPH result with ethyl acetate fraction maintaining the highest activity while n-hexane fraction the least (Table10).

As shown in figure 8, the reducing power of the extract and fractions increaded steadily with increasing concentration of the samples. The extract and the fractions showed reducing potentials through reduction of ferric ion to its ferrous form as observed by the increasing dark blue colouration.The extract gave $EC_{50}$of 166.67 µg/ml (Table 11). The n-hexane fraction showed poor reducing ability with EC_{50}above 200 µg/ml while the ethyl acetate and butanol fractions effectively reduced ferric ion with EC_{50}45.83 and 125 µg/ml respectively.

The total phenolic content of the extract was 121.4 mgGAE/g while that of the n-hexane, ethyl acetate and butanol fractions were 26.1, 305.2 and 247.6 mgGAE/g (Table 12).

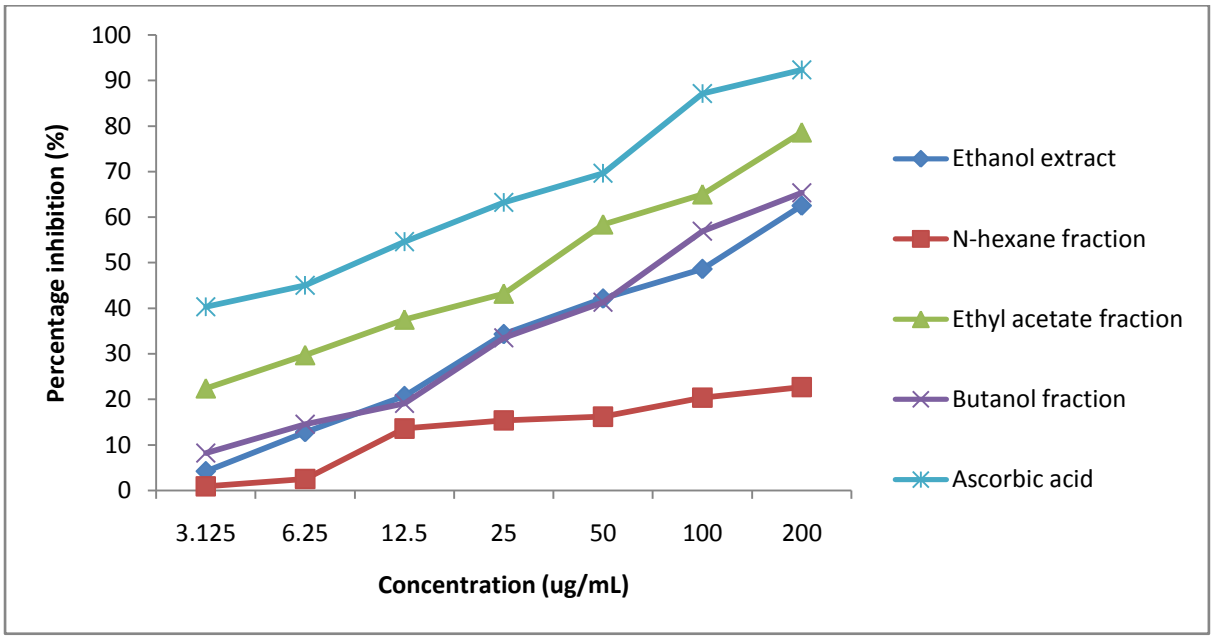


Figure 6: DPPH scavenging activity of the extract and fractions of *M. aboensis*

Table 9: EC_{50} of the extract and fractions against DPPH radical

Extract/Fractions	EC_{50}(µg/ml)
Ethanol extract	116.67
N-hexane fraction	>200
Ethyl acetate fraction	35.33
Butanol fraction	79.17
Ascorbic acid	9.38

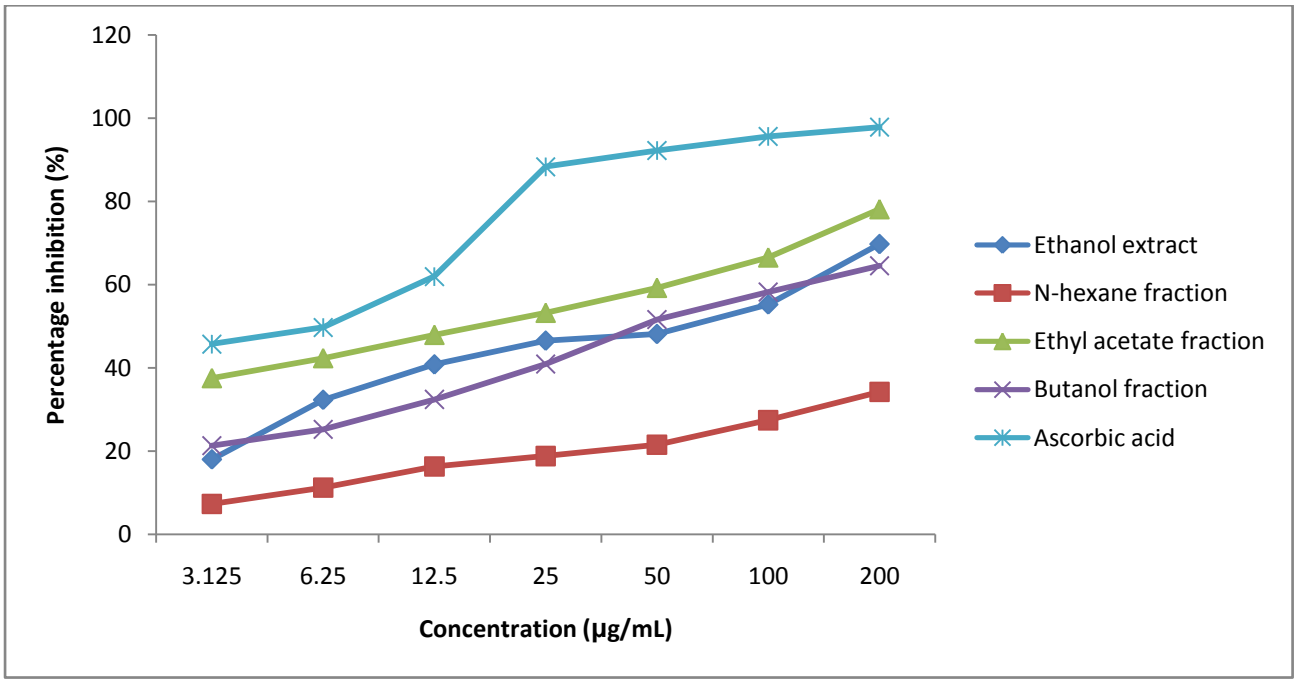


Figure 7: Hydrogen peroxide scavenging activity of the extract and fractions of *Millettia aboensis*

Table 10: EC_{50} of the extract and fractions against hydrogen peroxide radical

Extract/Fractions	EC_{50}(µg/ml)
Ethanol extract	62.5
N-hexane fraction	>200
Ethyl acetate fraction	16.67
Butanol fraction	43.75
Ascorbic acid	6.26

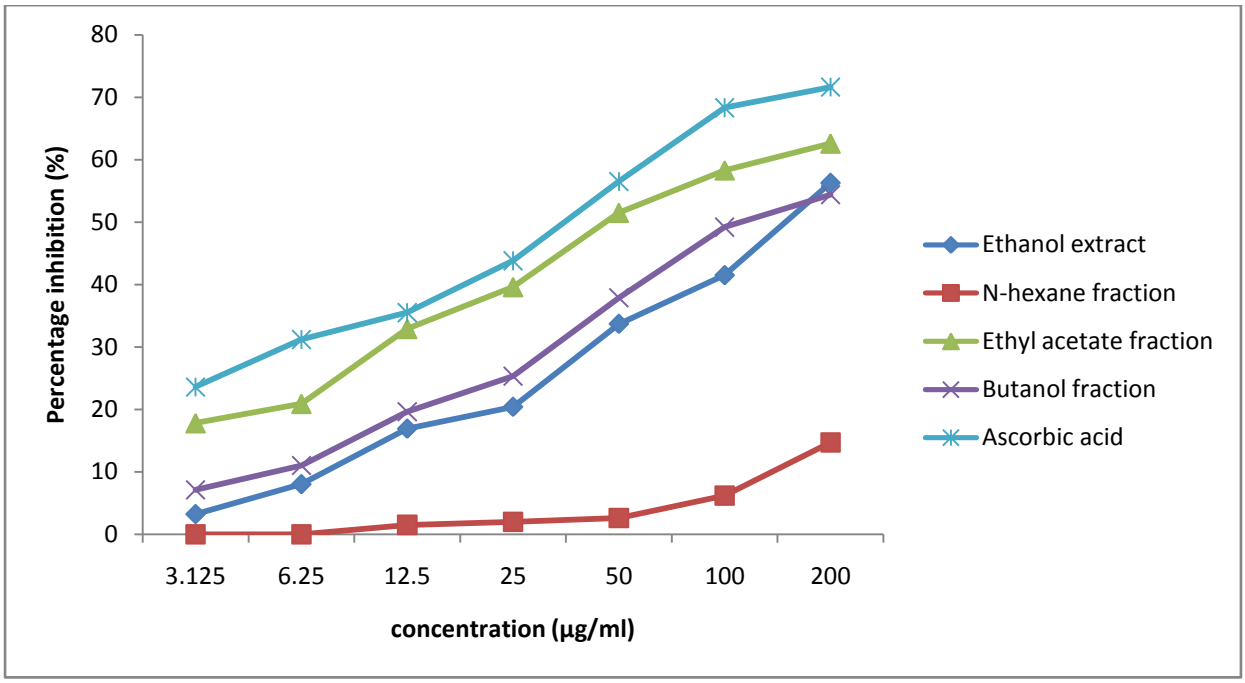


Figure 8: Ferric reducing antioxidant power (FRAP) of the extract and fractions of *M. aboensis*

Table 11: EC_{50} of the extract and fractions on FRAP

Extract/Fractions	EC_{50}(µg/mL)
Ethanol extract	166.67
N-hexane fraction	>200
Ethyl acetate fraction	45.83
Butanol fraction	125.00
Ascorbic acid	37.50

Table 12: Total phenolic content of the extract and fractions

Samples	Mean gallic acid equivalent (mgGAE/g)
Ethanol extract	121.4± 0.64
n-Hexane fraction	26.1± 0.42
Ethyl acetate fraction	305.2± 0.39
Butanol fraction	247.6± 0.50

4.4 *In vivo*median effectivedose

The median effective oral dose of the extract was determined based on its ability to inhibit ethanol induced lipid peroxidation.The ability of ethanol to induce lipid peroxidation was confirmed through high liver malondialdehyde – a product of lipid peroxidation – following oral ethanol administration (Table 13). The extract produced inhibition of ethanol induced liver lipid peroxidation in a dose dependent manner. The concentration of the extract that inhibited 50% of the lipid peroxidation was estimated to be approximately 300 mg/kg. Compared with the median lethal dose (4699 mg/kg) previously reported (Ajaghaku *et al*., 2012), the extract showed a wide therapeutic index. The ED_{50} dose and doses below and above formed the treatment doses in subsequent animal experiments.

Table 13: Effect of the extract on ethanol induced liver lipid peroxidation

Doses (mg/kg)	MDA µM/g
100	3.55± 0.42 (32.25)
200	3.10± 0.26 (40.84)
400	2.05± 0.21 (58.97)
800	1.86± 0.09 (64.50)
1600	1.71 0±.11 (67.37)
5 mL/kg 5% Tween 20	5.24± 0.07

N = 6 values in parenthesis represent percentage inhibition relative to control (5 mL/kg 5% tween 20). MDA - malondialdehyde

4.5 Local/Site specific oxidative damage

Serum ALT, ALP and AST which are markers of hepatocellular damage increased significantly in vehicle treated control group exposed to CCl_4. The extract and fractions of *M. aboensis* markedly reduces the activities of these liver function enzymes in a dose-dependent manner, of which ethyl acetate fraction was the most effective. Serum concentrations of alanine and aspartate aminotransferases extrapolated from their linear calibration curves showed that the extract at 300 and 400 mg/kg protected the liver against CCl_4 induced damage through significant ($p < 0.05$) reduction in aminotransferase enzymes activity (Figure 9 and 10). The effect of the extract on liver alkaline phosphatase enzyme activity was similar to that of the transaminase activity (Figure 11). Ethyl acetate fraction offered strong protection against liver damage as demonstrated by significant ($p < 0.05$) reduction in the liver marker enzymes at all tested doses. Significant ($p<0.05$) liver damage protection offered by butanol fraction was evident through reduction of the transaminases and alkaline phosphatase at 300 and 400 mg/kg. Serum liver enzyme activity of n-hexane treated groups were similar to the vehicle treated group an indication of poor ability of the fraction to ameriolate local liver oxidative damage produced by CCl_4.

The extract, ethyl acetate and butanol fractions demonstrated strong antioxidant activity through significant ($p < 0.05$) reduction in the formation of malondialdehyde – a product of lipid peroxidation (Figure 12). The liver malondialdehyde of the 200 mg/kg extract treated group was 3.15 µM/g liver tissue against 4.48 µM/g of the the vehicle treated group. The inhibition produced by the ethyl acetate fraction at 200 mg/kg was comparable to that produced by 100 mg/kg Silymarin with mean MDA values of 2.03 and 1.76 µM/g respectively (Appendix table 9A).

Pre-treatment with the extract and fractions protected the liver from the CCl_4 induced oxidative damage. Similar to Silymarin, the extract, ethyl acetate and butanol fractions produced significant ($p < 0.05$) increase of catalase enzyme at all doses compared with the vehicle treated group (Figure 13). Significant ($p < 0.05$) increase was also observed for the superoxide dismutase enzyme at 300 and 400 mg/kg for the extract and butanol fraction while ethyl acetate fraction maintained significant ($p < 0.05$) activity at all doses (Figure 14).

Necrosis, fatty liver, fibrosis and haemorrhage with filtration of inflammatory cells were evidence in the CCl_4 treated groups (Figure 15). Pretrearment with the extract, ethyl acetate and butanol fractions ameliorated the severity of the CCl_4 induced liver damage as assessed by H&E stained liver sections (Figuress 16, 18 and 19) compared with the vehicle treated control group. Silymarin treated group showed mild necrosis and inflammation (Figure 20) while little or no protection occurred in the n-hexane pre-treated group as expressed by presence of marked necrosis and interstitial haemorrhage(Figure 17).

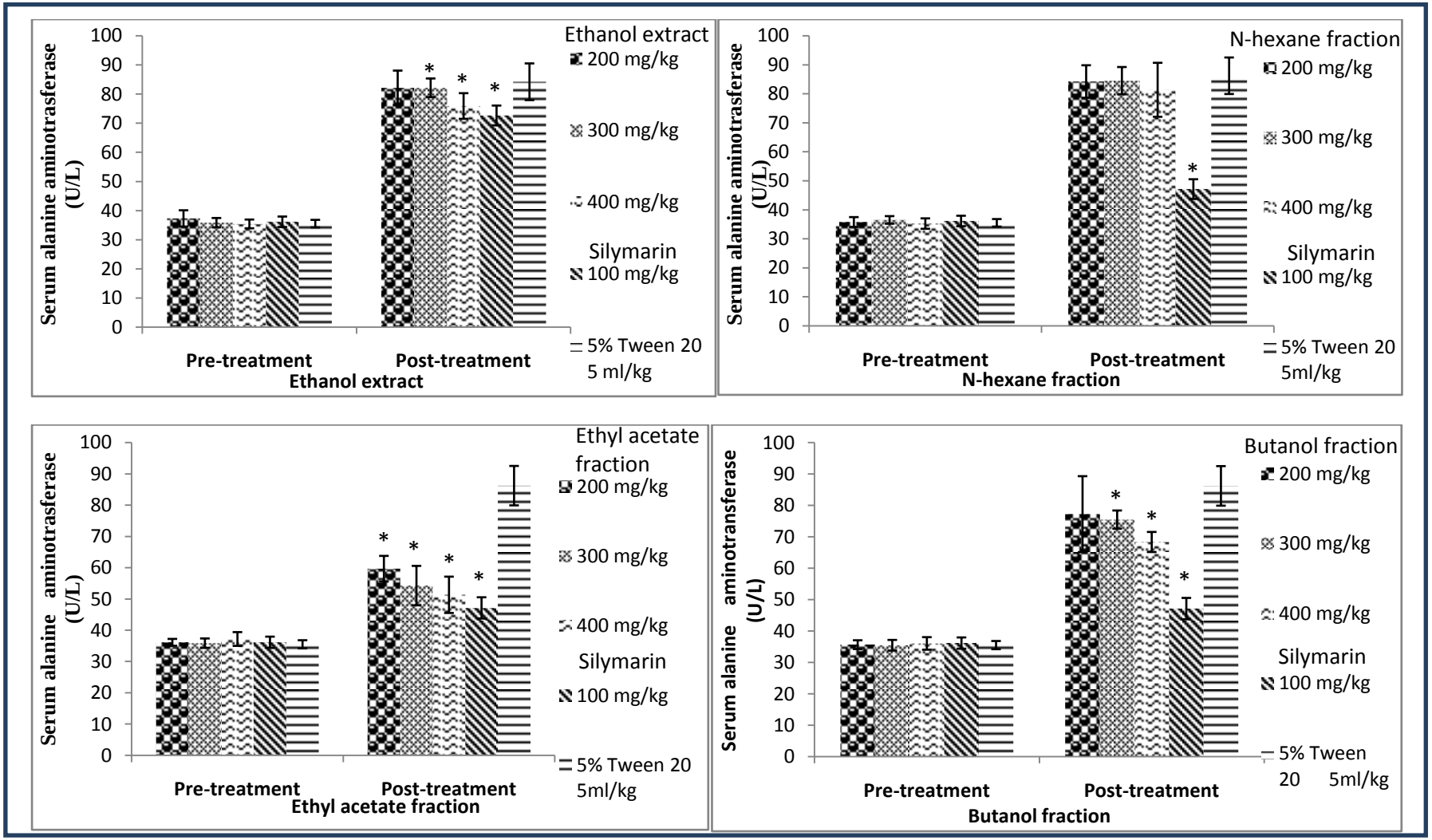


Figure 9: Effect of extract and fraction on alanine aminotransferase

* P<0.05 compared with 5% Tween 20 (control)

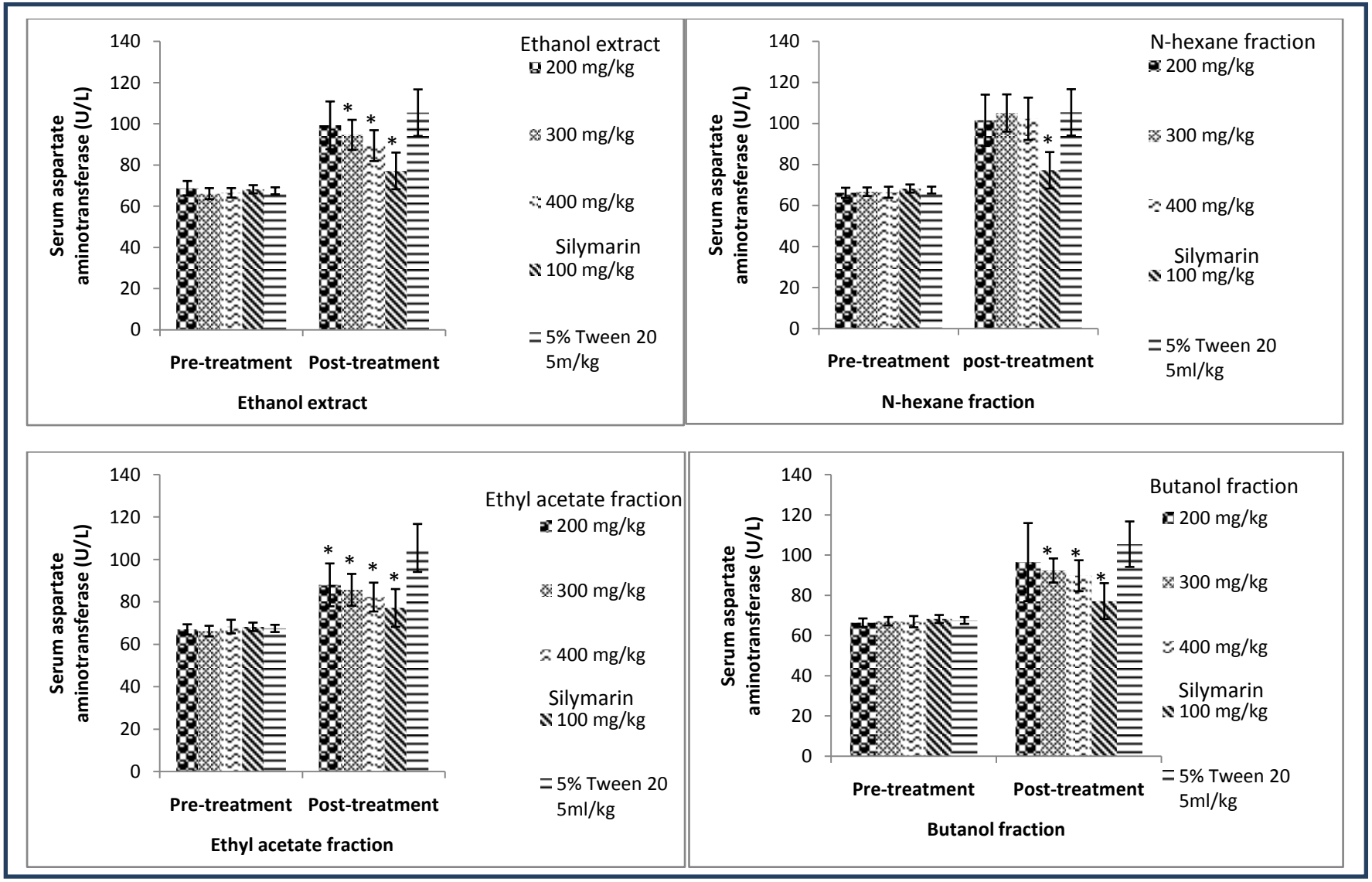


Figure 10: Effect of extract and fractions on aspartate aminotransferase

* P<0.05 compared with 5% Tween 20 (control)

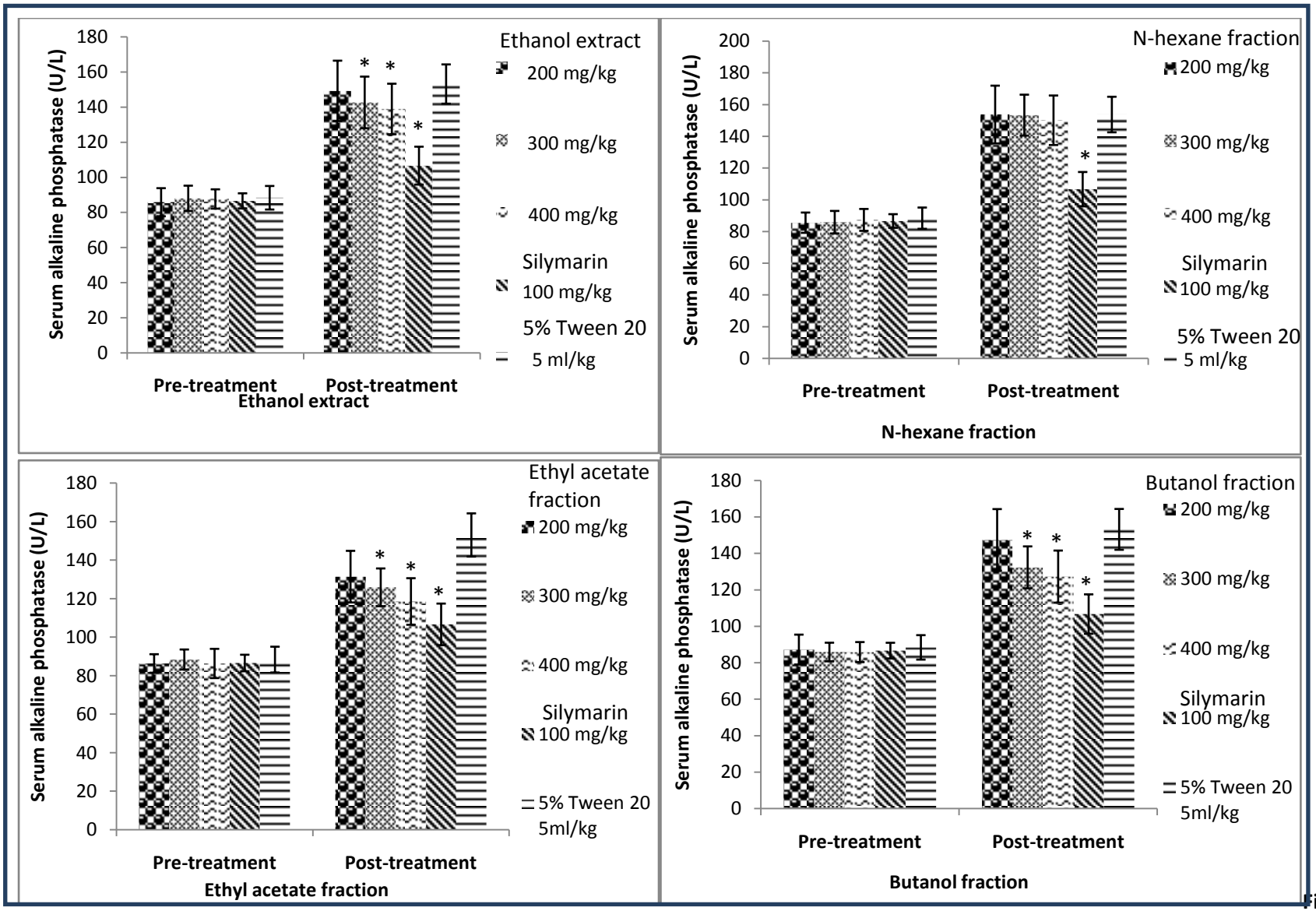


Figure 11: Effect of the extract and fraction on alkaline phosphatase

* P<0.05 compared with 5% Tween 20 (control)

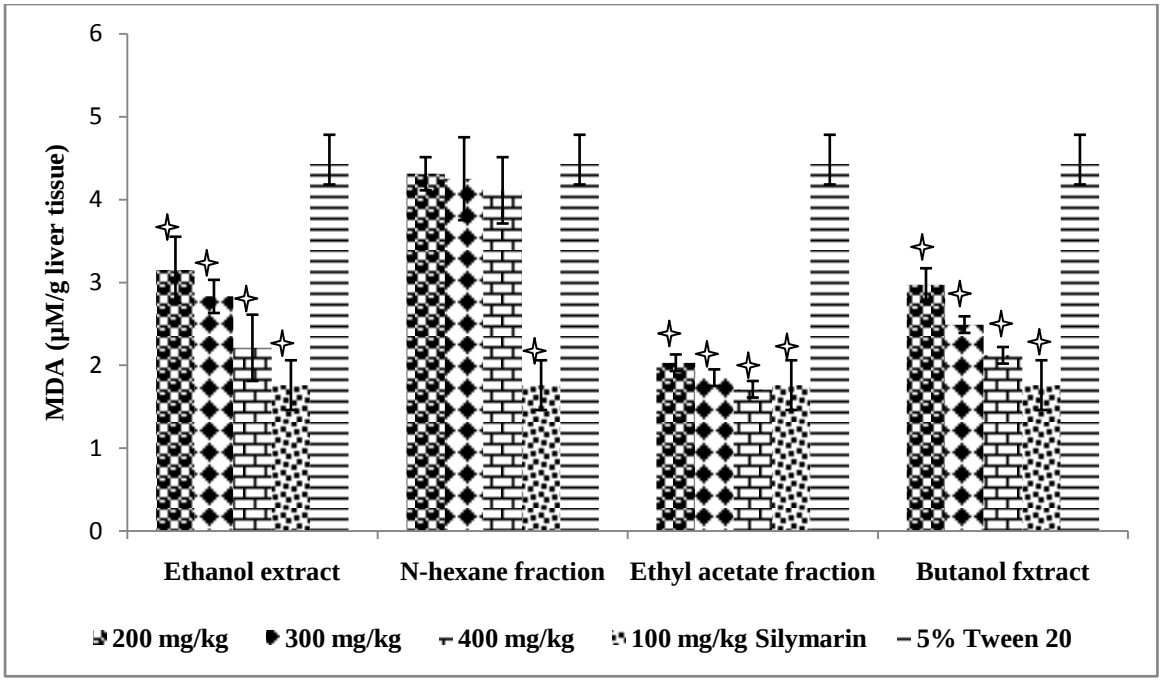


Figure 12: Effect of extract and fractions on liver lipid peroxidation

n = 6, * $p < 0.05$ compared with 5% Tween 20 (control)

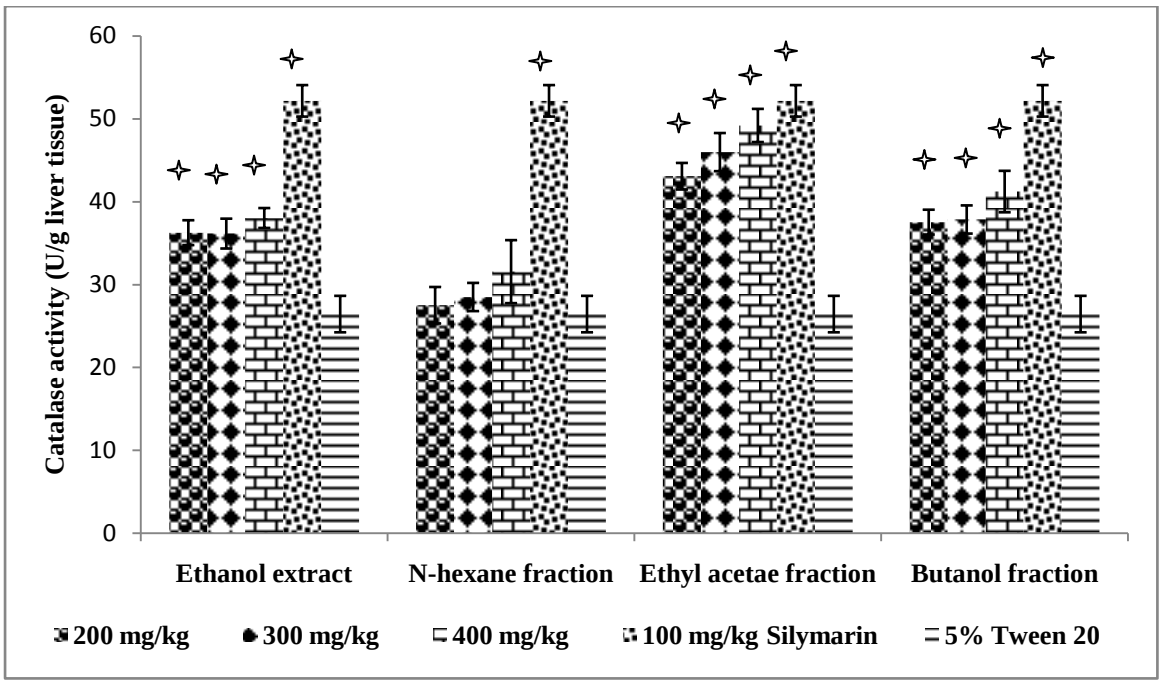


Figure 13: Effect of the extract and fractions on liver catalase enzyme activity

n = 6, * $p < 0.05$ compared with 5% Tween 20 (control)

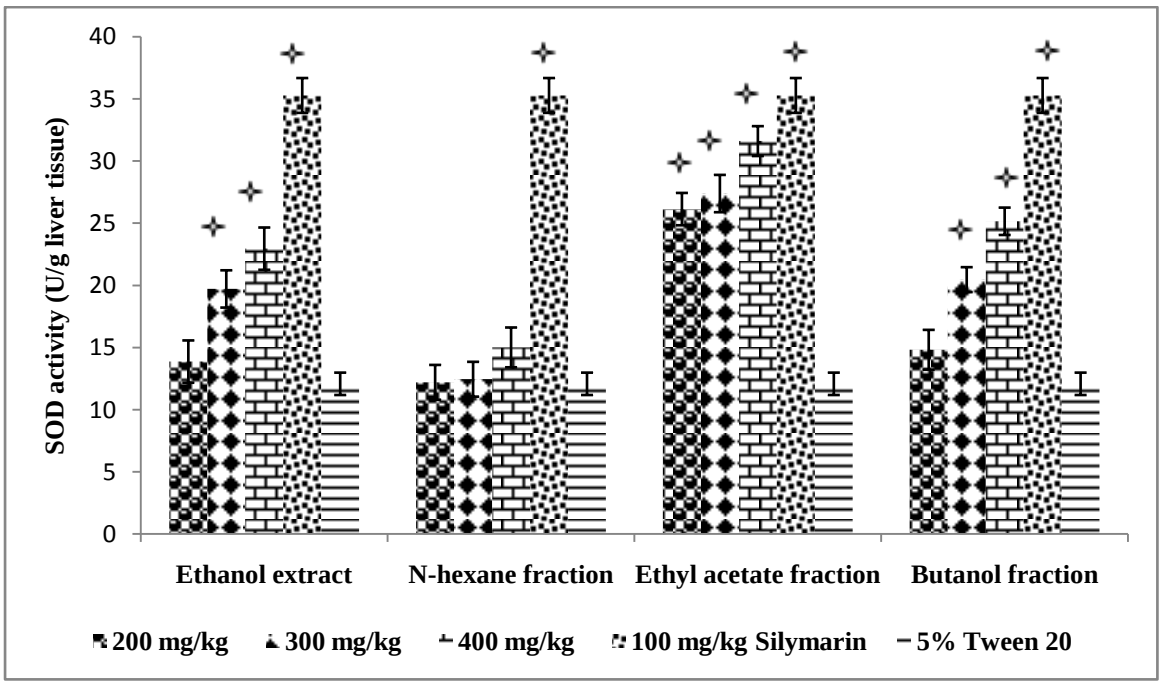
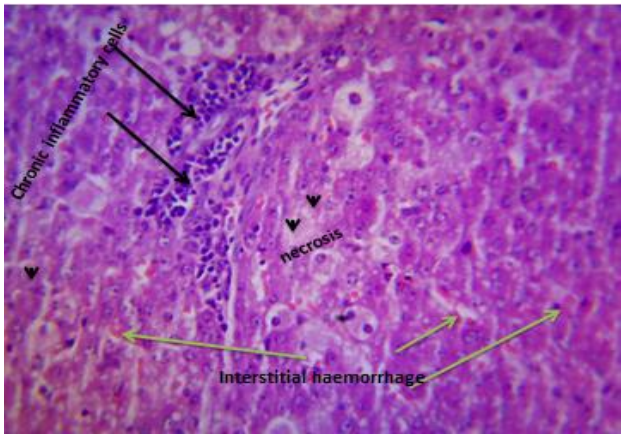


Figure 14: Effect of extract and fractionson liver superoxide dismutase enzyme activity.

n = 6, * $p < 0.05$ compared with 5% Tween 20 (control)



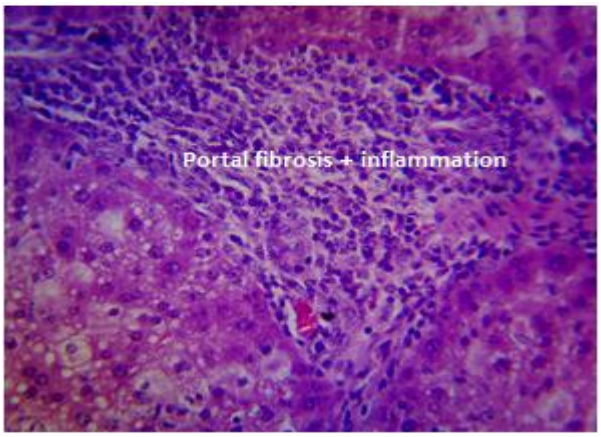
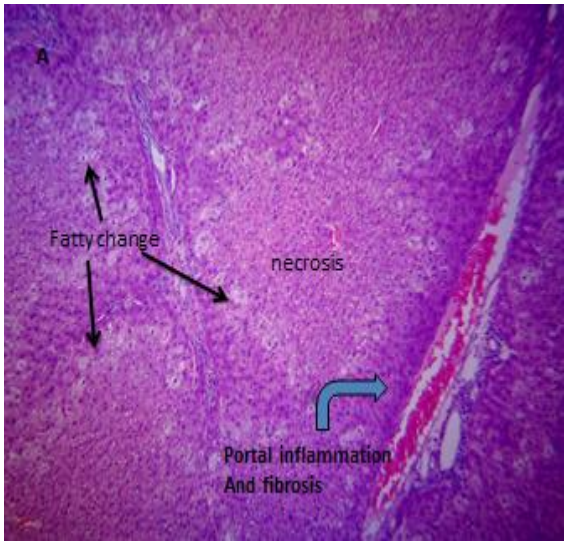


Figure 15: Photomicrograph of CCl_4 induced liver damageof control group.

Arrows showing different damages on the liver (H&E x400)



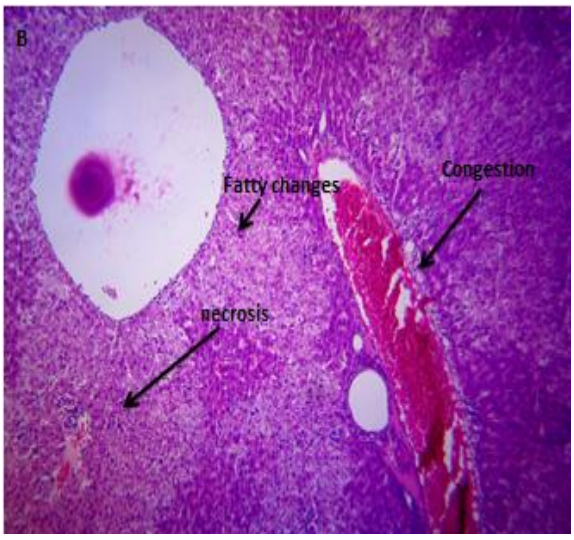
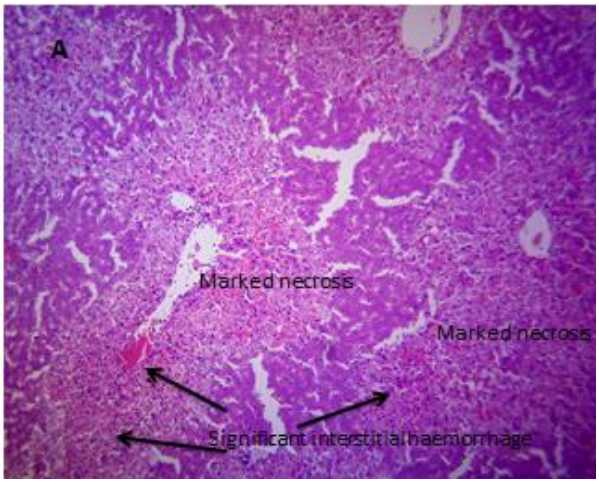


Figure 16: Photomicrograph of CCl4 induced liver damageof extract treated group

A – 200 mg/kg treated group, B – 400 mg/kg treated group. Arrows showing different damages on the liver. (H&E x 400)



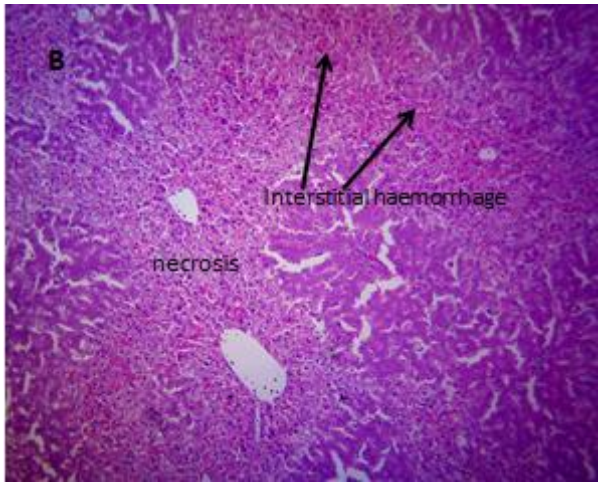


Figure 17: Photomicrograph of CCl_4 induced liver damageof n-hexane fraction treated group.

A – 200 mg/kg treated group, B – 400 mg/kg treated group. Arrows showing different damages on the liver (H&E x 400)

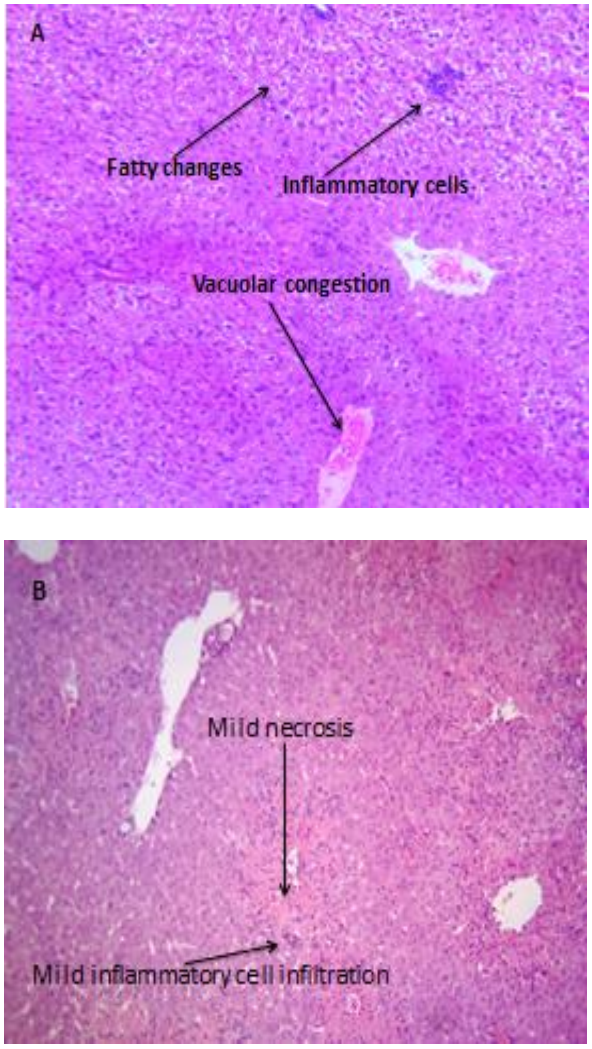
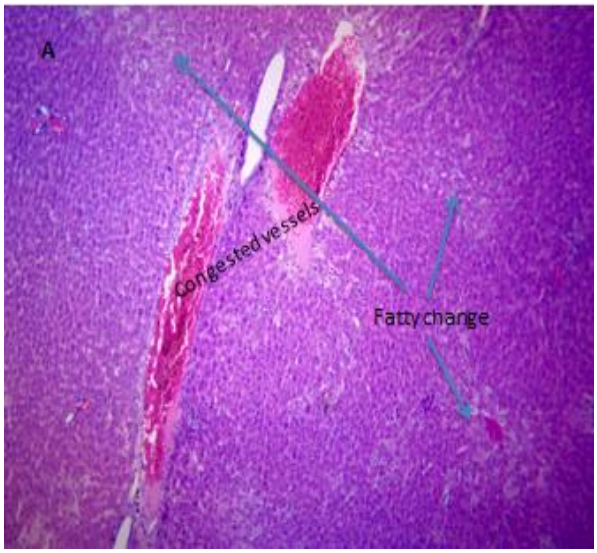


Figure 18: Photomicrograph of CCl_4 induced liver damage of ethyl acetate fraction treated group.

A – 200 mg/kg treated group, B – 400 mg/kg treated group. Arrows showing different damages on the liver (H&E x100)



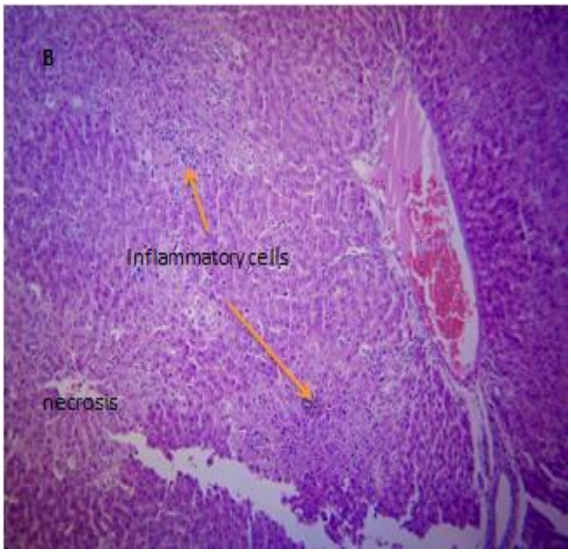
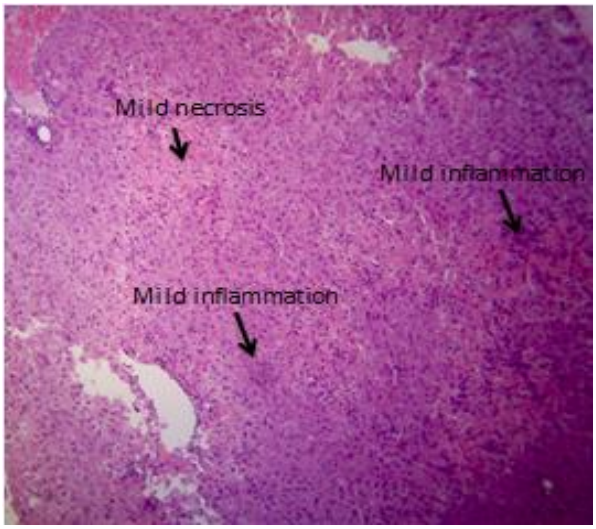


Figure 19: Photomicrograph of CCl_4 induced liver damage of butanol fraction treated group.

A – 200 mg/kg treated group, B – 400 mg/kg treated group. Arrows showing different damages on the liver (H&E x100)



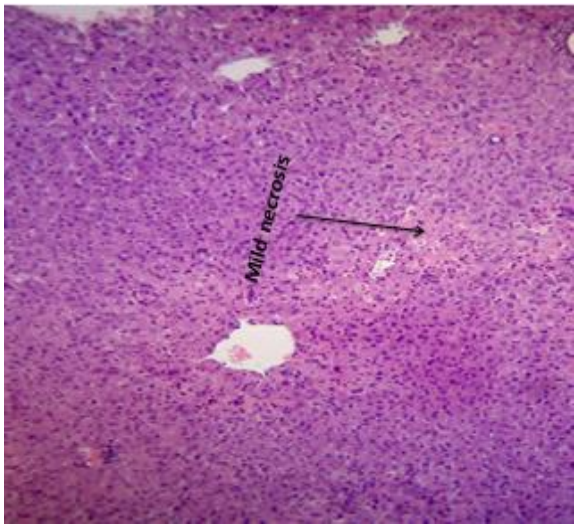


Figure 20: Photomicrograph of CCl_4 induced liver damage of silymarin treated group.

Arrows showing different damages on the liver (H&E x100)

4.6 Diabetes induced systemic oxidative stress

High blood glucose (> 160 mg/dl) recorded 72 h post administration of STZ-NAD was used an indication of a diabetic state. This hyperglycermic state was maintained throughout the 21 –days' study period (Table 14).On the 14th day of treatment, the extract treated group at 400 mg/kg showed significant ($p<0.05$) reduction in blood glucose compared with the vehicle treated group. The ethyl acetate fraction at 300 and 400 mg/kg produced significant ($p <0.05$) reduction in blood glucose from the 7th day compared with vehicle treated group (5% tween 20) justlike 400 mg/kg of butanol fraction and 5 mg/kg glibenclamide. However, when compared with 72 h post-induction diabetic state, significant ($p < 0.05$) effect of the ethyl acetate fraction was recorded from the 14th day unlike glibenclamide that maintained significant ($p < 0.05$) effect from the 7th day.

Reduction in serum SOD and catalase enzyme activities were indication of compromised systemic oxidative stress following STZ-NAD induced diabetes. The suppressed antioxidant enzymes were however significantly ($p < 0.05$) elevated after treatment with 300 and 400 mg/kg of the extract. Significant ($p < 0.05$) increase in antioxidant enzymes wereproduced by the ethyl acetate and butanol fractions at all doses (Figure 21 and 22).

Suppressed serum total antioxidant capacity was the hallmark of systemic oxidative stress induced by STZ-NAD (Figure 23). The extract and butanol fraction at 300 and 400 mg/kg significantly ($p < 0.05$) increased serum total antioxidant capacity. High total serum antioxidant capacity of the ethyl acetate fraction was demonstrated by its significant ($p < 0.05$) activity at lower concentration (200 mg/kg).

Table 14: Effect of extract and fractions of *M. aboensis* on STZ-NAD induced hyperglycemia

Treatment	Dose	Mean blood glucose (mg/dl)				
		Pre-induction	72h post-induction	Day 7	Day 14	Day 21
Ethanol	200	79.1± 2.8	234.4± 27.1	229.6 ± 24.3 (2.0)	215.6± 26.2 (8.0)	*208.3± 21.4 (11.1)
extract	300	77.0± 3.0	239.3± 16.3	233.2± 23.6 (2.5)	215.3± 19.7 (10.0)	*206.5± 21.9 (13.7)
	400	76.4± 2.5	231.5± 21.5	223.1± 27.7 (3.6)	*206.4± 22.0 (10.8)	*195.7± 23.5 (15.5)
N-hexane	200	72.5± 2.2	237.2± 25.2	231.7± 23.5 (2.3)	221.0± 20.3 (6.8)	217.5± 20.3 (8.3)
fraction	300	77.9± 2.4	241.8± 22.7	236.5± 26.4 (2.2)	223.3± 24.4 (7.7)	219.5± 25.9 (9.2)
	400	69.3± 3.3	245.1± 20.9	239.5± 24.1 (2.4)	227.8± 27.3 (7.1)	*219.4± 27.2 (10.5)
Ethyl acetate	200	76.1± 3.1	235.7± 15.6	229.2± 17.2 (2.8)	*210.1± 25.1 (10.9)	*194.2± 17.5 (17.6)
fraction	300	79.3± 2.7	232.5± 17.8	*220.8± 21.0 (5.0)	[a]*205.0± 21.6 (11.8)	[a]*181.9± 20.8 (21.8)
	400	73.4± 2.1	240.9± 26.4	*221.8± 19.2 (7.9)	[a]*201.8± 23.7 (16.2)	[a]*186.0± 19.4 (22.8)
Butanol	200	75.6± 3.6	229.0± 24.1	223.3± 25.9 (2.5)	209.2± 23.2 (8.6)	*198.9± 25.6 (13.1)
fraction	300	77.2± 1.9	238.6± 27.4	230.5± 23.8 (3.4)	216.7± 19.8 (9.2)	*204.3± 16.3 (14.4)
	400	73.8± 3.2	225.4± 19.3	*214.1± 21.5 (5.3)	[a]*200.6± 24.3 (11.5)	[a]*189.1± 20.7 (16.9)
Glibenclamide	5	79.1± 2.6	242.7± 17.2	[a]*197.6± 15.8 (18.6)	[a]*182.2± 17.5 (24.9)	[a]*161.2± 15.1 (33.6)
5% Tween 20	5 mL/kg	74.8± 3.4	236.9± 21.5	231.5± 23.2 (2.3)	222.1± 21.3 (6.2)	215.4± 19.9 (9.1)

N = 6, [a] $p < 0.05$ compared with 72 h post-induction, * $p < 0.05$ compared with control. Values in parenthesis represent percentage glucose reduction

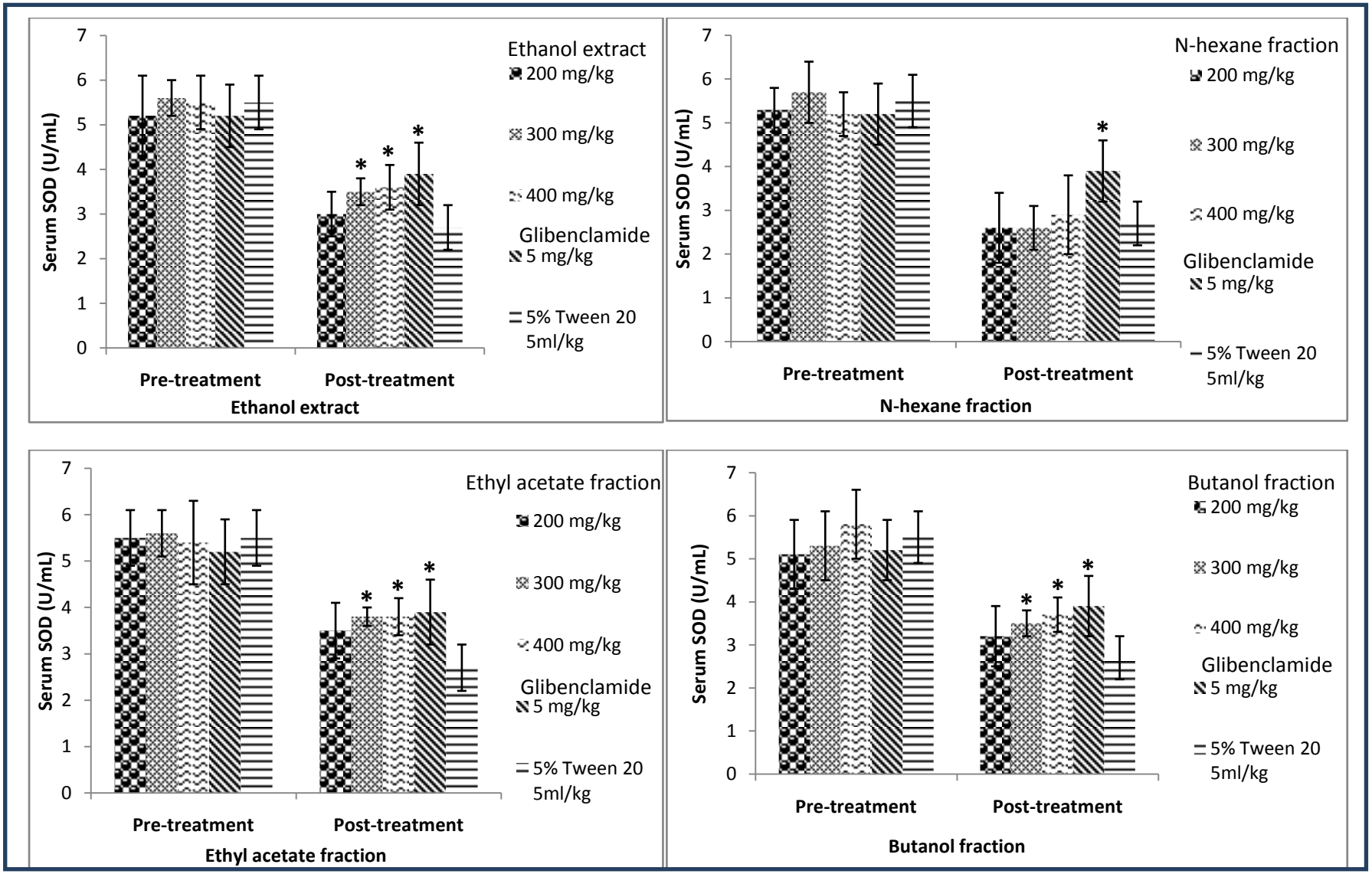


Figure 21: Effect of extract and fractions on serum superoxide dismutase (SOD) enzyme

*$p<0.05$ compared with 5% Tween 20 (control)

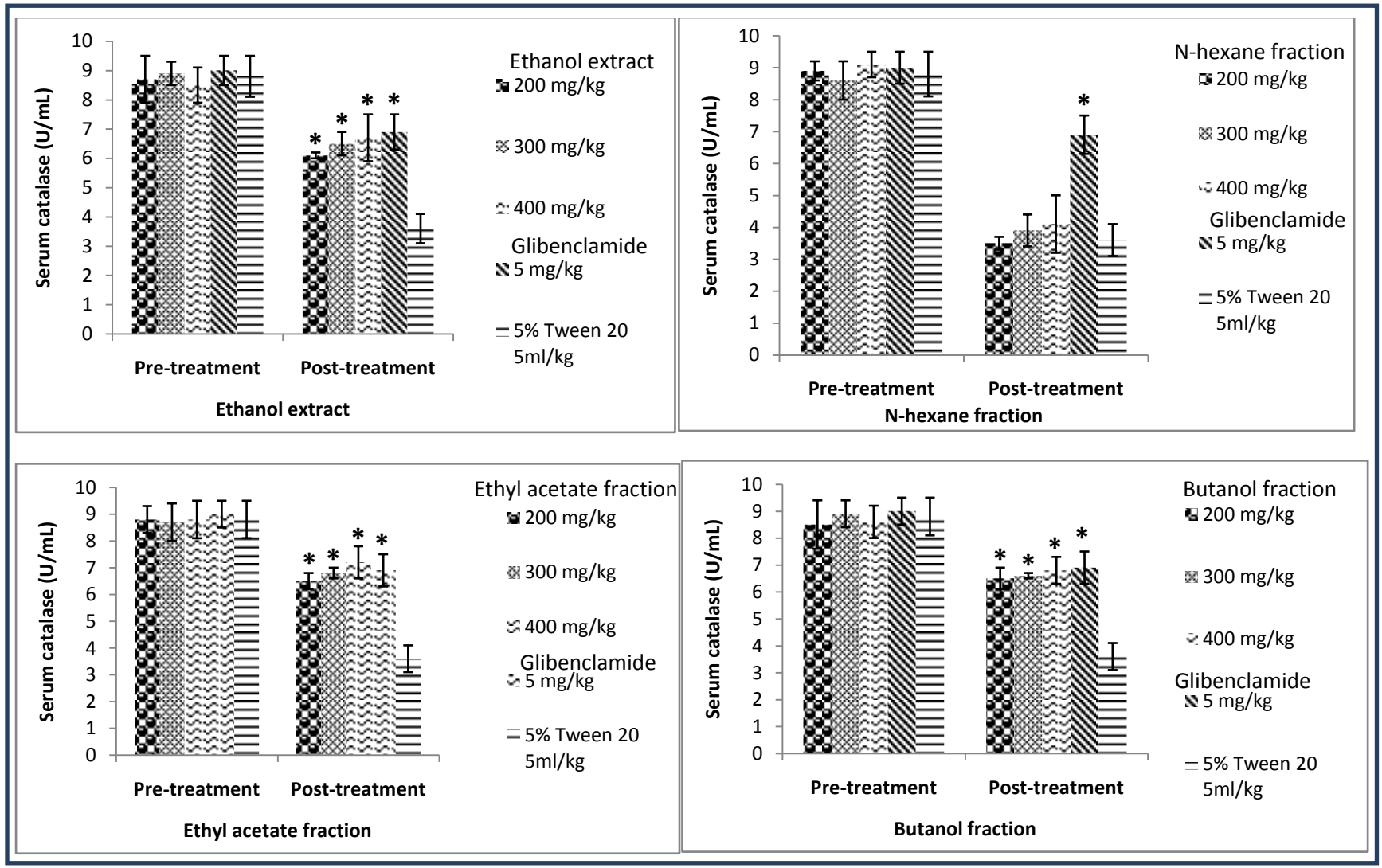


Figure 22: Effect of extract and fractions on serum catalase enzyme activity

*p<0.05 compared with 5% Tween 20 (control)

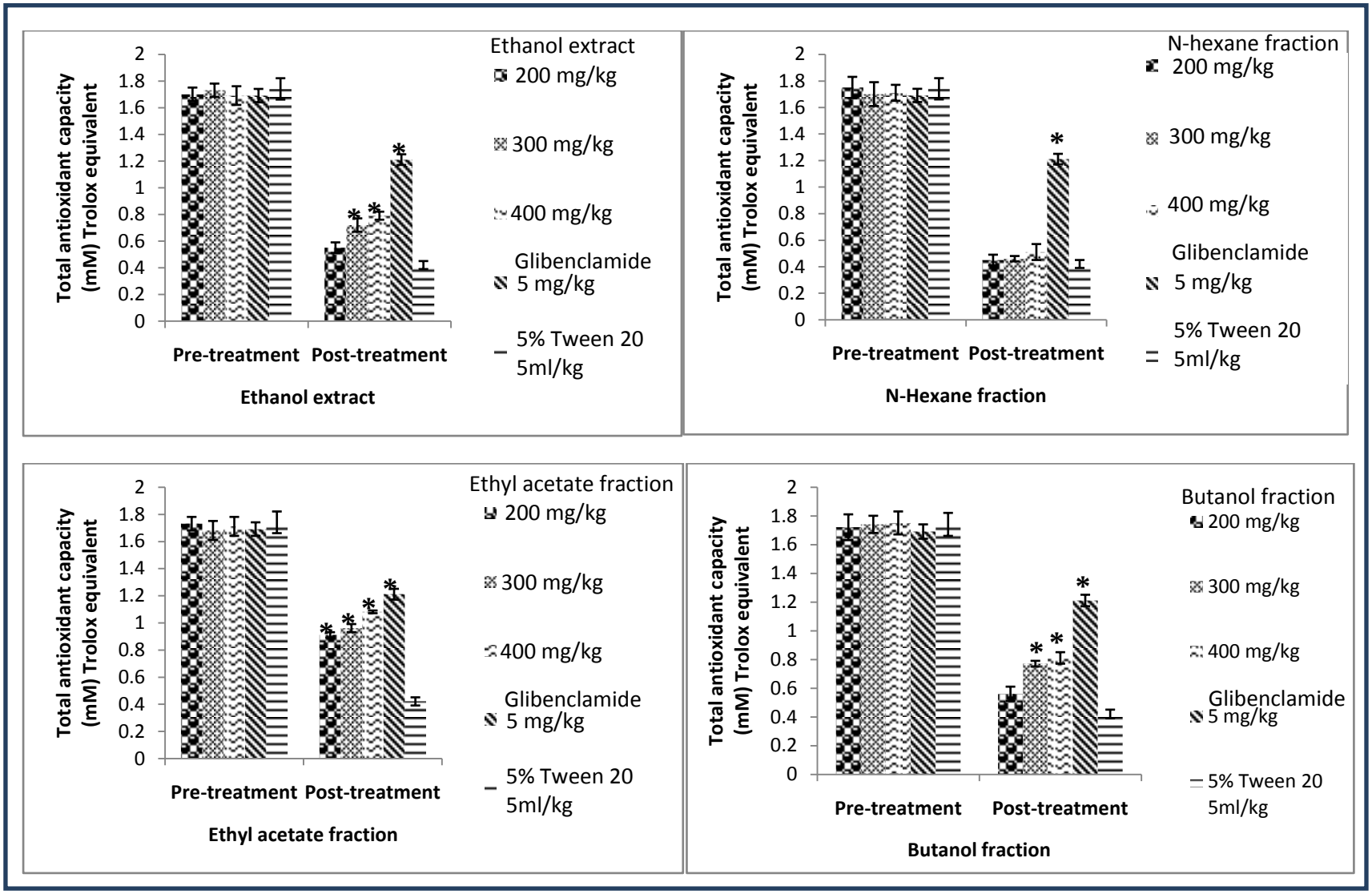


Figure 23: Effect of extract and fractions on serum total antioxidant capacity

* p<0.05 compared with 5% Tween 20 (control)

4.7 Immunomodulatory effect of the extract and fractions of *M. aboensis*

Administration of the extract, ethyl acetate and butanol fractions of *M. aboensis* caused an elevation of both primary and secondary humoral immune responses to tetanus toxoid antigen. In response to primary challenge with the tetanus toxoid (antigen), the extract evoked significant ($p < 0.05$) increase in serum IgG1 at 400 mg/kg while the ethyl acetate fraction at 300 and 400 mg/kg significantly ($p < 0.05$) elevated both IgG1 and IgG2a. Butanol fraction significantly ($p < 0.05$) elevated both antibodies at 200 mg/kg just like our reference standard (Noni capsule®) at 100 mg/kg (Table 15).

Secondary challenge of the animals with tetanus toxoid antigen produced higher response of both IgG1 and IgG2a. At 300 and 400 mg/kg, the extract and ethyl acetate fraction showed significant ($p<0.05$) response of both antibodies compared with the vehicle treated group while butanol fraction at 200 mg/kg produced similar significant ($p<0.05$) response. The nature of immunoglobulin stimulation by the extract and fractions of *M. aboensis* showed IgG1 as more expressed compared with IgG2a

For the cellular immunity, the extract (400 mg/kg) significantly ($p < 0.05$) elevated total leukocyte count in cyclophosphamide induced leukopenia mice (Figure 24). Ethyl acetate fraction produced significant ($p < 0.05$) elevation at both 300 and 400 mg/kg. The activity of butanol fraction was more pronounced with significant ($p < 0.05$) elevation in total leukocyte count at 200 mg/kg.

Differential leukocyte counts revealed that the significant response produced by the extract was largely contributed by the lymphocytes while that of ethyl acetate was accounted for by lymphocytes and monocytes. At 200 mg/kg, butanol fraction significantly ($p < 0.05$) stimulated the neutrophils while at higher concentrations, it produced significant ($p < 0.05$)

stimulation of the neutrophils, lymphocytes and monocytes similar to Noni capsule® (Table 16).

The stimulation of cellular immunity by the butanol fraction led to the determination of its rate of reactivation of immune cells on prolonged immunosuppressed state. The findings revealed that this fraction produced significant ($p < 0.05$) elevation of total leukocyte counts from the 10th day irrespective of dose (Figure 25). This was also observed in the differential leucocyte responses (Figures 26 - 28).

Findings from the nature of cytokine up-regulation by the butanol fraction revealed that IL12, IL17A, IFN-γ and IL4 showed 48.14, 41.37, 38.22 and 31.03% expression. G-CSF, GM-CSF, TNF-α, IL6, IL10 and IL1B were moderately expressed while IL1A and IL2 were the least expressed (Figure 29).

Table 15: Effect of extract and fractions of *M. aboensis* on primary and secondary immune response to tetanus toxoid

Treatment	Dose (mg/kg)	Primary immune response		Secondary immune response	
		IgG1IgG2a		IgG1	IgG2a
Ethanol extract	200	2.5± 1.07	1.9± 1.09	6.4 ± 1.46	4.8 ± 1.01
	300	2.7± 0.88	1.9± 1.19	*7.1 ± 1.46	*6.3 ± 1.33
	400	*3.0± 0.83	2.1± 0.78	*7.9 ± 1.47	*6.7 ± 1.25
N-hexane fraction	200	2.0± 0.58	1.7± 1.39	5.5 ± 2.81	4.3 ± 1.54
	300	2.1± 0.59	1.9± 0.80	5.9 ± 1.29	4.5 ± 1.05
	400	2.4± 1.73	1.9± 1.01	5.9 ± 1.87	4.7 ± 0.66
Ethyl acetate	200	2.8± 0.77	1.9± 0.90	6.6 ± 1.59	5.1 ± 0.84
fraction	300	*3.1± 0.73	*2.2 ± 0.69	*7.3 ± 0.90	*6.5± 0.68
	400	*3.3± 0.67	*2.2 ± 0.69	*8.2 ± 1.82	*7.1± 0.86
Butanol fraction	200	*3.1± 0.84	*2.2 ± 1.20	*8.1 ± 1.08	*6.8± 0.69
	300	*3.4± 0.71	*2.2 ± 0.71	*8.6 ± 1.07	*7.2± 0.77
	400	*3.5± 1.09	*2.5 ± 0.83	*9.0 ± 1.05	*7.7± 1.20
Noni capsule	100	*3.9± 0.80	*2.6 ± 1.14	*16.5 ± 3.18	*13.2± 3.20
5% Tween 20	5 mL/kg	2.1± 0.59	1.8 ± 1.05	5.7 ± 1.92	4.3± 1.19

n = 6, * $p < 0.05$ compared with 5% Tween 20 (control)

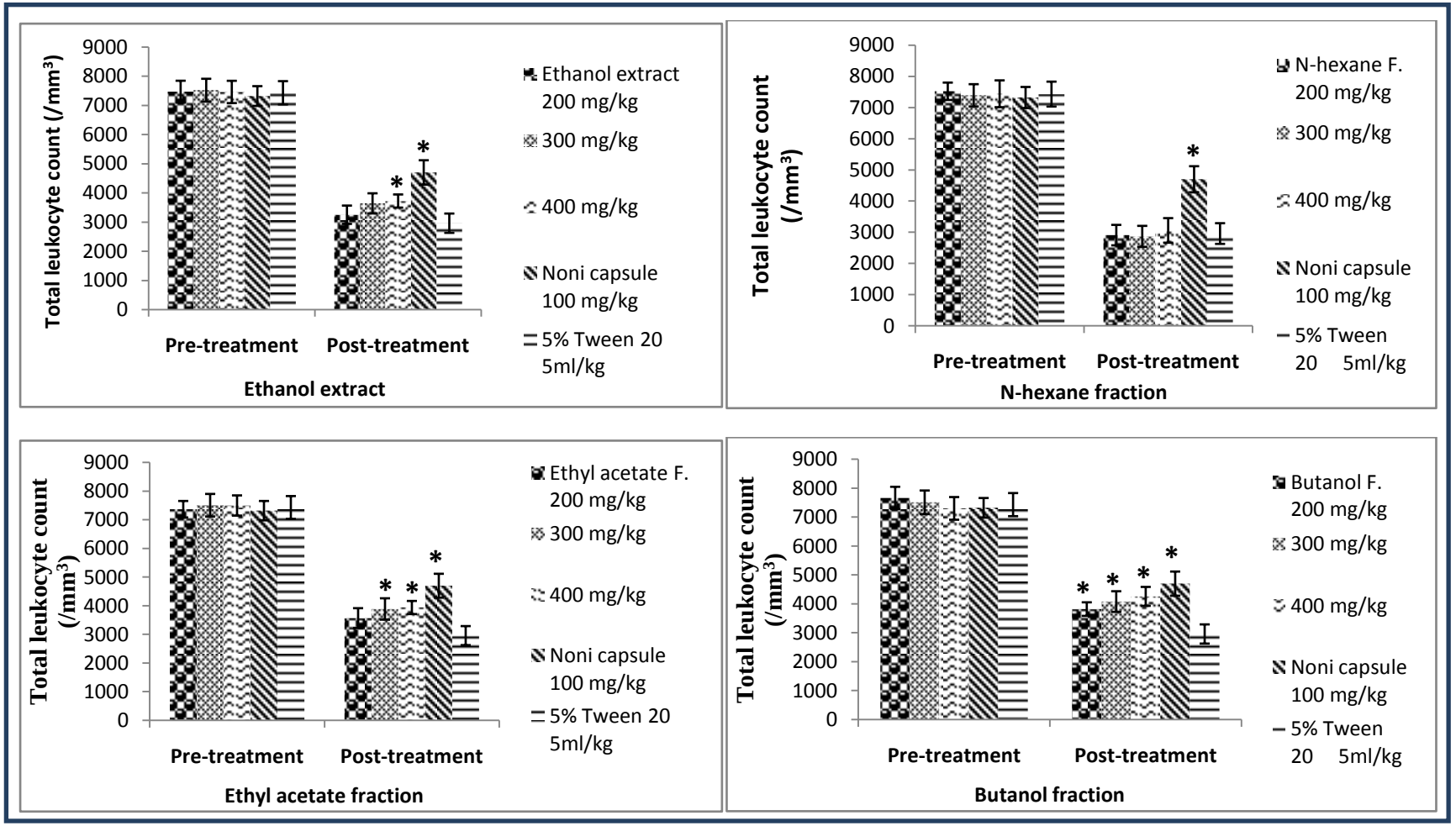


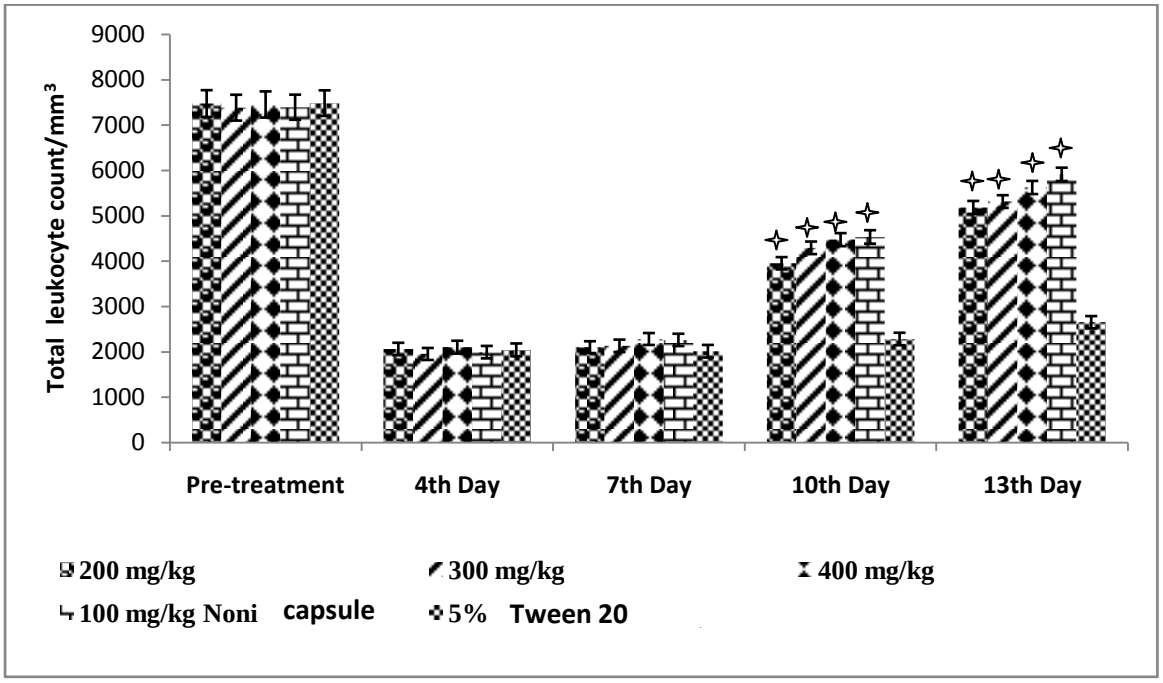
Figure 24: Effect of extract and fractions on total leukocyte count

*$p<0.05$ compared with 5% Tween 20 (control)

Table 16: Effect of extract and fractions of *M. aboensis* on differential leukocyte count

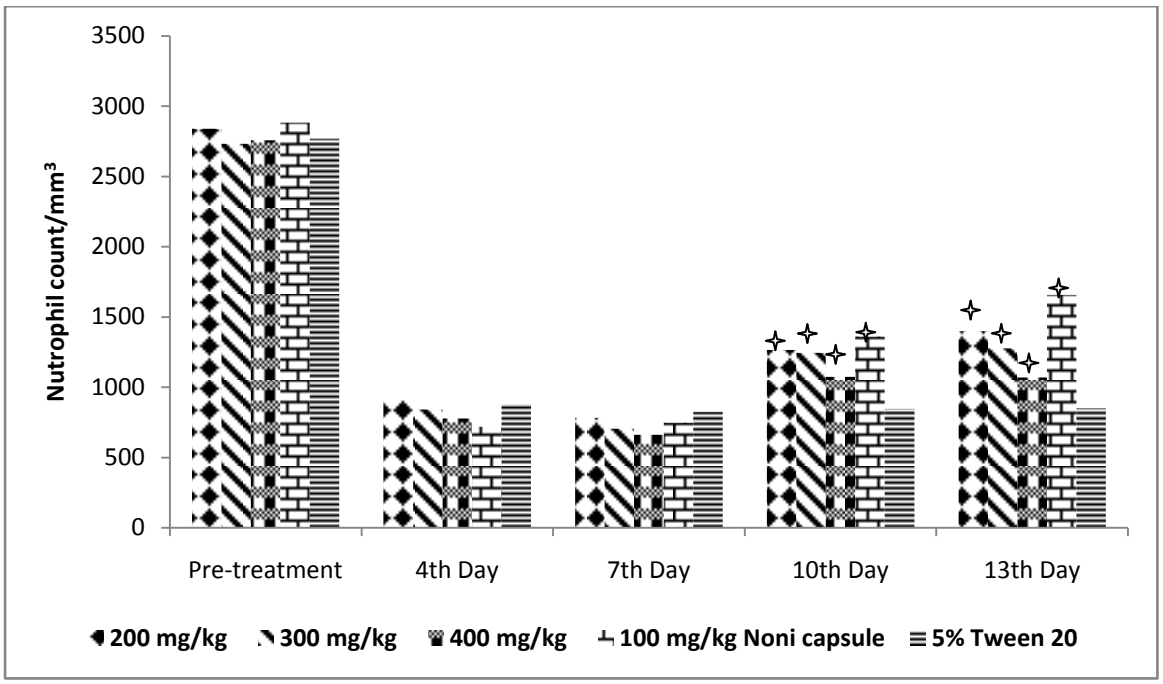
Treatment	Dose (mg/kg)	Pre-treatment			Post-treatment		
		Neutrophils	Lymphocytes	Monocytes	Neutrophils	Lymphocytes	Monocytes
Ethanol	200	2390±228.3	4930±341.7	149±7.4	1073±220.9	2113±234.2	65 ± 16.8
extract	300	2406±162.2	5038± 354.2	75± 3.6	1274± 199.4	2330±347.5	36 ± 1.1
	400	2611±190.6	4774±339.4	75± 4.2	1265± 201.7	*2418± 263.1	37 ± 1.2
N-hexane	200	2858±227.3	4211±344.8	451±16.7	1077± 230.2	1746± 211.5	87 ± 22.6
fraction	300	2290±143.7	5025± 353.3	74± 3.0	978±119.6	1894± 234.7	29 ± 1.2
	400	2604±225.9	4687±280.9	149± 11.5	1040±217.3	1958± 220.2	61 ± 25.9
Ethyl acetate	200	2580±232.0	4717±378.1	74± 9.3	1107± 210.7	2428±341.2	36 ± 7.1
fraction	300	2403±213.4	4882± 424.2	225±12.3	1400±231.8	2412±346.9	*78 ± 9.7
	400	2550±292.8	4800± 337.7	150±12.1	1379± 247.5	*2482±226.0	*79 ± 8.0
Butanol	200	2604±201.3	4979±313.5	77± 8.1	*1452±218.6	2330±220.6	38 ± 4.9
fraction	300	2253± 287.6	5181±345.9	75± 5.0	*1550± 213.9	*2489±221.1	*41 ±4.1
	400	2263± 229.1	4964± 373.3	73± 6.3	*1619± 241.4	*2599±223.3	*43 ±4.5
Noni capsule	100	2416±233.5	4831± 324.1	73± 7.4	*1739± 225.7	*2914±238.2	*47 ± 3.4
5% Tween 20	5mL/kg	2303± 257.2	4978± 391.5	223±10.5	1184±219.2	1746±222.6	30 ± 4.6

n = 6, * $p < 0.05$ compared with 5% Tween 20 (control)



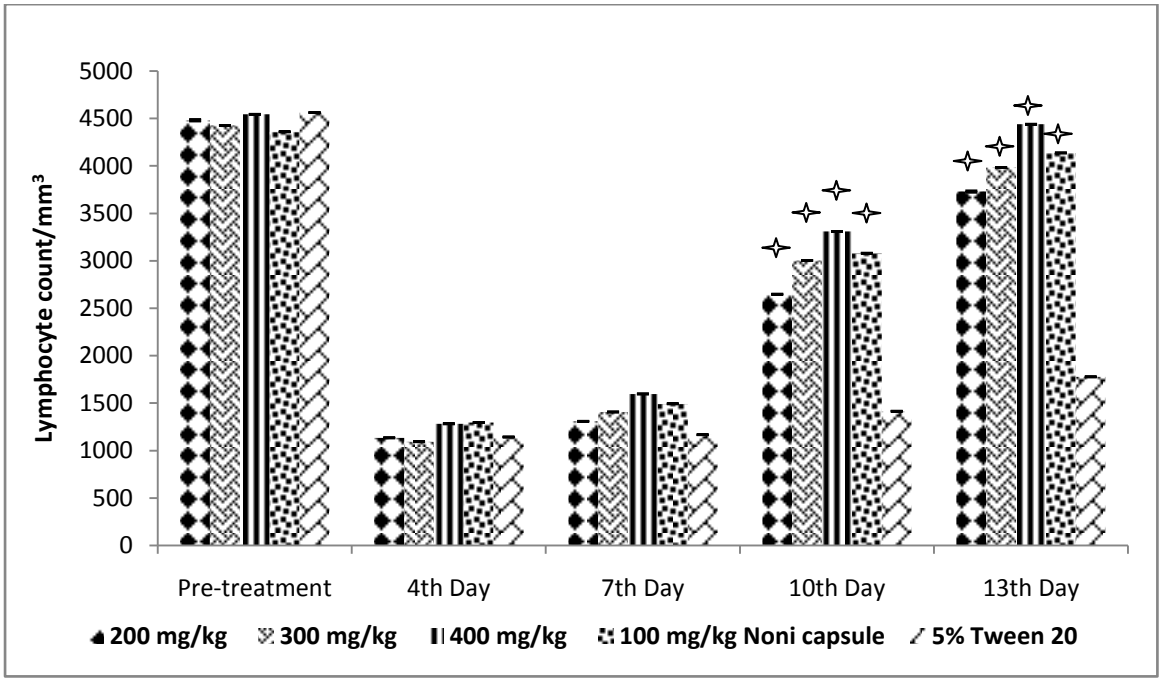
n = 6, * p < 0.05 compared with 5% Tween 20 (control)

Figure 25: Effect of butanol fraction on total leukocyte count



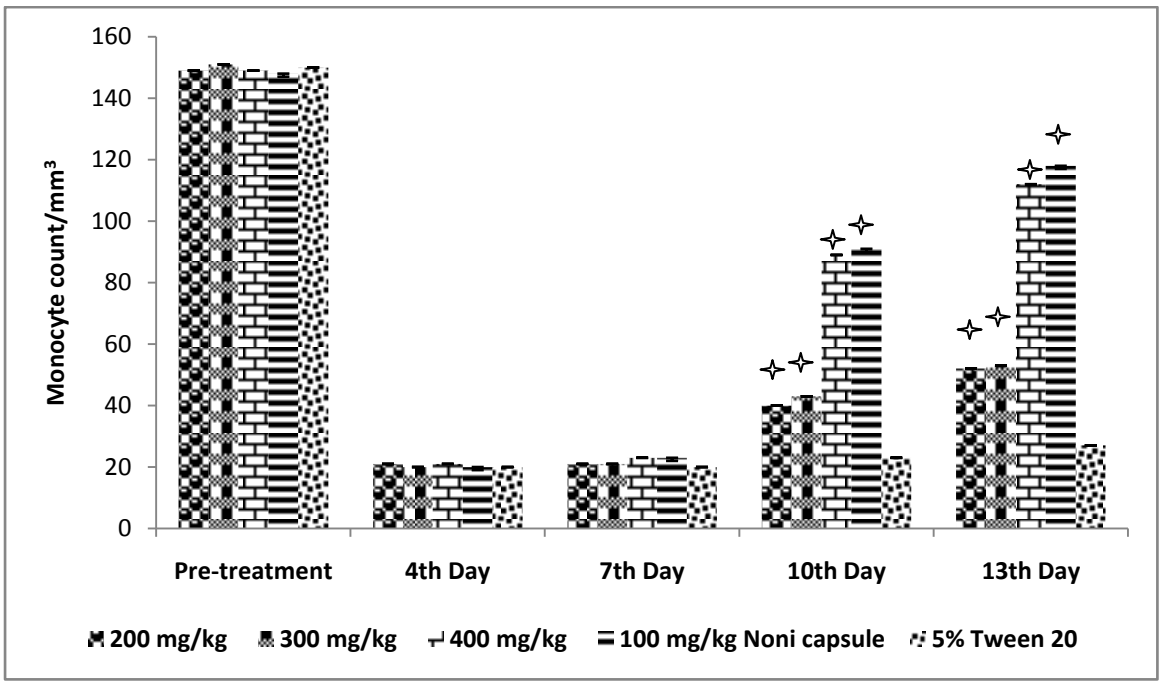
n = 6, * $p < 0.05$ compared with 5% Tween 20 (control)

Figure 26: Effect of butanol fraction on absolute nutrophil count



n = 6, * $p < 0.05$ compared with 5% Tween 20 (control)

Figure 27: Effect of butanol fraction on absolute lymphocyte count



n = 6, * $p < 0.05$ compared with 5% Tween 20 (control)

Figure 28: Effect of butanol fraction on absolute monocyte count

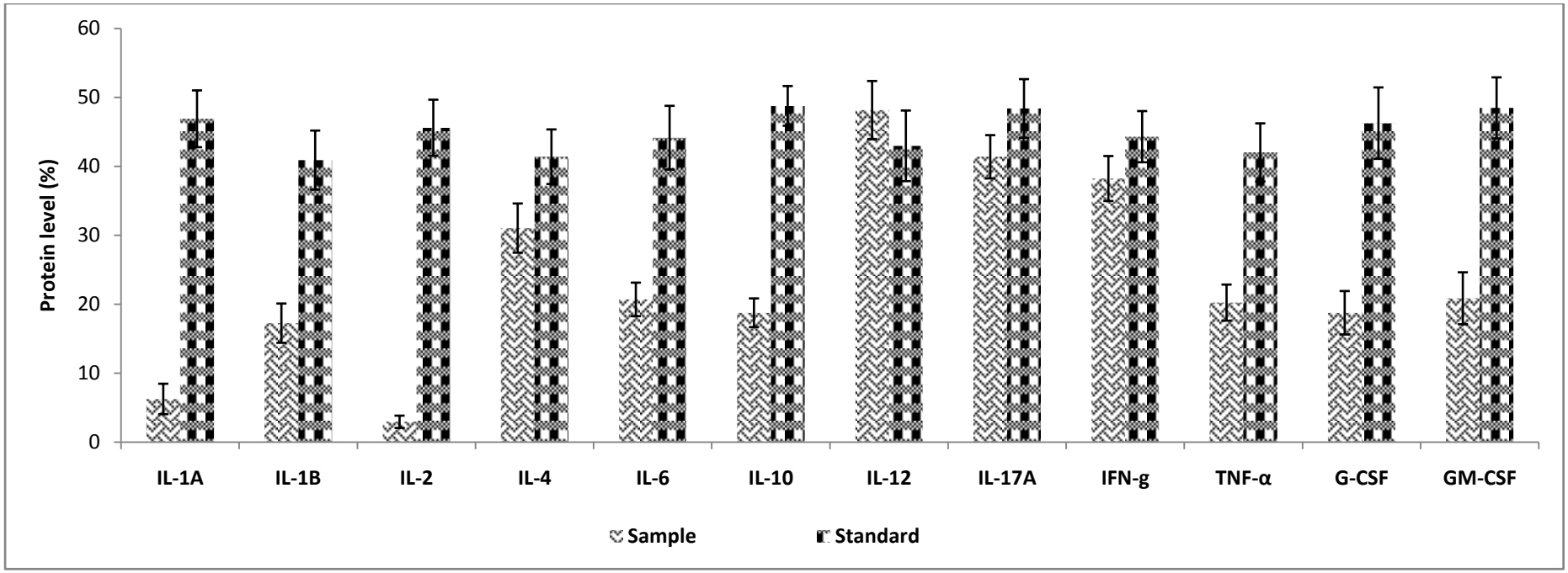


Figure 29: Effect of butanol fraction of serum cytokine expression

4.8 Antioxidant activity of ethyl acetate sub-fraction and isolated compounds.

DPPH scavenging activity of the ethyl acetate sub-fractions revealed that fractions D3 to D8 had EC_{50}below 200 µg/mL(Figure 30) with D5 possessing the highest activity (EC_{50} 15.63 µg/mL) followed by D6 (EC_{50} 21.88 µg/mL) (Table 17). High scavenging activity of D5 led to further purification of this sub-fraction with the isolation of compounds **1** and **2**.

The antioxidant activities of the isolated compounds were first evaluated by their ability to inhibit liver microsomal lipid peroxidation.Protein content of the isolated rat liver microsome was estimated to be561 mg/mL. *In vitro* lipid peroxidation assay of the isolated compounds from ethyl acetate sub-fractions showed a dose response inhibition of microsomal lipid peroxidation similar to Trolox® (standard water soluble vitamin E) (Figure 31). Compound **1** exhibited higher inhibition of lipid peroxidation with EC_{50} 46 µg/mL against 55 µg/mL exhibited by compound **2**. Higher antioxidant activities of compound **1** against compound **2** were also demonstrated in their scavenging of DPPH radical (Figure 32) with EC_{50}values of 9.75 µg/mL and 12.5 µg/mL respectively. Combination of the two compounds in equal ratios evoked synergistic activity with EC_{50} 7.0 µg/mL which is better thanascorbic acid (EC_{50} 9.10 µg/mL) (Table 18).

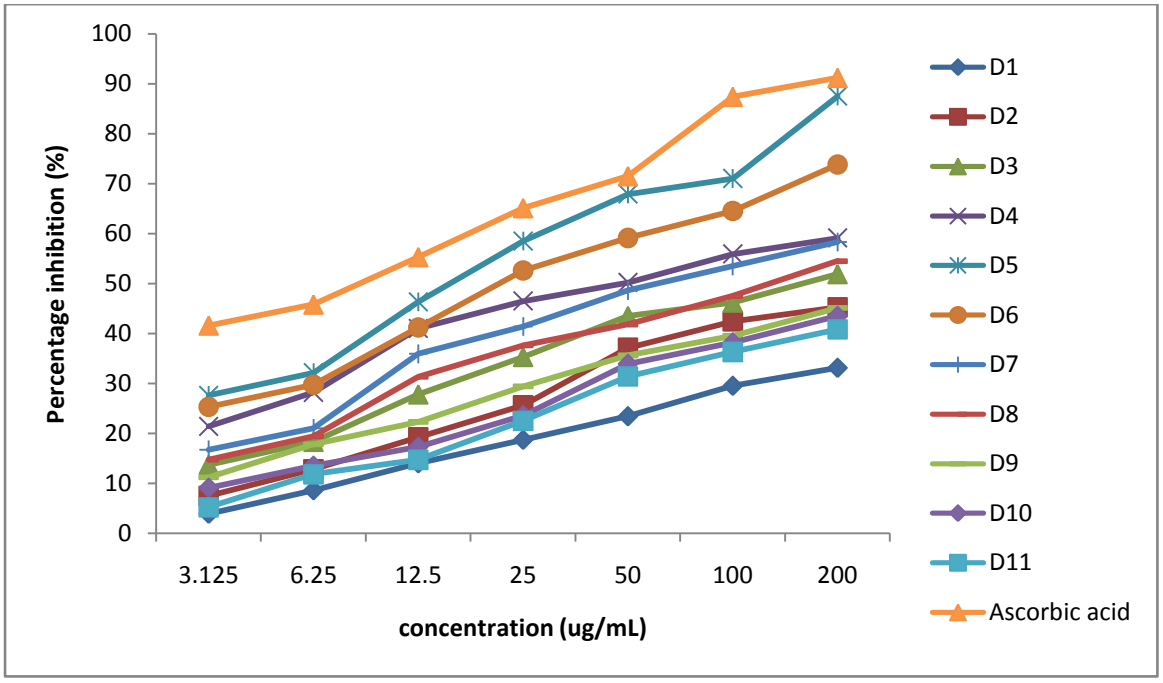


Figure 30: DPPH scavenging activity of ethyl acetate sub-fractions

Table 17: EC_{50} of ethyl acetate sub-fractions against DPPH radical

Sub-fractions	EC_{50}(µg/mL)
D1	>200
D2	>200
D3	166.67
D4	49.2
D5	15.63
D6	21.88
D7	62.5
D8	125.0
D9	>200
D10	>200
D11	>200
Ascorbic acid	8.85

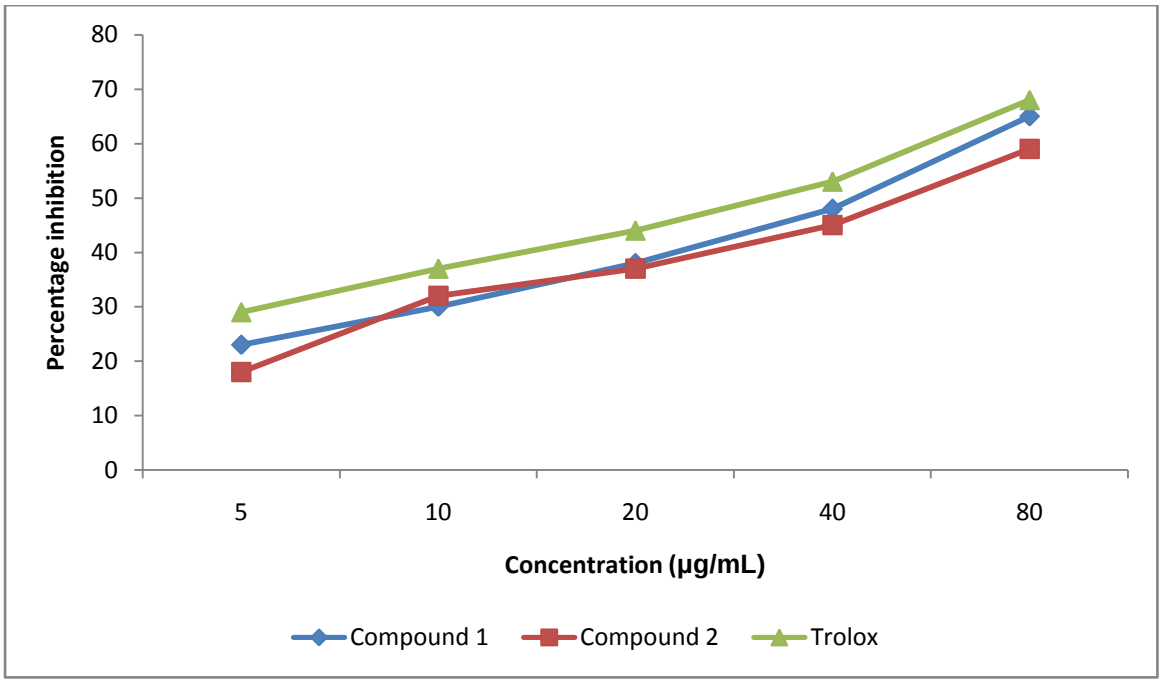


Figure 31: Effect of compounds 1 and 2 on microsomal lipid peroxidation.

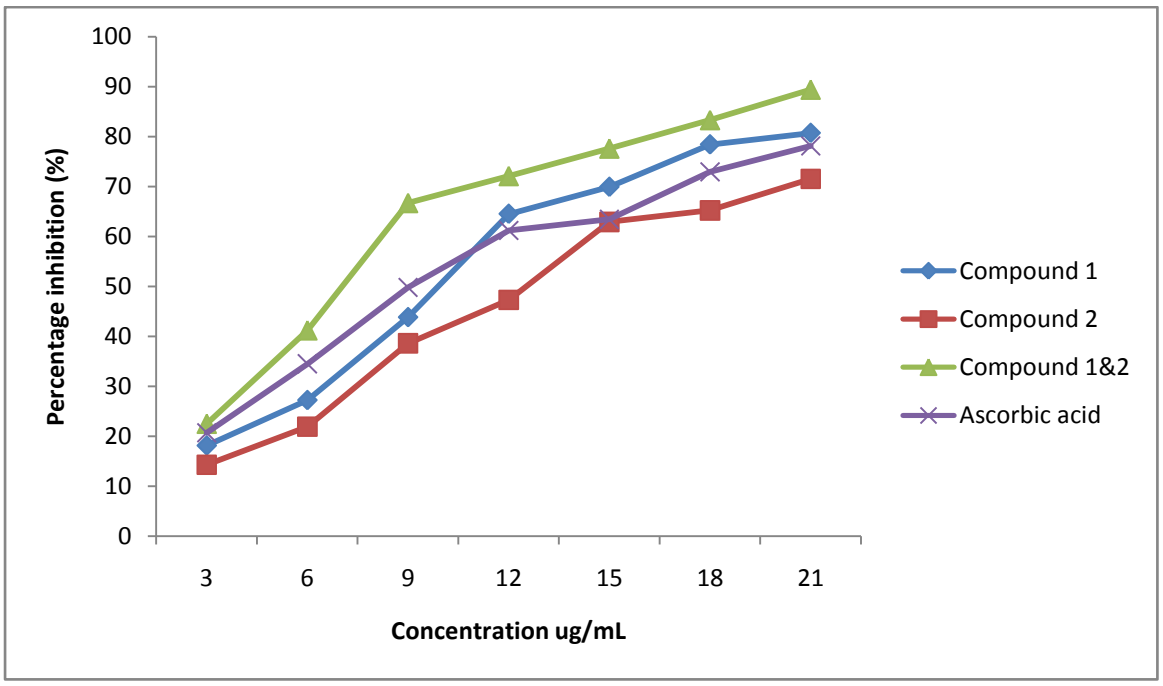


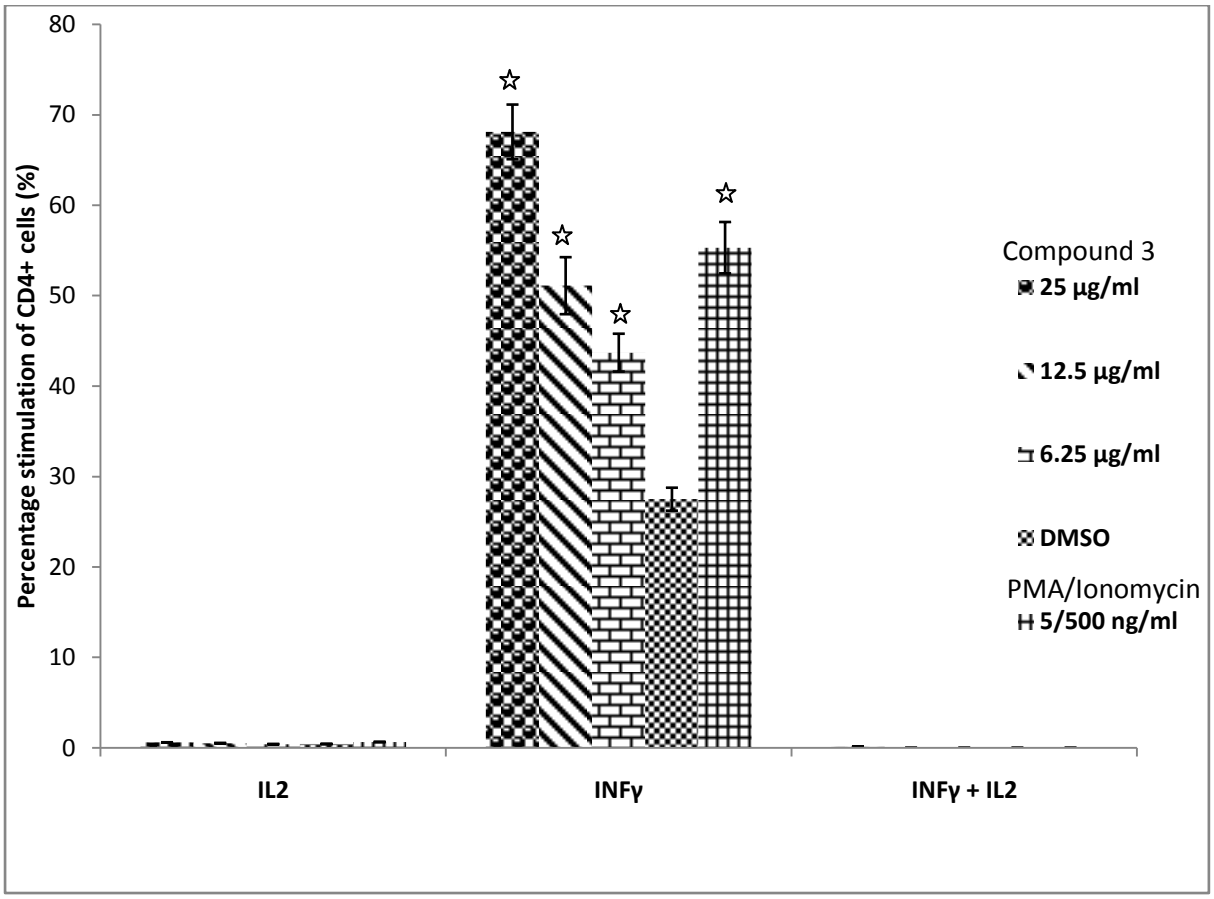
Figure 32: DPPH scavenging activity of compounds 1 and 2

Table 18: EC_{50} of compounds 1 and 2 against DPPH radicals

Compounds	EC_{50}(µg/mL)
Compound 1	9.75
Compound 2	12.5
Compounds 1 & 2	7.0
Ascorbic acid	9.10

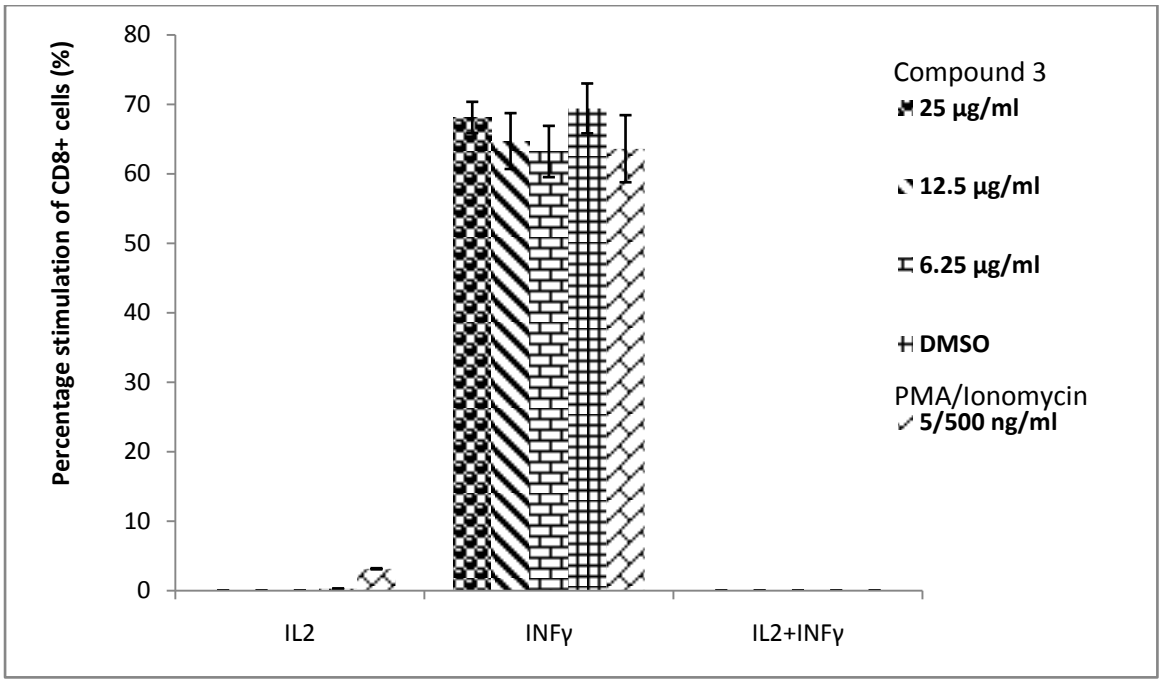
4.9 Immunomodulatory activity of compound 3

There was an up-regulation of specific activated CD_4^+ positive T-lymphocytes that were largely interferon-gamma-releasing in the compound **3** treated groups with only a baseline effect in the DMSO control group (Figure 33). The significant ($p<0.05$) up-regulation was also seen at 6.25 ug/mL. Interleukin 2 (IL2) release was barely seen. Specifically activated CD_8^+ T-lymphocyte was not evident in the compound **3**treated groups relative to control (Figure 34).



n = 3, * $p < 0.05$ compared with DMSO

Figure 33: Effect of compound 3 on CD_4^+ T lymphocytes.



n = 3

Figure34: Effect of compound 3 on CD_8^+ T lymphocytes.

CHAPTER FIVE

DISCUSSION AND CONCLUSION

5.1 Discussion

The great significance of plant secondary metabolites is not only limited to their ecological functions but also have bearing on potential medicinal effects for humans. These phytocompounds may be unique in particular plant species and differ in others. Their relative abundances in most cases as well affect their therapeutic strength (Briskin, 2000). Therapeutic uses of *M. aboensis* may be connected to its abundant phytocompounds most of which have been reported by others studies (Onyegeme-Okerenta *et al*., 2013) although with varying relative abundances which might be explained by time and location of sample collection.

Natural antioxidants have been demonstrated to be beneficial in maintenance of healthy living and in amelioration of various diseases (Mishra *et al*., 2012). Due to the rapid, simple and reproducibility of DPPH test, it has over time become a test of choice for screening potential antioxidant substances (Kedare and Singh, 2011). Hydrogen peroxide, though a weak oxidizing agent, can readily react with Fe^{2+} and Cu^{2+} to form hydroxyl radical which mediates most of its toxic effects (Kumaran and Karunakaran, 2007). Addition of hydrogen peroxide to cells in culture can lead to transition metal ion-dependent hydroxyl radical-mediated oxidative damage (Halliwell and Poulsen, 2006, Gulcin *et al*., 2010). Nevertheless, hydrogen peroxide can produce direct deleterious biological effects like oxidation of essential thiol (-SH) groups. Its rapid passage through biological membrane barriers increases it chances of exerting biological oxidative toxicity (Nishaa *et al*., 2012). Scavenging of these radicals by the extract and fractions of *M. aboensis* are all indications of their antioxidant potentials.

Direct correlation of the antioxidant activity of most substances to their reducing capacity has made ferric reducing antioxidant power assay a reliable method of studying the potential antioxidant activity of various compounds (Firuzi *et al*., 2005). The reducing ability of the extract and fractions demonstrate the electron donor properties of *M. aboensis* in neutralizing free radicals which is capable of terminating radical chain reactions that may otherwise lead to a very damaging effect to cells and other biomolecules.

Linear strong correlations have been established to exist between total phenolic content and free radical scavenging activities of antioxidant substances (Gorinstein *et al*., 2004; Rabeta and Nur, 2013). The extract contained abundant phenolic compounds that were found to concentrate in the ethyl acetate fraction – partitioned between the polar and non-polar fractions. The variable total phenolic content of the fractions demonstrated the influence of solvent on the extractability of phenolic antioxidant compounds. Several other studies indicated that phenols are moderately polar compounds that tend to accumulate in solvent fraction of medium polarity such as ethyl acetate (Wei *et al*., 2010; Mariem *et al*., 2014). Results from this study showed good association between the total phenolic content of the extract and fractions and their *in vitro* antioxidant activities suggesting that *M. aboensis* could owe its antioxidant activity to its abundant phenolic constituents. The antioxidant properties of phenolic compounds have been reported to be due to their redox potentials which allow them to act as reducing agents and hydrogen donors (Naguib *et al*., 2012). The amount of phenolic compounds and/or the nature of these compounds with respect to their redox potentials may have accounted for the *in vitro* differential antioxidant activities of the extract and fractions of *M. aboensis*.

For the determination of effective dose of the extract, lipid peroxidation assay was chosen because it is considered the main molecular mechanism involved in the oxidative damage to

cell structures and in the toxicity process that leads to cell death (Negre-Salvayre *et al*., 2010). One of the manifestations of ethanol induced oxidative stress is peroxidised lipids (Miller, 2013). Ethanol induced lipid peroxidation have been found not to be restricted to the liver, where ethanol is actively oxidized, but also at extra-hepatic tissues including the central nervous system, heart and the reproductive system (Beecroft *et al*., 2010).

The liver plays an important role in the toxic biotransformation of CCl_4 and as such a local specific site of CCl_4 induced oxidative damage. Among known hepatotoxicants, numerous studies have demonstrated CCl_4 as the best characterized model of xenobiotics and oxidative stress induced hepatotoxicity (Rahmat *et al*., 2014). Hepatic mediated metabolism of CCl_4 results in formation of radical intermediate such as trichloromethyl (CCl_3.) radical that induces liver damage particularly through lipid peroxidation. Elevation of serum liver function marker enzymes activities after oral administration of CCl_4 was an indication of cellular leakage and loss of the functional integrity of the cell membrane of the liver cells. Among the aminotransferases, serum alanine aminotransferase (ALT) originates rather specifically from hepatocytes (Nyblom *et al*., 2006) while aspartate aminotransferase apart from hepatocytes may also originate from heart and skeletal muscle tissues and as such is often seen as a supportive biomarker in liver damage (Alatalo, 2011). Alkaline phosphatase which is a liver membrane associated enzyme is also a liver damage marker enzyme and a specific indicator of biliary dysfuction. The findings from this study confirmed previous observations on hepatocellular damage in CCl_4 toxicity (Saba *et al*., 2010; Ozturk *et al*., 2012).

Liver lipid peroxidation is a known oxidative effect of CCl_4(Moreira *et al*., 2014). Trichloromethyl radical formed during hepatic biotransformation of CCl_4 in the presence of oxygen is transformed into trichloromethyl peroxyl radical (CCl_3OO.) which can bind covalently to macromolecules and induce peroxidative degradation of membrane lipids that

are rich in polyunsaturated fatty acids. This leads to the formation of lipid peroxides that give rise to products such as malondialdehyde. The increased MDA in the liver of CCl_4-treated rats suggested that the natural antioxidant defence mechanism of the liver to scavenge excessive free radicals has been compromised. This was demonstrated by the reduction in antioxidant defence enzymes (Catalase and superoxide dismutase). These protective enzymatic factors have been found to decrease under conditions of oxidative stress (Halliwell and Gutteridge, 2007).

The choice of liver microsome for the evaluation of inhibition of lipid peroxidation by the isolated compounds was informed by the fact that microsomal membranes are particularly susceptible to lipid peroxidation owing to the presence of high concentrations of polyunsaturated fatty acids coupled with the presence of the transition metal - iron (Devasagayam *et al*., 2003). High protein concentration of the isolated microsome was an indication that the handling and isolation processes did not denature residual proteins. The inhibition of liver lipid peroxidation by the isolated phenolic compounds from ethyl acetate fraction was an indication of their antioxidant activity.

Hepatoprotective effects of phenolic compounds against various toxicants particularly those mediating their toxicity through oxidative or reactive radical attack have been documented (Maheshwari *et al*., 2011; Fernando and Soysa, 2014). Not only that these compounds have been established to have direct protective effect through scavenging of reactive free radicals, they also have strengthened the hepatic antioxidant defence system. While hepatotoxicity by CCl_4 has been found to be partly due to CYP2E1 mediated free radical catalysis (Jeong *et al*., 2013), phenolic compounds have been shown to disrupt the upregulation of CYP450 isoenzyme thereby intervening in the early process in the cascade of events leading to CCl_4 induced liver injury (Ekstrand *et al*., 2015). Upregulation of mRNA transcriptional expression of SOD and CAT through activation of nuclear factor erythroid 2 – related factor

2 (Nrf2) has also been reported as part of the mechanisms of phenolic compound mediated antioxidant enzyme gene induction (Yeh and Yen, 2006). Elevation of hepatic antioxidant defence system by the extract and fractions in the order of their phenolic compound abundance may have been mediated through genetic induction of these enzymes.

Oxidative stress and oxidative damage to tissues are common end point of diabetes and have also been found to be implicated in pancreatic cell destruction by diabetogenic drugs like STZ (Aboonabi *et al*., 2014). STZ is known to cause pancreatic β-cell damage whereas NAD partially protects insulin secreting cells against STZ complete damage (Szkudelski, 2012). STZ-NAD – induced diabetes therefore mimics type II diabetes mellitus. Many biochemical pathways associated with hyperglycemia, such as glucose autooxidation, polyol pathway, prostanoid synthesis and protein glycation increases ROS production (Busik *et al*., 2008).

Phenolic compounds particularly catechins have been shown to increase plasma total antioxidant capacity, attenuate stress-sensitive signalling pathways and prooxidant enzymes and induced antioxidant enzymes like catalase and superoxidase dismutase (Crespy and Williamson, 2004). Similarly, there is increasing evidence that as antioxidants, polyphenols protect cell constituents against oxidative damage and therefore through such mechanism limit the risk of disease conditions associated with oxidative stress (Pandey and Rizvi, 2010). Pandey and Rizvi (2009) also reported that consumption of antioxidants has been associated with reduced levels of systemic oxidative damages. The results of the *in vivo* oxidative damage protection by the extract and fractions of *M. aboensis* just like in *in vitro* studies maintained the same trend of activity according to their phenolic compound abundance. These observations went further to support our earlier hypothesis that the antioxidant effect of this plant could be linked to their phenolic phyto-compounds.

Proanthocyanidines – oligomeric compounds formed from catechin and epicatechin molecules – are powerful antioxidant agents. The lower bond dissociation energies of their O-H groups and the several O-dihydroxy phenolic groups increase their reducing potentials and metal ion complexing capacity respectively (Porto *et al*., 2003). Also their corresponding oxidised forms acquire additional stabilization due to the extensive electron delocalization induced by the catechol unit on the aromatic β-ring (Rice-Evans *et al*., 1996). Catechin component of the procyanidines has been demonstrated to possess better activity than epicatechin (Liao and Yin, 2000) and may have accounted for the higher DPPH scavenging and inhibition of lipid peroxidation by compound **1** relative to compound **2**.

In contrast to most synthetic pharmaceuticals, phytomedicines exert their therapeutic effects through additive or synergistic action of several chemical compounds acting at single or multiple target sites or through related or different mechanisms associated with a physiological process. The synergistic antioxidant activity of the two compounds isolated from the ethyl acetate fraction of *M. aboensis* showed that the therapeutic effects of this plant may not depend entirely on a single phytocompound. Equal ratio combination of catechin and epicatechin has been documented to produce enhanced antioxidant activity (Kadoma and Fujisawa, 2011) which is in agreement with the observed synergism of the procyanidines isolated in this study. Enhanced antioxidant interaction of cathechin has also been documented with other antioxidant natural products. Synergistic anticancer activity was observed when it was combined with curcumin (Manikandan *et al*., 2012) and when combined with quercetin, it was shown to synergistically inhibit hydrogen peroxide mediated platelate formation (Pignatelli *et al*., 2000). Synergistic interaction of catechin and epicatechin gallate has also been demonstrated both *in vitro* and *in vivo* (Qin *et al*., 2013). Epicatechin interacts synergistically with epigallocatechin gallate producing enhanced antioxidant effects (Suganuma *et al*., 1999).

Procyanidines from grape, buckwheat and hop have all been reported to show strong antioxidant activities ranging from electron donation to mediating in lipid peroxidation processes (Jeong and King, 2004). Catechin and epicatechins have been shown to scavenge various forms of free radicals including DPPH, superoxide anion, hydroxyl, nitric oxde and alkyl peroxyl radicals and to supress array of signalling molecules that mediate endotoxin induced liver injury (Bharrhan *et al*., 2011, Yetuk *et al*., 2014). Thus, it is likely that these compounds combat oxidative stress through many mechanisms that may account for their overall effect on oxidative stress mediated disease conditions.

Oxidative stress adversely affects immune system which could manifest in disease state. The major agents of adaptive immunity are the lymphocytes, antibodies and the molecules they produce which mediate cellular and humoral immune responses (Monzavi-Karbassi *et al*., 2013).This up-regulatory activity of *M. aboensis* on both primary and secondary antibody synthesis may be responsible for its protective effect against disease causing organism thus its traditional use for general body healing. Subsequent (secondary) antigenic stimulation of priorly-sensitized animals resulted in higher secondary antibody production which was expected since there may be expanded clone of cells with memory of the original antigen available to proliferate into mature plasma cells (Uribe *et al*., 2011). These memory cells are capable of producing faster and more intense immune responses. Higher expression of IgG_1 relative to IgG2a is an indication that the *M. aboensis* is capable of modulating thymus mediated immune responses against proteins and polypeptide antigens. The chief function of antibodies is to protect against infection by either direct effect on the infectious agents and their toxic products or indirectly through activation of components of innate resistance including complements and macrophages (Casadevall and Pirofski, 2006). The extract and its fractions may thus be beneficial in immune disorders that arise secondary to infection or

other external agents and can thus minimize the risk of diseases associated with infectious agents.

Leukocytes are important part of body's defense against infectious organisms and foreign substances. Each of the several differential types of leukocytes provides specific and contributive function in defending the body against infections. Neutrophils defend against bacterial or fungal infections as the most common first responders to microbial infections (Goldsby *et al*., 2000). Lymphocytes on the other hand, perform multiple immune tasks through production of antibodies that protect against infections, blocking of pathogenic invasion, activation of complement system and enhancement of pathogenic destruction (USC, 2006). Monocytes basically engulf apoptotic cells and pathogens as well as produce immune effector molecules (USC, 2006).

Immunodeficiency resulting from decreased leukocytes have been found to occur in autoimmune disorders and also secondary due to infections and medications (Fried and Bonilla, 2009). The ability of the extract and fractions of *M. aboensis* to have increased total and differential leukocyte count following cyclophosphamide induced leukopenia is an indication that they may be beneficial in modulating immunodeficiency where leukopenia is implicated.

Polyphenols from different sources have been shown to up regulate immune function through modulation of gene expression of factors that play key roles in activation and differentiation of cell types involved in immune function (Cuevas *et al*., 2013). Also adjuvant activity of phenolic compounds has been demonstrated to be by both increase in cellular and humoral immune responses (Fischer *et al*., 2010). However, the presence of polyphenols alone may not explain the entire immunomodulatory activity of the fractions since butaol fraction which had lower total phenolic content relative to ethyl acetate fraction was found to exhibit better

activity. The additive or synergistic contributions of other phytocompounds in butanol fraction may explain higher immune-stimulating activity of the fraction. Alternatively, based on the phenolic compounds, the nature, type and class of phenolic compounds which differentially distributed in these solvents of varying polarities may account for the higher activity exhibited by butanol fraction.

Among the various cytokines use as signaling molecules in immune system, interleukin-12 has been shown to possess multiple biological properties that govern immune effector actions against a variety of infections and malignancies (Lasek *et al*., 2014). Its immunotherapeutic role is anchored on its central role in regulating innate and adaptive immune responses in addition to its ability to synergize with several other cytokines for increased immunoregulatory activities (Hanza *et al*., 2010). Animal and human studies have also shown improved outcomes in treating or preventing infections based on the mechanism of IL-12 dependent therapies (Hanza *et al*., 2010). IL-12 induces the production of INF-γ. This effect of IL-12 was obtained in this study with concomitant increase in both cytokines. IFN-γ is also known to regulate both innate and adaptive immunity (Schroder *et al*., 2004).

Numerous *in vivo* studies have substantiated the importance of IL-17A in antimicrobial responses involving bacterial, fungal and viral infections. The importance of this cytokine has been shown by the impairment of host defence pathways in the deficiency of IL-17A (Pappu *et al*., 2011). IL-4 produced by TH2 cells is well known for its protective immunity against helminths and other extracellular parasites. Antibodies to IL4 also inhibit allergic response.

The ability of the butanol fraction to up regulate the production of IL12 and TNF-γ which are Th1 cells and IL4 cytokines, secreted by Th2 cells, indicated that *M. aboensis* can promote both cell-mediated immunity that helps in the clearance of damaged cells and humoral immunity that protects against extracellular invaders. These findings which were supported

by the humoral and cellular activity of the extract and fractions particularly the butanol fraction coupled with the up-regulation of other cytokines that also mediate immune responses may in part explain the traditional use of *Millettia aboenis* for general body healing.

Quercetin-3O-rutinoside is among the major immunological leads from medicinal plants (Mukherjee *et al*., 2014). The identification and isolation of isomeric mixture of this compound and Quercetin-3O-robinobioside as the major immunomodulatory compounds of *Millettia aboensis* and its activity on interferon-gamma-releasing CD_4^+ T-lymphocytes agrees with other studies done with this compound. It has been reported to induce an increase in lymphocytes with intracytoplasmic IFN-γ as well as the synthesis and secretion of IFN-γ (Nair *et al*., 2002). Its stimulation of lymphocyte basal proliferation as well as supporting T cells have also been documented (Valentova *et al*., 2015). Long term administration of quercetin was reported to improve splenic immune cell proliferation as well as significant increase in $CD4^+/CD8^+$ response in radiation induced immunosuppression (Jung *et al*., 2012). Modulation of epigenetic factors is part of the documented mechanisms of quercetin induced upregulation of immune function (Lee *et al*., 2011).

The up-regulatory effect on the CD_4^+ T-lymphocyte population of the adaptive immunity by quercetin-3O-rutinoside may be expected to positively improve the functionality of other cells of the adaptive immunity, and some cells of the innate immunity due to its helper functions. This in turn would enable the host mount a better and more avid immune response in the fate of infections or other immune driven diseases.

5.2 Conclusion

The results of this study revealed that *M. aboensis* possesses antioxidant activity through both direct scavenging of reactive species and through boosting of endogenous antioxidant system in albino rats. Enhancement of both the innate and adaptive immune defense responses is suggestive of the immunostimulant potentials of this plant. Therefore, free radical scavenging and immunostimulation might be the mechanisms through which the general body healing properties of *M. aboensis*occurs. The active compounds responsible for the antioxidant effectswereoligomeric phenolic compounds epicathechin-(2β→O→7, 4β→8)-cathechin (Procyanidine A1)and epicathechin-(2β→O→7, 4β→8)-epicathechin (Procyanidine A2) that act in synergy while isomeric mixture of quercetin-3O-rutinoside and quercetin-3O-robinobiosidewas found to be responsible for the immunoenhancing activities.

5.3 Recommendation

Having established the antioxidant and immune enhancing potentials of *M. aboensis*, further studies on the effect of this plant extract and active compounds on disease conditions directly involving oxidative stress and compromised immune response as major part of their pathogenesis or progressionare recommended to be investigated. Comparative toxicity and efficacy studies with known drugs used in combating oxidative stress and in enhancing immune system is also advocated. Isolation of other minor compounds in the plant extract and their activities as well as interactions are also required to further understand their contributions in the general body healing.

5.4 Contribution to knowledge

This study initiated and provided the scientific basis ofuse of *M. aboensis* in traditional medicine for general body healing. Free radical scavenging and stimulation of immune system were identified as mechanisms of therapeutic effects of *M. aboensis*. The active

compounds responsible for these effects were isolated and charaterised.These compounds were isolated for the first time from the genus *Millettia*.

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Appendix