THE EFFECTS OF *Vernonia amygdalina(Asteraceae)* Delile METHANOLIC LEAF EXTRACT ON MARKERS OF OXIDATIVE STRESS IN MICE TREATED WITH HIGH DOSES OF ACETAMINOPHEN

By:

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A dissertation submitted to the University of Zambia in partial fulfilment of the requirements for the award of the degree of Master of Science in Pharmacology**.**

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APPROVAL

This dissertation of Sitanimezi Mweenda is approved as partial requirement for the award of the degree of Master of Science in Pharmacology by the University of Zambia.

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DEDICATION

This work is dedicated to my supportive and loving husband, Mr Lanco Moono Chikuta, my daughter, Chabilo Chikuta and my sons Namunjwebwa Musale Chikuta and Musa Akufuna Chikuta who have been very tolerant and allowed me long hours of time to dedicate to this work and thus make it a success. Also dedicated to my parents Mr and Mrs Mweenda, who tirelessly encouraged me and supported me to ensure that I can be who I am today.

CERTIFICATION OF COMPLETION

This work has been certified completed by my supervisors.

Supervisor:

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Co-supervisor:

Name:…….. Signature: …… Date:……

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ABSTRACT

Oxidative stress has been associated with many diseases, causing injury on cells, tissues and organs. Due the complications associated with it, conventional medicines have used antioxidants like minerals and vitamins in its management. Several plants have been shown to be rich in substances that possess antioxidant properties and have thus been used in the treatment of many ailments.

To determine the effects of methanolic leaf extract of *Vernonia amygdalina Delile* on Total antioxidant capacity and malondialdehyde levels in mice treated with toxic doses of acetaminophen**.**

The leaves of local varieties of *Vernonia amygdalina Delile* were extracted with 80% methanol, concentrated, dried and re-dissolved in distilled water for use. White albino mice of either sex, weighing between 20 – 31g were randomly separated into 6 groups of 6 mice each. 300 mg of acetaminophen was administered orally to all the groups except group 1(negative control) which received normal saline at 2ml/kg and group 2 (extract only). This was followed by administration of *Vernonia amygdalina Delile* extract to groups 2 and 4 at 50 mg/kg and group 5 at 100 mg/kg, while group 6 was given Vitamin C at 500 mg/kg, all treatments were given orally. After 8 hours blood samples were collected by cardiac puncture, the livers were excised from the animals, homogenised and centrifuged to obtain a supernatant. Whole blood and supernatant of liver were used for biochemical analysis, using Ferric Reducing Antioxidant Power Assay (FRAP) for Total antioxidant capacity and Thiobarbituric-Acid reactive substances (TBARS) Assay for estimating malondialdehyde levels. The results for the Total antioxidant capacity were compared by One-way analysis of variance, while Kruskal Wallis H test was used for the malondialdehyde levels; then these were followed by Turkeys HSD Post Hoc Test, with $p < 0.05$ considered significant.

The administration of *Vernonia amygdalina* Delile, methanolic leaf extract produced an increase in the total antioxidant capacity, Group1 (negative control) [mean blood 0.75U/mg, liver 0.81U/mg] as compared to Group 3 (positive control) [mean blood 0.54U/mg, liver 0.49U/mg, p=0.001]; Group 4 (acetaminophen + *Vernonia amygdalina* low dose) [mean blood 0.64U/mg, liver 0.68U/mg, p=0.001] ; Group 5 (acetaminophen + *Vernonia amygdalina* high dose) [mean blood 0.70U/mg, liver 0.77U/mg, p=0.001]; and Group 6 (acetaminophen + vitamin C)) [mean blood 0.69U/mg, liver 0.75U/mg, p=0.001], and a reduction in the malondialdehyde levels Group1 (negative control) [mean blood 0.75U/mg, liver 0.81U/mg] as compared to Group 3 (positive control) [mean blood 0.54U/mg, liver 0.49U/mg, p=0.001]; Group 4 (acetaminophen + *Vernonia amygdalina* low dose) [mean blood 0.64U/mg, liver 0.68U/mg, p=0.001] ; Group 5 (acetaminophen + *Vernonia amygdalina* high dose) [mean blood 0.70U/mg, liver 0.77U/mg, p=0.001]; and Group 6 (acetaminophen + vitamin C) [mean blood 0.69U/mg, liver 0.75U/mg, p=0.001].

In conclusion, the study showed that the methanolic leaf extract of *Vernonia amygdalina* Delile possesses significant antioxidant properties allowing it to attenuate oxidative stress and lipid peroxidation processes, thus this high antioxidant capacity justifies its use in folk medicine in the treatment of oxidative stress associated diseases like diabetes. This study has also contributed to the scientific validation of the antioxidant properties of *Vernonia amygdalina* plant growing in Zambia.

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ABBREVIATION

FRAP	Ferric Reducing Antioxidant Power
EDTA	Ethylenediaminetetraacetic Acid
BHT	Butylated Hydroxytoluene
DOPA	Dihydroxyphenylalanine
TPTZ	2,4,6-tripyridyl-s-triazine
PBS	Phosphate Buffered Saline
TCA	Trichloroacetic Acid
MDA	Malondialdehyde
TAOC	Total Antioxidant Capacity
TAS	Total Antioxidant Status

DEFINITIONS

Oxidative stress	a term that is used to denote a serious imbalance that occurs between the production of the Reactive Species(RS) and their elimination by the antioxidant mechanisms (Halliwell, 2007).
Antioxidant	is any substance that delays, prevents or removes oxidative damage to a target molecule (Halliwell, 2007).
Reactive Oxygen Species	is a collective term that is used to refer to species that originate from oxygen and are more reactive than Oxygen($O_{2)}$ itself (6).
Free radical	is any molecule which can exist independently and has one or two unpaired electrons (Halliwell, 2005).
Reactive Nitrogen Species	is a collective term that has been used to refer to any species of nitrogen origin and it also includes radicals like nitric oxide (NO) and nitrogen dioxide (NO_2) and non-radicals like HNO_2 and N_2O_3.$ONOO^-$ (Repetto et al, 2012).
Apoptosis	refers to a Programmed cell death (Halliwell, 2005).
Validity	refers to an extent to which a concept is accurately measured in a quantitative study (Heale and Alison, 2015).
Reliability	refers to the consistency of a measurement (DeVon et al, 2007).

CHAPTER ONE

INTRODUCTION

1.1 Background

Oxidative stress results from an imbalance in the rates of production and elimination of reactive species(RS)(Halliwell, 2007). These Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS) are products of normal aerobic metabolism, but in certain pathological conditions such as cardiovascular diseases, diabetes mellitus, chemical toxicity, hepatitis, and cancer(Bhattacharyya et al., 2014, Forbes et al., 2008, Mei et al., 2015), their production increases, and may overwhelm the normal antioxidant defence mechanisms of the body(Rahal et al., 2014). This may result in damage to macromolecules like proteins, carbohydrates, DNA, as well as triggering lipid peroxidation reactions(Halliwell, 2005). Maintaining a balance between the pro-oxidant and antioxidants is extremely important for optimal cellular and biological functions (Ayala et al., 2014).

Oxidative stress is an accompaniment of several disease processes, arising either from high rates of production of reactive species or depletion of the antioxidant stores or both; resulting in adaptation, tissue injury and/or cellular death by necrosis or apoptosis. In adaptation, the tissues upregulate the production of antioxidants; tissue injury results from damage to biological molecules(Halliwell, 2005); cell death may result from disruption of cellular membranes, production of dysfunctional proteins and enzymes, or activation of apoptotic mechanisms(Ayala et al., 2014). It also causes; oxidative biomolecules modification, interference with cellular metabolism, dysregulation of cell cycles due to alteration of signalling pathways, epigenic change, instability of genes and mutations(Thanan et al., 2014).

Uncontrolled Oxidative stress in the body has been shown to cause injury on cells, tissues and organs due to oxidative damage, and high levels of oxidative damage has been associated with direct damage to lipids, a process called Lipid peroxidation(Mei et al., 2015).

Lipid peroxidation reactions occur at the double bonds of polyunsaturated fatty acids (PUFAs), and are considered the major mechanism by which oxidative damage to cell structures takes place(Ayala et al., 2014, Repetto et al., 2012). Peroxidation reactions usually begin with the formation and propagation of lipid radicals, uptake of oxygen, rearrangement of double bonds of unsaturated fatty acids and eventual destruction of the double bonds, leading to the damage of the membrane structure and secondary products generation, like saturated aldehydes such as propanol and hexanal ; 2, 3-trans –unsaturated aldehydes such as hexenal and octenal and a series of 4-hydroxylated -2, 3-transunsaturated aldehydes including malondialdehyde which is now considered to be non-toxic and 4-Hydroxyneonenal (4-HNE) being the most important lipid peroxidation product which causes cellular damage by modulating gene expression, cell proliferation, differentiation, and apoptosis(Ayala et al., 2014).

In order to keep the activities of the ROS and RNS in check and maintain the redox potential of the cells, the body possesses biological and diet derived or exogenous antioxidants which include glutathione, Vitamin C and Vitamin E and also the enzymatic antioxidants (endogenous) which include the Superoxide Dismutase (SOD),Glutathione peroxidase and Catalase(Ayala et al., 2014, Niki et al., 2005, Repetto et al., 2012).

The antioxidants are substrate specific, for instance, superoxide dismutase (SOD) is responsible for the removal of the O^{2-} by converting it to H_2O_2 , and the H_2O_2 gets converted to H_2O and O_2 by Catalase. In humans however, the most important scavenger of the H_2O_2 is Glutathione peroxidase (GSHPX) which has been shown to require selenium for its action while GSH directly scavenges various types of free radicals, and is also a substrate for GSHPX enzymes(Forbes et al., 2008, Repetto et al., 2012).

Since oxidative stress has been found to play an important role in many pathogenic conditions, conventional medicine has used various vitamins and minerals that are available as tablets, as adjuncts to the treatment of diseases associated with oxidative stress. Many vitamins like vitamin C and E have been shown to give successful results (Bhattacharjee et al., 2013, Cichoż-Lach and Michalak, 2014, Di Mascio et al., 1991). However, the search of other sources of these antioxidants has become important as the search for better medicines with fewer side effects continues. Currently plant based antioxidants are now preferred to the synthetic ones as a result of safety issues(Wichi, 1988).

1.2 Pharmacological Properties of *Vernonia amygdalina* Delile

World Health Organisation (WHO) has recommended the use of medicinal plants especially in countries where accesses to conventional treatments are inadequate (WHO, 2008). In folk medicine treatments in Zambia, the use of *Vernonia amygdalina (Delile)* in the treatment of diseases associated with oxidative stress like Diabetes and Cardiovascular diseases has been a practise for a long time now.

Vernonia amygdalina (Asteraceae)Delile commonly called bitter leaf is a shrub that grows up to about 5 meters high, it is found in tropical Africa and other parts of

Africa(Farombi and Owoeye, 2011), including Zambia. It is a plant that possesses many medicinal benefits which have been shown to be due to its constituents which include sesquiterpene lactones like vernolide and vernodalol; phytochemicals like phenolics, flavonoids , hydroxycinnamic acid, steroidal saponins, tannins and alkaloids(Erasto et al., 2007a, Erasto et al., 2007b, Mahammed et al., 2015, Owolabi et al., 2008, Wong et al., 2014). The total phenolics are the compounds which act as free radical terminators or primary antioxidants(Oriakhi et al., 2013), thus very vital in the treatment of oxidative stress associated diseases.

It is the presence of these phytochemicals that gives it the antioxidant properties which have been shown to give it anti-diabetic effects, antibacterial effects, anti-inflammatory properties, analgesic effects, lipid lowering effects and the ability to mitigate irradiation damage in tissues(Adedapo et al., 2014, Bukar et al., 2013, Nwanjo, 2005, Owoeye et al., 2011).

Moreover the plant has been reported to have hypocholesterolmic effects produced by the micronutrients and antioxidant vitamins of the plant(Kate and Lucky, 2012). Hepatoprotective effects reported have been shown to be due to the combination of both the phytochemical and antioxidant vitamin content(Adesanoye and Farombi, 2010, Iwalokun et al., 2006).

Treatment with this plant has been shown to cause an improvement or prevention of ectopic/eczema dermatitis syndrome, due to the presence of Vernodalin the most potent anti-inflammatory(Ngatu et al., 2012). Literature has shown anticancer properties brought about by terpenoid and epivernodalol, the antitumour compounds found in the leaf extract of the plant(Gresham et al., 2008, Kupchan et al., 1969).

1.3 Ethnomedicinal Properties

The plant *Vernonia amygdalina Delile*, has found relevance in folk medicine in the treatment of ailments, in various countries in Africa, and Zambia in particular. Many herbalists and naturopathic doctors have used the aqueous extract of the plant in the treatment of malaria, sexually transmitted infections, emesis, loss of appetite, induced ambrosia, diabetes, gastrointestinal tract infections, dysentery, helminthiasis, loss of appetite, bacterial infections, anorexia, hypertension, fever, fungal infections, cancer, constipation, gynecological uses, and other infectious diseases(Adesanoye and Farombi, 2010, Asante et al., 2016, Farombi and Owoeye, 2011).

The leaves of this plant have been consumed as a vegetable in many African countries and used for treatment of many ailments; while roots are mixed with roots from other plants and are used in many homes for treatment of diseases like gingivitis and toothache(Ojiako, 2006, Kate and Lucky, 2012).

1.4 **Rationale for Study**

The treatment of many chronic diseases like diabetes and cardiovascular diseases has still remained a challenge in Zambia(Goma et al., 2011, Nsakashalo-Senkwe et al., 2011) and many other African countries, this is largely because these treatments are expensive, costly to maintain and thus not affordable for many poor people in developing countries(Bosi, 2009). WHO has estimated that about 80% of people worldwide use traditional medicines as a source of their primary health care(Demain and Sanchez, 2009). Apart from the costly nature of conventional medicines, they are usually not readily available especially in poor developing countries of Africa,

and some have also been shown to possesses a lot of side effects(Igile et al., 1994). In Zambia, Ghana and Nigeria, as in other African countries about 60% of children with fever are given herbal medicines as the first line of treatment, as they are considered to be effective, and cheaper sources of drugs(Bukar et al., 2013). Many medicinal plants have been shown to possess secondary metabolites that confer the preventive and therapeutic uses that they possess. One of the plants that have been used in folk medicine in the treatment of many diseases in Zambia is *Vernonia amygdalina Delile*. Being a medicinal plant that freely grows in many parts of Zambia and has found wide spread use in Zambia over a long period of time in the treatment of chronic diseases associated with oxidative stress. There is need to assess the purported medicinal properties that has led to its use in folk medicine. This plant has also been eaten as a vegetable in some West African countries(Lolodi and Eriyamremu, 2013), and has been shown in literature to have a high safety profile(Ojiako and Nwanjo, 2006). However, despite the plant having been shown to possess many health benefits(Owolabi et al., 2008), literature has shown that the pharmacological properties of this plant differ with location(Oyugi et al., 2009). To the best of my knowledge no study has been done on this plant in Zambia. This study was projected to provide scientific evidence that will justify its use in folk medicine in Zambia as well as provide scientific evidence concerning the antioxidant effects that this plant possesses. The provision of this information is of great value in future prospects for drug development

1.5 Problem Statement

There is a high prevalence of diseases like Hypertension (32%)(Goma et al., 2011), diabetes (4%)(Nsakashalo-Senkwe et al., 2011), HIV/AIDs (12.4%)(2016) and Cancer (3.81%)(2014) in Zambia, all of which are affected by oxidative stress

processes, and their treatment still remains a challenge. Because of the deleterious effects of oxidative stress(Gardner et al., 2014, Halliwell, 2005) on the disease pathology, conventional medicines have used synthetic antioxidants in the treatment of such diseases(Asante et al., 2016, Karahan et al., 2005).

However, significant evidence is available showing the use of natural products like plants as antioxidants, in the treatment of such diseases(Iwalokun et al., 2006). Currently these plant based drugs are preferred over the conventional ones because they are readily available, cheaper, biodegradable, safe and with less side effects (Bukar et al., 2013, Mahammed et al., 2015, Rizvi and Mishra, 2013, Udaya kumar et al., 2009, Malik et al., 2009). A medicinal plant called *Vernonia amygdalina Delile,* has been used in folk medicine in Zambia, in the treatment of diseases associated with oxidative stress from time in memorial(Gresham et al., 2008). There is yet no evidence of the antioxidant properties of Vernonia amygdalina grown under Zambian climatic conditions. This study attempts to provide scientific evidence of its antioxidant properties.

1.6 Research Questions

Based on the above problem statement, the following research questions were raised:

1. Does the methanolic leaf extract of *Vernonia amygdalina* enhance the total anti-oxidant capacity in blood and liver samples of acetaminophen induced toxic models of mice?
2. Can the methanolic leaf extract of *Vernonia amygdalina* reduce levels of malondialdehyde, a marker of lipid peroxidation, in acetaminophen induced toxic mice models?

3. Is the effect of *the* methanolic leaf extract of *Vernonia amygdalina Delile* on markers of oxidative stress, dose dependent**?**

1.7 Hypothesis

This study is determined to prove the following hypothesis:

Leaf extract of *Vernonia amygdalina Delile* enhances antioxidant capacity and reduces markers of lipid peroxidation during acetaminophen overdose in mice.

1.8 Significance

The significance of this study is as follows:

Oxidative stress is an accompaniment for many diseases. Its management currently depends on the use of refined medicines like Vitamin C, E or antioxidant chemicals like selenium. Given the number of diseases associated with oxidative stress and also the chronic nature of most of these diseases, heavy economic burdens of their management are quite significant on the individuals and the society as a whole. This is especially relevant in the poor developing countries of Africa. Therefore, cheaper natural sources such as medicinal plants could provide economic relief and reduce out of pocket spending for many patients. This study seeks to demonstrate the potential relevance of *Vernonia amygdalina Delile,* a plant used in folk medicine, in the attenuation of markers of oxidative stress induced by acetaminophen toxicity. Thus justifying its wide spread use in folk medicine in Zambia, and also provide not only a cheaper but also a readily available, and effective source of treatment(Iyamah et al., 2014). This might be useful

information in the design of interventions to prevent these processes and their deleterious effects in disease.

1.9 Aim

The aim of this study was to determine the effects of *Vernonia amygdalina* Delile leaf extract on the total antioxidant capacity and the malondialdehyde levels in mice treated with high doses of acetaminophen.

1.10 Specific Objective

The specific objectives of this study are:

1. To determine the effects of leaf extracts of *Vernonia amygdalina* Delile on total antioxidant capacity in the blood and liver of mice exposed to high doses of acetaminophen.
2. To determine the effects of *Vernonia amygdalina* Delile leaf extract on liver and blood concentrations of malondialdehyde as a marker of lipid peroxidation in mice exposed to high doses of acetaminophen.
3. To determine if these effects are dose dependent.

1.11 Theoretical Grounding and Conceptual Framework

1.11.1 Theoretical Grounding

Oxidative stress has been shown in cases of disease, drug overdose and other stressors, leads to an increase in the production of the reactive species (RS) with a resultant depletion of the antioxidant species (Repetto et al., 2012). The oxidation of lipids causes a destruction of the cell membranes contributing adversely to disease, as a result of cell injury and cell death(Ayala et al., 2014, Halliwell, 2005). In

conventional medicines, antioxidants like vitamin E, C and selenium have been used for the treatment of diseases associated with oxidative stress like diabetes and cardiovascular diseases (Di Mascio et al., 1991).

Nevertheless, plants are still being used as sources of alternative drugs, as they have an advantage of being cheaper and effective sources of treatment (Demain and Sanchez, 2009, Mahammed et al., 2015). The plant *Vernonia amygdalina* has been used for the treatment of oxidative stress associated diseases (Asante et al., 2016, Sies et al., 1992). This plant, has been shown to possesses phytochemicals such as phenolics, flavanoids, saponins, tannins which gives the plant the pharmacological properties it possesses (Adedapo et al., 2014, Iwalewa et al., 2007). The flavanoids and phenolic compounds are consistent with the antioxidant effects that the plant possesses (Mahammed et al., 2015).

In cases of oxidative stress, administration of the plant extract of *Vernonia amygdalina* causes an increase in the antioxidants, which leads to a decrease in the ROS/RNS by wiping them out (Iwalokun et al., 2006, Offor, 2014). This then leads to decreases in oxidative stress and lipid peroxidation processes, which decreases the cell injury and cell death, as a result of cell repair (Atangwho et al., 2009, Iwalewa EO et al., 2005). This then prevents the adverse effects on disease. For the illustration of this information, see figure 1 below.

1.11.2 Conceptual Framework

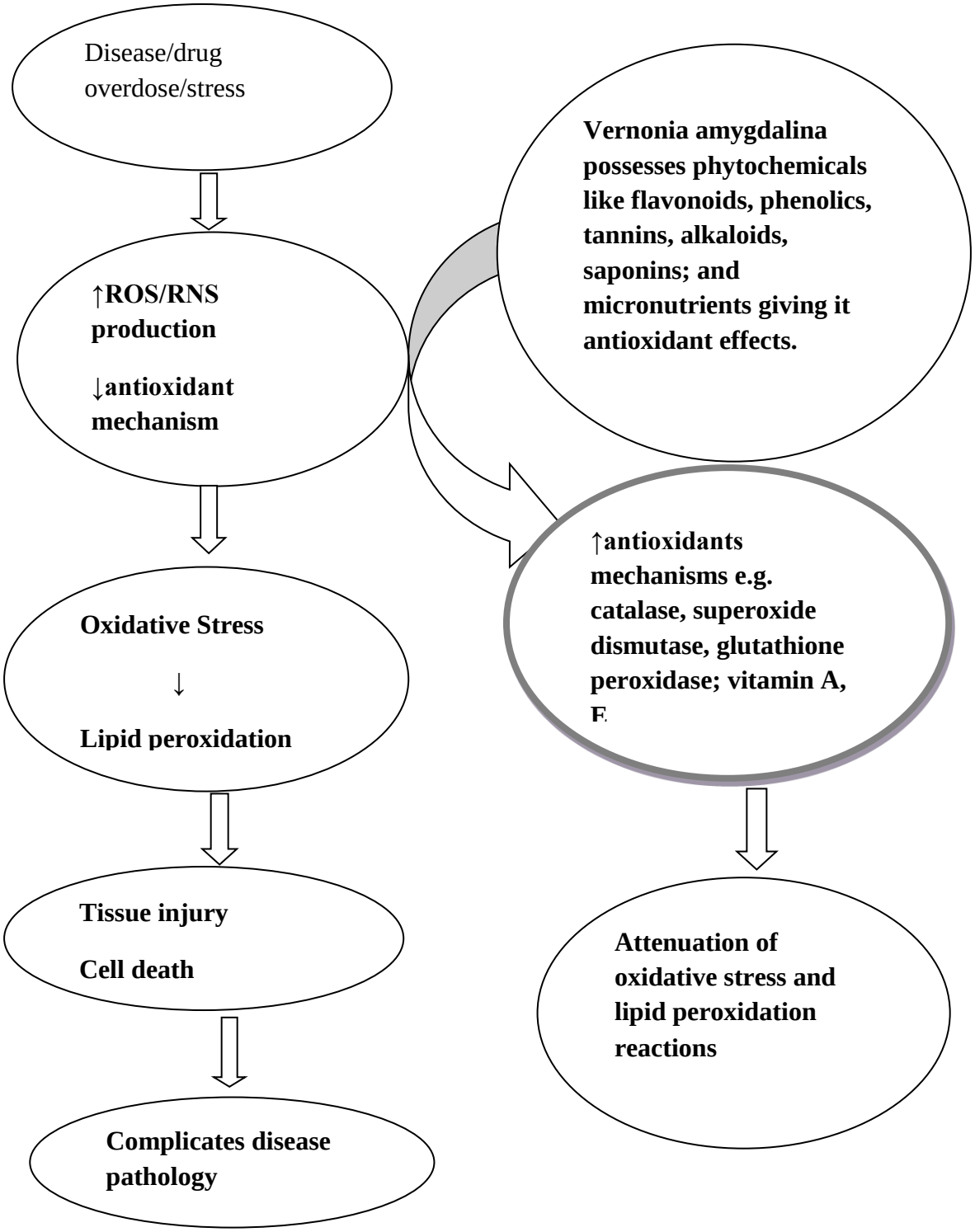


Figure 1 - Consequences of oxidative stress on disease and the attenuating effects of *Vernonia amygdalina Delile* on oxidative stress. As adapted from Gil-Mohapel et al(Gil-Mohapel et al., 2014), Ayala et al (7)Iwalewa et al(Iwalewa et al., 2007), Ojieh et al(Ojieh et al.), Atangwho et al(Atangwho et al., 2009), Adaramoye et al(Adaramoye Oluwatosin et al., 2008).

1.12 Validity and Reliability

Validity is the extent to which the measurements or conclusions of the experiment is well founded and corresponds to the real situation (Heale Roberta and Alison, 2015). **Reliability** on the other hand refers to the ability of an instrument to consistently measure an attribute (DeVon et al., 2007). In this study validity and reliability were ascertained as the following:

The study used tried and tested procedures from literature which have been clearly elaborated in the methodology section. This study used controls as part of the experimental design to ensure precision in the results obtained. Moreover, the study included both the positive and negative controls, for comparison with the experimental groups. The study was made up of two experimental groups, which included the one that received a high dose and the one that received a low dose of the extract. The cause-effect principle was established in that an increment in the dose caused a higher response. Randomization in sampling was done, which allows an equal distribution of any confounding factors. Acclimatizing the mice to the environment prior to the start of the study ensures that the testing environment does not influence the behaviour of the study subjects. Since research is designed to test statistical significance of the results with minimal participants or animals, the sample size used in this study was obtained from published data(Iwalokun et al., 2006). Statistical tests were used to maximize the data generated from each participant.

1.14 Ethical Considerations

The study was approved by ERES Converge in Lusaka, an independent ethical review board. The ethical review reference number was 2015-May-013.

Permission to do the study was granted by the University and departmental management of the University of Zambia.

All the animals were handled humanely by qualified personnels who are all members of the University of Zambia staff. Moreover the mice were kept in an hygienically good environment, monitored by a trained veterinarian, kept in well ventilated cages, and the pain and sufferings were minimised, and the most minimal number that allows for inference of the causal-effect relationship was used in accordance with internationally acceptable regulations for the care and use of laboratory animals as given by the National Institute of Health (NIH) (Care et al., 1985).

CHAPTER 2

LITERATURE REVIEW

2.1 Overview

Oxidative stress is a tilt towards the reactive species in the oxidant-antioxidant balance that takes place in cases of human diseases, and other stressors like ingestion of non-steroidal anti-inflammatory drugs (NSAIDs), cigarette smoking, alcohol consumption and ultraviolet radiation(Bhattacharyya et al., 2014, Halliwell, 2005). This is of great importance as tissue injury is important in some diseases and also in prospects of development of drug intervention(Halliwell, 2005).

Oxidative stress occurs as a result of accumulation of the free radicals at a rate that is higher than the rate of their removal from the system by the antioxidant system) (Halliwell, 2007). Various free radical species that are responsible for biological toxicity of oxygen are intermediates of oxygen partial reduction and these include the following radicals, superoxide (O_2^-), hydrogen peroxide (H_2O_2), peroxyl (ROO^-), hydroxyl(OH^-) , nitric oxide (NO), peroxynitrite ($ONOO^-$) and singlet Oxygen (O_2^-)(Repetto et al., 2012). These highly reactive species are produced as a normal consequence of very important biochemical processes in the body(Ayala et al., 2014).

2.2 Mechanisms of Lipid peroxidation

When free radicals are in high levels they cause damage to lipids, by attacking the lipids containing Carbon-Carbon double bonds, more so the polyunsaturated fatty acids, giving rise to lipid hydroperoxides (LOOH) as the main primary products; and

secondary products which include various aldehydes(Ayala et al., 2014, Repetto et al., 2012). The free radicals cause harm to macromolecules like DNA, proteins and lipids(Halliwell, 2005). DNA reactive aldehydes such as malondialdehyde, acrolein and 4-HNE cause damage to the DNA directly by reacting with the DNA bases or indirectly by producing more reactive bifunctional intermediates that form exocyclic DNA adducts(Repetto et al., 2012).

Various mechanisms have been involved in the lipid peroxidation reactions. The first stage in the lipid peroxidation chain reaction is the initiation stage, which involves the attack of a free radical causing the abstraction of a hydrogen ion from the methylene group of an unsaturated fatty acid, which is left with one electron and is stabilised by rearrangement of the molecule forming a conjugated diene(Ayala et al., 2014, Kohen and Nyska, 2002). This fatty acid radical so formed in the presence of oxygen reacts to form an $ROO^{\cdot}$ in a propagation stage(Niki et al., 2005). These radicals so formed are capable of undergoing the same process by abstracting a hydrogen ion from neighbouring unsaturated fatty acids that undergo the same reaction allowing for the continuity of the lipid chain reaction(Ayala et al., 2014). $ROO^{\cdot}$ becomes a lipid hydroperoxide that decomposes to an aldehyde, hydrocarbon or form cyclic endoperoxides and hydrocarbons(Kohen and Nyska, 2002, Repetto et al., 2012). With a single initiation reaction all the unsaturated lipids in the membrane, undergo peroxidation. The end stage called the Termination stage only takes place when the ROO- interacts with another radical or with an antioxidant(Kohen and Nyska, 2002).

The polyunsaturated fatty acid (PUFAs) are oxidised using both enzymatic and non-enzymatic pathways(Niki et al., 2005). Once the lipid hydroperoxides have been formed they are targeted by different reduction reactions which result in either

inhibition of peroxidation reaction using enzymes like selenium dependent Glutathione peroxidation (GPx) and selenoprotein P (SeP) or induction of the peroxidation reaction, in which lipid hydroperoxides are converted to intermediates of oxygen radicals like lipid peroxyl radical(Ayala et al., 2014). These radicals lead to activation of the lipid peroxidation chain reactions(Di Mascio et al., 1991).

Non-enzymatic pathways involves autoxidation in which a chain reaction driven by a free radical, induces the oxidation of lipids, especially phospholipids containing polyunsaturated fatty acids, (PUFAs) and this occurs by homolysis of endogenous hydroperoxides through the production of RO^- and ROO^- from ROOH(Repetto et al., 2012).

In the Fenton reaction transition metals like Fe(2^+) and copper (Cu) are oxidised generating HO^- and OH^- from H_2O_2, and it is the generation of OH^- that accelerates the lipid peroxidation reaction(Bucher et al., 1983).

2.3 Occurrence of Oxidative Stress

It is important to note that occurrence of oxidative stress has been associated with disease, smoking, use of certain drugs, and in drug overdoses. Moreover, in all these cases it has been associated with deleterious effects on disease pathology.

2.3.1 Oxidative Stress and Disease

Imbalance in oxidant and anti-oxidants, results in the pathophysiological mechanisms of diseases.

The effects of oxidative stress in pulmonary artery disease (PAD), have shown in the decrease in antioxidants with an increase in ROS, and an associated impairment of the endothelial function due to apoptosis and endothelial inflammation(Gardner et

al., 2014). As for systemic lupus erythematosus, many oxidative modifications of biomolecules have been evident in biological fluids of the patients and this has been proportional to the organ and disease damage(Shah Dilip et al., 2014). Similarly, in chronic obstructive pulmonary disease increased inflammation and excessively high levels of ROS from mitochondrial, have contributed to the hyperproliferation and inflammation that occurs(Wiegman et al., 2015). Similarly for Huntington's it has been noted that mitochondrial malfunction, high levels of ROS, oxidation of proteins, lipids and DNA as important factors in its pathology(Gil-Mohapel et al., 2014).

Furthermore, in Alzheimer Disease (AD), evidence of oxidative stress has shown by the presence of modified macromolecules in the brain and CSF, reduced antioxidants in the blood, brain and CSF and large numbers of redox-active metals in the brain enhancing formation of ROS(Persson et al., 2014) .

Oxidative stress has been linked to the glycaemic variability that occurs between diabetes and Cardiovascular disease; with high levels of ROS and oxidative stress being more pronounced in hyperglycaemia(Saisho, 2014). Literature has also shown that glucose deprivation of myocytes of the cardiac tissue leads to overproduction of ROS by the dysfunctional mitochondria, triggering oxidative stress; and the resulting oxidative stress in diabetes and hypertension, sensitizes the heart to rennin-angiotensin-aldosterone system that leads to autophargic apoptosis, causing an increase in cardiac remodelling and dysfunctional(Mei et al., 2015).

Chronic Kidney disease (CKD), on the other hand has lipid peroxidation chain reactions causing the production of abundant oxidized particles which disseminates oxidative stress and leads to the promotion of cardiovascular disease, progression of

CKD, cachexia, malnutrition, anaemia, inflammation and other morbidities associated with this disease(Vaziri, 2014).

2.3.2 Effects of Drugs on Oxidative Stress

Another important cause of oxidative stress has been the mere use of certain drugs in the treatment of disease, this has been shown e.g. antitumor drugs(Forrester et al., 2007, Pillai et al., 2007), and also antibiotics for instance Gentamycin(Derakhshanfar et al., 2013, Karahan et al., 2005).

Certain drugs, which are quiet helpful in the treatment of cancer, have led to the development of oxidative stress in the patients taking them. Concerning drug induced stress it has been noted that out 132 anticancer drugs that have been approved for use by the Food and Drug Agency (FDA) about 52 have been reported to induce oxidative stress including anthracyclines, cyclophosphamide, cisplatin, busulfan and mitomycin(Chen et al., 2007). Drugs used in cancer chemotherapy and radiotherapies work through generation of ROS which causes destruction of malignant cells through apoptosis; and this is shown by increases in lipid peroxidation, reductions in the antioxidants like GSH during therapy(Bhattacharyya et al., 2014).

2.3.3 Poisoning and Oxidative Stress

Poisoning in animals has shown increases in specific end products of peroxidation such as isoprostanes in their body fluids(Halliwell, 2005). Drug overdoses have also been implicated in causing oxidative stress. Overdoses of Acetaminophen have been shown to cause oxidative stress, through induction of hepatotoxicity(Chun et al., 2009).

In normal therapeutic doses about 90% of the acetaminophen is metabolised by the liver to phenolic glucuronide and sulphate, by glucuronyltransferases and sulfotransferases, and is eventually excreted in urine; meanwhile of the remaining 10% , 2% is excreted unchanged(Larson, 2007). About 5 – 10% of the acetaminophen is metabolised to N-acetyl-p-benzoquinoneimine (NAPQI)(Nelson, 1990), by cytochrome P450, mostly the enzyme CYP2E1(Rumack, 2002). NAPQI is an electrophilic molecule that is highly reactive and causes damage by formation of covalent bonds with other intracellular proteins(Nelson, 1990). Prevention of this reaction in the body is by conjugation of this molecule with glutathione, generating a product that is water-soluble and is excreted in bile(Jaeschke and Bajt, 2005).

However, in cases of acetaminophen toxicity, saturation of glucuronyltransferases and sulfotransferases occurs diverting the metabolism of the drug to cytochrome P450 thus leading to the generation of NAPQI in amounts that causes depletion of glutathione(Chun et al., 2009). With depleted glutathione, NAPQI accumulates in hepatocyte, forming covalent bonds with cellular proteins and causing modification of their structure and function(Lee, 1995). With the resulting cellular disturbance, a decrease in the activity of calcium ATPase and increase in cytosolic calcium levels occurs(Jaeschke and Bajt, 2005, Jaeschke et al., 2003); and the abnormal cellular calcium homeostasis present leads to altered cell permeability, with formation of blebs in the cell membrane and loss of membrane integrity(Lee, 1995).

2.4 The Antioxidant Defence System

Oxidative stress although an important factor in disease situations has been seen to occur in healthy individuals, in low levels, as evidence of its occurrence have been brought forward in literature(Halliwell, 2007). DNA isolated from aerobic cells of

health individuals have shown evidence of oxidative base damage products e.g. carbonyls, allantoin and other products of oxidation of uric acid, have also been found in healthy individuals(Halliwell, 2005). In order to protect the body from the effects of oxidative stress that continues to occur and ensure that the oxidatively damaged molecules like DNA are repaired; the body possesses an antioxidant defence system.

This antioxidant defence system maintains the free radicals and antioxidants balance, keeping this balance tightly regulated, as it is specific for each organelle and organic site; thus the balance is essential to allow for maintenance of important cellular and biochemical functions(Repetto et al., 2012).

The antioxidant defence system is made up of endogenous and exogenous (diet derived) antioxidants which are enzymatic and/or non-enzymatic, and are responsible for the elimination of free radicals from the system and hence maintenance of the redox potential of the organism(Halliwell, 2005, Sies, 1991) .

The endogenous antioxidants are enzymes, superoxide dismutase (SOD), catalase, and Glutathione peroxidase (GSHPX), and also low molecular mass free radical scavengers exist namely Glutathione (GSH), endogenous Vitamin C and E; whereas the diet derived antioxidants include Vitamin E and C and flavonoids(Di Mascio et al., 1991, Halliwell, 2005) . . These antioxidants are found either in the lipophilic or in hydrophilic phases(Niki et al., 2005).

Vitamin E is made up of the tocopherols and tocotrienols, these as antioxidants react with organic peroxyl radicals, without reacting in further chain propagating

reactions, and this is believed to be their major biological functions(Di Mascio et al., 1991, Sies et al., 1992). Vitamin E breaks the chain propagation in lipid peroxidation by scavenging lipid peroxyl radicals, giving rise to a stable lipid hydroperoxide and the tocopheroxyl radical which actually interrupts the radical chain reaction(Halliwell, 2005, Niki et al., 2005, Sies et al., 1992).

Vitamin C or ascorbic acid renders protection to the membranes against peroxidation damage by trapping peroxyl radicals while still in the aqueous phase before initiation of lipid peroxidation(Kohen and Nyska, 2002). It has been shown to enhance the activity of Vitamin E in that the tocopheroxyl radical formed reacts with ascorbate giving rise to tocopherol and ascorbyl acid thus maintaining the radical scavenging potential of the membrane as the tocopherol is regenerated transferring the oxidative potential to the aqueous phase(Niki et al., 2005, Sies et al., 1992).

The antioxidant activity of beta carotene just like with the other carotenes, contributes to the membrane protection against lipid peroxidation, in which it may protect biological systems against free radical mediated damage by quenching(Machlin and Bendich, 1987). The importance of a particular antioxidant, in a steady state does not only rely on its reactivity or concentration but also on its capacity to react with regenerating systems; because when these micronutrients have undergone oxidation, there is need for them to be regenerated in the biological settings, as such these are further coupled to non-radical reducing systems like $NADPH/NADP^+$ and $NADH/NAD^+$ (Sies et al., 1992).

The antioxidants are very important in the body for the normal functioning of the organism, however, it is important that the levels of antioxidants are maintained to certain level; otherwise, they may lead to deleterious effects.

2.5 Pharmacological Management of Oxidative Stress

The imbalance in the oxidant-antioxidant mechanisms causing oxidative stress is very important in disease situations, and so much more for some diseases than others, currently prospects for therapeutic interventions are underway in which rationally designed antioxidants are being worked on for use in such diseases(Halliwell, 2005).

The antioxidants work in various ways which include, prevention of formation of oxidants by scavenging, quenching, or removal of the active oxidants; repairing damaged molecules; ensuring the excretion of toxic oxidants from the system and by adaptive mechanisms or responses; inhibition or inactivation of enzymatic chain reactions; inhibition of chain initiation; or by accelerating chain termination(Niki et al., 2005).

Scientific evidence has been able to show that Vitamin C caused a decrease in the levels of oxidative stress and lipid peroxidation induced by drug treatment, by decreasing the levels of MDA which was in association with an increase in the SOD levels(Derakhshanfar et al., 2013). Other antioxidants that have been used in the treatment of diseases associated with oxidative stress include minerals like selenium, and these have been made synthetically.

These synthetic antioxidants used are believed to possess complications which includes bad side effects, for instance butylated hydrotoluene (BHT) and Butylated Hydrooxyanisole (BHA) used in food preservation causes liver damage, thus the search for the plant based and non-toxic ones is seriously starting to gain much attention(Iyawe and Azih, 2016). The plant-derived ones possess an advantage of having minimal side effects associated with them, as compared to the synthetic

counterparts(Wong et al., 2014, Wichi, 1988). The majority of the people in the developing countries live in poverty, and the cost of buying these synthetically made drugs is (Igile et al., 1994).

These drugs may not be readily available in some remote areas of the developing countries making access to them a challenge. As such, evidence has shown that about 80% of people in developing countries of Africa use medicinal plants as the source of their health care needs(Bukar et al., 2013).

Many foods are believed to contain the radical scavenging capacity and most synthetic and natural supplements and drugs with the radical scavenging properties are being studied(Niki et al., 2005).

2.6 Antioxidant Properties of Medicinal Plants

Folk medicine has since time in memorial been using plants as sources of medicines for the treatment of many ailments like diabetes, gastrointestinal diseases, gynaecological diseases, cardiovascular diseases, and bacterial infections, among others(Farombi and Owoeye, 2011). Some of these plants have also been used as food substances and thus consumed in most communities. Therefore, medicinal plants offer a reservoir of therapeutic and preventive medicines.

There is enough evidence that has been brought forward which has been able to show that various plants possess secondary metabolites that give them antioxidant properties.

In a study done by Ayoola et al(Ayoola et al., 2008), plant extracts that were obtained from the plants *Carica papaya, Mangifera indica, Vernonia amygdalina* and *Psidium guajava* were seen to possess antioxidant properties that were varying

in strength. In this study these plants' antioxidant activity was compared with that obtained from vitamin C, and it was shown that their antioxidant was found in increasing order from V. *amygdalina, C. papaya, M. indica*, vitamin C and lastly *P. guajava*. It showed that the pronounced anti-malarial content of these plants could be due to its antioxidant properties.

Crassocephalum rubens, and *Solanum americanum* showed more antioxidant activity than, Solanum macrocarpon, Telfaria occidentalis, Amaranthus hybridus, and *Jatrophata njorensis*(Iwalewa EO et al., 2005, Olusola et al., 2015).

Azadirachta indica, also possesses antioxidant properties which have been seen to be made better when used in combination with *V. amygdalina*, and also was able to prevent atherosclerosis(Ebong et al., 2011).

Morinda lucida ,Psidium guajava L, Chrysophyllum albidum, Anacardium occidentalis L. Mangifera indica L, Carica papaya L, Chromolaena odorata (L) King & robinson, Rauwolfia vomitoria Afzel, Khayasene galensis Dest, Citrus aurantifolia L, Citrus paradise Rutaceae, Tithoni adiversifolia, Nauclea latifolia and *Adansonia digitata* are believed to possess various amount of antioxidant activity (Adedosu et al., 2014, Grace et al., 2014, Iyamah et al., 2014).

In a study done by Atangwho *et al*(Atangwho et al., 2009) and also other researchers, the proximate vitamin, mineral element and phytochemical composition of *Azadirachta indica*, and *Gongronema latifolium* were determined using standard methods and compared, and were found to possess mineral elements, vitamins and phytochemicals, which are responsible for the antioxidant and ant-diabetic properties that they possess.

According to a study done by Adaramoye *et al* (Adaramoye Oluwatosin et al., 2008), the radioprotective efficacy of methanolic extracts of leaves of *Hibiscus sabdariffa and Vernonia amygdalina*; and vitamin C, against gamma radiation induced liver damage were studied in male wister albino rats. Treatments were given as follows: Vitamin C at 250 mg/kg body weight, *Hibiscus sabdariffa* and *Vernonia amygdalina* extracts at 200, 400 and 800mg/kg body weight orally for 4 weeks prior to irradiation and 5 weeks after irradiation. The plants *Vernonia amygdalina* and *Hibiscus sabdariffa*, were shown to have hepatoprotective effects in that, when the mice that had irradiation induced liver damage were given the extracts of the plants, the rats showed a reduction in the levels of lipid peroxidation and an increase in the antioxidant levels. Thus it was suggested that these plant were able to give protection against irradiation induced liver damage by increasing the antioxidant defence system.

Ocimum gratissimum was also seen to possesses antioxidant properties as it was able to cause a reduction in lipid peroxidation levels in the kidneys and liver of the treated rats(Iyawe and Azih, 2015). Standard analytical assays were used to assess the effects of this plant and it was shown in this study to possess high levels of phenolic compound and alkaloids and had a very high free radical scavenging power.

2.7 *Vernonia amygdalina* Delile

2.7.1 Classification of *Vernonia amygdalina* Delile

Table 1: Botanical classification of *Vernonia amygdalina* (Delile)

Subdivision	**Name**
Kingdom	*Plantae*
Division	*Angiosperm*
Genus	*Vernonia*
Family	*Asteracea*
Species	*V. amygdalina*
Class	*Dycotyledone*
Order	*Asterales*
Botanical Name:	*Vernonia amygdalina*
Common Names:	Iron weed **English** – Bitter Leaf **African** - Grawa (Amharic), muluuza (Luganda), etidot (Ibibio), ewuro (Yoruba), oriwo (Edo), onugbu (Igbo), ityuna (Tiv), labwori (Acholi), chusar-doki (Hausa) and olusia (Luo). **French** - Ndole

As adapted from Muhammed *et al*(Mahammed et al., 2015), Bukar *et al*(Bukar et al., 2013), Adedapo *et al*(Adedapo et al., 2014), Agbogidi and Akpomorine(Agbogidi and Akpomorine, 2013), Johnson *et al*(Johnson et al., 2014), Adebayo *et al*(Adebayo et al., 2014)

Figure 2: *Vernonia amygdalina* Delile growing in a residential area in Olympia, Lusaka province, Zambia.

Figure 3: The flowers of *Vernonia amygdalina Delile* growing on a mature plant, in a residential area of Olympia in Lusaka Province of Zambia.

Figure 4: The leaves and flowers of *V. amygdalina* in the residential area in Olympia of Lusaka Zambia.

2.7.3 Ecological Distribution

This plant is very popular in west African countries like Nigeria, Cameroon, Gabon and Democratic Republic Congo(Agbogidi and Akpomorine, 2013). However, this plant is commonly found in many parts of Africa and Asia(Mahammed et al., 2015) and naturally grows in Zambia, the plant grows also in East, West and South of Tropical Africa(Owoeye et al., 2010). The genus *Vernonia* is believed to possess over 1,000 species of which 500 are in Africa and Asia, 300 in Mexico, central and south America, and 16 in North America(Yeap et al., 2010).

2.7.4 Composition of the *V. amygdalina*

This plant possesses many biological components that are purported to be responsible for many pharmacological properties it has.

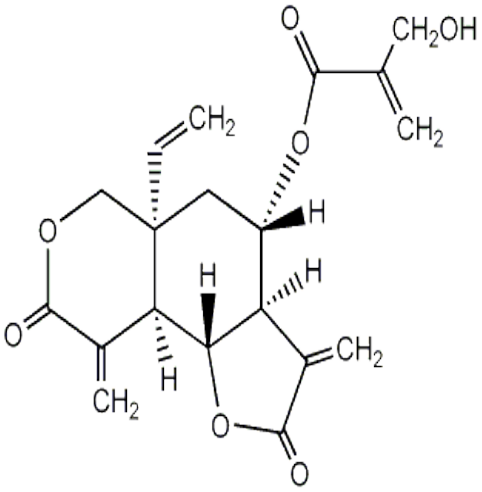
In a study done by Sha'a *et al*(Sha'a et al., 2011), the results of the phytochemical screening was able to show the presence of saponins, carbohydrates, tannins, flavonoids, steroids, cardiac glycosides, alkaloids, and anthraquinones, consistent with other studies(Adedapo et al., 2014, Atangwho et al., 2009).

The plant has been shown to possess the following compounds:

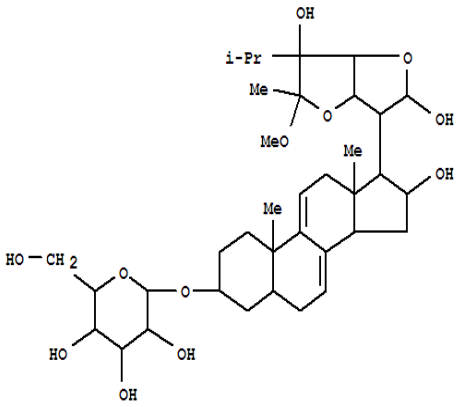
Caffeoylquinic acid, Chlorogenic acid, Rutin, Luteolin 7-O-glu, Luteolin 4'-O-rut, Flavonoid, Luteolin-7-O- glucur§, 1,5 dicaffeoyl-Qac, dicaffeoyl-QAc, dicaffeoyl-QAc, Apigenin 6-O or 7-O, glucur., Luteolin and Flavonoid(Salawu et al., 2007). The plant *Vernonia amygdalina* is believed to possess phenols, tannins, flavonoids, beta carotenes, leucopene, anthocyanin, Vitamin C, Vitamin E(Eseyin et al., 2015) and epivernodalol which is responsible for it anticancer activity(Owoeye et al., 2010).

2.7.5 Structure of Phytochemicals from the leaves of *Vernonia amygdalina Delile*

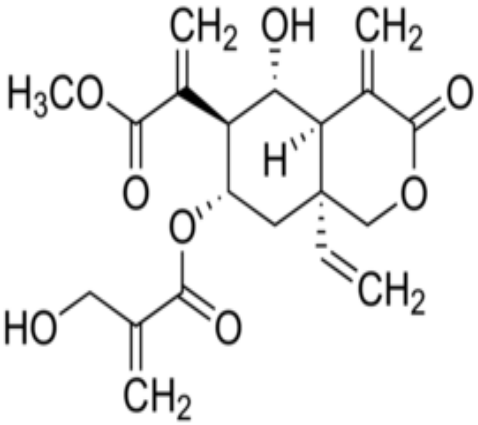
The plant *Vernonia amygdalina* possesses phytochemicals that are purported to be responsible for most of the pharmacological properties it possess; it has both nutritive and non-nutritive factors. It has been shown to possess sesquiterpene lactones namely Vernodalin, Vernodolol, Vernoleptin and Stigmastane-type saponin, Vernonioside D, and flavanoid namely *Luteolin* (see figure 5 (a) – (e)), which with other phytochemicals gives the plant the antioxidant properties it possess (Erasto et al., 2006, Erasto et al., 2007a, Jisaka et al., 1993, Koshimizu et al., 1994, Kupchan et al., 1969, Salawu et al., 2007).



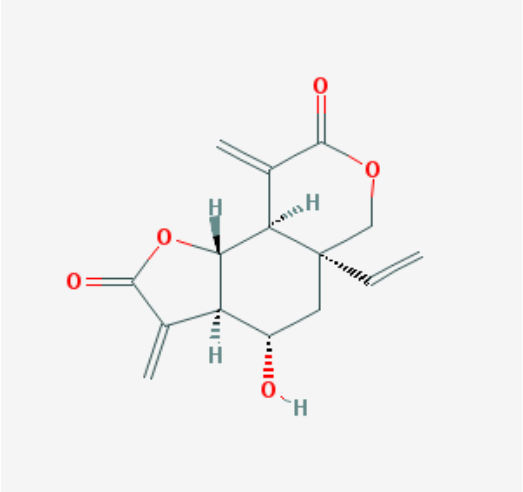
5 (a) Vernodalin



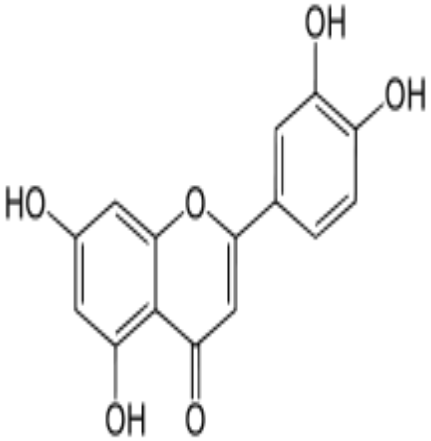
(b) Vernonioside D



(c) Vernodolol



(d) Vernolepin



(e) Luteolin

Figure 5 (a) – (e): The Structures of some phytochemicals that have been shown to be present in the plant extract of *Vernonia amygdalina*. As adapted in Ijeh and Ejike (Ijeh and Ejike, 2011).

2.7.6 Documented Research Findings on *Vernonia amygdalina Delile*

Literature has shown this plant to be safe to use both as a medicine and a nutrient (Adedapo et al., 2014). Various studies have been done which show the various pharmacological properties of Vernonia amygdalina plant related to its antioxidant properties.

2.7.6.1 Anthelminthic Effects

The extract of this plant has been shown to have anthelminthic effects, consistent with its tannins content, effected by binding to the glycoprotein on the cuticle, it has therefore been shown to possibly possess weak GABA mimetic effects similar to that of piperazine citrate(Danquah et al., 2012). This has also shown the other effects of the phytochemicals of this plant that have been associated with its antioxidant effects.

2.7.6.2 Anti-diabetic Effects

In a study done by Atangwho *et al*(Atangwho et al., 2009), it was suggested that this plant possesses ant-diabetic effects, as was shown by other researchers also(Asante et al., 2016), and that these effects maybe as a result of the micronutrients and antioxidant vitamins that this plant possesses. The study also suggested that the developmental nutritional disorders and their complications in diabetes may be due to an imbalance in these micronutrients and antioxidant vitamins, which are present in the plant and thus relevant for the plant to give its hypoglycaemic effects and also attenuation of the macrovascular complications that presents(Atangwho et al., 2009).

2.7.6.3 Antiparasitic Effects

.According to a study done by Bashir *et al(Bashir and Alhaji, 2012),* it was shown that the methanolic leaf extract possessed activity against the *Trypanasoma brucei brucei*. The mice treated with the extract survived longer because the extract kept

the parastemia of these mice low as compared to the control which was not treated. Triglycerides for the other untreated mice were increased while that of the extract treated mice was reduced following administration of the extract. And the extract treated mice appeared physically good as compared to the untreated groups. The extract probably possesses the capacity to prevent lipid peroxidation as was shown by reduced MDA levels, and also increase the GSH and SOD levels in the treated mice. The increase in the GSH could have been by the extract stimulating *de novo* synthesis or sparing the endogenous GSH and using its own antioxidants to scavenge the free radicals.

2.7.6.4 Anti-inflammatory Effects

A study done by Iwalewa *et al* (Iwalewa et al., 2007), has shown that plants that possesses alkaloids, tannin, saponins, anthraquinones, and probably other constituents possesses the anti-inflammatory effects that could probably be due to these elements. The plant extract was also considered to have an *in vitro* antimalarial effects that were moderate(Sha'a et al., 2011). And these effects are believed to be brought about by the antioxidant constituents of the plant.

2.7.6.5 Analgesic Effects

In a study done by Adedapo *et al* (Adedapo et al., 2014), the acetone leaf extract of *Vernonia amygdalina* at both doses caused a reduction in the number of writhes as compared to the control. The extract of this plant was shown to exhibit significant antinociceptive effects, which were comparable with that shown by indomethacin. Therefore, the plant was able to show a significant analgesic effect relative to the standard drug. These pharmacological effects were suggested to be due to the presence of the phytochemicals that confers it the antioxidant effects.

In a study done by Njan *et al*(Njan et al., 2008), this plant extract was administered to the mice that had undergone visceral nociception which was induced by acetic acid, and the effect which was suggested was that this plant reduced the numbers of writhing in the treated mice in a dose dependent manner. These effects were shown to be from the flavonoids, alkaloids, tannins, saponins, steroids and phenols that this plant possesses.

2.7.6.6 Anticancer Effects

In a study done by Lolodi and Eriyamremu (Lolodi and Eriyamremu, 2013), the plant was shown to possess the ability to increase the antioxidant levels in the colon and thus was believed to be able to have chemoprotective effects on the colon-rectal cancer. In this study cycasin was used to induce the oxidative stress, where administration led to a significant reduction in the colonic-superoxide dismutase (SOD) as compared with the controls, whereas administration of *V. amygdalina* was shown to significantly restore the levels of the colonic SOD to levels significantly different from the controls. However, it was shown that pre-treatment of the mice with *V. amygdalina* for 5 weeks followed by treatment with 5% Cacystin produced a better response. The restoration of the antioxidant levels by the plant *V. amygdalina* was probably coming from the total flavonoids, the micronutrient like zinc, copper and manganese that it possesses(Eyong et al., 2011).

2.7.6.7 Antioxidant Effects

Vernonia amygdalina has been shown to possesses antioxidant properties, according to a study done by Ho *et al* (Ho et al., 2012), which showed that this plant was able to significantly increase the levels of an enzyme SOD present in the body as an antioxidant, this was comparable to the levels see with vitamin C. In this study, the plant was able to increase the total antioxidants and the levels of malondialdehyde

were reduced, this was also shown by other researchers(Ho et al., 2015). Therefore, it was observed in this study that the plant possesses both in vivo and in vitro antioxidant properties and enhanced both the plasma and red blood cells antioxidant levels and also gave protection to the liver by entering the cells, and this information was also shown by other researchers (Usunobun et al., 2015).

In a study done by Minari (Minari, 2012), it was suggested that the plant possesses components that are hepato-protective as was shown by the significant increase in AST and ALT activities after administration of the extract as compared with those that received only the carbon tetrachloride (CCL_4). These hepato-protective effects are believed to be due to the antioxidant properties of the plant coming from the phenolic and flavonoids it possesses (Ho et al., 2015).

According to the study by Bashir *et al* (Bashir and Alhaji, 2012), the plant was also shown to possess antioxidant effects which were believed to be responsible for the antiparasitic effects which were observed in the study. The plant increased the antioxidant levels, reduced the MDA levels of the mice which were treated, and these effects were believed to be due to the antioxidant vitamins C and E and also the presence of phenolic compounds and flavonoids, which is in agreement with other researchers (Lolodi and Eriyamremu, 2013, Owolabi et al., 2008).

In a study done by Adaramoye *et al* (Adaramoye Oluwatosin et al., 2008), *V. amygdalina* extract was shown to possesses hepatoprotective effects on an irradiation induced hepato-damage. The study also had shown that the phenolic compounds and flavonoids that it possesses were able to reduce the levels of lipid peroxidation while increasing GSH and SOD, it was also observed to cause a reduction in the gamma irradiation induced increase in serum alanine

aminotransferase (ALT) and aspartate aminotransferase (AST)(Owoeye et al., 2011).

According to the results found by Ojieh *et al (Ojieh EA and Njoku IP, 2016),* the plant extract of *V. amygdalina* was shown to cause a reduction in oxidative stress by bringing back a balance in the oxidant-antioxidant levels and also causing a restoration of the deranged renal function.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Chemicals and Reagents

Below is a list of chemicals and reagents used in the study.

Sodium acetate trihydrate, trichloroacetic acid, phosphate buffered saline (PBS), methanol, vitamin C, ethylenediaminetetraacetic acid (EDTA), sodium hydroxide (NaOH), and ferric chloride (FeCL3.6H2O) were purchased from Sigma (USA), while butylated hydrooxytolene (BHT), thiobarbituric acid and iron reagent (TPTZ (2,4,6-tripyridyl-s-triazine)) were obtained from Himedia.

Methods for the preparation of the reagents for FRAP(Benzie and Strain, 1996) and TBARS assays(Ho et al., 2012) are presented in Appendix A.

3.2 Plant Material and Preparation of Extract

Vernonia amygdalina (asteraceae) Delile, was obtained locally, from a garden in Olympia residence of Lusaka, Zambia. These leaves were collected in June, 2016. The plant was then authenticated at the School of Natural Sciences, Department of Biological Sciences, University of Zambia. A specimen was also kept at the herbarium in the Department.

The process of preparation of the extract was done according to the procedure of Kate and Lucky (Kate and Lucky, 2012). The leaves were air dried in the shade at room temperature for two weeks. Then they were ground to a uniform powder with a mortar and pestle. 100g of powder was macerated in 1 litre of 80% methanol at room temperature (22 - 26 ˚C) and allowed to stand for 72 hours with periodic

agitation; this was done to allow for as much of the chemical constituents of the plant to be extracted. The extract was then filtered through the Whatmann no. 42 (125 mm) filter paper, and the filtrate was evaporated to a paste with the aid of a rotavapour and an oven at 40 ˚C. The residue was weighed and the percentage yield determined. This residue was then stored in the refrigerator until required for experimental use. For use, the residue was reconstituted in distilled water.

The percentage yield was determined by the following calculation:

$$\text{Percentage Yield} = \frac{\text{Weight of sample extract obtained (g)}}{\text{Weight of the powdered sample used (g)}} \times 100$$

$$= \frac{20.62\text{g}}{100\text{g}} \times 100$$

$$= 20.62\%$$

3.3 Working Solutions:

3.3.1 *Acetaminophen:*

A working solution of 100mg/ml acetaminophen was prepared by dissolving 1000mg APAP in 10ml using warm normal saline.

3.3.2 *Vernonia amygdalina solution*

Five grams of *Vernonia amygdalina* was dissolved in 100ml of distilled water, to make 50mg/ml.

3.3.3 *Vitamin C solution:*

One gram of Vitamin C was dissolved in 10ml solution with distilled water making a final concentration of 100mg/ml.

3.4 Experimental Animals

Experimental animals, Albino mice of both sexes (weighing between 20 – 31g), were obtained from the animal house of the Department of Physiological Sciences at the Ridgeway campus. These were habituated in metal cages for 2 weeks at room temperatures (22 – 26˚C), 12-hour light/dark cycle, and free access to food and water. The mice were fed mice shells obtained from Livestock Services Cooperation, Showgrounds, Lusaka. The mice were handled by qualified animal care personnel of the Department. The mice were handled in compliance with the NIH guidance for care and use of laboratory animals(Care et al., 1985).

3.5 Experimental Design

A total of 36 white albino mice were randomly assigned to one of the six groups each having six mice. After an overnight fast, each group received treatments according to the information given below.

Group 1 (Negative Control) received 2ml/kg normal saline, orally(Adedapo et al., 2014).

Group 2 (Extract Only) received 50mg/kg *Vernonia amygdalina Delile* extract(Iwalokun et al., 2006).

Group 3(Positive control) received 300mg/kg body weight of acetaminophen orally.

Group 4 (Test (low dose)) received 300mg/kg acetaminophen and *Vernonia amygdalina Delile* methanolic leaf extract at the dose of 50mg/kg both orally.

Group 5(Test (high dose)) received acetaminophen at the dose of 300mg/kg and *Vernonia amygdalina Delile* at the dose of 100mg/kg both orally(Iwalokun et al., 2006).

Group 6 (Standard drug) received 300mg/kg acetaminophen and vitamin C at the dose of 500mg/kg, both orally(Ajith et al., 2007).

Table 2 - Titration of the doses for the treatments given to the mice

Weight(g)	Treatments				
	Normal saline(ml)	*Vernonia amygdalina* (ml)		Vitamin C(ml)	Acetaminophen (ml)
	2ml/kg	50mg/kg	100mg/kg	500mg/kg	300mg/kg
20	0.04	0.02(1mg)	0.04(2.0mg)	0.1(10mg)	0.06(6mg)
21	0.042	0.021(1.05mg)	0.042(2.10mg)	0.105(10.5mg)	0.063(6.3mg)
22	0.044	0.022(1.1mg)	0.044(2.20mg)	0.11(11mg)	0.066(6.6mg)
23	0.046	0.023(1.15mg)	0.046(2.30mg)	0.115(11.50mg)	0.069(6.9mg)
24	0.048	0.024(1.2mg)	0.048(2.40mg)	0.12(12 mg)	0.072(7.2mg)
25	0.05	0.025(1.25mg)	0.050(2.50mg)	0.125(12.5mg)	0.075(7.5mg)
26	0.052	0.026(1.30mg)	0.052(2.60mg)	0.13(13 mg)	0.078(7.8mg)
27	0.054	0.027(1.35mg)	0.054(2.70mg)	0.135(13.50mg)	0.081(8.1mg)
28	0.056	0.028(1.40mg)	0.056(2.80mg)	0.14(14mg)	0.084(8.4mg)
29	0.058	0.029(1.45mg)	0.058(2.90mg)	0.145(14.5mg)	0.087(8.7mg)
30	0.06	0.030(1.50mg)	0.06(3.00mg)	0.15(15 mg)	0.09(9.0mg)
31	0.062	0.031(1.55mg)	0.062(3.10)	0.155(15.5mg)	0.093(9.3mg)

Data are volumes of normal saline, *Vernonia amygdalina* extract, vitamin C and acetaminophen given to the mice according to the weight of the mice.

Following the administration of all the treatments as shown above, the mice were allowed to fast for another four hours before being put back on food. Then another four hours was allowed to pass before samples could be collected for analysis.

Blood samples were collected 8 hours after acetaminophen administration, by cardiac puncture into EDTA tubes. Each animal was sacrificed by decapitation after blood collection, and the liver was removed, homogenised and centrifuged (Hitachi centrifuge 05P-21), at 2,500 rpm for 5 minutes. The supernatant from the liver collected was separated into vials and stored in the refrigerator before use. The whole bloods from the EDTA tubes were used as samples for analysis.

3.6 Biochemical Analysis

3.6.1 *Total Antioxidant capacity*

This was measured by evaluating the ability of the plasma to reduce ferric by using Ferric Reducing Antioxidant Power (FRAP) Assay(Benzie and Strain, 1996).

Principle: The assay measures the ability of an antioxidant or a substance to reduce Fe^{3+} to Fe^{2+}. Therefore, at low PH the Ferric-tripyridyltriazine (Fe^{III}-TPTZ) form is reduced to the ferrous (Fe^{II}) form with an intense blue colour formation of that has an absorbance maximum at 593nm.

The FRAP Assay was done according to the method of Benzie and Strain(Benzie and Strain, 1996). Absorbances of the FRAP reagent (consisting of 300 mmol/L acetate buffer with pH 3.6; 10 mmol/L TPTZ in 40 mmol/L HCL and 20 mmol/L FeCl3.6H2O) mixed with the sample (liver and whole blood) were taken. Six different concentrations of Fe (II) standard solutions were tested in parallel.

Procedure:

- Aqueous solutions of known Fe(II) concentration in the range of) 0.1mM, 0.2mM, 0.4mM, 0.6mM, 0.8mM and 1.0mM, were used for calibration. The actual dilutions are as shown in appendix B.
- 300µl of freshly prepared FRAP reagent was warmed at 37ºC and its absorbance reading was taken (M1) at 593nm.
- 10µl of sample was added along with 30µl of water, making a sample dilution in a mixture of 1/34.
- Readings of absorbance were initially taken after 5 seconds and then after every 15 seconds making up to 4 readings. Then one of the readings was taken.
- The change in absorbance between the final volume selected and M1 absorbance reading was calculated for each sample and these were related to the absorbance of Fe (II) standard measured in parallel.

A standard curve was constructed and used to determine FRAP values in mM Fe(II)/L(Atawodi et al., 2007, Benzie and Strain, 1996).

3.6.2 *Determination of Malondialdehyde (MDA)*

Principle: Malondialdehyde through an acid catalysed nucleophilic addition reaction reacts with two molecules of Thiobarbituric acid (TBA), giving rise to a pinkish-red chromagen with an absorption maximum at 532nm(Janero, 1990, Kappus, 1985).

Procedure:

- 250µl sample were placed in 250µl of phosphate buffer (PBS), this was done to keep the sample in slightly alkaline conditions to allow for the release of bound MDA.

- Then 25µl of butylated hydroxytoulene was added to the sample to allow for prevention of further lipid peroxidation.
- Proteins were precipitated with the addition of 1ml of Trichloroacetic acid.
- Then the sample was incubated on ice for 2 hours and pelleted, and then the supernatant was obtained.
- To supernatant was added 75µl of 0.1M EDTA and 250µl of 1% Thiobarbituric acid in 0.05M NaOH, and this was placed on boiling water for 15 minutes.
- After cooling to room temperature, the absorbance was measured at 532nm and 600nm(Ho et al., 2012).
- The concentrations of samples were obtained using the method of Heath and Packer(Heath and Packer, 1968). This method has been shown in appendix C.

These protocols were done from the biomedical laboratories at the Departments of Biomedical sciences and disease control, at the school of Veterinary Medicine at the University of Zambia.

3.7 Statistical Analysis

Data analysis was done using the SPSS version 21 software. Descriptive statistics included Mean ± SEM for all the six groups. Data of the groups were tested for normality using the Shapiro Wilk test and the homogeneity of variances was tested by using the Levene's test. The One Way Analysis of Variance was used to analyse the data of the total antioxidant capacity and malondialdehyde levels thereafter the Turkeys HSD post Hoc test was used for the multiple comparisons.

Tests with a probability value, $P < 0.05$ were considered significant(Nwanjo, 2005).

3.8 Ethical Issues

The study was approved by ERES Converge, Lusaka, an independent ethical review board. The ethical review reference number was 2015-May-013. Approval was also obtained from the University and departmental management of the University of Zambia.

The laboratory animals were handled humanely, such that there was minimisation of pain inflicted on them. Moreover, these were kept in well ventilated cages, being handled by qualified personnel who are a member of staff of the University of Zambia. These received veterinary care from qualified veterinarians.

The experimental design was properly planned to ensure that the most minimum numbers of animals were used which were able to provide necessary information and ensure precise inference using the statistical data packages used. And the exact tests animals with their controls were used and also minimisation of environmental variables to ensure uniformity in health, genetics and experimental variables was done. Therefore the protocol conformed to the guidelines of the National Institute of Health (NIH) for laboratory animal care and use(Care et al., 1985).

CHAPTER FOUR

RESULTS

This chapter presents the findings of the study including the descriptive statistics (Mean ± SEM) of both the Total Antioxidant Capacity (TAOC) and malondialdehyde (MDA) levels in both the blood and liver homogenates for the six groups, including the statistical analysis (using one-way ANOVA) to compare data between groups and (the Turkey' HSD post hoc tests) for multiple comparisons.

4.1 Determination of Percentage Yield:

The percentage yield of the macerated *Vernonia amygdalina* leaves was 20.62%.

4.2 Analysis of Results

The data obtained was tested for normality and homogeneity of variances which must not be violated in order for the one-way analysis of variances to be used. The test for normality was carried out using the Shapiro-wilk test, which is the most appropriate way of testing for normality with small sample sizes. All the data for the total antioxidant capacity and the malondialdehyde levels of the blood and liver samples were normally distributed refer to appendix D for more information.

The homogeneity of variances was tested using the Levene test, and this showed the equality of variances for the blood and liver samples of both the total antioxidant capacity and the malondialdehyde levels, this has been further clarified in appendix E.

Table 3- The mean and standard error of the Total antioxidant capacity for the blood samples of the mice.

Groups	Treatment	Mean	Standard error	p-value(1-sided)
1	Normal saline at 2ml/kg (negative control)	0.75	0.01	0.001*≠
2	V. amygdalina at 50mg/kg (Extract only)	0.81	0.01	0.001*≠
3	Acetaminophen at 300mg/kg (positive control)	0.54	0.004	0.001*≠
4	Acetaminophen at 300mg/kg + extract at 50mg/kg	0.64	0.004	0.001*≠
5	Acetaminophen at 300mg/kg + extract at 100mg/kg	0.70	0.01	0.001*≠
6	Acetaminophen at 300mg/kg + Vitamin C at 500mg/kg	0.69	0.01	0.001*≠
	Mean	0.69	0.01	

Data are the Mean, Standard error of the mean and p-values of the six different treatment groups (n=6). Differences in means were analysed using One-way analysis of Variance, followed by Turkey HSD Post Hoc test. p<0.05 was considered significant. And significant comparison of Group 1 with each of the other groups was shown by *, while significant comparison of Group 3 (positive control) with the other groups was shown by ≠, and the actual p values are as shown in the p-value columns.

Table 4- The mean and standard error of the Total antioxidant capacity for the liver samples of the mice.

Groups	Treatment	Mean	Standard error	p-value(1-sided)
1	Normal saline at 2ml/kg (negative control)	0.60	0.01	0.001*≠
2	V. amygdalina at 50mg/kg (Extract only)	0.86	0.01	0.001*≠
3	Acetaminophen at 300mg/kg (positive control)	0.49	0.004	0.001*≠
4	Acetaminophen at 300mg/kg + extract at 50mg/kg	0.68	0.004	0.001*≠
5	Acetaminophen at 300mg/kg + extract at 100mg/kg	0.77	0.01	0.001*≠
6	Acetaminophen at 300mg/kg + Vitamin C at 500mg/kg	0.75	0.01	0.001*≠
	Mean	0.69	0.01	

Data are the Mean, Standard error of the mean and p-values of the six different treatment groups (n=6). Differences in means were analysed using One-way analysis of Variance, followed by Turkey HSD Post Hoc test. p<0.05 was considered significant. And significant comparison of Group 1 with each of the other groups was shown by *, while significant comparison of Group 3 (positive control) with the other groups was shown by ≠, and the actual p values areas shown in the p-value columns respectively.

4.2.1 The Total Antioxidant Status in various groups of the mice

The following was observed from the results in Table 3 and 4:

The administration of the plant extract produced a statistically significant difference in the mean values of the Total antioxidant capacity of the blood and liver samples of Group 4 (acetaminophen + low extract dose - endline)- [mean blood 0.64U/mg, p=0.001 and liver 0.68U/mg of protein, p=0.001 and] and Group 5 (acetaminophen + high extract dose - endline) – [Mean blood 0.70U/mg, p=0.001 and liver 0.77U/mg of protein, p=0.001] as compared to those in Group 1(Negative control - baseline) – [Mean blood 0.75U/mg and liver 0.81U/mg of protein].

A statistically significant difference was observed in the mean values of the Total antioxidant capacity of the blood and liver samples of Group 4 (acetaminophen + low extract dose) and Group 5 (acetaminophen + high extract dose) as compared to those in Group 3(Positive control) – [Mean blood 0.54U/mg and liver 0.49U/mg of protein].

A statistically significant difference is evident in the mean values of the Total antioxidant capacity of the blood and liver samples of Group 1(Negative control - baseline) as compared to Group 2 (Extract only - endline) – [Mean blood 0.81U/mg, p=0.001 and liver 0.86U/mg of protein, p=0.001]; Group 3 (Positive control - endline) and Group 6 (Acetaminophen + Vitamin C - endline) – [mean blood 0.69U/mg, p=0.001 and liver 0.75U/mg of protein, p=0.001].

These results have further shown that there was a statistically significant difference in the mean values of the Total antioxidant capacity of the blood and liver samples

of group 3 (Positive control) as compared to group 2 (Extract only) and group 6 (Acetaminophen + Vitamin C).

A statistically significant difference in the mean values of the total antioxidant capacity of both the blood and liver samples was shown in group 2 as compared to group 6.

4.2.1.1 One-way Analysis of Variance (one-way ANOVA)

In order to assess if there were at least any two groups from the six that are statistically significantly different, the one-way ANOVA was done for both the total antioxidant capacity and malondialdehyde levels, the results are as shown in tables 5 and 6 below.

One-way ANOVA for the total antioxidant capacity of blood and liver samples

Table 5: One –way Analysis of variance for Total antioxidant capacity of blood and liver samples for the between group differences.

Source	SS	df	MS	F	p-value
Between groups (blood)	0.258	5	0.052	141.622	0.001
Between groups (liver)	0.519	5	0.104	1,530.787	0.001
Total	0.767	10			

There was a statistically significant difference in the Total antioxidant capacity of the blood samples as determined by the one-way ANOVA ($F(5,30) = 141.622$, $p=0.001$.

There was a statistically significant difference in the Total antioxidant capacity of the liver samples as determined by the one-way ANOVA ($F(5,30) = 1,530.787$, $p=0.001$.

4.2.1.2 Multiple comparisons

Since the data for the blood and liver samples of the Total antioxidant capacity did not violate the assumption of homogeneity of variances, the Turkey HSD Post Hoc Test was used to find out which of the six groups were statistically significantly different from the others.

The results from the post Hoc test of the total antioxidant capacity of the blood samples are as shown in table 6 and graphically in the figure 6 below.

4.2.1.2.1 Multiple comparisons for the total antioxidant capacity of the blood samples

Table 6: Total antioxidant capacity from Turkey Post Hoc Test of Blood samples.

Group	Mean(U/mg)	Comparison groups	Mean(U/mg of protein)	p-value(1-sided)	Confidence interval	
					Lower bound	Upper bound
Negative control	0.75	Extract only	0.81	0.001	0.78	0.83
		Positive control	0.54	0.001	0.53	0.55
		Low dose extract	0.64	0.001	0.63	0.65
		High Dose extract	0.70	0.001	0.68	0.72
		Vitamin C	0.69	0.001	0.67	0.72
Extract only	0.81	Positive control	0.54	0.001	0.53	0.55
		Low dose extract	0.64	0.001	0.63	0.65
		High dose extract	0.70	0.001	0.68	0.72
		Vitamin C	0.69	0.001	0.67	0.72
Positive control	0.54	Low dose extract	0.64	0.001	0.63	0.65
		High dose extract	0.70	0.001	0.68	0.72
		Vitamin C	0.69	0.001	0.67	0.72
Low Dose Extract	0.64	High Dose extract	0.70	0.001	0.68	0.72
		Vitamin C	0.69	0.001	0.67	0.72
High Dose Extract	0.70	Vitamin C	0.69	0.990	0.67	0.72

Data are mean ± SEM (n=6). The groups were as follows: Group 1 (negative control)- received normal saline at 2ml/kg, Group 2 (Extract only) – received Vernonia amygdalina Delile extract at 50mg/kg orally, Groups 3 – 6 received acetaminophen at 300mg/kg orally; Group 3 (Positive control) – received only acetaminophen; Group 4 (experimental low extract dose) – also received Vernonia amygdalina at 50mg/kg; group 5 (experimental high extract dose) – also received 100mg/kg Vernonia amygdalina Delile extract; Group 6 (Known antioxidant) – also received Vitamin C at 500mg/kg orally. Differences in means were analysed using one-way ANOVA, followed by Turkey HSD Post Hoc test. $p<0.05$ were considered significant.

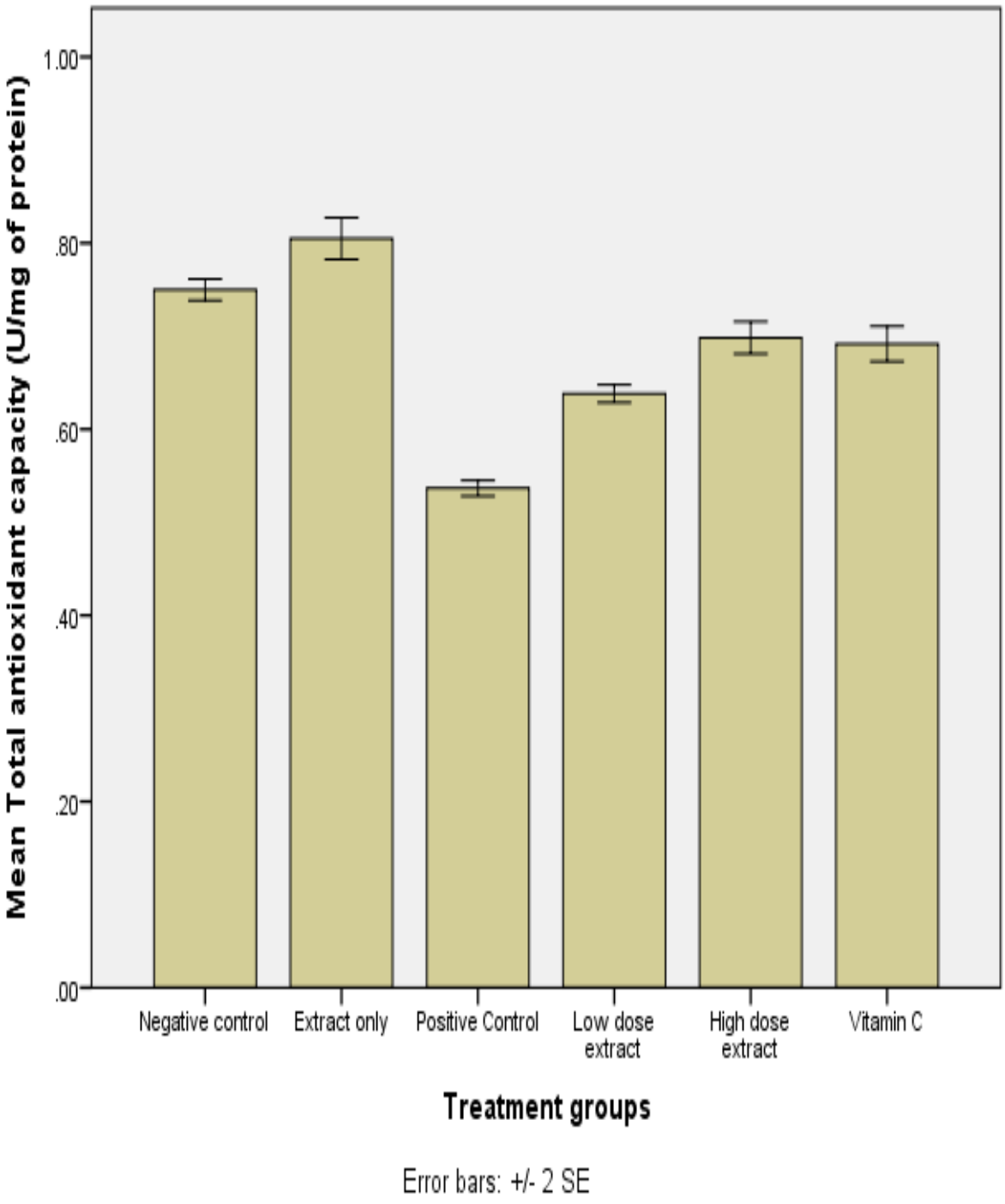


Figure 6- Mean and SEM values of the Total antioxidant capacity of the blood samples of the six groups (n=6).

Reporting the results of the Post Hoc Test for the blood sample:

The results from table 6 and figure 6 have shown the following;

There is a statistically significant difference in the Total antioxidant capacity of blood samples between the group that received normal saline only (group 1), as compared to all the other groups. When compared to that which received plant extract only (group 2), p=0.001; that which received acetaminophen only (group 3), p=0.001; that which received acetaminophen followed by low dose of *Vernonia amygdalina* methanolic extract (groups 4), p=0.001; that which received acetaminophen followed by high dose of *Vernonia amygdalina* methanolic extract (group 5), p=0.001; and also that which received acetaminophen followed by Vitamin C (group 6), p=0.001.

A significant difference in the Total antioxidant capacity of blood samples between the group that received acetaminophen only (group 3), as compared to the other groups as follows: that which received plant extract only (group 2), p=0.001; that which received acetaminophen followed by low dose of *Vernonia amygdalina* methanolic extract (groups 4), p=0.001; that which received acetaminophen followed by high dose of *Vernonia amygdalina* methanolic extract (group 5), p=0.001; and also the group that received acetaminophen followed by Vitamin C (group 6), p=0.001.

A significant difference in the Total antioxidant levels of blood samples between the group that received plant extract only (group 2), when compared to the other groups was seen. These include: that which received acetaminophen followed by low dose of *Vernonia amygdalina* methanolic extract (groups 4) p=0.001; that which received acetaminophen followed by high dose of *Vernonia amygdalina* methanolic extract

(group 5), p=0.001; and also the group that received acetaminophen followed by Vitamin C (group 6), p=0.001.

A significant difference was also seen, when the Total antioxidant capacity of blood samples between the group that received acetaminophen followed by low dose of *Vernonia amygdalina* methanolic extract (group 4) was compared to that which received acetaminophen followed by high dose of *Vernonia amygdalina* methanolic extract (group 5), p=0.001; and also the group that received acetaminophen followed by Vitamin C (group 6), p=0.001. However, there was no significant difference between the group that received acetaminophen followed by high dose of *Vernonia amygdalina* methanolic extract (group 5), as compared to that which received acetaminophen followed by Vitamin C (group 6), p=0.990.

The results from the Turkeys HSD post hoc test at 95% confidence interval have been graphically represented in the bar chart in the figure 6 above. The figure has shown that there was a significant difference in the Total antioxidant capacity of the various groups which are represented by the columns in the bar graph. The graph shows that the Total antioxidant capacity of the blood sample was highest in group 2 (extract only) and is reducing in the following order: group 1(Normal saline), group 5 (acetaminophen + high extract dose), group 6 (acetaminophen + vitamin C), group 4(acetaminophen + low extract dose), and lastly group 3 (acetaminophen only).

4.2.1.3.2 Multiple comparisons for the total antioxidant capacity of the liver samples

Table 7: Total antioxidant capacity from Turkey Post Hoc Test of Liver samples.

Group	Mean(U/mg)	Comparison groups	Mean (U/mg of protein)	p-value (1-sided)	Confidence interval	
					Lower bound	Upper bound
Negative control	0.81	Extract only	0.86	0.001	0.85	0.87
		Positive control	0.49	0.001	0.48	0.50
		Low dose extract	0.68	0.001	0.67	0.69
		High Dose extract	0.77	0.001	0.76	0.78
		Vitamin C	0.75	0.001	0.74	0.76
Extract only	0.86	Positive control	0.49	0.001	0.48	0.50
		Low dose extract	0.68	0.001	0.67	0.69
		High dose extract	0.77	0.001	0.76	0.78
		Vitamin C	0.75	0.001	0.74	0.76
Positive control	0.49	Low dose extract	0.68	0.001	0.67	0.69
		High dose extract	0.77	0.001	0.76	0.78
		Vitamin C	0.75	0.001	0.74	0.76
Low Dose Extract	0.68	High Dose extract	0.77	0.001	0.76	0.78
		Vitamin C	0.75	0.001	0.74	0.76
High Dose Extract	0.77	Vitamin C	0.75	0.084	0.74	0.76

Data are mean ± SEM (n=6). The groups were as follows: Group 1 (negative control)- received normal saline at 2ml/kg, Group 2 (Extract only) – received Vernonia amygdalina Delile extract at 50mg/kg orally, Groups 3 – 6 received acetaminophen at 300mg/kg orally; Group 3 (Positive control) – received only acetaminophen; Group 4 (experimental low extract dose) – also received Vernonia amygdalina at 50mg/kg; group 5 (experimental high extract dose) – also received 100mg/kg Vernonia amygdalina Delile extract; Group 6 (Known antioxidant) – also received Vitamin C at 500mg/kg orally. Differences in means were analysed using Kruskal Wallis H Test, followed by Turkey HSD Post Hoc test. $p<0.05$ were considered significant.

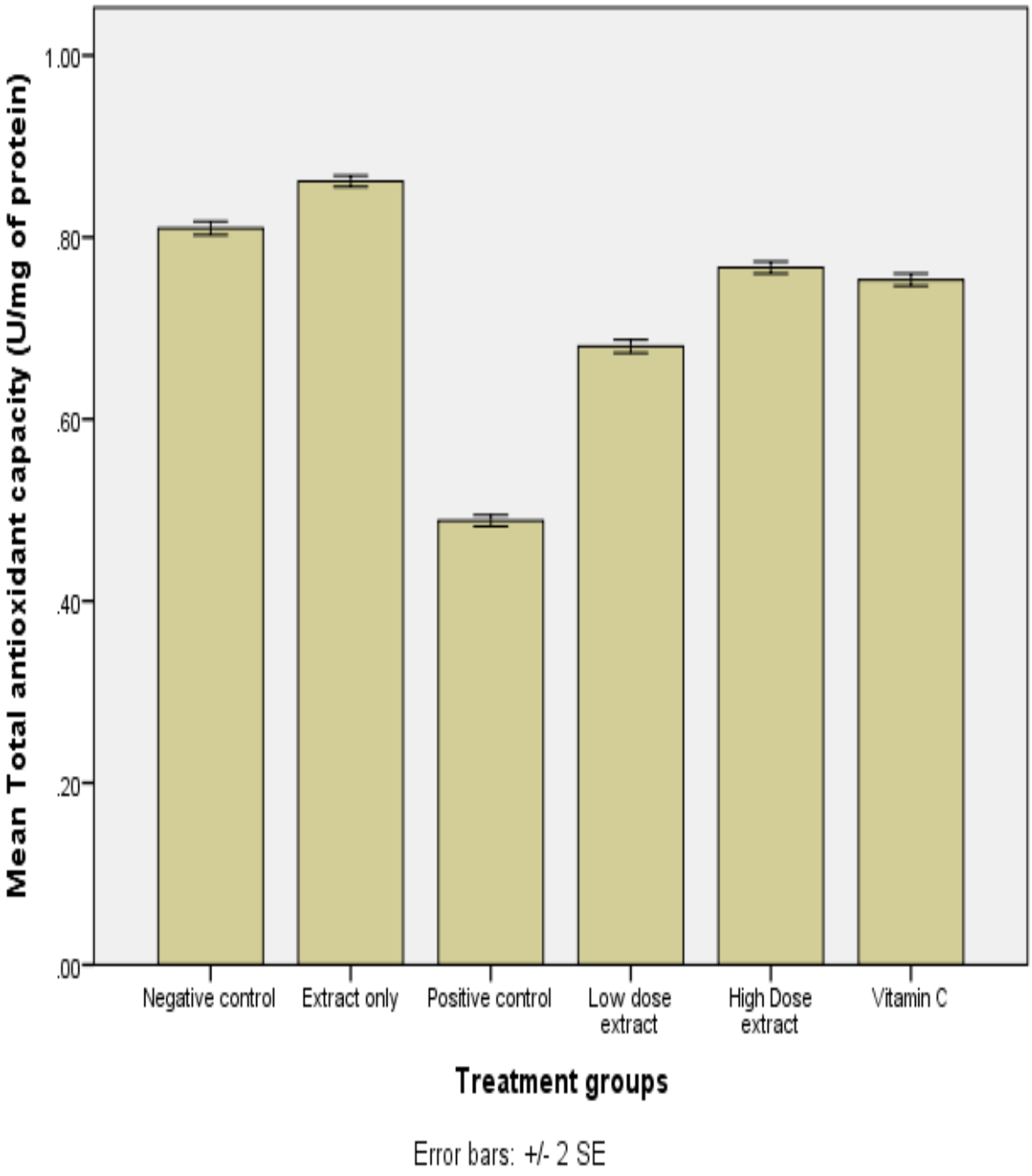


Figure 7: The mean SEM values of the Total antioxidant capacity of the liver samples of the six different groups (n=6).

Reporting the results of the Post Hoc Test for the liver samples:

The results from table 7 and figure 7 above have shown the following:

A significant difference in the Total antioxidant capacity of liver samples between the group that received normal saline only (group 1), as compared to the other groups was observed. When compared to: that which received plant extract only (group 2), p=0.001; that which received acetaminophen only (group 3), p=0.001; that which received acetaminophen followed by low dose of *Vernonia amygdalina* methanolic extract (groups 4), p=0.001; that which received acetaminophen followed by high dose of *Vernonia amygdalina* methanolic extract (group 5), p=0.001; and also the group that received acetaminophen followed by Vitamin C (group 6), p=0.001.

A significant difference in the Total antioxidant capacity of liver samples between the group that received acetaminophen only (group 3), as compared to the other groups were as follows: that which received plant extract only (group 2), p=0.001; that which received acetaminophen followed by low dose of *Vernonia amygdalina* methanolic extract (groups 4), p=0.001; that which received acetaminophen followed by high dose of *Vernonia amygdalina* methanolic extract (group 5), p=0.001; and also the group that received acetaminophen followed by Vitamin C (group 6), p=0.001.

A significant difference in the Total antioxidant capacity of liver samples was observed between the group that received plant extract only (group 2), as compared to the other groups as follows: that which received acetaminophen followed by low dose of *Vernonia amygdalina* methanolic extract (groups 4), p=0.001; that which received acetaminophen followed by high dose of *Vernonia amygdalina*

methanolic extract (group 5), p=0.001; and also the group that received acetaminophen followed by Vitamin C (group 6), p=0.001.

A significant difference was also observed in the Total antioxidant capacity of liver samples between the group that received acetaminophen followed by low dose of *Vernonia amygdalina* methanolic extract (groups 4) as compared to that which received acetaminophen followed by high dose of *Vernonia amygdalina* methanolic extract (group 5), p=0.001 and also to the group that received acetaminophen followed by Vitamin C (group 6), p=0.001. However, there was no significant difference between the group that received acetaminophen followed by high dose of *Vernonia amygdalina* methanolic extract (group 5), as compared to that which received acetaminophen followed by Vitamin C (group 6), p=0.08.

A graphical representation of the Turkeys HSD post hoc test at 95% confidence interval is in the bar chart in the Figure 7 above. The graph shows that the Total antioxidant capacity of the liver sample was highest in group 2 (extract only) and was reducing in the following order: group 1 (Normal saline), group 5 (acetaminophen + high extract dose), group 6 (acetaminophen + vitamin C), group 4 (acetaminophen + low extract dose), and lastly group 3 (acetaminophen only).

4.2.2 The Malondialdehyde Levels in Various Groups

The results from Table 8 and 9 below have shown the following:

A statistically significant difference in the mean values of Malondialdehyde levels of both the blood and liver samples of Group 4 (acetaminophen + low extract dose - endline) - [mean blood 1.91nmol/mg, p=0.001 and liver 1.65nmol /mg of protein, p=0.01 and] and Group 5 (acetaminophen + high extract dose - endline) – [Mean blood 0.56nmol/mg, p=0.001 and liver 0.63nmol /mg of protein, p=0.001] as

compared to those in Group 1(Negative control - baseline) – [Mean blood 0.44nmol /mg and liver 0.37nmol/mg of protein].

Administration of the plant extract produced a statistically significant difference in the mean values of the Malondialdehyde levels of the blood and liver samples of Group 4 (acetaminophen + low extract dose - endline) and Group 5 (acetaminophen + high extract dose – endline) as compared to those in Group 3(Positive control - endline) – [Mean blood 4.07nmol /mg and liver 5.37nmol /mg of protein].

A statistically significant difference was observed in the mean values of the Malondialdehyde levels of both the blood and liver samples of Group 1(Negative control - baseline) as compared to Group 2 (Extract only - endline) – [Mean blood 0.23nmol /mg, p=0.001 and liver 0.20nmol /mg of protein, p=0.001]; Group 3 (Positive control – endline); and Group 6 (Acetaminophen + Vitamin C - endline) – [mean blood 0.61nmol /mg, p=0.001 and liver 0.59nmol /mg of protein, p=0.001].

A statistically significant difference was also shown in the mean values of the Malondialdehyde levels of both the blood and liver samples of Group 3 (Positive control) as compared to Group 1(Negative control); Group 2 (Extract only); and Group 6 (Acetaminophen + Vitamin C)

Table 8- The mean and standard error of the Malondialdehyde levels for the blood samples of the mice.

Groups	Treatment	Mean	Standard error	p-value(1-sided)
1	Normal saline at 2ml/kg(negative control)	0.44	0.01	0.001*≠
2	V. amygdalina at 50mg/kg (Extract only)	0.23	0.02	0.001*≠
3	Acetaminophen at 300mg/kg (positive control)	4.07	0.02	0.001*≠
4	Acetaminophen at 300mg/kg + extract at 50mg/kg	1.91	0.02	0.001*≠
5	Acetaminophen at 300mg/kg + extract at 100mg/kg	0.56	0.02	0.02*/0.001≠
6	Acetaminophen at 300mg/kg + Vitamin C at 500mg/kg	0.61	0.02	0.001*≠
	Mean	0.69	0.01	

Data are the Mean, Standard error of the mean and p-values of the six different treatment groups (n=6). Differences in means were analysed using One-way analysis of Variance, followed by Turkey HSD Post Hoc test. p<0.05 were considered significant. And significant comparison of Group 1 with each of the other groups was shown by *, while significant comparison of Group 3(positive control) with the other groups was shown by ≠, and the actual p values areas shown in the p-value columns respectively.

Table 9- The mean and standard error of the Malondialdehyde for the liver samples of the mice.

Groups	Treatment	Mean	Standard error	p-value(1-sided)
1	Normal saline at 2ml/kg(negative control)	0.37	0.02	0.001*≠
2	V. amygdalina at 50mg/kg (Extract only)	0.20	0.02	0.001*≠
3	Acetaminophen at 300mg/kg (positive control)	5.37	0.02	0.001*≠
4	Acetaminophen at 300mg/kg + extract at 50mg/kg	1.65	0.02	0.001*≠
5	Acetaminophen at 300mg/kg + extract at 100mg/kg	0.63	0.02	0.001*≠
6	Acetaminophen at 300mg/kg + Vitamin C at 500mg/kg	0.59	0.03	0.001*≠
	Mean	1.47	0.3	

Data are the Mean, Standard error of the mean and p-values of the six different treatment groups (n=6). Differences in means were analysed using One-way analysis of Variance, followed by Turkey HSD Post Hoc test. p<0.05 were considered significant. And significant comparison of Group 1 with each of the groups was shown by * while that of Group 3(positive control) with the other groups was shown by ≠, and the actual p values areas shown in the p-value columns respectively.

4.2.2.1 One-way ANOVA for the malondialdehyde levels of blood and liver samples

Table 10: One –way Analysis of variance for malondialdehyde levels of blood and liver samples for the between group differences.

Source	SS	df	MS	F	p-value
Between groups (blood)	65.82	5	13.164	3225.172	0.001
Between groups (liver)	117.4	5	23.480	7211.045	0.001
Total	183.22	10			

There was a statistically significant difference in the malondialdehyde levels of the blood samples as determined by the one-way ANOVA ($F(5,30) = 3225.172$, $p=0.001$.

There was a statistically significant difference in the malondialdehyde levels of the liver samples as determined by the one-way ANOVA ($F(5,30) = 7211.045$ $p=0.001$.

These p-values obtained in both the blood and liver samples of the total antioxidant capacity and the malondialdehyde levels were 1-sided alpha measurements, which were in one direction.

4.2.2.2 Multiple Comparisons - Turkey HSD Post Hoc Test for malondialdehyde of blood and liver samples

The tables 11 and 12 below are showing the multiple comparisons of the mean values of the six different treatment groups of the malondialdehyde levels of the blood and liver samples respectively. While the figures 8 and 9 are showing the graphical representation of the MDA values of both the blood and liver samples respectively.

4.2.2.2.1 Multiple comparisons of the malondialdehyde levels of blood Samples

Table 11. The multiple comparisons of Malondialhyde levels Turkey Post Hoc Test of Blood samples.

Group	Mean (nmol/mg of protein)	Comparison groups	Mean (nmol/mg of protein)	p-value(1-sided)	Confidence interval	
					Lower bound	Upper bound
Negative control	0.44	Extract only	0.23	0.001	0.85	0.87
		Positive control	4.07	0.001	0.48	0.50
		Low dose extract	1.91	0.001	0.67	0.69
		High Dose extract	0.56	0.02	0.76	0.78
		Vitamin C	0.61	0.001	0.74	0.76
Extract only	0.23	Positive control	4.07	0.001	0.48	0.50
		Low dose extract	1.91	0.001	0.67	0.69
		High dose extract	0.56	0.001	0.76	0.78
		Vitamin C	0.61	0.001	0.74	0.76
Positive control	4.07	Low dose extract	1.91	0.001	0.67	0.69
		High dose extract	0.56	0.001	0.76	0.78
		Vitamin C	0.61	0.001	0.74	0.76
Low Dose Extract	1.91	High Dose extract	0.56	0.001	0.76	0.78
		Vitamin C	0.61	0.001	0.74	0.76
High Dose Extract	0.56	Vitamin C	0.61	0.777	0.74	0.76

Data are mean ± SEM (n=6). The groups were as follows: Group1(negative control)- received normal saline at 2ml/kg, Group 2 (Extract only) – received Vernonia amygdalina Delile extract at 50mg/kg orally, Groups 3 – 6 received acetaminophen at 300mg/kg orally; Group 3 (Positive control) – received only acetaminophen; Group 4 (experimental low extract dose) – also received Vernonia amygdalina at 50mg/kg; group 5 (experimental high extract dose) – also received 100mg/kg Vernonia amygdalina Delile extract; Group 6 (Known antioxidant) – also received Vitamin C at 500mg/kg orally. Differences in means were analysed using Kruskal Wallis H Test, followed by Turkey HSD Post Hoc test. $p<0.05$ were considered significant.

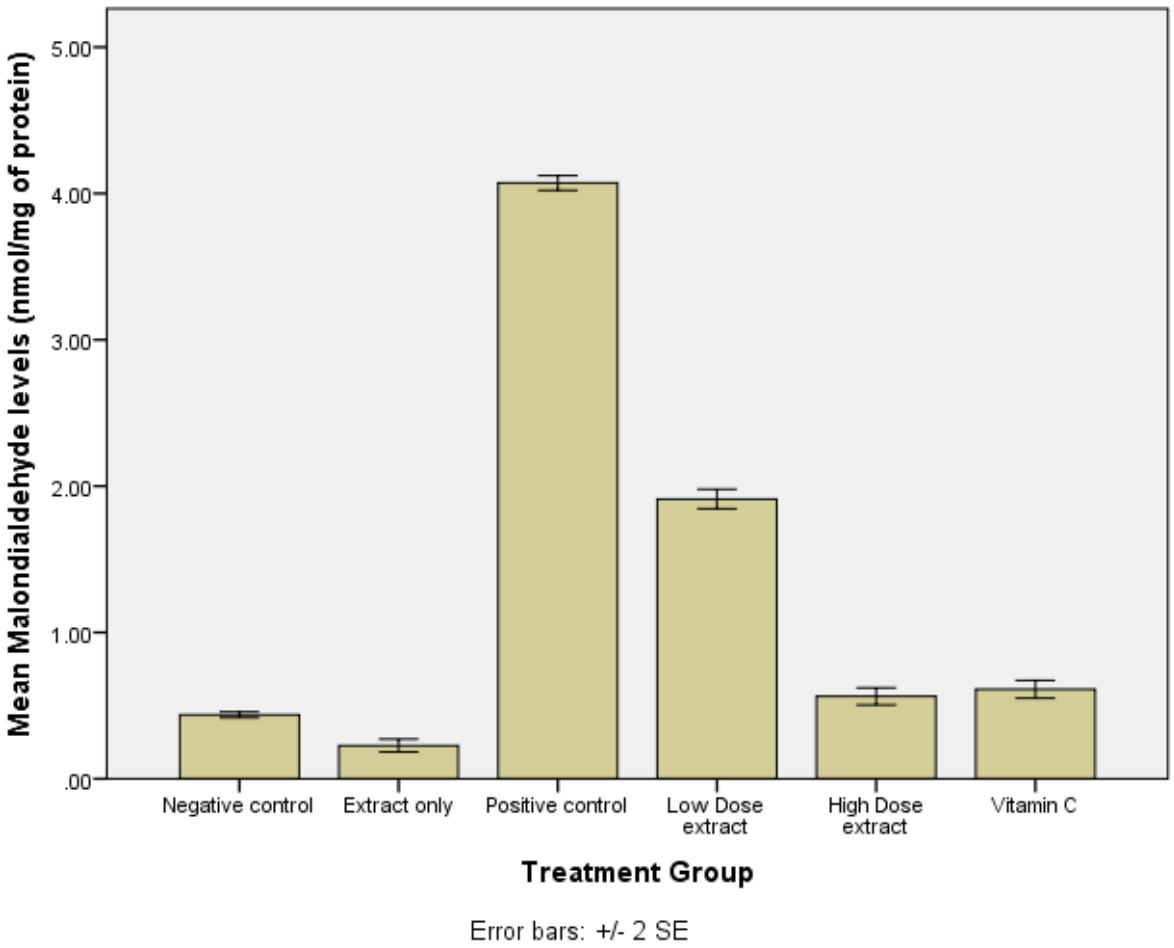


Figure 8 – The mean and SEM values of the Malondialdehyde levels of the blood samples of six different groups (n=6).

Reporting the results of the Post Hoc Test for malondialdehyde levels of the blood samples:

The results from table 11 and figure 8 have shown the following:

A significant difference in the Malondialdehyde levels of blood samples between the group that received normal saline only (group 1), as compared to the other groups as follows: that which received plant extract only (group 2), p=0.001; that which received acetaminophen only (group 3), p=0.001; that which received acetaminophen followed by low dose of *Vernonia amygdalina* methanolic extract (groups 4), p=0.001; that which received acetaminophen followed by high

dose of *Vernonia amygdalina* methanolic extract (group 5), p=0.001; and also the group that received acetaminophen followed by Vitamin C (group 6), p=0.001.

A significant difference was also observed in the Malondialdehyde levels of blood samples between the group that received acetaminophen only (group 3), as compared to the other groups as follows: that which received plant extract only (group 2), p=0.001; that which received acetaminophen followed by low dose of *Vernonia amygdalina* methanolic extract (groups 4), p=0.001; that which received acetaminophen followed by high dose of *Vernonia amygdalina* methanolic extract (group 5), p=0.001; and also the group that received acetaminophen followed by Vitamin C (group 6), p=0.001.

A significant difference was also seen in the Malondialdehyde levels of blood samples between the group that received plant extract only (group 2), as compared to the other groups as follows: that which received acetaminophen followed by low dose of *Vernonia amygdalina* methanolic extract (groups 4), p=0.001; that which received acetaminophen followed by high dose of *Vernonia amygdalina* methanolic extract (group 5), p=0.001; and also the group that received acetaminophen followed by Vitamin C (group 6), p=0.001.

A significant difference was evident in the Malondialdehyde levels of blood samples between the group that received acetaminophen followed by low dose of *Vernonia amygdalina* methanolic extract (groups 4) as compared to that which received acetaminophen followed by high dose of *Vernonia amygdalina* methanolic extract (group 5), p=0.001; and also the group that received acetaminophen followed by Vitamin C (group 6), p=0.001. However, there was no significant difference between the group that received acetaminophen followed by high dose of *Vernonia amygdalina* methanolic extract (group 5), as compared to that which received acetaminophen followed by Vitamin C (group 6), p=0.797.

A graphical presentation of the data from the Turkeys HSD post hoc test at 95% confidence interval in figure 8 has shown that the malondialdehyde levels of the blood sample was lowest in group 2 (extract only) and was increasing in the following order: group 1 (Normal saline), group 5 (acetaminophen + high extract dose), group 6 (acetaminophen + vitamin C), group 4 (acetaminophen + low extract dose), and lastly group 3 (acetaminophen only).

4.2.2.2.2 Multiple comparisons for the malondialdehyde levels of Liver Samples

Table 12: The multiple comparisons of malondialdehyde levels from Turkey Post Hoc Test of liver samples.

Group	Mean (nmol/mg of protein)	Comparison groups	Mean (nmol/mg of protein)	p-value(1-sided)	Confidence interval	
					Lower bound	Upper bound
Negative control	0.37	Extract only	0.20	0.001	0.15	0.25
		Positive control	5.37	0.001	5.32	5.43
		Low dose extract	1.65	0.001	1.59	1.72
		High Dose extract	0.63	0.001	0.56	0.69
		Vitamin C	0.59	0.001	0.51	0.68
Extract only	0.20	Positive control	5.37	0.001	5.32	5.43
		Low dose extract	1.65	0.001	1.59	1.72
		High dose extract	0.63	0.001	0.56	0.69
		Vitamin C	0.59	0.001	0.51	0.68
Positive control	5.37	Low dose extract	1.65	0.001	1.59	1.72
		High dose extract	0.63	0.001	0.56	0.69
		Vitamin C	0.59	0.001	0.51	0.68
Low Dose Extract	1.65	High Dose extract	0.63	0.001	0.56	0.69
		Vitamin C	0.59	0.001	0.51	0.68
High Dose Extract	0.63	Vitamin C	0.59	0.927	0.51	0.68

Data are mean ± SEM (n=6). The groups were as follows: Group1(negative control)- received normal saline at 2ml/kg, Group 2 (Extract only) – received Vernonia amygdalina Delile extract at 50mg/kg orally, Groups 3 – 6 received acetaminophen at 300mg/kg orally; Group 3 (Positive control) – received only acetaminophen; Group 4 (experimental low extract dose) – also received Vernonia amygdalina at 50mg/kg; group 5 (experimental high extract dose) – also received 100mg/kg Vernonia amygdalina Delile extract; Group 6 (Known antioxidant) – also received Vitamin C at 500mg/kg orally. Differences in means were analysed using Kruskal Wallis H Test, followed by Turkey HSD Post Hoc test. $p<0.05$ were considered significant.

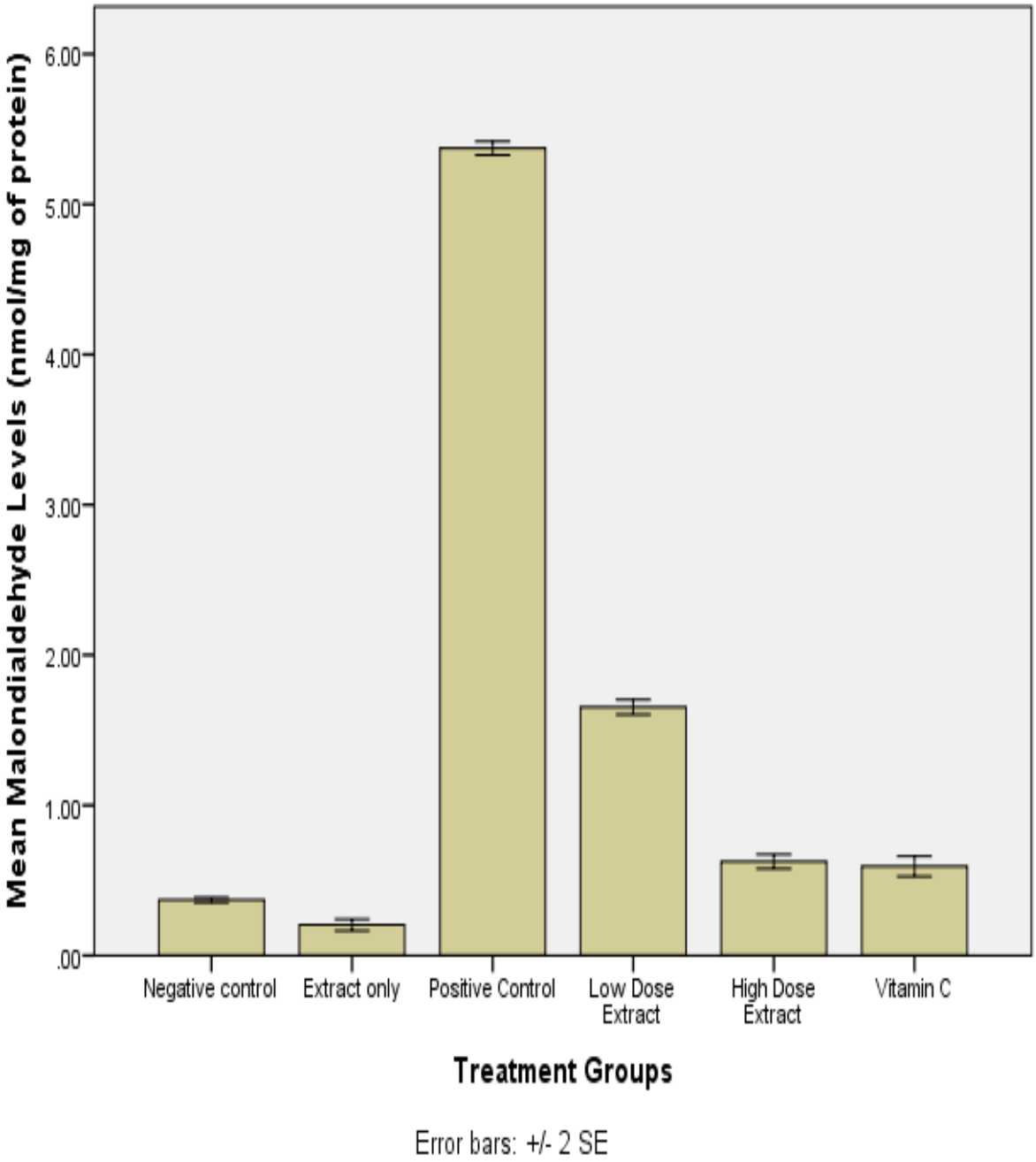


Figure 9 – The mean and SEM values of Malondialdehyde levels of the liver samples of six different groups (n=6).

Reporting the results of the Post Hoc Test of malondialdehyde levels for liver samples:

From results in table 12 and figure 9 above, the following was observed:

A significant difference was seen in the Malondialdehyde levels of liver samples between the group that received normal saline only (group 1), compared to the other groups. The significance level, when compared with plant extract only (group 2), was p=0.001; with the group which received acetaminophen only (group 3), p=0.001; that which received acetaminophen followed by low dose of *Vernonia amygdalina* methanolic extract (groups 4), p=0.01; for the group that received acetaminophen followed by high dose of *Vernonia amygdalina* methanolic extract (group 5), p=0.001; and for the group that received acetaminophen followed by Vitamin C (group 6), p=0.001.

A significant difference was evident in the Malondialdehyde levels of liver samples between group 3, as compared to the other groups as follows: group 2, p=0.001; group 4, p=0.001; group 5, p=0.001 and group 6, p=0.001.

Also observed was a significant difference in the Malondialdehyde levels of liver samples between group 2, as compared to the other groups as follows: group 4, p=0.001; group 5, p=0.001; and group 6, p=0.001.

A significant difference was seen in the Malondialdehyde levels of liver samples between the group that received acetaminophen followed by low dose of *Vernonia amygdalina* methanolic extract (groups 4) as compared to that which received acetaminophen followed by high dose of *Vernonia amygdalina* methanolic extract (group 5), p=0.001; and also the group that received acetaminophen followed by Vitamin C (group 6), p=0.001. However, there was no significant difference between the group that received acetaminophen followed by high dose of *Vernonia*

amygdalina methanolic extract (group 5), as compared to that which received acetaminophen followed by Vitamin C (group 6), p=0.945.

From the graphical representation of the Turkeys HSD post Hoc test at 95% confidence interval as shown in figure 9 a significant difference was observed in the Malondialdehyde levels of the liver samples of the different groups where the levels were lowest in group 2 (extract only) and was increasing in the following order: group 1(Normal saline), group 5 (acetaminophen + high extract dose), group 6 (acetaminophen + vitamin C), group 4(acetaminophen + low extract dose), and lastly group 3 (acetaminophen only).

CHAPTER FIVE

DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 Discussion

The main objective of this study was to determine the effects of *Vernonia amygdalina* methanolic leaf extract on the Total Antioxidants and Malondialdehyde levels in an acetaminophen induced hepatotoxicity.

This study showed that the administration of Vernonia amygdalina extract to mice treated with toxic doses of acetaminophen was able to attenuate oxidative stress by causing an increase in the total antioxidant capacity of the mice. This therefore implies that the plant possess constituents that are able to cause an increase in the antioxidant levels in the body by either increasing their synthesis or by using its own antioxidants to scavenge the free radicals(Adedosu et al., 2014, Ho et al., 2015, Iwalokun et al., 2006). This plant has been shown to possesses phytochemicals which include flavonoids, phenols, alkaloids, saponins and terpenoids; which have been demonstrated to produce antioxidant activities(Atangwho et al., 2009, Ayoola et al., 2008, Salawu et al., 2007). The plant contains high amounts of phenolics and flavonoids which have been shown to be free radicals scavengers and may be responsible for the free radical scavenging properties of the plant(Adedosu et al., 2014). This was consistent with the findings of Adedapo *et al*(Adedapo et al., 2014), who demonstrated that pretreatment with *Vernonia amygdalina* extract is able to attenuate oxidative stress, this was also shown by other researchers (Adedosu et al., 2014, Bashir and Alhaji, 2012, Iwalokun et al., 2006, Iyawe and Azih, 2015). Moreover, the findings of Iwalokun et al(Iwalokun et al., 2006), suggests that the plant produces

hepatoprotective effects which are due its antioxidant properties, this was consistent with the findings of other researchers (Ho et al., 2015).

Another study by Usunamena *et al* (Usunobun et al., 2015), also demonstrated that pretreatment with extract of *V. amygdalina* led to a significant attenuation of oxidative stress by increasing the antioxidant enzymes. This was also shown by other researchers (Adaramoye Oluwatosin et al., 2008, Huang et al., 2011, Iroanya et al., 2014). The hydroxyl group of the phenols which is bonded to the aromatic hydrocarbon gives them the antioxidant activity which they possesses as they donate the electrons to the free radicals which are seeking electrons thus reducing the damage to living cells (Uyoh et al., 2013).

Despite these findings, a study done by Ebong *et al*(Ebong et al., 2011), demonstrated that pretreatment with the extract of Vernonia amygdalina only increased the levels of catalase but not the rest of the antioxidants. This could probably be due to variations in the antioxidant activity in the plant extracts which has been shown to depend on the model involved, with one type giving a high activity and another giving a lower activity of the same plant(Halliwell et al., 1987).

Being a plant that has been used in folk medicine successfully in the treatment of various diseases like malaria and diabetes(Farombi and Owoeye, 2011), this information will be useful in clinical practice as individuals with diseases associated with oxidative stress could be treated with this plant extract. This information will also justify its use in folk medicine in the treatment of oxidative stress related diseases.

The study was also able to show that treatment of the mice with *Vernonia amygdalina* extract caused significant attenuation of lipid peroxidation processes, as was shown by the reduction of

MDA levels. This implies that this plant extract contains some elements that are probably radical chain reaction terminators, which leads to the termination of the lipid peroxidation chain reactions(Ngatu et al., 2012, Ugwu et al., 2011). Since lipid peroxidation occurs due to the oxidation of polyunsaturated fatty acids in the cells membranes, the plant extract may have attenuated it by the presence of antioxidant Vitamins namely A, E, and probably other substances which protect and stabilize the plasma membrane and prevent the leakage of the enzymes through the membrane structure of the cells(Huang et al., 2011, Ojiako and Nwanjo, 2006, Owoeye et al., 2010).

These findings are similar to the findings of Oriakhi *et al* (Oriakhi et al., 2013), who demonstrated that the extract of *V. amygdalina* significantly inhibited lipid peroxidation through a reduction in MDA levels, this was also shown by other researchers(Iwalokun et al., 2006, Kate and Lucky, 2012, Nwanjo, 2005, Saisho, 2014). The reduction in the MDA levels is attributed to the composition of phytochemicals like tannins, flavonoids, phenols, alkaloids, and saponins which are found in the extract of *Vernonia amygdalina*, and gives rise to the antioxidant properties that it portrays(Ayoola et al., 2008).

These findings are different from those found in a study done by Ebong *et al*(Ebong et al., 2011), who demonstrated that pretreatment with the extract of *Vernonia amygdalina* did not reduce the levels of MDA, thus did not attenuate lipid peroxidation. This could probably have been due to a method that was used in this study which could have given the plant low activity, thus showing an insignificant activity (Eseyin et al., 2015).

The clinical applications of these results, following further studies are the possible use of this plant in treatment of diseases complicated by lipid peroxidation reactions and also possible manufacturing of drugs for treatment of such conditions.

The attenuation of oxidative stress was dose dependent, indicating that the effects shown are probably due to the plant extract and that its effect can be increased or reduced by altering the dose given. These results are consistent with the findings of Iwalokun *et al* (Iwalokun et al., 2006), who demonstrated that the suppression of lipid peroxidation and oxidative stress in the mice pretreated with Vernonia amygdalina extract was dose dependent, being lower at a low dose and higher at a high dose, this was also shown by other researchers(Ojiako and Nwanjo, 2006). This in clinical practice will allow for proper dosage of the medications when it is used in the treatment of patients with oxidative stress and lipid peroxidation associated diseases like malaria and diabetes. It will also allow for scientific validation of the antioxidant properties of this plant in Zambia.

The antioxidant activity produced by the high dose extract was comparable with that produced by a known antioxidant, Vitamin C. This shows that *Vernonia amygdalina* is a potent antioxidant as it has a similar activity as for a known antioxidant. These results are in agreement with the findings of Adaramoye *et al*(Adaramoye Oluwatosin et al., 2008), who showed that the extract of *V. amygdalina* and vitamin C both produced potent antioxidant activity, as they were both able to increase antioxidant enzymes. The relevance of this finding is the possible use of this plant as a natural source of antioxidants, like vitamin C is used.

This study has also shown that administration of the *Vernonia amygdalina* plant extract to normal animals also increased their antioxidant properties. This was consistent with the findings

of Iyawe and Azih (Iyawe and Azih, 2016), who demonstrated that *Vernonia amygdalina* extract possessed antioxidant properties that allows it to quench free radicals in normal conditions. The clinical relevance of this finding is the possibility of using this plant as a chemoprophylactic agent, in the prevention of oxidative stress related diseases.

An overdose of acetaminophen causes an increased production and accumulation in the hepatocytes, of the toxic metabolite of acetaminophen, called N-acetyl-p-benzo- quinoneimine (NAPQI), an eletrophillic molecule that causes harm by covalently binding with other intracellular proteins modifying their structure and function and lead to a cascade of events that lead to the formation of blebs and cause loss of integrity in the cell membrane (Black, 1984, Lee, 1995, Nelson, 1990). Apart from the NAPQI formation, this oxidative reaction of cytochrome P450 also leads to formation of free radicals that covalently binds to proteins and unsaturated fatty acids causing lipid peroxidation (Farrell, 1994). Therefore the reduction in the total antioxidant status following the administration of the acetaminophen overdose, could be due to the increase in the amount of the antioxidants being used in the mopping up of the ROS brought about by the process of oxidative stress (Imafidon et al., 2015). The decrease in oxidative stress suggests that constituents in the extract of *V. amygdalina* could probably complex with the NAPQI or inhibit the CYP 450, which may render protection to the liver. However, further studies are required to assess the liver enzyme levels and its histology.

5.2 Conclusion

The hypothesis that drove this study states that' leaf extracts of *Vernonia amygdalina* enhances antioxidant capacity and reduces levels of lipid peroxidation reaction products during acetaminophen overdose in mice'. The methanolic leaf extract of *Vernonia amygdaliana* showed

a significant dose dependent attenuation of oxidative stress induced by acetaminophen drug toxicity by enhancing the total antioxidant capacity and reducing the malondialdehyde levels, in blood and liver samples.

In conclusion the study showed that the methanolic leaf extract of *Vernonia amygdalina Delile* possesses significant antioxidant properties allowing it to attenuate oxidative stress and lipid peroxidation processes, thus the anti-oxidant properties of the plant justify its use in folk medicine in the treatment of oxidative stress associated diseases like diabetes. This study has also contributed to the scientific validation of the antioxidant properties of *Vernonia amygdalina* plant growing in Zambia. Further studies are recommended to study other medicinal uses of this plant grown in Zambian environment and also on the safety of its use.

5.3 Recommendations

The decrease in oxidative stress in acetaminophen overdose following administration of *Vernonia amygdalina* extract suggests that there could be constituents in this plant that probably complexes with the NAPQI or inhibit the CYP 450, therefore there is need for further studies to be done in this area.

I recommend that the another study should be done to assess and determine the quantities of other biologically active components of the plant *Vernonia amygdalina* Delile, as this will provide more comprehensive information on the medicinal properties of this plant, grown in Zambian climatic conditions.

I also recommend that more studies be done to determine the active components of *V. amygdalina* responsible for the antioxidant effects shown and whether they act singly or

synergistically to produce these found effects. There is therefore need for another study to be done in this area.

5.4 Limitations

The major limitation of this study is that the none availability of the HPLC-MS system that gives a more specific measurement of the MDA levels in the system, thus a less specific test was used although having shown that the plant extract of *Vernonia amygdalina* possesses antioxidant properties by which it attenuates the effects of oxidative stress, it has not been able to show which components of the plant are responsible for these effects and whether they act singly or synergistically to produce these found effects. There is therefore need for another study to be done in this area.

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APPENDIXES

APPENDIX A Stock Solutions Preparation

Reagents for the Total Antioxidant Capacity (TAOC)

Preparation of 300mmol Acetate Buffer Solution

This was prepared by adding 1.68g of glacial acetic acid to a 100ml volumetric flask, and then 0.3g of sodium acetate trihydrate was also added, and this was made to the mark after dissolving the solids to 100ml. Then the PH was adjusted to 3.6 using glacial acetic acid.

Preparation of 10mmol TPTZ (2,4,6-tripyridyl-s-triazine (Iron Reagent)) in40mmol of Hydrochloric acid (HCl) Solution

0.0312g of the TPTZ was placedinto a 100ml volumetric flask and to it 0.36ml of HCl was added, after dissolving the solids, the solution was made up to 100ml with distilled water.

Preparation of 20mmol of Ferric Chloride (FeCl3.6H2O) solution

3.24g of ferric chloride was placed into a 1000ml volumetric flask and after dissolving it with distilled water, it was made to the mark using distilled water.

Preparation of the FRAP (Ferric Reducing Power of Plasma) reagent

This was made by adding 25ml of acetate buffer, 2.5ml of TPTZ solution and 2.5ml of ferric chloride (FeCl3.6H2O).

Preparation of Fe (II) Standard solution

69.50g of Ferrous Sulphate (FeSO4.7H2O) was placed in a 250ml volumetric flask and after dissolving with distilled water, it was made up to the mark with distilled water. Then 0.1mM, 0.2mM, 0.4mM, 0.6mM, 0.8mM, and 1.0mM dilutions of the standard solution were made as shown in Appendix B below.

Malondialdehyde (MDA) Levels Reagents:

Preparation of the Phosphate buffered saline (PBS) Solution

This was prepared by mixing 8.1g NaCl (Sodium Chloride), 2.302g Na2HPO4and 0.194g NaH2PO4in a 1000ml volumetric flask, these were dissolved into distilled water and then it was made to the 1000ml mark with distilled water. The PH of the solution was adjusted downwards using NaOH solution, to PH 7.4.

Butylated Hydroxytoulene Solution

This was prepared by adding 88mg of butylated hydroxytoulene into 10ml of absolute alcohol.

30% Trichloroacetic Acid(TCA) solution

This was prepared by adding 30g of TCA into a 100ml volumetric flask and dissolving using distilled water, then it was made to the mark with distilled water.

EDTA (Ethyl-diamianetetraacetic Acid) solution

This was prepared by adding 29.224g in 1000ml volumetric flask, and it was dissolved in distilled water by slightly raising the temperature, then this was made up to the mark (1000ml) in the volumetric flask.

1% Thiobarbituric Acid in 0.05M NaOH solution

1g of thiobarbituric acid was placed into 100ml volumetric flask, after dissolving it was made up to the mark. Then o.2g of NaOH pellets were placed in 100ml of 1% Thiobarbituric acid.

Appendix B – Dilution

The dilutions for the standard solution

Concentration (mM)	Fe2SO4.7H2O (ml)	Distilled water (ml)
0.1	1	9
0.2	2	8
0.4	4	6
0.6	6	4
0.8	8	2
1.0	1.0	0

Appendix C - MDA concentrations

The method of Heath and Parker(Heath and Packer, 1968), was used to obtain the concentrations of the sample. This method uses the following formular:

$$\mathbf{MDA}_{\text{equivalents (nmol/ml)}} = [(A_{532} - A_{600}) / 155000]\, 10^6$$

Where:

- 532 nm - maximum absorbance of the TBA-MDA complex
- 600 nm - correction for nonspecic turbidity, and
- 155 000 - the molar extinction coefficient for MDA.

Appendix D Test for Normality of the data:

This was carried out using the Shapiro-wilk Test, because it is the most appropriate way of assessing normality with small sample sizes.

(a) Total Antioxidant Capacity of the plasma samples were analysed and the dependent variable,' treatment', as was given in groups 1, 2, 3, 4, 5 and 6 were normally distributed, as their probability values were 0.960, 0.801. 0.473, 0.421, 0.875 and 0.801 respectively. These were greater than 0.05 meaning that the data were normally distributed.

(b) Total Antioxidant Capacity of the liver samples were analysed and the dependent variable,' treatment', as given in groups 1, 2, 3, 4, 5 and 6 were normally distributed, their probability values were, 0.167, 0.212, 0.212, 0.167, 0.91 and 0.91, respectively. The data were normally distributed, as for all the probability was greater than 0.05.

(c) Malondialdehyde levels of the plasma samples were analysed and the dependent variable,' treatment', as given in groups 1, 2, 3, 4, 5 and 6 were normally distributed, 0.415, 0.447, 0.961, 0.637, 0.586 and 0.661. All were greater than 0.05 meaning that the data was normally distributed.

(d) Malondialdehyde levels of the liver samples were analysed and the dependent variable,' treatment', as given in groups 1, 2, 3, 4, 5 and 6 were normally distribution and they gave the probability values of 0.320, 0.672, 0.839, 0.771, 0.781 and 0.870, all these were greater than 0.05.

Appendix E Test for homogeneity of Variances:

(a) The Levene test for homogeneity of variances showed a non-significant value of p=0.144, this means that the population variances of the total antioxidant capacity of the blood sample in each group are equal.

(b) The levene test for homogeneity of variances showed a non-significant value of p=0.991, this means that the population variances of the total antioxidant capacity of the liver sample in each group are equal.

(c) The levene test for homogeneity of variances showed a non-significant value of p=0.229, this means that the population variances of the Malondialdehyde levels of the blood sample in each group are equal.

(d) The levene test for homogeneity of variances showed a non-significant value of p=0.241, this means that the population variances of the Malondialdehyde levels of the liver sample in each group are equal.