PROPHYLACTIC EFFICACY OF *MORINGA OLEIFERA* LEAF EXTRACTS AGAINST LIVER INJURY INDUCED BY ARTESUNATE-AMODIAQUINE ANTIMALARIAL COMBINATION

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DECLARATION

This thesis is my original work and has not been presented for the award of a degree in any University.

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DEDICATION

I dedicate this work to a flower, a gem and the most influential person in my life to date, my beloved mother, the late Alice Nyang'or. Your tenacity, hardwork, dedication and industry in all that you did set a benchmark for me to follow. Your memory lives on in my heart.

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Mitchel Otieno Okumu

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LIST OF ACRONYMS

AAE-Ascorbic Acid Equivalents

ALT-Alanine Amino Transferase

AQ-Aqueous

AQ-ME-Aqueous Methanol

ASAQ-Artesunate-Amodiaquine

AST-Aspartate Amino Transferase

BHT-Butylated Hydroxy Toluene

CE-Catechin Equivalents

CSD- Clinical Studies Department

EDTA-Ethylene Diamine Tetra Acetic Acid

GAE-Gallic Acid Equivalents

HIV-Human Immunodeficiency Virus

LD50-Lethal dose responsible for the death of 50% of a population of animals

MO-Moringa oleifera

OECD- Organization for Economic Co-operation and Development

PHPT-Public Health, Pharmacology and Toxicology

TB-Total Bilirubin

TFC-Total Flavonoid Content

TPC-Total Phenolic Content

VPMP- Veterinary Pathology, Microbiology and Parasitology

WHO-World Health Organisation

ABSTRACT

Malaria is a mosquito-borne disease caused by *plasmodium falciparum* parasites. It is treated using antimalarial agents. In malaria endemic countries such as Kenya, self-medication with antimalarial agents is rife. This increases the likelihood of occurrence of severe adverse effects associated with drug misappropriation. Artesunate-amodiaquine (AS-AQ) is an antimalarial combination currently in use for malaria therapy. This combination causes liver damage, neurotoxicity, agranulocytosis and haemolytic anaemia as adverse effects. These effects have been shown to be due to the free radicals generated by the drug. Plants with antioxidant effects may be used to alleviate the adverse effects of AS-AQ. *Moringa oleifera (MO)* is a plant that originates in Asia but is nowadays extensively cultivated in the tropics and sub-tropics. The plant is claimed to have antioxidant properties and other medicinal values. The antioxidant and` hepatoprotective effects of this plant are some of the medicinal claims that have not been fully investigated although scanty literature is available on these claims. The objective of the current study was to evaluate the antioxidant capacity and hepatoprotective potential of leaf extracts of *MO* against liver degradation caused by AS-AQ antimalarial combination. The leaves of *MO* were obtained from a plantation of the plant in a farm of the University of Nairobi located at Kibwezi in Makueni county. Water (AQ) and aqueous-methanol (AQ-ME) leaf extracts were prepared. Qualitative phytochemical analysis was performed on both extracts to identify compounds of pharmacological value. The antioxidant capacity of these extracts was then assessed *in vitro* by preparing calibration curves of the antioxidant standards; gallic acid, catechin, ascorbic acid, ethylene diamine tetra acetic acid and butylated hydroxy toluene using a UV-Visible

spectrophotometer and thereafter extrapolating the concentration of the antioxidants in the extracts from these curves. The results of these *in vitro* assays informed the decision of the extract to be selected for testing *in vivo* hepatoprotective efficacy in female albino rats. Acute oral toxicity of the selected extract was evaluated using the limit test dose of the up and down procedure based on OECD guidelines. From acute oral toxicity results, a safe dose (1000mg/kg) was selected and evaluated for hepatoprotective activity against AS-AQ induced toxicity in female rats using the biochemical parameters aspartate amino transferase (AST), alanine amino transferase (ALT) and total bilirubin (TB) as well as histopathological examination of rat liver sections. The extraction yield of the AQ and AQ-ME extracts was 14 % and 18 % respectively. Qualitative phytochemical screening of the extracts revealed the presence of alkaloids, cardiac glycosides, flavonoids, phenolics, saponins, tannins and ascorbic acid in both extracts. The total content of phenolics in the AQ and AQ-ME extracts was 35.42 ± 5.80 and 52.04 ± 3.13 milligrams of gallic acid equivalents (GAE) per gram of the dry plant material respectively and the total content of flavonoids was 78.69 ± 13.04 and 365.52 ± 86.76 milligrams of catechin equivalents (CE) per gram of the dry plant material respectively while the content of ascorbic acid was 2.02 ± 0.66 and 3.04 ± 2.06 milligrams of ascorbic acid equivalents (AAE) per gram of the dry plant material respectively. The LD_{50} of the AQ-ME extract was found to be > 2000mg/kg. A 1000mg/kg dose of the AQ-ME extract lowered the serum ALT and total bilirubin non-significantly *($p>0.05$)* but significantly *($p<0.05$)* lowered the AST levels to values comparable to those of the standard hepatoprotectant, siliphos®. The AQ-ME extract reduced the histological distortion of liver cells in the experimental rats induced by high doses of AS-AQ. The findings of this study suggest

that the bio protective activity of the leaves of *MO* may have some relation to its antioxidant properties which are attributable to the phenolic, flavonoid and ascorbic acid contents. Thus, leaves of *MO* may be useful in the mitigation of free-radical initiated disease conditions.

Keywords; *Moringa oleifera*, *antioxidant potential*, *hepatoprotective capacity*, *artesunate-amodiaquine, albino rats, liver*

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background information

The liver is an organ with intimate proximity to the small intestine and blood circulation. As such, it is exposed to a multitude of foreign substances thereby making it one of the most susceptible organs to injury by these substances (Devlin, 1997; Roy and Bhattacharya, 2006). There has been an upsurge in the incidences of medication-induced liver failure for a good long time (Mcnally, 2005). Moreover, hepatic failure that is induced by medication is one of the major reasons for the removal of a drug from the market by drug-regulatory bodies throughout the world. It is also a significant stumbling block in the development of new chemical entities intended for therapeutic benefit (Masabuchi *et al.,* 2011).

A high rate of malaria infection in the African region has increased the access to antimalarials (Ruebush *et al.,* 1995). Consequently, unwarranted self-medication practices make these drugs prone to abuse (Breman, 2001). The antimalarial drugs such as artesunate-amodiaquine currently in use are combination drugs and work in different ways to bring about eradication of the parasites responsible for malaria (Ruttiman *et al.,* 2006).

Antimalarial agents are selectively distributed in major body organs (liver, lungs, nervous system) and the blood plasma. The distribution is subsequently followed by free radical generation which makes these organs susceptible to deleterious effects of free radicals (Davis *et al.,* 2003).

Free radicals are electron deficient chemical entities that may either be produced within body cells during normal cell metabolism (endogenous free radicals) or from external sources such as smoking, radiation, pollution or medication (exogenous free radicals) (Valko *et al.,* 2007). The concentration of these chemical entities coupled with the prevailing conditions in the body determine whether the outcome of their effects is harmful or beneficial (Lien *et al.,* 2008).

At low to moderate concentrations, they facilitate cellular response and functioning of the immune system. However, when their rate of generation in the body is greater than their rate of destruction, they build-up to levels which induce a state in which radicals with an oxygen moiety dominate the internal homeostatic environment of cells. This phenomenon is significant in the pathophysiology of several chronic degenerative disorders including rheumatoid arthritis, osteo-arthritis, cardiac ischemia, cancer, cataracts, autoimmune diseases as well as impairment of the liver and kidney (Bahorun *et al.,* 2006).

Antioxidant substances are involved in the maintenance of a homeostatic balance between the two antagonistic effects of these chemical entities. Antioxidants are substances which suppress the initial excess production of free radicals. They are synthesized naturally and form part of the defense system of both man and animals. However, they may also be obtained from diet (Adikwu and Deo, 2013). *Moringa oleifera* is a plant associated with antioxidant properties (Santos *et al.,* 2012). Moreover, positive correlations have been made between the antioxidant properties of medicinal plants and hepatoprotective activity (Hiraganahalli *et al.,* 2012).

However, the antioxidant and hepatoprotective effects of this plant have not been adequately defined. The present study aims to provide a burden of scientific proof to the

claims of antioxidant and hepatoprotective properties of *Moringa oleifera* that have originated from oral tales, traditional medicinal folklore and native *materia medica.*

1.2 Problem statement

In areas where malaria is endemic, access to antimalarial drugs remains unrestricted and measures to curb the use of these drugs are inadequate or all together non-existent. Thus, self-medication in such regions of the world is rife and increases the potential danger likely to result from misappropriation of such drugs.

1.3 Justification of the study

In view of the facts above, studies of the potential of antimalarials such as artesunate-amodiaquine (AS-AQ) to cause toxic manifestations are important as are the studies of the methods aimed at mitigating the toxicity brought about by these drugs. In light of this, some of the phytochemicals reported in *Moringa oleifera* have been associated with antioxidant value (Santos *et al.*, 2012). The antioxidant effects of *Moringa oleifera* may be used for prophylaxis against AS-AQ induced liver injury but scientific data to support its use is currently not available.

1.4 Objectives

1.4.1 General objective

To investigate the hepatoprotective effect of *M. oleifera* leaf extracts against liver damage induced by artesunate-amodiaquine (AS-AQ) antimalarial combination.

1.4.2 Specific objectives

1.To determine the effect of variation of solvent systems on the extraction yield and phytochemical composition of leaves of *M. oleifera*.

2. To determine the *in vitro* antioxidant potential of leaf extracts of *M. oleifera* with a view to selecting the best extract for use in *in vivo* studies.

3. To determine the acute oral toxic potential of the *M. oleifera* extract identified in (2) above.

4. To determine the dose-response hepatotoxic effect of AS-AQ combination in rats

5. To investigate the protective capacity of the leaf extract of *M. oleifera* identified in (2) above against AS-AQ induced liver damage.

1.5 Hypothesis of the study

Ho; The leaves of *M. oleifera* have no protective effect on the rat liver

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Malaria; etiology, symptoms, epidemiology

Malaria is a vector-borne disease caused by protozoan parasites of the genus *Plasmodium*. These parasites gain entry into the body of a human host via the bite of a female anopheles' mosquito. There are five different strains of these parasites that have been found to infect humans (*vivax, ovale, malariae, falciparum* and recently *knowlesi*). These strains are found in the tropics and subtropics around the world (Marsh and Makani, 2004).

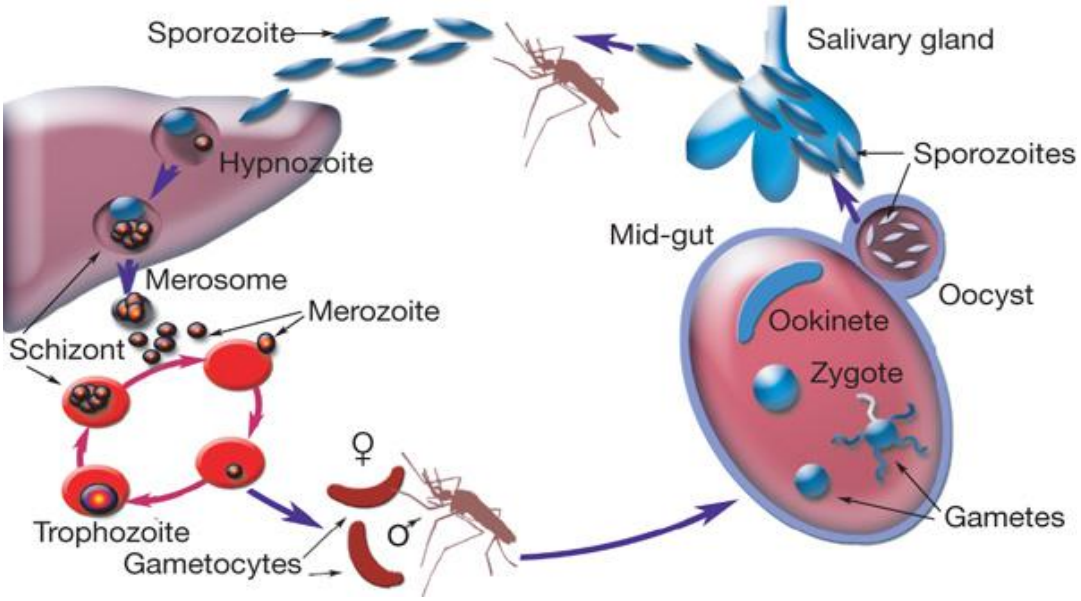


Figure 2.1; Stages of malarial parasite development
Source; Winzeler, 2008

Symptoms of malaria appear after the causative parasites undergo a complex life cycle as shown in Figure 2.1. The proboscis (mouth part) of a female anopheles' mosquito breaks the surface of the skin of the host which subsequently offers an entry point for parasitic substances known as sporozoites to gain access to the blood. These then travel on to the liver rapidly where they begin to reproduce to become solid masses known as liver

schizonts. These then go on to rupture releasing other parasitic entities known as merozoites into the blood. These entities infiltrate the red blood cells and cause malarial symptoms such as fever. This process takes 48 hours and is called the asexual life cycle. (Marsh and Makani, 2004).

When a female anopheles' mosquito which is uninfected bites an infected human and consumes blood that contains both male and female gametocytes, the mosquito thus becomes infected (Ashley *et al.,* 2006). These gametes then interact and fuse in the gut of the mosquito and a zygote is formed. This zygote then develops into new sporozoites. This is the sexual life cycle (Ashley *et al.,* 2006).

Manifestations of malaria in humans may vary from fever which is self-limiting to illness which is severe. In adults infected with this parasite, the disease manifests as headache, fever, joint pains, hepatomegaly, jaundice, multiple organ failure and coma (Bartolini and Zammarchi, 2012). However, the disease manifests slightly differently in children with anaemia being a hallmark symptom. Other symptoms range from fever, headache and vomiting to impaired consciousness, prostration, multiple convulsions and hypoglycemia in severe cases (Greenwood *et al.,* 2005).

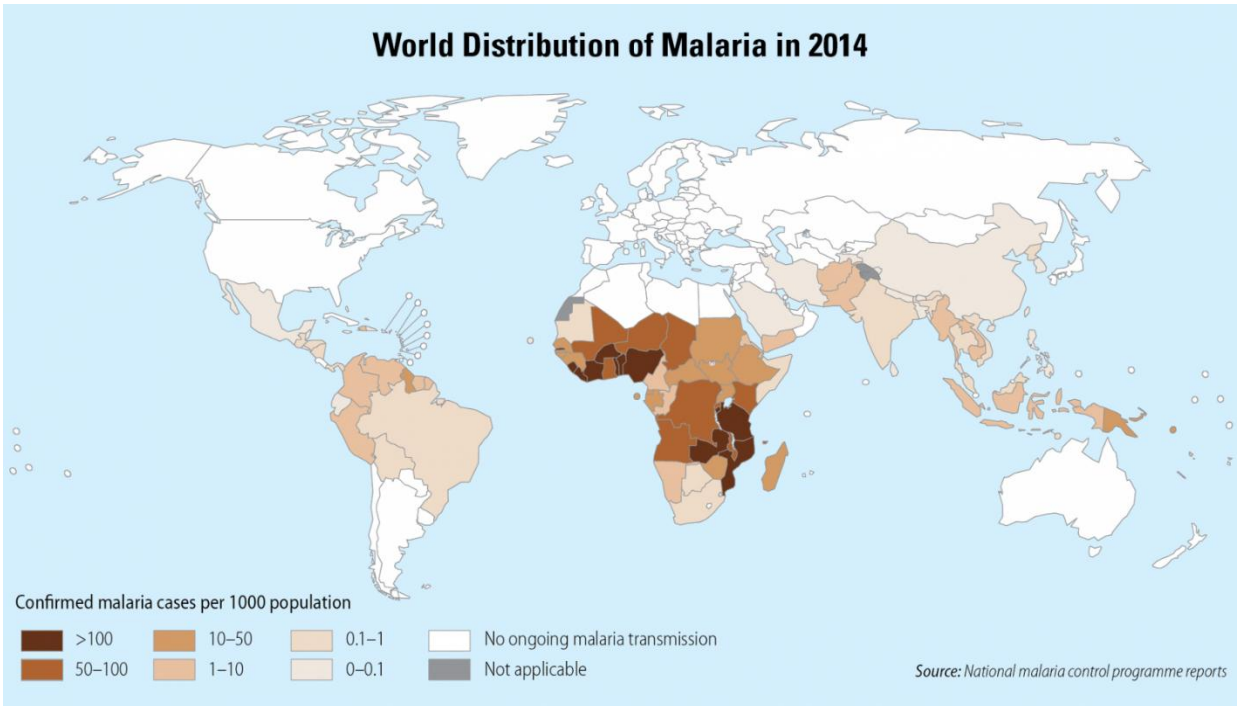


Figure 2.2; Prevalence of malaria in the world
Source; World Malaria Report, 2014

Malaria is a disease which occurs in all the six WHO regions. Statistics estimate that up to thirty percent of the cumulative population of the world is susceptible to infection by *plasmodium falciparum*. However, it is endemic predominantly in Sub-Saharan Africa, Asia and Latin America (Autino *et al.*, 2012).

Africa bears the heaviest brunt of this dangerous disease with about 9 in 10 deaths reported globally occurring in the region. Seventy-eight percent (78%) of this statistic are children under the age of five (5) (World Malaria Report, 2014). Furthermore, travellers and migrants from areas with generally little transmission of malaria, first time pregnant women and patients with immunosuppressive diseases are also vulnerable to the disease (World Malaria Report, 2014). On the backdrop of these facts, malaria has earned the

reputation of being one of the deadly parasitic infections that afflict mankind (Nicholas and Breman, 2005).

According to a 2006 report by the Ministry of Health, Kenya has four major malaria zones namely; the low-risk zone, seasonal malaria transmission zone, highland epidemic-prone zone and the malaria endemic zone as shown in Figure 2.3

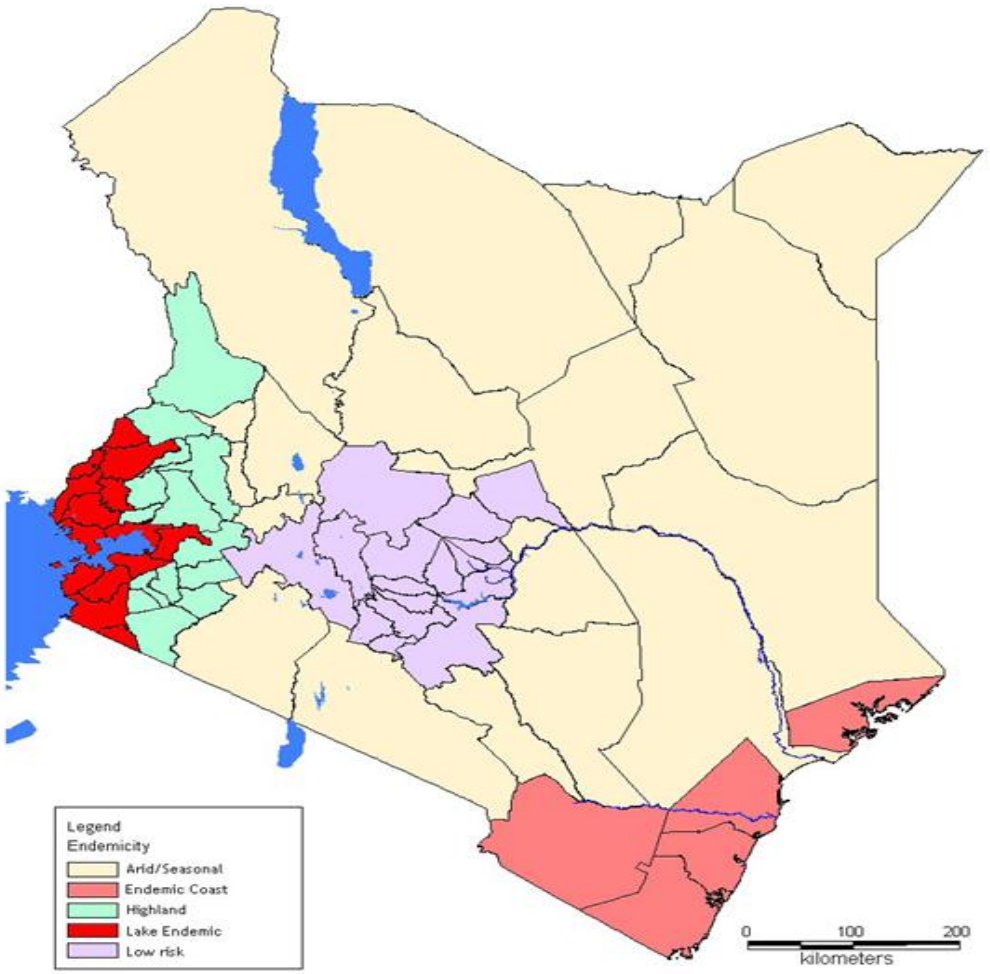


Figure 2.3; Distribution of malaria in Kenya
Source; Ministry of Health 2006

2.2 Chemotherapy of malaria; past and present

Since malaria shows several different manifestations, there is need to separately address each of these in its treatment and management. The earliest record of malaria therapy was in the 1st century A.D. by the Roman physician Galen (Karamanou *et al*., 2013). At around the same time, the *materia medica* of traditional Chinese medicine had the wormwood plant in its arsenal as an effective means of treating swamp fever. This went on to become the mainstay in Europe for 1600 years, until quinine was discovered in the 17th century (Petterson, 2005).

2.2.1 Aminoquinolines; Quinine and its analogues

The effectiveness of the bark of Cinchona (Peruvian bark) in the treatment of episodes of fever was first identified by the Indians and later adopted by European settlers (Greenwood, 1992). On her visit to the Indian sub-continent, the countess of Chincón (from Peru) fell ill to a fever and this bark was found to be effective in treating the fever. Hence, the bark was renamed Peruvian bark and the tree was named 'Cinchona'. This took place in the 17th century and by the 19th century the pharmacophore quinine had been isolated from the Cinchona bark (Eiden, 1998). Since then, it has served as an ideal model for developing synthetic antimalarials.

Chloroquine is a derivative of quinine and was developed during World war II (Hempelmann *et al*., 2009). It was found to be ideal; short-acting, having high efficacy as well as tolerable side effects and low cost when compared to quinine. However, towards the late 1940's there were reports of chloroquine treatment failures that emerged from South America and Asia. Today, the entire Asian continent and vast areas of Sub-Saharan Africa are adversely affected by the emergence of resistance to this drug (Farooq and Mahajan, 2004).

Resistance in Africa seems to have been imported from South-East Asia and is now commonplace in many countries on the continent. Owing to this phenomenon, the use of quinine has re-emerged and the drug is now part of the arsenal of antimalarial agents (Butler *et al*., 2010). Moreover, plasmodium resistance accelerated the discovery of other aminoquinolines such as amodiaquine (1946) and Primaquine (1950) (Ramachandra *et al.*, 2004). These drugs work by inhibiting haempolymerase enzyme effectively resulting in the accumulation of haem. Molecules of this substance are toxic to the parasite and result in the death of blood stages of the parasite (Petterson, 2005).

Antimicrobials such as clindamycin and tetracyclines enhance the activity of aminoquinolines by acting in synergism with them (Petterson, 2005).

2.2.2 Antifolates

The effectiveness of the aminoquinolines was limited by the emergence of resistance. This created a platform for the development and use of antifolates which include proguanil and sulfadoxine-pyrimethamine (SP) (Farooq and Mahajan, 2004). Initially, the latter was a first line drug in malaria therapy. However, the emergence of resistance limited its use (Farooq and Mahajan, 2004). On the other hand, proguanil is still being used in patients with sickle-cell anaemia.

Antifolates exert their action on both the blood and liver stages of the parasite. They do so by inhibiting the enzyme dihydrofolate reductase. This results in depletion of pyrimidines, inhibition of DNA replication and eventual death of the parasite (Tripathi, 2003).

2.2.3 Artemisinin derivatives

Prior to its use for the treatment of malaria, *Artemisia annua* was used for fever and hemorrhoid therapy by the Chinese. However, during the Vietnam war of 1967 and on the backdrop of numerous malaria related casualties on both sides of the conflict, it became a matter of strategic importance to find a potent antimalarial drug (Kuhn and Wang, 2008). While America failed in its quest to get a suitable candidate drug, the Vietnamese turned to the Chinese for help in this regard. The Chinese then set out on a systematic and elaborate exploration and screening of traditional Chinese medicine in a bid to develop a highly effective antimalarial drug that was safe (Kuhn and Wang, 2008). This project was dubbed 'Project 523' and in 1971, the artemisinin (*Qinghaosu*) was isolated and shown to have outstanding antimalarial activity (Kuhn and Wang, 2008). The structure of the pharmacophore was then resolved several years later (Klayman, 1985).

Artemisinin has profound activity against blood stages of the parasite including gametocytes. Haem is a component of blood that is toxic to the malarial parasites. These parasites adapt by producing a metabolite which detoxifies the haem. The pharmacophore of artemisinin inhibits this process by generating free radicals which have a high affinity for parasite infected red blood cells (Bhisutthibhan *et al.*, 1998). The free radical species then form covalent bonds with the membranes of the parasite which compromises the integrity of the parasite membranes (Bhisutthibhan *et al.*, 1998; Cazelles *et al.*, 2002).

There are several derivatives of artemisinin such as dihydroartemisinin, artemether, arteether and artesunate (Jansen, 2002). These have been shown to be safe and effective, acting faster than the older antimalarials and also the parent compound (Jansen, 2002).

Rectal applications of artemisinins have now been developed. This has significantly improved treatment outcomes in clinical settings where interventions with intravenous quinine is not possible (Butler *et al*., 2010).

Owing to their short plasma half-lives, rapid clearance from the body, as well as a fear of the risk of malarial parasites developing resistance to these drugs, they are now formulated in a combination with older antimalarial agents (Butler *et al*., 2010).

2.2.4 Combination therapies

The strategy of using several drugs with different mechanisms of action in disease targeting has been successful thus far in HIV, tuberculosis and cancer therapy (Timothy *et al*., 2005). Combination of therapeutic agents serves to accelerate the response to therapy, improve the rate of cure by the drugs, preserve the efficacy of the individual drugs and delay the development of resistance. This is the approach taken into consideration in the development of the current antimalarials (Timothy *et al*., 2005).

Antimalarial combinations should have the following ideal characteristics; the components should have different modes of action, there should be no interaction between the individual drugs, the drug regimen should be sufficient for a 3-day period and one of the component drugs should be fast in clearing asexual forms of the parasite (Davis *et al*., 2005). Additionally, one of the component drugs should have a half-life of more than 4 days and the combination should have good tolerability, minimal side effects and high efficacy against gametocytes of the parasites. The combination should also be cost effective (Davis *et al*., 2005; Medicines for malaria Venture, 2010).

2.2.5 Non-artemisinin combinations

These are antimalarial combinations that are devoid of the artemisinin pharmacophore. These combinations are now ineffective courses of therapy against malaria with the exception of a few cases. This is because malarial parasites have developed resistance to the individual drugs used in these combinations (Mayxay *et al.*, 2004). In addition, these combinations had several shortcomings. A summary of the non-artemisinin combinations and their shortcomings is as provided on Table 2.1;

Table 2.1 Non-artemisinin combinations and their shortcomings

Non-artemisinin antimalarial	Shortcoming
Quinine+Sulfadoxine-Pyrimethamine (Q-SP)	Costly, requires long treatment periods
Quinine+Doxycycline (Q-D)	Costly, severe adverse events
Sulfadoxine Pyrimethamine+Chloroquine (SP-C)	Rendered ineffective due to parasitic resistance
Sulfadoxine-Pyrimethamine+Amodiaquine (SP-A)	Limited by amodiaquine resistance
Atovaquone+Proguanil(A-P)	Suitable for prophylaxis only

Source; Mayxay *et al.*, 2004

In view of these facts, WHO recommends combinations of short half-life artemisinin derivatives with long half-life arylmethanols or quinolones. Such formulations are referred to as artemisinin-based combination therapy (ACT) (Mutabingwa *et al.,* 2005).

2.2.6 Artemisinin-based combination therapies (ACT's)

These are combinations that comprise of artemisinin or its derivatives and the older conventional antimalarials (Nosten and White, 2007). Artemisinin and its structural analogues rapidly clear malarial parasites and resolve symptoms of the disease.

These agents are active against all five species of the *Plasmodium* parasite and are generally well tolerated (Falade *et al.,* 2014; Taylor *et al*., 2006).

These drugs are rapidly eliminated from the body thus when used with drugs of a similar elimination profile such as tetracyclines the course of treatment is adjusted to several days (Petterson, 2005).

However, when the short half-life antimalarial drugs are combined with long half-life companion drugs such as aminoquinolines (amodiaquine, piperaquine) or arylmethanols (lumefantrine) the period of therapy is reduced to about 3 days (Nosten and White, 2007).

These combinations of antimalarials rapidly reduce the parasite biomass, resolve clinical symptoms in a timely manner, reduce gametocyte carriage and have few reported adverse effects (Taylor *et al.,* 2006).

They have been shown to have wide safety margins and act faster than the older antimalarials as well as the parent compound-artemisinin (Nosten and White, 2007).

They are presently recommended as the drugs of choice in the treatment of uncomplicated malaria (Whitty and Staedke, 2005).

Recently, more formulations of artemisinins have been developed with the aim of significantly improving treatment outcomes in clinical settings where interventions with intravenous quinine is problematic (Wells *et al*., 2009). Owing to their short plasma half-lives, rapid clearance from the body, and possible future emergence of resistance, artemisinin and its derivatives are preferably used in combination therapies with other drugs (Nosten and White, 2007). Based on the available data on safe and efficacious combination therapies, the following antimalarial drugs are available;

Table 2.2; Artemisinin based combination therapy (ACT) endorsed by the World Health Organization (WHO)

Artemisinin based combination therapy	**Description**
Artemether-Lumefantrine	The 1st combination to meet the World Health Organization criteria on safety. Launched in the 1st quarter of 2001
Artesunate-Amodiaquine	Found to be effective in regions with an amodiaquine cure rate of 80%. Launched in the 4th quarter of 2008
Artesunate-pyronaridine	Combines well with primaquine To produce radical cure of parasitaemia. Launched in 2011
Artesunate-mefloquine	Effective against *P.* vivax. Suitable for intermittent preventive therapy in infants
Dihydroartemisinin-Piperaquine Phosphate	Efficacious in malaria therapy in infants

Source; Wells, *et al*., 2009

2.3 Pharmaceutical profile of artesunate-amodiaquine antimalarial

2.3.1 Artesunate

Molecular formula: $C_{19}H_{28}0_8$

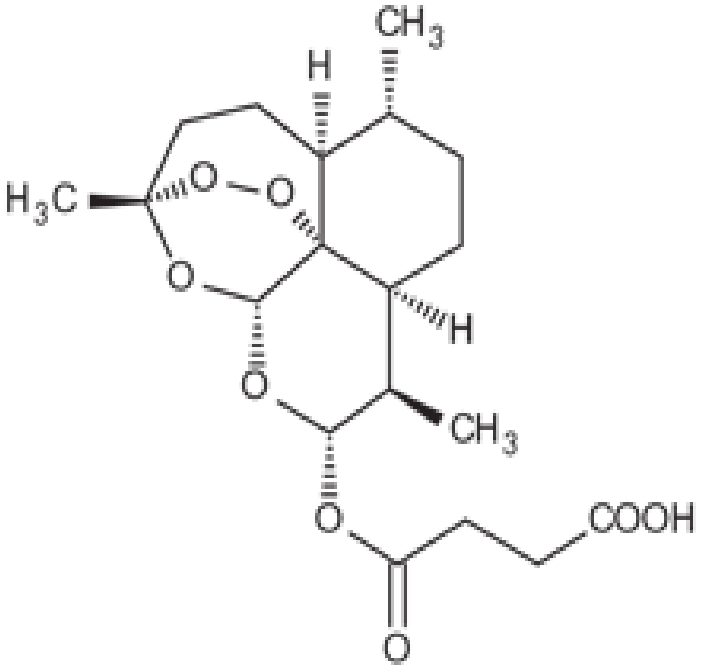


Figure 2.4; Structure of artesunate

Chemical name;

3R,5aS,6R,8aS,9R,10S,12R,12aR)-Decahydro-3,6,9-trimethyl-3,12-epoxy-12H-pyranol[4,3-j]-1,2-benzodioxepin-10-ol, hydrogen succinate

Characteristics: It is a white powder with a crystalline nature. It is synthesized by the reduction of artemisinin. It has slight solubility in water and good miscibility with organic solvents.

pH value: Artesunate acts as a weak acid. 10 mg of this drug in 1 ml of water gives a pH value of between 3.5 to 4.5 and a pKa value of 4.6.

Formulation:

Artesunate is available as an oral tablet with 50 or 200 mg in the form of a salt (sodium artesunate), an intramuscular/intravenous ampoule with 60mg of artesunic acid or a capsule meant for rectal administration with a 100 or 400mg content of sodium artesunate (Haynes *et al.*, 2006).

However, combination therapy of artesunate with a partner antimalarial drug is only possible for oral dosage forms (Haynes *et al.,* 2006; Lacaze *et al.,* 2011).

Storage: Should be stored in containers made of light protective materials with appropriate closures as the drug is photosensitive

Therapeutic indications and adverse effects

The dose that is currently recommended for artesunate is 2-10 mg/kg per day over a period of 3 days. Artesunate is an ideal antimalarial agent useful in the event of severe malaria. In ancient China, it was useful in treatment of haemorrhoids and fever. Generally, it is well tolerated. However, its distribution in major organs including the liver, central nervous system, lungs and plasma makes such organs susceptible to toxic effects (Davis *et al.,*2003). Moreover, acute anaphylaxis (Vugt *et al.,* 2000), acute intravascular haemolysis (Bethel *et al.,* 1997), cardiotoxicity, neutropenia and severe neurotoxicities have been reported when high doses are administered (Looareesuwan *et al.,* 1992).

Clinical Pharmacology

At low pH, artesunate is metabolized to an active metabolite known as dihydroartemisinin. This molecule is the main antimalarial pharmacophore. Both the drug and the metabolite are selectively distributed to infected erythrocytes where they bind to the membranes of the malaria parasite. Dihydroartemisinin exhibits potent action on

blood schizonticides and is particularly efficacious against the ring stages of *Plasmodium*. Peak plasma concentration (C_{max}) is attained after an hour and persists for up to 4 hours. Artesunate binds poorly to proteins and is incompatible with basic quinolones. These drugs have rapid rates of clearance from the body. In this way, contact between the drug and the parasites is kept at a minimum to avoid the risk of development of resistance (Whitty and Staedke, 2005; Wells, *et al*., 2009).

2.3.2 Amodiaquine hydrochloride

Molecular formula: $C_{20}H_{22}ClN_3O.2HCl.2H_2O$

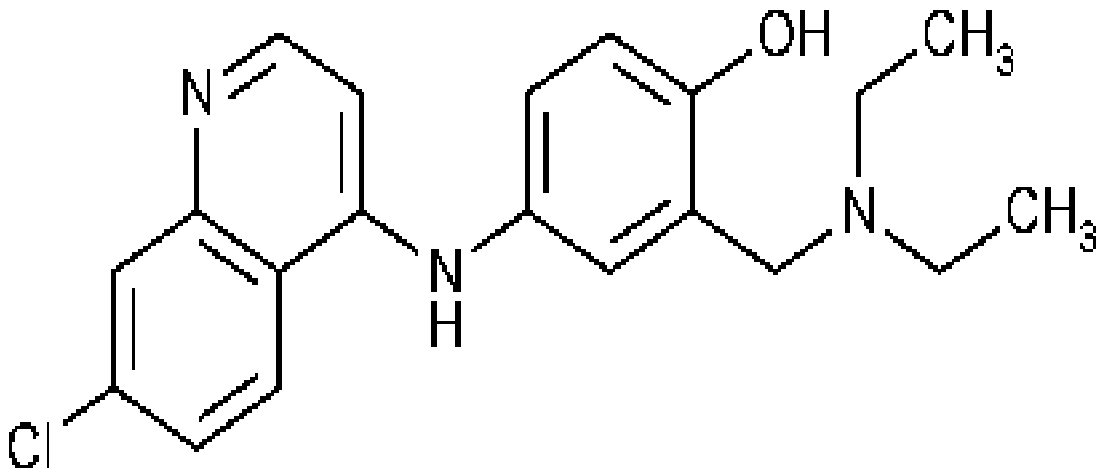


Figure 2.5; Structure of amodiaquine hydrochloride

Chemical name: 4-[7-Chloro-4-quinolyl) amino]-α-(diethyl amino)-o-cresol dihydrochloride dihydrate; 4-[7-chloro-4-quinolinyl) amino]-2- [(diethyl amino)-methyl] phenol dihydrochloride dehydrate.

Characteristics: It is a yellow crystalline and odorless powder. It has structural similarity with chloroquine. It has fair solubility in water and organic solvents. 20mg of this drug in 1 ml of water registers a pH of 4.0-4.8.

Formulation: An oral tablet containing 200mg of the drug as a base or 153.1mg of the drug as a chlorohydrate.

Storage: Should be kept in tightly closed containers as the drug is hygroscopic in nature.

Therapeutic indications and side effects

This is an antimalarial which shares both structural and mechanistic similarity with chloroquine. It is used at 25-30mg/kg dose over a period of 3 days (Olliaro and Mussano, 2003). When used prophylactically (400mg/kg weekly) amodiaquine causes severe neutropenia and hepatitis which may be fatal (Neftel *et al.,* 1986). Various mechanisms have been hypothesized to be responsible for the adverse events of amodiaquine treatment. One mechanism implicates the metabolite of amodiaquine to be responsible for causing a hypersensitivity reaction while another postulates that the drug itself is responsible for inducing direct toxicity to precursor cells of the liver and bone marrow (Winstanley *et al.,* 1990; Naisbit *et al.,* 1997).

Agranulocytosis, other blood dyscrasias, fulminant hepatic failure, haemolytic anaemia and severe neurotoxicities have also been reported when amodiaquine is used alone for malaria prophylaxis (Markham *et al.,* 2007). These adverse effects are very similar to those of chloroquine and have largely contributed to the low uptake of this drug (Adjuik *et al*., 2002).

Pharmacokinetics

On oral administration, absorption of the drug occurs rapidly. Thereafter, the drug is metabolized by CYP2C8 isoenzymes to its active metabolite monodesethylamodiaquine (AQm_1). The AQm_1 metabolite is then further metabolized through de-ethylation to form bis-ethylamodiaquine (AQm_2) (Winstanley *et al*., 1987). Studies have shown that the primary metabolite AQm_1 contributes to the antimalarial activity significantly more than amodiaquine (Krishna and White, 2007). Moreover, complete elimination of AQm_1 from the body takes a long time (Ornell *et al*., 2008).

The prodrug amodiaquine is eliminated after biotransformation in the liver. However, some portion is excreted via the kidneys in an unchanged form (WHOPAR, 2011).

2.3.3 Artesunate-Amodiaquine (AS-AQ)

This is an artemisinin based combination that comprises of an artemisinin derivative (artesunate) and an aminoquinoline (amodiaquine). The first randomized clinical trials on this combination showed that it had promising malaria cure rates and cleared gametocytes more effectively than amodiaquine monotherapy (Adjuik *et al.,* 2002).

Artesunate and amodiaquine are both blood schizonticides and are combined in a fixed ratio. Each of the individual drugs have different mechanisms of action. They also target different molecules within the parasite (Walter *et al.,* 2006). Both drugs clear the ring stages of the malarial parasite though at different rates. Artesunate rapidly clears the ring stages while amodiaquine acts more slowly, ensuring that artesunate is never exposed to the parasites on its own. Thus, there is an improvement in patient compliance as well as a reduction in the risk of resistance (Walter *et al.,* 2006).

The combination is recommended for uncomplicated malaria but has also been shown to be effective against other species of *Plasmodium*. In the administration of this drug, both age and weight are taken into consideration as depicted in Table 2.3 below;

Table 2.3: Dosage administration of artesunate-amodiaquine antimalarial combination based on age and weight

Body weight ranges	**Fixed dose combination tablet of artesunate-amodiaquine**
≥4.5 kg to <9kg	25mg artesunate/67.5 mg amodiaquine single tablet daily for 3 days
≥9kg to <18kg	50mg artesunate/135mg amodiaquine single tablet daily for 3 days
≥18kg to < 36kg	100mg artesunate/270mg amodiaquine single tablet daily for 3 days
≥36kg	100mg artesunate/270mg amodiaquine two tablets daily for 3 days

Source; SANOFI AVENTIS 2010

The formulation of artesunate-amodiaquine as a fixed dose combination was done to ensure that both drugs are taken at correct doses especially for paediatric patients (Falade *et al.*, 2014).

2.3.4 Toxicity and adverse effects of artesunate-amodiaquine

When toxicity related manifestations occur following administration of artemisinin-based combinations, the partner drug is usually the one that is suspected to be the cause rather than the artemisinin derivative (Adjuik *et al.*, 2004). Dosages of antimalarial drugs are usually calculated on the basis of body weight. However, most countries where malaria is endemic are resource constrained, with poor or limited access to healthcare as well as inadequacy or absence of functioning weighing scales. This coupled with the fact that treatment mostly takes place at home using antimalarial drugs obtained from local shops and vendors, makes weight based dosing impractical (Ruebush *et al.*, 1995; Breman, 2001). Thus, most doses of antimalarials are administered using age as a reference point. In view of this, both age and weight have been taken into consideration in formulating the current antimalarials (Beeson *et al.*, 2015). In as much as the practicability of age based dosing is not in doubt, it however results in incidences where individuals are under or over dosed. Consequently, the risk of failure of the regimen or attendant toxicity significantly increases (Breman, 2001). The results of adverse events from two pivotal studies on the artesunate-amodiaquine combination are as summarized in Table 2.4;

Table 2.4: Toxic manifestations of artesunate-amodiaquine antimalarial drug

Organ	Frequency	Adverse effects
Blood, circulatory and lymphatic system disorders	Uncommon	Anaemia, arrhythmia, bradycardia
Disorders of the eye and ear	Uncommon	Vertigo, ocular icterus
Gastro-intestinal disorders	Common	Nausea, abdominal pain
	Uncommon	Diarrhoea, vomiting
Disorders of metabolism and nutrition	Uncommon	Hypoglycemia
Disorders of the musculo-skeletal system and connective tissue	Uncommon	Arthralgia
Disorders of the nervous system	Common	Somnolescence
	Uncommon	Paresthesia
Disorders of the respiratory system	Common	Cough
Disorders of the skin	Uncommon	Oedematous face, skin, pruritus

Source: WHOPAR 2011

2.4 Characteristics of the silybin-phosphatidylcholine complex (Siliphos®)

This is a complex of the silybin flavonoid molecule and natural phospholipid substances. It is also known as siliphos and was formulated as an improvement of silymarin from the milk thistle plant (*Silybium marianum*) which had problems of low solubility and poor intestinal absorption (Indena, 2004). Siliphos shows a good ability to move from an environment that is hydrophilic (water-loving) through the membranes of cells and ultimately into their internal environment (Kidd *et al*., 2005). This drug has good oral absorption (Savio *et al*., 1999) and attains peak plasma levels within an hour which remains elevated for several hours. It is then eliminated in urine (Morazzoni *et al*., 1992). This drug acts as a hepatic detoxifier by conserving glutathione in the liver and repairs or

replaces damaged cell membranes (Kidd, 1996). Several studies have evaluated the hepatoprotective efficacy of siliphos in animal (Hikino *et al*.,1984; Conti *et al*.,1992; Enjalbert, 2002) and human models (Indena, 1992; Marena and Lampertico, 1999; Indena ,2004).

2.5 The profile of *Moringa oleifera*

2.5.1 Classification, geography and use

M.oleifera is a drumstick (horse radish) tree which has a rapid rate of growth and development. It is the most popular of all the species of the *Moringaceae* family and is also the most cultivated. Moringa traces its origin to the sub-Himalayan area of the Indian sub-continent particularly in countries such as India, Pakistan, Sri Lanka, Bangladesh and Afghanistan where it is available as 13 species of trees and shrub (Ramachandran *et al.*, 1980; Jahn *et al.*, 1986). However, it has now been found to do well in other areas of the world. Thus, the plant is identified by different names some of which include horseradish, nebeday, benzolive tree, sajhan and mlonge (Jed, 2005).

The geographical distribution of *Moringa oleifera* is as shown on the map below;

Figure 2.6; Distribution of *Moringa oleifera* in the world
Source; *Moringa oleifera* BIO 203

The plant propagates in a twofold manner; sexually or asexually and has a low requirement for not only water but nutrients as well. Thus, its management and subsequent cultivation is easy. Introducing this plant in a farm may result in benefits for the surrounding eco-system as well as for the farm owner (Foidl et al., 2001). The tree is perennial and of softwood variety but produces timber of low quality. Its fruits have on average 12 seeds which are dry and brown in colour when mature. It has an oleaginous embryo, two cotyledons and undergoes hypogeal germination. (Bezzera et al., 2004; Ramos et al., 2010). This plant has been associated with nutritional, medicinal or miscellaneous value (Singh et al., 2014). Nutritionally, the pods and seeds taste better while they are still in the early stages of growth before they become brown in colour. In parts of Asia, pods which are in the early stages of growth find utility as food additives (Abdul et al., 2005). Owing to high content of calcium, Philipino women use the leaves in soup to improve the production of breast milk (Iqbal and Bhanger, 2006). When powdered, they give a spicy flavor similar to the horseradish hence the name ``Horseradish Tree''. These leaves have also exhibited an enormous potential to be used as animal feed but this is still underexplored. Powdered leaves of Moringa have been used as animal feed for a variety of animals such as fish, chicken and sheep (Richter et al., 2003; Kakengi et al., 2003; Murro et al., 2003). Figure 2.7 is a representation of the different parts of *Moringa oleifera;*

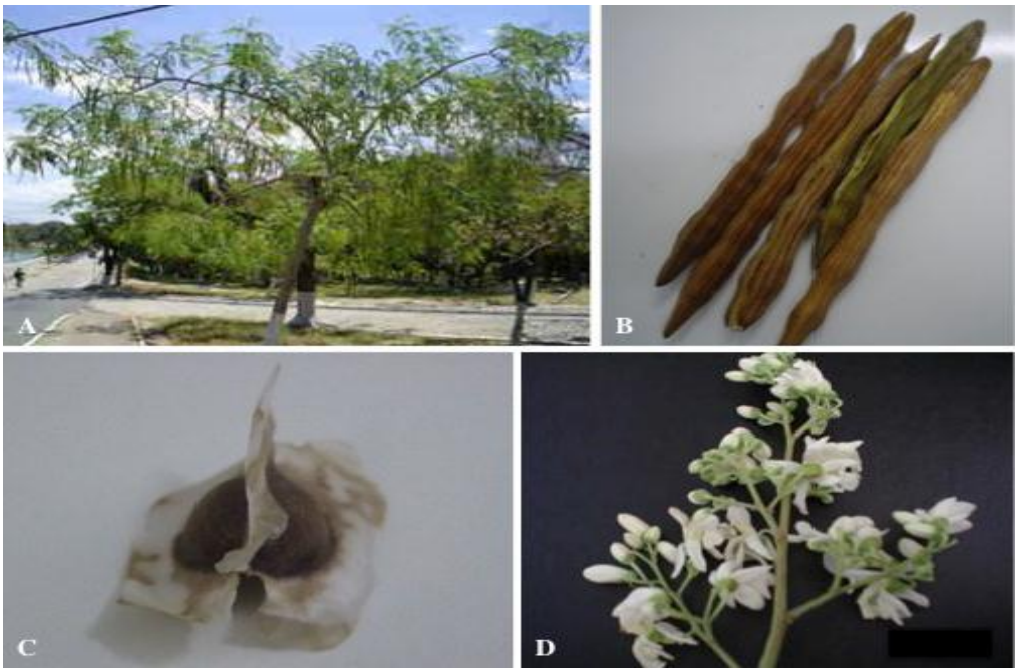


Figure 2.7: parts of the Moringa plant

A-Whole Tree B-Pods C- Mature Seed D-Flower

Source; Ferreira *et al.,* 2014

2.5.2 Pharmacological properties/medicinal activity

Medicinally, the antioxidant (Santos *et al.*, 2012), wound healing, hypotensive, and diuretic effects of this plant have been reported (Faizi, 1995; Guevara *et al.,* 1999). Additionally, native *materia medica* from the sub-continent of India provides an elaborate description of how the roots of Moringa are used in mitigating several disease conditions (Fuglie, 1999). Many other folkloric medicinal properties of this plant continue to be corroborated by sound analytical techniques. The various uses of the parts of this plant are as summarized on Table 2.5;

Table 2.5; Pharmacological activities of *Moringa oleifera*

Effect on body systems	Specific pharmacological activity	Part of the plant used
Antimicrobial	Anti-tubercular	Stem bark
	Bactericidal	Leaves, stems, pods
	Pupicidal	Seeds
	Larvicidal	Seeds
	Fungicidal	Leaves, seeds
	Antimalarial	Seeds
	Anti-schistosomatic	Seeds
Alteration of metabolic functions	Antioxidant, anti-tumour	Leaves, seeds, stems
	Hepatoprotective	Seeds, leaves
	Lipid lowering	Leaves, roots, seeds
Relief of pain, inflammation and fever	Analgesic	Roots
	Anti-pyretic	Leaves
	Anti-inflammatory	Seeds, leaves, roots
Alteration of digestive system functions	Purgative	Leaves
	Cholagogue	Flower
	Carminative	Root
	Anti-ulcerogenic	Leaves, seeds
Alteration of smooth muscle activity	Abortifacient	Leaves, roots
	Anti-spasmodic	Leaves, seeds
Alteration of the nervous system activity	Aphrodisiac	Flower
	Anti-convulsant	Leaves, roots
Alteration of cardiac System activity	Anti-atherosclerotic	Leaves
	Bradycardic	Seeds
	Circulatory tonic	Roots
Alteration of the immune system functions	Immunosuppression	Seeds
	Immunostimulation	Flower
Miscellaneous	Repellent	Seeds

Source; Toma and Deyno, 2014, Ferreira *et al.*,2014

2.5.3 Toxicology

2.5.3.1 Leaves, roots and flowers

The consumption of the leaves of *M. oleifera* is encouraged as they contain trace quantities of tannin and phytic acid. Stems and pods have negligible amounts of tannins. However, despite leaves and stems having significant amounts of alkaloids they are considered non-toxic to ruminants (Makkar and Becker, 1997).

Various solvent extracts of the roots and flowers of Moringa at 200mg/kg/day maintained the level of activity of transferases and bilirubin and also provided protection to the liver against hepatotoxicity induced by toxic metabolites of acetaminophen (Asare *et al*., 2012). However, intraperitoneal administration of high doses of methanolic root extracts of this plant resulted in hepato-renal toxicity manifested by hematological and plasma changes (Mazumder *et al.,* 1999).

Examinations of liver sections of guinea pigs previously administered with the methanolic extract of the roots of *Moringa oleifera* at 3.5, 4.6 and 7.0 mg/kg dose levels exhibited toxic manifestations. Balloon degeneration, micro and macrovesicular steatosis, intestinal inflammation, renal tubular damage and amorphous eosinophilic matter in the kidneys have been reported following the use of this extract (Paul and Didia, 2012). Awodele *et al.,* 2012 reported that the exposure of rodents and man to short-term and extended oral dosing with the aqueous leaf extract of *Moringa oleifera* at dose ranges 400 to 6400mg/kg was devoid of any deleterious effects, thus the extract was considered safe. These results compare well with those of Adedapo *et al.,* 2009 and Bakre *et al.,* 2013 who performed acute oral toxicity studies on *M. oleifera* extracts derived from water and ethanol respectively. Moreover, Asare *et al.,* 2012 tested the acute oral toxicity of a 3000mg/kg dose of the extract of *M. oleifera* obtained by using ethanol as a solvent and

reported that the extract lowered the levels of albumin and urea. However, the dose tested showed some genotoxic potential as shown by the increase in the polychromatic micro nucleated red blood cells from the bone marrow of rodents (Asare *et al.,* 2012).

2.5.3.2 Seeds

A low dose of *M. oleifera* seed extract (400mg/kg/day) showed remarkably low toxicity when used in the clarification of water (Eze and Ananso, 2014). There were no alterations in the biochemical, histological or hematological parameters. Similarly, there were no observed toxic effects from the consumption of the water extract at doses in the range of between 1200-1700mg/kg/day over a one-month period of treatment (Ferreira *et al.,* 2009). Other workers found out that this extract recovered physiological measures of haemoglobin, red blood cells and glutathione transferase to normal levels in an arsenic intoxicated animal model (Gupta *et al.,* 2005).

As far as seed extracts of *M. oleifera* obtained by the use of multiple solvent systems are concerned, Bharali *et al.,* 2003 found that an orally administered *M. oleifera* hydro alcoholic fresh pod extract increased the levels of the enzymes that take part in phase I and II reactions in the liver. These observations were in agreement with those of Hamza, 2010 who reported that 1g/kg of the hydro-ethanolic seed extract avoided carbon tetrachloride induced liver fibrosis and reduced characteristic histopathologic as well as biochemical indicators of inflammatory hepatocytic necrosis. The findings from these studies further underscored the chemo preventive character attributable to the antioxidant chemicals in the seeds (Siddhuraju and Becker, 2003).

2.5.4 Phytochemistry of *Moringa oleifera*

This plant has a high composition of rhamnose, a simple sugar as well as glucosinolates and isothiocyanates, which are a group of unique compounds (Fahey *et al.,* 2001). Moringine and moringinine have been reported in the stem bark of the plant (Kerharo 1969). Studies on the isolation of compounds from the stem bark has revealed the presence of some compounds of note such as vanillin, hydroxymellin, β-sitosterol, octacosanoic acid and β-sitostenone. Minerals such as calcium and potassium have been identified in the ash of burnt stems (Faizi, 1994). The flowers of this plant contain several amino acids, alkaloidal substances, wax and quercetin. The acetate phase of pods has been found to contain thiocarbamate and isothiocyanate (Faizi *et al.,* 1998).

2.6 Causes and pathophysiology of toxicant induced liver injury

The human body is exposed to a vast array of different types of chemicals; food additives, chemicals for industrial use, pesticides as well as other contaminants present in the environment. At high doses, these chemicals may cause liver injury and are thus referred to as hepatotoxins or hepatotoxicants (Navarro and Senior, 2006). The susceptibility of the liver to injury is borne on the fact that this organ has a high perfusion rate, close proximity to the gastro intestinal system and the spleen and a high metabolic activity. Damage to the liver may arise from the direct toxic effect of primary compounds in a drug or from secondary products from the process of metabolism. It may also be a response that is mediated by hepatocytes, biliary epithelial cells or blood vessels (Saukkonen *et al.,* 2006; Deng *et al.,* 2009). Free radical chemical species have been implicated in general cell, tissue and organ injury (Halliwell and Gutteridge, 2002). These reactive species form covalent bonds with biological membranes consequently altering the functioning of vital components of the body (Sheweita *et al.,* 2001). Similarly, lipid

peroxidation forms an integral component of the pathology of disease conditions hypothesized to be free-radical oriented such as hepatic dysfunction, inflammatory diseases and arthritis (Vuillaume, 1987). The response of cells of the body to toxic insult varies from cell to cell. The most severe response results in cell death. A less severe response may involve the cells adapting to the injury by various mechanisms such as protein aggregation, fibrosis or autophagy (Leak, 2014). Adaptive responses are a reliable indicator of the extent of exposure of an organ to the toxin (Singh *et al.,* 2011). Pathologic analysis of organs such as the liver is an important tool in the identification and characterization of organ (liver) injury (Singh *et al.,* 2011). On the basis of the serological picture of the liver after toxic challenge by a chemical, liver injury may broadly be classified as hepatocellular, cholestatic or mixed (Tsui, 2003). The following are some common patterns of liver injury that result from the use of drugs;

2.6.1 Hepatic necrosis

Hepatic necrosis is the process by which liver cells form large rounded outgrowths of plasma membranes that are devoid of cellular organelles (Hotchkiss *et al.*, 2009). In an experimental setup, necrosis is identified by the use of dyes such as trytan blue, eosin, propidium iodide and sytox green. The rupture of plasma membranes of hepatic cells results in release of cellular components into the extracellular environment. This is a pathological process that can produce an inflammatory response (Hotchkiss *et al.,* 2009). Necrosis is usually accompanied by clinico-biochemical changes (Singh *et al.,* 2011). Paracetamol (Boyd and Bereczky, 1966), carbon tetrachloride (Mochizuki *et al.,* 2009), amatoxins (Vetter, 1998) and some plants of herbal origin such as *Atractylis gummifera* (Larrey, 1997) have been implicated to cause this type of liver injury.

2.6.2 Apoptosis

This is a highly regulated process that is internally programmed within cells and results in cell death. In the case of liver cells, the process serves to maintain liver health (Jacobson *et al.,* 1997).

It is characterized by distinct morphological alterations which include shrinkage, condensation, marginalization of cells and rounded outgrowths of plasma membranes accompanied by biochemical features (DNA fragmentation, alteration of membranes, protein degradation) (Wyllie *et al.,* 1980). In the ultimate stages, the dying cells fragment into membrane bound vesicles with relatively intact cellular organelles and chromatin residues referred to as apoptotic bodies (Wyllie *et al.,* 1980).

The apoptotic masses formed are either phagocytosed by the surrounding hepatocytes to form acidophilic and councilman bodies (Ishak *et al*., 1995) or engulfed by the kupffer cells in the liver (Guicciardi *et al.,* 2005).

2.6.3 Hepatic lipidosis/steatosis (fatty liver)

This injury is caused by the accumulation of triglycerides within hepatocytes (Saukkonen *et al*., 2006). This accumulation is the result of a discrepancy between the uptake of fatty acid substances and their subsequent secretion as lipoproteins. When the liver cells are observed, there is a displacement of the nucleus to the periphery of the cells by the vacuoles. This is known as large droplet fatty liver or macro vesicular lipidosis (Lee and Farrel, 1997).

Substances that may cause macro vesicular lipidosis include acetaminophen, methotrexate. However, in some instances, the vacuoles of liver cells are not sufficiently large to displace the nucleus. This phenomenon is thus known as small droplet fatty liver

or micro vesicular lipidosis. It may be caused by aspirin, herbal plants such as *Scutellaria spp* and expired tetracyclines (Itoh *et al*., 1995).

2.6.4 Cholestasis

Involves disruption in the flow of bile resulting in yellowing of the skin and mucous membranes. Here, drugs interfere with the transport polypeptides on the surface of canaliculi of hepatocytes (Saukkonen *et al.,* 2006). Individuals who experience genetic polymorphism of transporter polypeptides may be pre-disposed to this condition. Moreover, the failure of both canaliculi pumps and intracellular transport processes in liver cells exacerbate the accumulation of bile acids within hepatocytes (Picciotti *et al.,* 1998). Cholestasis may be classified as bland cholestasis, cholestatic hepatitis, chronic hepatitis vanishing bile syndrome or bile duct sclerosis (Picciotti *et al.,* 1998).

Bland cholestasis is mainly caused by anabolic/contraceptive steroids (Picciotti *et al.,* 1998). Cholestatic hepatitis is accompanied by liver cell damage and necrosis. Portal tracts show variable inflammation and bile duct injury. Chlorpromazine, allopurinol and the antibiotic combination of amoxicillin and clavulanic acid have been implicated to cause cholestatic hepatitis (Benninger *et al.,* 1999).

Chronic cholestasis and the vanishing bile duct syndrome have jaundice and pruritus as hallmark features. Here, early biopsies of the liver tend to show severe bile duct injury with swollen and necrotic cells. Later biopsies (9-14 months) show ductopenia (vanishing bile duct syndrome) with ductular proliferation and persistent inflammation. Drugs such as chlorpromazine, flucloxacillin and tolbutamide are incriminated to cause these injuries to the liver (Brind, 2007).

In bile duct sclerosis, lesions that mimic primary sclerosis cholangitis of the extrahepatic bile ducts are observed. Hepatic arterial infusion of fluxoridine in patients with metastatic colon cancer has been shown to produce this liver injury (Sallie *et al.,* 1991).

2.6.5 Hepatitis

This is an inflammatory condition of the liver caused by several therapeutic agents. In this injury, liver cell death is accompanied by the accumulation of inflammatory cells. This phenomenon is due to the production of reactive metabolites or an immunological hypersensitivity to the challenge drug (Teshke, 2009). Often, there is the formation of an adduct between the metabolites and the intracellular macromolecules. The resultant product serves as an antigen which elicits an immunological response. This injury is characterized by multinucleated giant hepatocytes, many eosinophils and neutrophils and poorly developed portal reaction (Murray *et al.,* 2008). Isoniazid, acetaminophen, nevirapine, troglitazone (Kaplowitz, 2004), phenytoin (Saukkonen *et al.,* 2006), herbal plants such as *Chelidonium majus* (Benninger *et al.,* 1999) have been incriminated to cause this pattern of liver injury.

Focal hepatitis is non-specific and features scattered necrotic bodies which have been infiltrated by lymphocytes. Aspirin is one drug that has been implicated to bring about this type of injury. Chronic hepatitis is clinically, serologically and histologically similar to autoimmune hepatitis. Drugs which may cause chronic hepatitis include methyldopa, diclofenac, minocycline, nitrofurantoin (Kaplowitz, 2004), herbal plants such as *Larrea tridentate* (Lee and Farrell, 1997) and *Lycopodium serratum* (Picciotti *et al.,* 1998)

2.6.6 Cirrhosis

This pattern of liver injury occurs in response to chronic liver injury. Histologically, it is characterized by the development of hepatic fibrosis and regenerative nodules which are surrounded by fibrous bands (Schuppan and Afdhal, 2008). The condition is a pre-cursor of portal hypertension and end stage liver disease (Schuppan and Afdhal, 2008).

Liver cirrhosis compromises the exchange between sinusoids of the liver and the hepatocytic parenchyma by shunting the portal and arterial blood supply directly into the central veins (Schuppan and Afdhal, 2008).

The initial phase of this disease is accompanied by hepatomegaly. Further exacerbation of this condition may lead to hepatic failure (Polson and Lee, 2005). Excessive consumption of alcohol and hepatitis C have been identified as the main causative agents (Schupan and Afdhal, 2008).

2.6.7 Vascular changes

Drug therapy may bring about abnormal vascular changes in the liver. Some of these morphological alterations include veno-occlusive disease, sinusoidal dilatation and nodular regenerative hyperplasia (Gitlin, 1998).

Veno-occlusive disease (sinusoidal obstruction disease) is a type of liver injury that is characterized by the loss of endothelial cells of the sinusoids, impairment of the microcirculation in the liver and hepatic necrosis (Rollins, 1986). It involves toxic destruction of the endothelial cells of the sinusoids and is accompanied by rounding up and sloughing (Kumar, 2003). This injury has been associated with herbal teas (*Crotalaria sp*) containing pyrrolizidine alkaloids and the antineoplastic drugs cyclophosphamide and busulfan (King and Perry, 2001; Kaplowitz, 2004).

Sinusoidal dilatation refers to an increase in the diameter of the hepatic capillaries and may involve the entire lobule or predominantly affect the central, Periportal or medial areas (Degott and Potet, 1984). Drugs such as oral contraceptives, azathioprine, vinyl chloride, vitamin A have been identified as causative agents of this liver injury (Zafrani *et al.,* 1983: Ishak and Zimmerman, 1995).

Nodular regenerative hyperplasia is a condition of the liver that is a rare occurrence. It is characterized by the transformation of parenchymatous cells in the liver into small nodules (Hartleb *et al.,* 2011). It may lead to the development of non-cirrhotic portal hypertension. Autoimmunity, infection, neoplasms and drugs are causal factors. Histopathologically, it manifests as diffuse micronodules that are devoid of fibrous septa (Hartleb *et al.,* 2011).

2.6.8 Neoplasms

The prolonged exposure of the liver to some toxins or drugs such as arsenic, vinyl chloride, thorotrast and anabolic steroids may result in neoplasms such as liver adenomas, angiosarcoma or hepatocellular carcinoma (Brind, 2007).

2.7 Assessment of liver injury

Hepatotoxicants produce a range of clinical and histopathological indicators when the liver is injured. Clinical indicators involve the use of liver-specific parameters which leak from hepatocytes to the blood circulation raising not only their concentration in blood but also their activity as well (Nyblom *et al*., 2004). Significant elevations in the serum liver enzymes are an appropriate means of identifying the emergence of toxic conditions in the liver. Moreover, the measurement of clinical biochemical parameters together with macroscopic and histo-pathological observations are useful in investigating liver injury

(Reuben, 2004). Some biochemical markers of hepatotoxicity in major organs and blood plasma are represented on Table 2.6;

Table 2.6: Biochemical markers of hepatotoxicity

Biochemical marker	Tissue localization	Cellular localization	Lesion
Alanine amino transferase	Liver, skeletal muscles, heart (trace amounts)	Cytoplasm, mitochondria	Necrosis
Aspartate amino transferase	Liver, kidney, brain	Cytoplasm, mitochondria	Necrosis
Alkaline phosphatase	Liver, placenta, intestines	Cell membrane	Cholestasis
γ-Glutamyl transferase	Kidney, bile ducts, pancrease	Cell membrane	Cholestasis
Total bilirubin	Direct (bile fluid, intestines) Indirect (reticuloendothelium of spleen and serum	Extracellular membrane	Cholestasis
Lactate dehydrogenase	Peroxisomes of the liver, heart and kidney cells	Mitochondria, sarcoplasmic reticulum	Necrosis
Malate dehydrogenase	Liver, heart, muscle, brain	Cytoplasm, mitochondria	Necrosis
Total protein	Hepatic and immune system	Blood plasma	Hepatic dysfunction
Albumin	Liver	Blood plasma	Hepatic dysfunction
Glutamate dehydrogenase	Liver, kidney	Mitochondrial matrix	Necrosis

Source; Singh *et al.,* 2011.

In addition to these parameters, a relationship has also been identified between the organ (liver) weight and toxicological effects (Amacher *et al.,* 1998). Thus, the weight of the liver *post humous* as well as the weight of the liver relative to the overall body eight are important in assessing organ toxicity.

2.8 Hepatospecific parameters used in this study and their working principles

2.8.1 Alanine aminotransferase (ALT/SGPT)-the standard clinical biomarker of hepatotoxicity

This is one of the liver specific enzymes which can be used to accurately diagnose the occurrence of liver injury (Schuman *et al.,*2002). It is also known as serum glutamate pyruvic transaminase. The normal levels of this enzyme in healthy human individuals is in the range of between 5 and 50 units per litre. Increased activities of this enzyme may be indicative of dystrophy of the muscles and may also point to the occurrence of infarcts of the heart as well as diseases of the hepatobiliary system (Schuman *et al.,* 2002).

It is thus an important index in diagnosing both liver and heart dysfunction as well as monitoring treatment of these conditions. It's activity in serum is measured by using end point colorimetric diagnostic assays with specific manufacturer protocols. The bioassay of ALT follows the following reaction (Schuman *et al.,* 2002).

$$\text{alpha-oxoglutamate + Levorotatory-alanine} \xleftrightarrow{\text{ALT (SGPT)}} \text{Levorotatory-glutamate + pyruvate}$$

$$\text{Pyruvate + Reduced-NAD + } H^{+} \xleftrightarrow{\text{LDH}} \text{Levorotatory-lactate + } NAD^{+}$$

Key; ALT-Alanine Amino Transferase, NAD-Nicotinamide Adenine Dinucleotide, LDH-Lactate Dehydrogenase

This reaction process begins by the reaction of alpha-oxoglutamate (a salt of glutamic acid) and the amino acid levorotatory-alanine. This takes place in the presence of serum glutamic-pyruvic transaminase (SGPT/ALT) enzyme which functions as a catalyst (Schuman *et al.,* 2002).

Amino acid units are transferred from the levorotatory alanine to the alpha-oxoglutarate molecules resulting in the formation of the levo-rotatory glutamate amino acid as well as pyruvate (a salt of pyruvic acid) (Schuman *et al.,* 2002).

The latter then reacts with reduced nicotinamide adenine dinucleotide (NADH) in the presence of hydrogen ions. This reaction is catalyzed by another enzyme known as lactate dehydrogenase (LDH) and two products are formed; levorotatory lactate and nicotinamide adenine dinucleotide (Schuman *et al.,* 2002). This is a redox reaction which is accompanied by a change in colour in the reaction tubes. This colour change is measured as absorbance on a spectrophotometer at a pre-determined wavelength. The rate of change of the colour reaction observed directly corresponds to the concentration of the enzyme in the sample under investigation (Schuman *et al.,* 2002).

2.8.2 Aspartate aminotransferase (AST/SGOT)

This is a liver enzyme which helps in the synthesis of proteins. It is found in the cytoplasm and mitochondria of cells and is also known as serum glutamic oxaloacetate transaminase (SGOT). It belongs to the transaminase family and is an important indicator of liver cell injury. However, it is less specific (Ozer *et al.,* 2008) as it may also imply the occurrence of dysfunction in other body organs such as the heart and skeletal muscles (Dufuor *et al*., 2000). It is for this reason that this enzyme may be useful in monitoring not only the condition of these organs but also their treatment. It's concentration in blood

is usually low in a healthy individual (7-40 units per litre). However, in the event of injury or damage to these tissues, AST is released into blood circulation resulting in high AST activity in blood (Nathwani *et al.*, 2005). This activity is measured by using end point colorimetric diagnostic kits with specific manufacturers protocols. The bioassay of AST follows the following reaction;

$$\text{Levorotatory-aspartate} + \text{alpha-oxoglutamate} \xleftrightarrow{\text{AST/SGOT}} \text{Oxaloacetate} + \text{Levorotatory-glutamate}$$

$$\text{Oxaloacetate} + \text{Reduced-NAD} + H^{+} \xleftrightarrow{\text{MDH}} \text{Levorotatory-malate} + NAD^{+}$$

Here, levorotatory aspartate (amino acid) reacts with alpha-oxoglutamate (a salt of glutamic acid) to form oxaloacetate and levorotatory glutamate (amino acid). This reaction is catalyzed by serum oxalo acetate transaminase (SGOT/AST) (Nathwani *et al.*, 2005). The oxaloacetate molecule then reacts with reduced nicotinamide adenine dinucleotide (NADH) in the presence of hydrogen ions to form levorotatory malate and nicotinamide adenine dinucleotide (NAD) (Schuman *et al.*, 2002). This process is catalyzed by the enzyme malate dehydrogenase (MDH). This is a redox reaction which is accompanied by a change in colour in the reaction tubes. This colour change is measured as absorbance on a spectrophotometer at a pre-determined wavelength (Schuman *et al.*, 2002). The rate of change of the colour reaction observed directly corresponds to the activity of the enzyme in the sample under investigation (Schuman *et al.*, 2002; Singh *et al.*, 2011).

2.8.3 Total bilirubin: a marker molecule of hepatobiliary injury

This substance is an endogenous anion that is produced by the normal breakdown of haemoglobin from erythrocytes and is excreted from the liver via bile (Saukkonen *et al.*, 2006). This chemical is present in trace quantities in blood and is utilized in the synthesis of bile fluid by the liver. The levels of bilirubin in healthy individuals varies from between 0.2 to 1.2 mg/dL (Dufuor *et al.,* 2000). Elevated levels of bilirubin (hyperbilirubinemia) may be the result of damaged hepatocytes which have lost the ability to excrete bilirubin, increased activity of albumin in serum which consequently results in bilirubin shifting from sites within the tissue to the blood circulation, decreased hepatic clearance which precipitates to jaundice as well as other symptoms of hepatotoxicity (Saukkonen *et al.,* 2006), pre-hepatic jaundice (e.g. haemolysis), intrahepatic jaundice (e.g. viral hepatitis) or post-hepatic jaundice (e.g. gall stone). The measurement of bilirubin in plasma is useful in the diagnosis of hepatobiliary diseases such as jaundice. Moreover, the severity of acute liver injury can be diagnosed more accurately using total bilirubin than ALT (Dufuor *et al.,* 2001). The activity of this endogenous anion in serum is measured by using end point colorimetric diagnostic kits with specific manufacturer protocols. The bioassay of bilirubin follows the following reaction;

$$\text{Bilirubin + Diazo salt} \xleftrightarrow{H^+,\ \text{surfactant}} \text{Azobilirubin}$$

In this assay, unconjugated bilirubin reacts with a diazo salt in the presence of hydrogen ions and a special surfactant. The hydrogen ions create an acidic medium which accelerates the solubility of bilirubin and a red product called azobilirubin is formed. The intensity of the colour of this product is measured as absorbance on a spectrophotometer.

The absorbance obtained from the reaction directly corresponds to the activity of bilirubin in the sample under investigation (Schuman *et al*., 2002; Singh *et al*., 2011)

2.9 General measures of mitigating toxicity of the liver.

The most effective method of mitigating organ (liver) injury usually involves ceasing the administration of the challenge drug or agent followed by the provision of general supportive care. Tapering the dose of the challenge drug may be a secondary alternative. In the event of liver damage, the prompt use of specific antidotes such as N-acetyl cysteine (Polson and Lee, 2005), carnitine (Bohan *et al.,* 2001) and dimercaprol have proven successful in managing acute liver injury. Diuretic agents such as furosemide and hydrochlorothiazide may also be useful in preventing or alleviating fluid buildup in the body. Nutrapharmaceuticals such as vitamins (A, B-complex, C, E), amino acids (taurine, methionine and arginine) and natural phospholipid substances (phosphatidylcholine, alpha-lipoic acid, methylsulfonylmethane) function as hepatoprotective agents. Recently, several authors have reported that feeding on antioxidant substances galvanizes the antioxidant defense system of body cells (Kiefer, 2004; Elias and Oputiri, 2013). Moreover, there has been a concerted effort to promote the use of synthetic agents with antioxidant properties such as butylated hydoxytoluene (BHT), butylated hydroxy anisole (BHA) and ethylene diamine tetra acetic acid (EDTA) in mitigating organ toxicities (Khan, 2005). However, safety considerations and resource-constraints have limited the use of these substances (Bonilla *et al.,* 1999). This has intensified the search for alternative substances with antioxidant properties. Scientists have thus turned to nature for solutions (Beeguni and Devi, 2003).

2.10 Plants with antioxidant activity

The antioxidant substances occurring naturally in plants have varying phytochemical composition, diverse physical and chemical properties and different mechanisms and sites of activity (Naik *et al.*, 2003). Some plants with antioxidant activity are as shown on Table 2.7;

Table 2.7; Some plants with antioxidant activity

Botanical identity	Family name	Common name	Part with antioxidant properties
Curcuma domestica Valeton	Zingiberacea	Tumeric	Leaf
Glycyyrhiza glabra Linn	Fabacea	Liquorice	Root
Momordica charantia Linn	Cucurbitaceae	Bitter lemon	Root, leaf, fruit, seed
Mangifera indica Linn	Anacaridiacea	Mango	Root, leaf, fruit
Santalum album Linn	Santalaceae	Sandal	Heartwood, bark
Foeniculum vulgare Mill	Apiacea	Fennel	Fruit oil
Embilica officinalis	Euphorbiaceae	Amla	Fruit
Daucus carota	Apiaceae	Carrot	Root
Solanum nigrum	Solanaceae	Nightshade	Leaf
Withania somnifera	Solanaceae	Ashwagandha	Root, leaf, seed

Source; Vivek *et al*., 2006

Some plants with antioxidant effects have also been shown to have hepatoprotective activity as shown in the Table 2.8;

Table 2.8; Plants with both antioxidant properties and hepatoprotective activity

Plant	Active component
Achillea millefolium	Caffeic acid
Eclipta alba	Ecliptine
Garcinia mangostana	Garcinone E
Bacopa monniera	Bacoside A
Phyllanthus amarus	Phyllanthine
Colchicum autamnale	Cochicine
Curcuma longa	Curcumin
Andrographis paniculata	Andrographolide
Equisetum arvense	Ornitine, kaempferol-3-o-glucoside
Anoectochilus formosanus	Kinsenoside
Glycyrrhiza glabra	Glycyrrhizin
Cassia tora	Ononitol monohydrate

Source; Singh *et al.*, 2011

2.11 Measurement of the antioxidant capacity of plants

The unique antioxidant activities of natural substances have captured the interest of scientists and the public alike. As a result, various methodologies have been advanced for studying phytoconstituents with promising antioxidant potential (Charles, 2013). Moreover, the diverse nature of phytochemicals present in plants dictates the use of multiple methodologies (assays) to give an elaborate characterization of the substance being investigated (Takeshita and Ozawa, 2004). Two major mechanisms are involved in these assays. One mechanism involves the transfer of hydrogen atoms while another involves the transfer of single electrons (Prior *et al.,* 2005).

2.11.1 Total phenolic content (Folin-Ciocalteau) assay

Compounds of a phenolic nature make up a significant proportion of secondary metabolites in plants. These include simple acids, hydrolysable and condensed tannins, phenols, lignans, lignins, tannins and stilbenes. They act as antioxidants, plant pigment precursors, UV light protective agents and also attract insects for pollination (Gottlieb and Borin, 2000). The quantitative determination of these compounds has been made problematic due to diversity and complexity of their structures (Pieroni *et al.,* 2010). However, spectrometry in the ultra-violet region has been found to be a useful tool in establishing the quantity of these compounds in plants. Polyphenols in plant extracts contain a pharmacophore that has the ability to absorb light in the ultraviolet to visible light range of the spectrum (Folin, 1927). This assay involves single electron transfer and measures the change in colour of the Folin-Ciocalteau reagent (redox reagent) from yellow to a dark blue complex in the presence of antioxidant samples. The maximum wavelength (λ_{max}) for the product of the reaction is 765nm and is measured using a UV-Visible spectrophotometer. Colour changes brought about by the different

compounds within the phenolics class depends on reaction kinetics and the unit mass of the individual compounds (Folin, 1927; Glasl ,1983).

2.11.2 Total flavonoid content (TFC) assay

Flavonoids are naturally occurring secondary metabolites having a C_6-C_3-C_6 backbone. These compounds are diverse in nature and widespread in vegetables, fruits, barks, roots, stems and flowers. They are subdivided into several structural categories; flavones, flavanones, anthocyanidins, flavonols and flavan-3-ols (catechins). There are over 5000 different flavonoids whose identity has been confirmed thus far (Bruneton, 1999). The aluminum chloride colorimetric assay is used to measure the amount of flavonoid substances in medicinal plants. The assay involves mixing an aliquot of the sample under investigation with a reagent made up of a mixture of sodium nitrite, aluminium chloride, sodium hydroxide and distilled water. This sets up a chemical reaction which is observed in the form of a colour change. The intensity of this colour is measured using a spectrophotometer at 510nm (Hazra *et al.,* 2010).

2.11.3 Iron (II) chelating activity assay

This is an assay designed to measure the binding power of antioxidant substances with iron II (Fe^{2+}) to form complexes. The absorbance of this complex is measured at 520nm and compared to the absorbance produced by a positive control of ethylene diamine tetra acetic acid (EDTA) (Vicente *et al.,* 2006). The intensity of colour of the complex reduces in the presence of substances with antioxidant properties. This is because the antioxidant agent disrupts the complex (Minotti and Aust, 1987) resulting in a decrease in absorbance.

2.11.4 Hydroxyl radical scavenging activity assay

Hydroxyl radicals have a high reactivity and have thus been implicated to cause harm in living organisms. When the activity of these radicals goes unchecked, they interact with biological components of cells such as proteins, nucleic acids and lipids potentially causing cellular damage (Ogasawara *et al.,* 2007). The assay reaction involves incubation of ferrous sulphate, hydrogen peroxide, sodium salicylate and different concentrations of the antioxidant substance at room temperature. A hydroxylated salicylate complex is formed whose absorbance is read at a wavelength of 562nm.

2.11.5 Hydrogen peroxide scavenging activity assay

In this assay, a phosphate buffer solution of pH 7.4 is prepared. This solution is then used to prepare serial dilutions of the sample under investigation. Hydrogen peroxide solution is then added to each of the concentrations prepared. The reaction proceeds by colour formation. The intensity of the colour so formed is measured as absorbance on a UV-VIS spectrophotometer at 230nm and the result compared with that of a positive reference compound such as butylated hydroxy toluene (Ruch *et al*., 1989).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Materials

3.1.1 Drugs and Chemicals

Pure artesunate drug powder (SIGMA A3731), pure amodiaquine drug powder (FLUKA A2799), phosphate buffer saline tablets, gallic acid, butylated hydroxy toluene, catechin, ascorbic acid and ethylene diamine tetra acetic acid were purchased from Sigma chemical company (St Louis MO, USA). Siliphos® was purchased from iherb, California, USA and diagnostic kits (MIND-RAY) were purchased from SHENZEN MINDRAY BIOMEDICAL ELECTRONICS, Shenzhen China. All other reagents and chemicals were of analytical grade and high purity.

3.1.2 Instruments

Analytical balance Mettler PM 4600 Delta Range®, analytical balance Mettler AE 163, memmert water bath apparatus (Germany), spectronic 21D Milton Roy UV-VIS Spectrophotometer (USA), búchi-Rota vapor-RE (Switzerland), labofuge A-heraeus separations technik GmbH (Germany), heidolph magnetic stirrer and magnetic bar (Germany), power sonic 405-microprocess controlled bench-top ultrasonic cleaner.

3.1.3 Ethical approval

Before commencement of the study, ethical approval was obtained from the Faculty of Veterinary Medicine Biosafety, Animal Use and Ethics Committee (BAUEC) of the University of Nairobi and a reference number assigned BAUEC/J56/76385/2014.

3.1.4 Experimental animals

Forty-six (46) healthy, 8-10 week old female *wistar* albino rats weighing 180 ± 20 grams were used for the study. These animals were nulliparous and non-pregnant and were purchased from the animal house of the department of PHPT of the University of Nairobi (UoN). They were then housed at a temperature of 25±3°c and 56-60% relative humidity. A 12-hour day and night cycle was maintained and the animals were fed on standard rat pellets from a commercial feed supplier (Unga feeds). Water was provided ad *libitum.*

3.2 Methods

3.2.1 Collection and authentication of plant material

Fresh aerial material of the *Moringa oleifera* plant was collected from the UoN Kibwezi farm. The leaves were manually separated from the plant and soaked in 1% w/v sodium chloride (NaCl) for 5 minutes to remove microbes. Further washing was done with aqueous-ethanol and later the leaves were rinsed using distilled water. They were then exposed to ambient air to allow surface moisture to dry. The botanical identity was authenticated at the National Museum Herbarium and a voucher specimen was submitted for future reference. Voucher number MO/001/16.

3.2.2 Preparation of plant material

The leaves were thinly spread on a mesh tied on racks in a well-ventilated, insect, rodent and dust free room. They were allowed to air dry for 10 days. Once dry, the leaves were ground to powder using an electric mill at the department of PHPT.

3.2.3 Preparation of plant extracts

The slightly modified method of Anwar *et al.,* 2013 was used to prepare two extracts from the dried powder of *Moringa oleifera* leaves.

3.2.3.1 Aqueous Extract

One hundred (100) grams of dried *M. oleifera* leaf powder was accurately weighed on an analytical balance (Mettler PM 4600, Germany) and poured into a 1 litre conical flask wrapped in aluminium foil. Eight hundred (800) ml of distilled water was then gradually added to the powder and the contents shaken until a slurry of uniform consistency was formed. Phytochemicals present in the leaf powder were extracted using the stirring technique. For this purpose, a magnetic bar and magnetic stirrer (Heidolph, Germany) were used. The magnetic stirrer was set to operate at 200 revolutions per minute (RPM) for 48 hours. This process was repeated again for another batch of 100 grams of leaf powder. The resultant slurry was then centrifuged (Heraeus technik centrifuge, Germany) at 3000 revolutions per minute (RPM) for 5 minutes, the supernatant collected into light-resistant bottles and stored at -4°C. After 24 hours, the mouth of the bottles was covered with muslin cloth and attached to a freeze drier (Virtis, New York). The set up was left overnight to obtain a freeze dried product. The percentage yield of the freeze dried product was then calculated as percentage weight by weight (%w/w).

Calculation of percentage yield;

% of crude extract yield= $(M_2\text{-}M_1/M_0) \times 100$,

Where;

M_2=mass of container + extract

M_1=mass of empty container

M_0=mass of the initial leaf powder sample

This product was then stored under refrigeration in well closed, light resistant bottles awaiting analysis.

3.2.3.2 Aqueous-Methanol Extract

One hundred (100) grams of dried leaf powder of *Moringa oleifera* was accurately weighed on an analytical balance (Mettler PM 4600, Germany) and poured into a 1 litre conical flask wrapped in aluminium foil. Eight hundred (800) ml of a methanol-water co-solvent system in the ratio 80:20 v/v ratio was then gradually added to the powder with gentle agitation until a slurry with uniform consistency was formed. The phytochemicals present in the leaf powder were then extracted by stirring technique using a magnetic bar and stirrer (Heidolph, Germany). The stirrer was set to operate at 200 revolutions per minute for 48 hours and this process was repeated for another batch of 100 grams of leaf powder. The resultant slurry was then centrifuged (Heraeus technik centrifuge, Germany) at 3000 revolutions per minute (RPM) for 5 minutes and the supernatant collected and subsequently transferred to a rotary evaporator (Búchi-technik AG, Switzerland) set at a temperature of between 40-45°c to get rid of excess organic solvent and concentrate the extract. Further solvent removal and concentration of the extract was done in a sandbath for 24 hours. The percentage yield of the dried product was then calculated as percentage weight by weight (%w/w).

Calculation of percentage yield;

% of crude extract yield= $(M_2\text{-}M_1/M_0) \times 100$,

Where;

M_2=mass of container + extract

M_1=mass of empty container

M_0=mass of the initial leaf powder sample

This product was then stored under refrigeration in well closed, light resistant bottles awaiting analysis.

3.2.4 Phytochemical screening of leaf extracts of *Moringa oleifera*

Qualitative methods (Harborne, 1998, Kokate, 2007; Trease and Evans, 2009) were used to identify the phytoconstituents in the prepared leaf extracts. The criteria for grading the phytochemicals was done on the basis of the intensity of colour produced from reactions observed in the test tubes. High concentration was denoted as (++), moderate concentration as (+) and nil (-) represented no observable reaction.

3.2.4.1 Test for alkaloids (Dragendorrf test)

Approximately fifty (50) mg of each of the extracts was dissolved in a sufficient amount of distilled water. Concentrated hydrochloric acid (HCl) was then added to each of the solutions and the mixture filtered. Two (2) ml of this filtrate was collected in a test tube and one (1) ml of dragendorrf's reagent was added along the inner wall of the test vessels.

3.2.4.2 Test for anthraquinones (Borntraggers test)

Five (5) mg of each of the extracts was added with ten (10) ml of benzene and the resulting mixture shaken and filtered. To each filtrate, five (5) ml of ammonia solution (10%) was added and the mixture agitated.

3.2.4.3 Test for cardiac glycosides (Keller-Killiani test)

About half (0.5) a gram of each of the extracts was diluted with five (5) ml of water. Two (2) ml of glacial acetic acid was then added followed by two (2) drops of Ferric chloride solution ($FeCl_3$). Thereafter, one (1) ml of concentrated sulphuric acid (H_2S0_4) was added along the inner walls of the reaction vessels.

3.2.4.4 Test for coumarins (Fluorescence test)

Approximately one (1) mg of each individual extract was dissolved in two (2) ml of water and the formed solutions were then divided into two (2) portions. To portion a) half (0.5) ml of ammonia solution (NH_3) was added while portion b) acted as reference.

3.2.4.5 Test for flavonoids (Alkaline reagent test)

Five (5) drops of five (5) % sodium hydroxide solution was added to one (1) ml of each of the extracts. Thereafter, 2M hydrochloric acid was added.

3.2.4.6 Test for phenolics (Ferric chloride test)

Two (2) ml of distilled water was added to about one (1) mg of each of the extracts. Thereafter, a few drops of 10% aqueous Ferric chloride solution was added to each test-tube.

3.2.4.7 Test for phytosterols (Liebermann-Burchard's test)

Two (2) mg of each of the extracts was dissolved in acetic anhydride and the mixture boiled then allowed to cool. A volume of one (1) ml of concentrated sulphuric acid was then added along the inner walls of the test vessels.

3.2.4.8 Test for saponins (Foam test)

Five (5) ml of the each of the test extract solutions were taken in a test vessel and vigorously agitated for a period of five (5) minutes.

3.2.4.9 Test for tannins (Ferric chloride test)

Five (5) % Ferric chloride solution was added to two (2) ml of each of the test extract solutions.

3.2.4.10 Test for triterpenes (Salkowski test)

About two (2) mg of the extracts were shaken with a volume of one (1) ml of chloroform and thereafter a few drops of concentrated sulphuric acid were added along the inner walls of the reaction vessels.

3.2.4.11 Test for ascorbic acid (Dichlorophenol indophenol-DCPIP test)

Fifty (50) ml of the dichlorophenol indophenol was filled in a burette. Five (5) ml of the sample extracts were then poured in one hundred (100) ml beakers. Titration was then performed and the number of milliliters required to decolorize the sample extract was noted.

3.2.5 Quantification of phenolic compounds in *Moringa oleifera* leaf extracts.

The method of Harnafi *et al.,* 2008 was adopted with slight modifications. This assay involved the use of gallic acid as standard substance. This also served as a representation of phenolic compounds in the *Moringa* plant. Ten (10) mg of this standard was mixed with on hundred (100) ml absolute methanol to dissolve and form a stock solution (100µg/ml).

3.2.5.1 Preparation of the calibration curve of gallic acid.

The method of Singleton *et al.,* 1999 was adopted with slight modifications. From the above stock solution, aliquots ranging from 0.25-2.0 ml were taken in five (5) different ten (10) ml volumetric flasks. Then, two and a half (2.5) ml of a tenfold dilution of Folin-Ciocalteau phenol reagent and two (2.0) ml of 7.5% w/v sodium carbonate were added to each of the flasks and the mixture made up to ten (10) ml using distilled water. A mixture of reagents and water was used as blank. The resultant solutions were then kept on a 45°C water bath for fifteen (15) minutes and thereafter the intensity of the colour produced was read at a wavelength of 765nm using a Milton Roy Spectronic 21D

UV-VIS spectrophotometer (USA). A calibration plot of absorbance (y) against concentration (x) was then computed using MS regression analysis.

3.2.5.2 Preparation of a solution of the extracts for estimation of phenols

Ten (10) ml of methanol was mixed with ten (10) mg of each of the extracts to dissolve. One (1) ml of each of these solutions were then transferred to ten (10) ml volumetric flasks and the development of colour allowed to take place in the same manner as of the standard (Folin, 1927). The intensity of the developed colour was then measured at a wavelength of 765nm using a Milton Roy Spectronic 21D UV-VIS spectrophotometer (USA). Distilled water was used as reagent blank. Phenolic concentration (in mg/ml) for each of the extracts was then determined by inferring readings from the calibration curve of the standard gallic acid (extrapolation). Further calculation was done to express this concentration as an equivalent of the gallic acid standard substance per gram of the extract. The formula of Gouveia and Castilho, 2011 was used to calculate the antioxidant capacity in terms of milligrams of gallic acid per gram of the plant material as below;

Total phenolic content in mg/g = Concentration of gallic acid established from the calibration curve in mg/ml × $\frac{\text{volume of the extract solution in (ml)}}{\text{weight of the extract in grams(g)}}$

3.2.6 Quantification of flavonoids in *Moringa oleifera* leaf extracts

The method of Atanassova *et al.,* 2011 was adopted with slight modifications. This assay involved the use of catechin as standard substance. This also served as a representation of flavonoid compounds in the *Moringa* plant. Ten (10) milligrams of this standard was dissolved in ten (10) ml absolute methanol to give a stock solution of 100µg/ml concentration.

3.2.6.1 Preparation of standard calibration curve of catechin

From the above solution, aliquots ranging from 0.1-1.0 ml were taken in five (5) different ten (10) ml volumetric flasks containing a mixture of distilled water (4ml) and 5% sodium nitrite (0.3ml). Five (5) minutes later, 10% w/v aluminium chloride (0.3ml) was added and six (6) minutes later 1M sodium hydroxide (2ml) was added and the total volume made up to ten (10) ml with distilled water. The intensity of colour produced was then read at 510nm using a Milton Roy Spectronic 21D UV-VIS spectrophotometer (USA). Distilled water was used as reagent blank (Atanassova *et al.,* 2011). The calibration curve was prepared by plotting a graph of absorbance (y) against concentration (x).

3.2.6.2 Preparation of the solution of the extracts for estimation of flavonoids

Ten (10) ml of methanol was added to ten (10) mg of each of the extracts to dissolve. An aliquot (1ml) of each extract was then transferred to ten (10) ml volumetric flasks and the addition of reagents followed the same protocol as for the standard substance. The intensity of the developed colour was then measured at 510nm using a Milton Roy Spectronic 21D UV-VIS Spectrophotometer. Distilled water was used as reagent blank (Atanassova *et al.,* 2011). The concentration of flavonoids (mg/ml) for each of the extracts was then determined by inferring readings from the calibration curve (extrapolation). Further calculation was done using the method of Gouveia and Castilho, 2011 to express the antioxidant capacity of the *Moringa oleifera* leaf extracts in terms of milligrams of catechin per gram of the plant material as below;

Total flavonoid content in mg/g = Concentration of catechin established from the calibration curve in mg/ml × $\frac{\text{volume of the extract solution in (ml)}}{\text{weight of the extract in grams (g)}}$

3.2.7 Quantification of ascorbic acid contents in *Moringa oleifera* leaf extracts

The method of Benderritter *et al.,* 1998 was adopted with slight modifications. This assay involved the use of ascorbic acid as a standard substance. This substance also served as a representation of vitamin C in the *Moringa* plant. Ten (10) mg of this standard was dissolved in one hundred (100) ml distilled water to give a stock solution of 100µg/ml concentration.

3.2.7.1 Preparation of standard calibration curve of ascorbic acid

From the above stock solution aliquots ranging between 0.05-1.5 ml were taken in six (6) different ten (10) ml standard flasks. A two hundred (200) µl mixture of trichloroacetic acid (13.3% w/v) and distilled water were then added followed by seventy-five (75) µl of dinitrophenyl hydrazine. The resulting mixtures were then incubated at 37°c for three (3) hours and thereafter 65% v/v of sulphuric acid (0.5ml) was added. The intensity of the developed colour was then measured at 520 nm using a Spectronic 21D Milton Roy UV-VIS spectrophotometer (USA). Distilled water was used as reagent blank (Benderritter *et al.,* 1998). The calibration curve was prepared by plotting a graph of absorbance (y) against concentration (x).

3.2.7.2 Preparation of solutions of the extracts for estimation of ascorbic acid

Ten (10) ml of distilled water was added to ten (10) mg of each of the extracts to dissolve. Colour development was carried out for 0.3 ml of these solutions following the same protocol as for the standard substance. Distilled water was used as reagent blank and the concentration of ascorbic acid (in mg/ml) for each of the extracts was determined by inferring from the standard calibration curve (extrapolation) (Benderritter *et al.,* 1998). The formula of Gouveia and Castilho, 2011 was to calculate the antioxidant capacity in terms of milligrams of ascorbic acid per gram of the plant material as below;

Ascorbic acid content in mg/g = Concentration of ascorbic acid established from the calibration curve in mg/ml × volume of the extract solution in (ml)

weight of the extract in grams (g)

3.2.8 Determination of the capacity of *Moringa oleifera* leaf extracts to scavenge for hydroxyl radicals

Extract concentrations ranging from between 10 and 100 µg/ml were added with a 3 ml mixture containing 1.5mM Ferrous sulphate, 6mM hydrogen peroxide and 20mM sodium salicylate. The resulting solution was then incubated at 37°C for 1 hour. The intensity of the colour developed was measured at 532nm using a Spectronic 21D Milton Roy UV-VIS Spectrophotometer (USA) (Klein *et al*., 1991). The capacity of the samples to scavenge hydroxyl free radicals was calculated using the formula of Singh *et al*., 2008 as described below;

$$\% \text{ Scavenging capacity of sample extracts on hydroxyl radicals} = \frac{A_{Control} - A_{Sample}}{A_{Control}} \times 100$$

Where;

$A_{Control}$ = Absorbance of the control (Ferrous sulphate, hydrogen peroxide and sodium salicylate)

A_{Sample} = Absorbance of the sample (Ferrous sulphate, hydrogen peroxide, sodium salicylate and varied concentrations of leaf extracts of *Moringa oleifera.*

3.2.8.1 Calculation of the concentration of extract samples that inhibit 50% of the hydroxyl radicals (IC_{50})

The concentration of the extracts which inhibited 50% of hydroxyl radicals was calculated using regression analysis in MS Excel 2007.

3.2.9 Determination of the capacity of *Moringa oleifera* leaf extracts to scavenge for hydrogen peroxide free radicals

The method of Ruch *et al*., 1989 was used with modifications. Concentrations ranging from between 10 and 100 µg/ml of each of the extracts were prepared in a pH 7.4 phosphate buffer. One (1) ml of each of these solutions were transferred to ten (10) ml standard flasks and to these a mixture of pH 7.4 phosphate and 45mM hydrogen peroxide were added to make a five (5) ml volume. The intensity of colour developed in the reaction mixture was measured at 230nm using a Spectronic 21D Milton Roy UV-VIS Spectrophotometer. A reagent blank of phosphate buffer without hydrogen peroxide was used. The capacity of the extracts to scavenge hydrogen peroxide free radicals was calculated using the formula of Kamkar *et al*., 2010 as described below;

$$\% \text{ Scavenging capacity of sample extracts} = \frac{A_{Control} - A_{Sample}}{A_{Control}} \times 100$$

Where;

$A_{Control}$ = Absorbance of the control (Phosphate buffer + hydrogen peroxide)

A_{Sample} = Absorbance of the sample (Phosphate buffer + hydrogen peroxide + varied concentrations of the leaf extracts of *Moringa oleifera.*

3.2.9.1 Calculation of the concentration of extract sample that inhibits 50% of the hydrogen-peroxide free radicals (IC_{50})

The concentration of the extracts which inhibited 50% of hydrogen-peroxide radicals (IC_{50}) was calculated using regression analysis in MS Excel 2007.

3.2.9.2 Determination of *in vitro* iron (Fe^{2+}) chelating capacity of *Moringa oleifera* leaf extracts.

The method of Minnoti and Aust, 1987 was used with slight modifications. One hundred and fifty (150) µl of varied concentrations of each of the extracts ranging from

(10-100µg/ml) were mixed with nine hundred (900) µl of 500 µM aqueous Ferrous sulphate and incubated at room temperature for 5 minutes. 1,10 phenanthroline (0.25% w/v) was then added to each of the samples and the intensity of the coloured product measured at 510nm using a Spectronic 21D Milton Roy, USA UV-VIS spectrophotometer. The capacity of the sample extracts to chelate iron II *in vitro* was then calculated using the formula of Wang and Li, 2007 as described below;

$$\% \text{ Chelating activity} = \frac{A_{Control} - A_{Sample}}{A_{Control}} \times 100$$

Where;

$A_{Control}$ = Absorbance of the control ($FeSO_4$ +1, 10 Phenanthroline)

A_{Sample}=The absorbance of the reaction mixture (Sample + FeSO4 +1, 10 Phenanthroline)

3.2.9.3 Calculation of iron II (Fe^{2+}) inhibitory concentration at 50% (IC_{50})

The concentration of each of the extracts which chelated 50% of iron II (IC_{50}) was calculated using regression analysis in MS Excel 2007.

3.2.10 Animal model

The protocol previously described in section 3.1.4 was adopted.

3.2.11 Occupational health and personal protective equipment (PPE)

Established protocols (OECD 2000) for handling laboratory animals were followed throughout the study. Latex hand gloves and protective masks were used at all times. In addition, anti-tetanus and anti-rabies vaccines were made available and stored under refrigeration.

3.2.12 Housing and feeding conditions

Rat cages measuring 35 (L) ×25 (W) ×18 (H) were used to house the animals. The cages were lined with wood shavings which served as beddings for the animals. The temperature in the animal house was maintained at 25 ± 3°C and relative humidity at 56 ± 4 %. Rat pellets from a commercial feed supplier (Unga feeds) were used to feed the animals. Water was provided *ad libitum* and a 12-hour light and dark cycle was maintained.

3.2.13 Preparation of animals

Random selection of the animals was done and each animal was marked to enable identification. They were then kept in rat cages measuring 35 (L) ×25 (W) ×18 (H) for a period of 10 days to facilitate acclimatization to the laboratory conditions.

3.2.14 Acute oral toxicity study of *Moringa oleifera* leaf extract

OECD test guideline 425 was used with slight modification. Ten (10) animals were used for this study. The animals were randomly assigned to two groups of five (5) rats each. Each animal was individually weighed and marked to enable identification. They were then fasted overnight before commencement of the study. One group served as the control and animals in this group were administered with two (2) ml of physiological buffer saline once daily. The other group served as the treatment group and were administered with a test dose (2000mg/kg) of AQ-ME *MO* based on the limit test dose of the up and down procedure for determining LD_{50} (OECD-TG 425, 2000). Each of the rats in this group was dosed once daily over a 48-hour period to identify the concentration of the extract responsible for mortality of 50% of the animals. Animal 1 in this group was orally dosed with the test dose of 2000mg/kg of the extract using physiological buffer saline as vehicle. The survival of this animal dictated the sequential administration of the same

dose to four (4) other animals. Physical changes in the mucous membranes, appearance of the fur and skin were monitored over a 48-hour period as were clinical signs of the rate of respiration, perspiration, incidence of urinary incontinence, blood pressure changes and the heart rate. Moreover, central nervous system effects such as locomotor activity, sensitivity to sound, drowsiness, tremors, convulsions were also used as indices for assessing the acute oral toxic effect of the *Moringa oleifera* leaf extract. The guidance document on humane endpoints was used to limit the overall suffering of the animals (OECD-TG 424, 2000). Blood was collected from each animal 48 hours after extract administration by holding them with a warm cloth to dilate the blood vessels. A 21-gauge needle attached to a 2ml syringe was then used to puncture the lateral tail vein and sufficient amount of blood allowed to collect in the syringe. One (1) ml of this blood was placed in stoppered cryo-vials and capped to enable clot formation for 30-45 minutes. Serum was extracted from the clotted blood by centrifugation at 3000 revolutions per minute for a period of 10 minutes. This was stored at -20°C and used for biochemical analysis of the activity of serum liver enzymes. Aspartate amino transferase (AST), alanine amino transferase (ALT) and total bilirubin (TB) were assayed for each animal using commercial kits according to the protocol of the International Federation of Clinical Chemists (Schuman *et al.,* 2002). A spectrophotometer was used to measure the activity of these enzymes in the serum samples. Thereafter the animals were humanely euthanized using intravenous sodium pentobarbital injection (150mg/kg bwt) and death was confirmed by using the following indicators; absence of heartbeat, no respiration, absence of corneal reflex, lack of response to pinch and greying of the mucous membranes. The

rats were then dissected, their livers excised, weighed and the hepatic index calculated using the formula of Peters and Boyd, 1966 as described below;

$$\text{Hepatic index} = \frac{\text{Liver weight of the rat}}{\text{Absolute weight of the rat}}$$

The livers were then preserved in 10% formalin awaiting histopathological work.

3.2.15 Experimental design for assessing the hepatoprotective potential of *Moringa oleifera* against artesunate-amodiaquine combination induced liver injury.

From the acute oral toxicity study of the extract, a safe dose (x mg/kg) was selected for use in the experimental groups. Thirty-six (36) female rats were used in this study and were randomly assigned to 12 groups (3 animals per group) and marked to enable identification (OECD-TG 423, 2000). All the test substances were prepared by dissolving them in physiological buffer saline and administered by oral gavage. Group one was used as the negative control (physiological buffer saline only) while groups two and three were used as *Moringa oleifera* and siliphos® control groups respectively. Groups four, five and six were treated with clinical, 2×clinical and 4×clinical dose of the antimalarial combination respectively. Female rats in the clinical dose group were administered with the antimalarial combination over a 5-day period at doses of 4, 2, 2, 2, and 2 mg/kg of artesunate and 10.8, 5.4 ,5.4 ,5.4 and 5.4 mg/kg of amodiaquine on the respective days. This dose is equivalent to the clinical dose of this combination sufficient in managing uncomplicated malaria over a 3-day period in man as described by Angus *et al.*, 2002 with modifications). The animals in the 2×clinical dose group were administered with 8, 4, 4, 4 and 4mg/kg of artesunate and 21.6, 10.8, 10.8, 10.8 and 10.8 mg/kg of amodiaquine over a 5-day period. Animals in the 4×clinical dose group were administered with 16, 8, 8, 8 and 8 mg/kg of artesunate and 43.2, 21.6, 21.6, 21.6 and

21.6 mg/kg of amodiaquine over a period of 5 days. Groups seven, eight and nine were first pre-treated with a 200mg/kg dose of the standard hepatoprotectant, siliphos ® an hour before treatment with clinical, 2× clinical and 4×clinical dose of the antimalarial combination respectively. Groups ten, eleven and twelve animals were first pre-treated with a 1000mg/kg dose of the *Moringa oleifera* leaf extract one hour before treatment with clinical, 2×clinical and 4×clinical dose of the antimalarial combination respectively.

3.2.16 Collection of blood, confirmation of death, biochemical analysis and calculation of hepatic index

The animals were evaluated for signs that point to the occurrence of pain or distress. Changes in weight, coat texture and colour as well as the changes related to the rate of respiration and external stimuli were monitored. Blood was collected from each animal after the period of study (5 days) by holding each animal with a warm cloth to dilate the blood vessels and puncturing the lateral vein of the tail using a 21-gauge needle attached to a 2ml hypodermic syringe. Three (3ml) of blood was allowed to collect in the syringe and one (1.0) ml of the collected blood was then placed in stoppered cryo-vials and capped and allowed to stand for 30-45 minutes to facilitate clotting. It was then centrifuged at 3000 revolutions per minute for 10 minutes to extract serum which was stored at -20°C and later used for biochemical analysis. Aspartate amino transferase (AST), alanine amino transferase (ALT) and total bilirubin (TB) were assayed for each animal using commercial kits according to the manufacturers protocols (Schuman *et al*., 2002). The activity of these enzymes in the serum sample was measured using a spectrophotometer. Thereafter the animals were humanely euthanized using intravenous sodium pentobarbital injection (150mg/kg body weight) and death was confirmed by the same indices as used in acute oral toxicity studies of the extract. The rats were then

dissected, livers excised, weighed and the hepatic index calculated using the formula of Peters and Boyd, 1966 as described below;

Hepatic index = Liver weight of the rat

Absolute weight of the rat

The livers were then preserved in 10% formalin awaiting histopathological work.

3.2.17 Disposal of rodent carcasses

The carcasses were placed in special non-polyvinylchloride (non-PVC), sealable, transparent plastic bags. They were then incinerated.

3.2.18 Histopathology

For liver histopathological analysis, mid-sections of the left lobes of the liver were used. The sections were processed and embedded in paraffin wax. The tissue blocks were then sectioned at 5 microns (5µ) using a microtone. The sections were then stained using haematoxylin and eosin (H&E) and observed under the light microscope.

3.2.19 Statistical analysis

Data from the analysis of the antioxidant parameters of the extracts were expressed as mean ± standard deviation and analysed using analysis of variance (ANOVA) by GenStat statistical software 4th edition. The results of the evaluation of parameters of toxicity in acute toxicity and prophylactic studies were expressed as mean ± standard deviation and analysed using analysis of variance by GenStat statistical software 4th edition. The level of significance, $(p \leq 0.05)$ was maintained at all times.

CHAPTER FOUR

4.0 RESULTS

4.1 Effect of variation of solvent systems on the extraction yield of leaves of Moringa oleifera

The percentage yield of the aqueous and aqueous-methanol leaf extracts of *M. oleifera* was 14 and 18% respectively. The appearance of the extracts is as described in Table 4.1 below.

Table 4.1; Extraction yield and appearance of *M. oleifera* leaf extracts

Solvent	Weight taken	Percentage yield (%w/w)	Appearance of the extract
Water	200 grams	14 %	Light green powder and a dark-green semi solid mass
80% methanol	200 grams	18%	Dark-brown semi solid mass

4.2 Phytochemical profile of the *Moringa oleifera* leaf extracts

The results of preliminary phytochemical analysis of the extracts are as shown in Table 4.2. The results revealed the presence of pharmacologically active chemical compounds such as alkaloids, cardiac glycosides, flavonoids, phenolics, phytosterols, saponins, tannins and vitamin C.

Table 4.2; Results of preliminary phytochemical analysis of Moringa oleifera leaf extracts

Phytoconstituent/metabolite	AQ powder	AQ gummy exudate	AQ-ME
Alkaloids	+	+ +	+ +
Anthraquinones	_	_	_
Cardiac glycosides	+	+	+ +
Coumarins	_	_	_
Flavonoids	+	_	+ +
Phenolics	+	+	+ +
Phytosterols	+ +	+	_
Saponins	+	_	+ +
Tannins	+	+	+ +
Triterpenes	_	_	_
Vitamin C	+ +	+ +	+ +

Key;

+: low concentration (trace but detectable amounts)

++: high concentration (high amount)

-: Absent

4.3 In *vitro* antioxidant capacity of *Moringa oleifera* leaf extracts

The *in vitro* antioxidant capacity of the *Moringa oleifera* leaf extracts was expressed in terms of several parameters; phenolic content, flavonoid content, ascorbic acid content, concentration of the extracts effective in inhibiting hydroxyl and hydrogen-peroxide free radicals and the concentration of extracts effective in forming complexes with iron (II).

4.3.1 Total phenolic content of *Moringa oleifera* leaf extracts

In plotting the standard calibration curve of gallic acid, a concentration dependent increase in the absorbance values was observed. Moreover, of the two *Moringa oleifera* leaf extracts tested, the AQ-ME extract registered higher absorbance values than the AQ extract as shown in Table 4.3

Table 4.3; Absorbance values of varied concentrations of gallic and *Moringa oleifera* leaf extracts

S.no	Concentration of gallic acid (µg/ml)	Absorbance			Mean ± SEM
1	1.0	0.123	0.149	0.138	0.137 ± 0.013
2	2.5	0.327	0.297	0.315	0.313 ± 0.015
3	5.0	0.611	0.614	0.595	0.607 ± 0.010
4	10.0	1.185	1.500	1.295	1.327 ± 0.160
5	15.0	1.940	1.980	1.920	1.947 ± 0.031
6	AQ extract	0.538	0.421	0.396	0.452 ± 0.076
7	AQ-ME extract	0.718	0.643	0.650	0.670 ± 0.041

The standard calibration curve (y=0.1311x-0.0123) of gallic acid showed linearity in the range 1-15 µg/ml with a coefficient of determination (r^2) of 0.9989 (Figure 4.1). Extrapolation of the phenolic content of the *Moringa oleifera* leaf extracts from the gallic acid standard curve revealed that the AQ-ME extract had significantly higher ($p<0.05$) total phenolic content than the AQ extract as shown in Table 4.4.

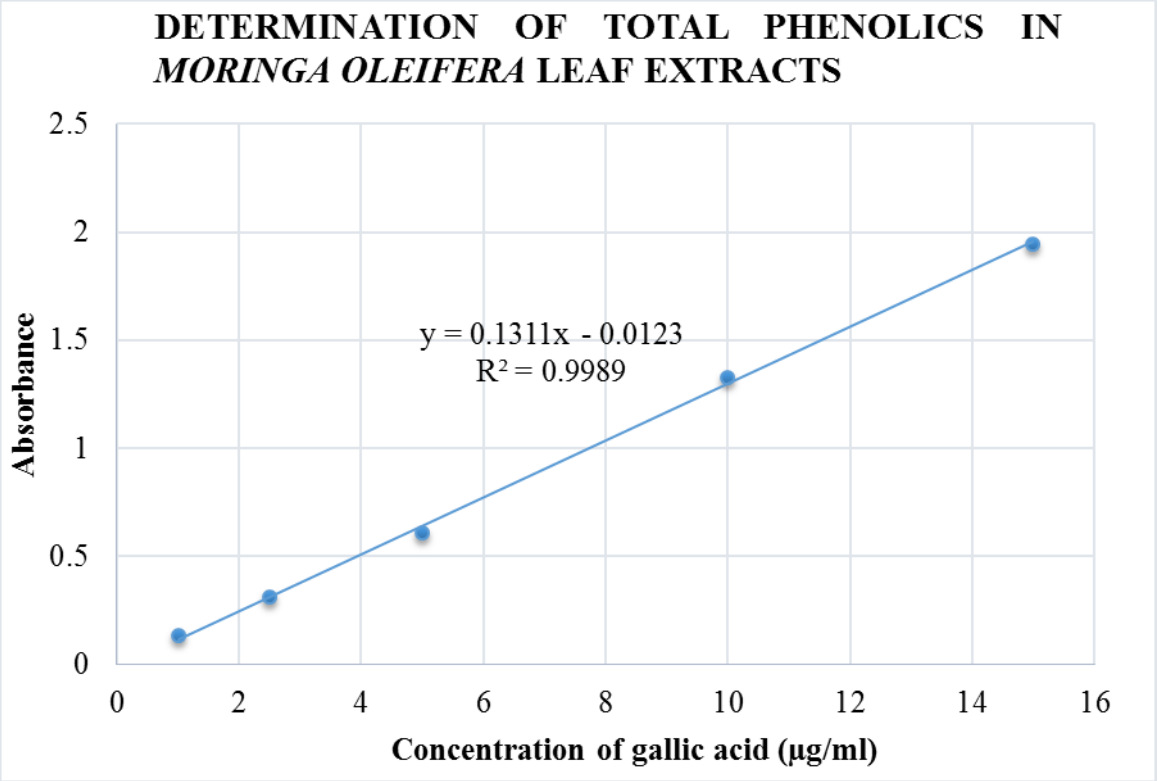


Figure 4.1; Standard calibration curve of gallic acid

Table 4.4; Calculated total phenolic contents of Moringa oleifera leaf extracts

Extract	Total phenolic content (mg.GAE.g^{-1}DW)			Mean ± SEM
AQ	29.62	35.42	41.21	35.42 ± 5.80
AQ-ME	48.92	52.04	55.17	52.04 ± 3.13

Key;

AQ: aqueous, AQ-ME: aqueous-methanol, GAE: gallic acid equivalents, DW: dry weight of plant material, SEM: standard error of the mean.

4.3.2 Total flavonoid content of *Moringa oleifera* leaf extracts

There was an increase in absorbance values with increasing concentration of the standard substance (catechin). Moreover, the AQ-ME extract had higher absorbance values as shown in Table 4.5.

Table 4.5; Absorbance values of varied concentrations of catechin and Moringa oleifera leaf extracts

S.no	Concentration of catechin (µg/ml)	Absorbance			Mean ± SEM
1	10	0.025	0.024	0.030	0.026 ± 0.003
2	20	0.033	0.056	0.056	0.048 ± 0.013
3	40	0.083	0.099	0.116	0.099 ± 0.017
4	80	0.152	0.178	0.233	0.188 ± 0.041
5	100	0.147	0.242	0.307	0.232 ± 0.080
6	AQ extract	0.020	0.022	0.025	0.022 ± 0.003
7	AQ-ME extract	0.074	0.080	0.111	0.088 ± 0.020

The standard calibration curve of catechin y=0.0023x+0.0039 showed linearity in the range of 10-100 µg/ml. The curve had a coefficient of determination (r^2) of 0.9995 (Figure 4.3.2). Extrapolation of total flavonoid content in the *Moringa oleifera* leaf extracts from the catechin standard curve revealed that the AQ-ME extract had higher total flavonoid contents; 365.52 ± 86.76 milligrams of catechin equivalents per gram of the extract compared to 78.69 ± 13.04 milligrams of catechin equivalents per gram of the extract in the AQ extract (Table 4.6).

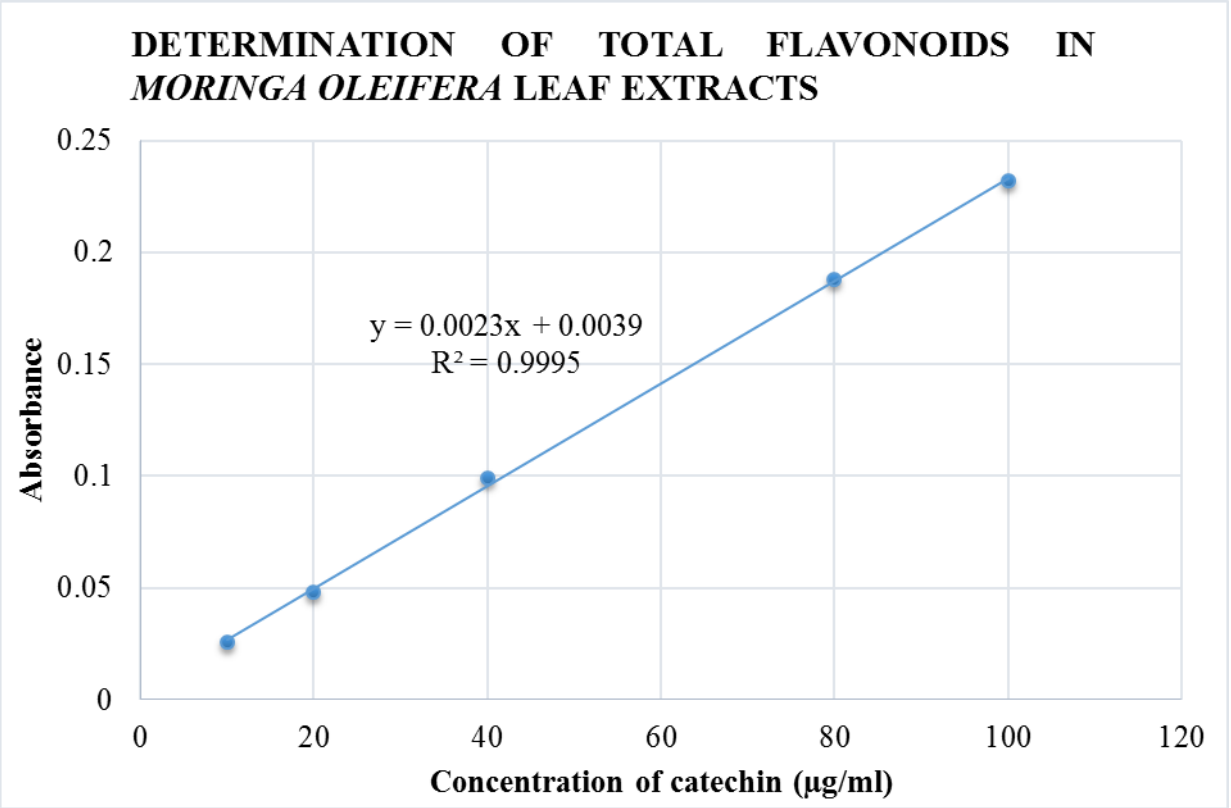


Figure 4.2; Standard calibration curve of catechin

Table 4.6 Calculated total flavonoid contents of Moringa oleifera leaf extracts

Extract	Total flavonoid content (mg.CE. g^{-1} DW)			Mean ± SEM
AQ	65.65	78.69	91.73	78.69 ± 13.04
AQ-ME	278.69	365.65	452.21	365.52 ± 86.76

Key;

AQ: aqueous, AQ-ME: aqueous-methanol, CE: catechin equivalents, DW: dry weight of plant material, SEM: standard error of the mean

4.3.3 Ascorbic acid contents in *Moringa oleifera* leaf extracts

There was an increase in absorbance values with increasing concentration of the ascorbic acid standard substance. Moreover, the AQ-ME extract had higher absorbance values as shown in Table 4.7

Table 4.7; Absorbance values of varied concentrations of ascorbic acid and Moringa oleifera leaf extracts

S.no	Concentration of ascorbic acid (µg/ml)	Absorbance			Mean ± SEM
1	0.05	0.022	0.038	0.026	0.029 ± 0.008
2	0.1	0.029	0.052	0.026	0.036 ± 0.014
3	0.25	0.187	0.343	0.184	0.238 ± 0.091
4	0.5	1.280	1.070	1.700	1.017 ± 0.294
5	1.0	1.420	1.354	1.125	1.300 ± 0.155
6	AQ extract	0.192	0.374	0.229	0.265 ± 0.096
7	AQ-ME extract	0.185	0.306	0.752	0.414 ± 0.299

The standard calibration curve y=1.4551x-0.029 of ascorbic acid showed linearity in the range of 0.05-1.0 µg/ml. The curve had a coefficient of determination (r^2) 0.9054 (Figure 4.5). AQ-ME extract contained higher ascorbic acid contents; 3.04 ± 2.06 compared to 2.02 ± 0.66 milligrams of ascorbic acid equivalents (Table 4.3)

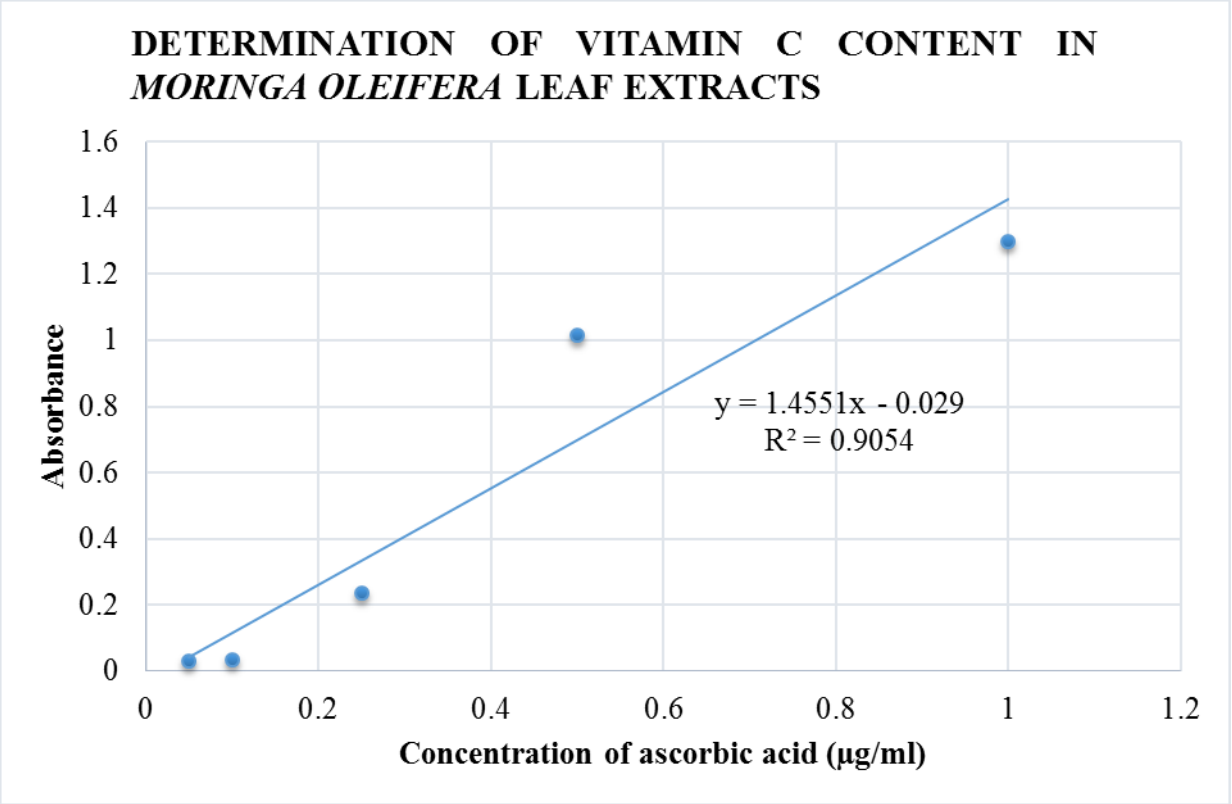


Figure 4.3; Standard calibration curve of ascorbic acid

Table 4.8; Calculated ascorbic acid contents of *Moringa oleifera* leaf extracts

Extract	Ascorbic acid content (mg.AAE.g^{-1} DW)			Mean ± SEM
AQ	1.36	2.02	2.68	2.02 ± 0.66
AQ-ME	0.99	3.04	5.10	3.04 ± 2.06

Key; AQ: aqueous, AQ-ME: aqueous-methanol, AAE: ascorbic acid equivalent, DW: dry weight of plant material, SEM: standard error of the mean.

A statistical analysis of the antioxidant parameters of *Moringa oleifera* leaf extracts revealed that there was a significant (p<0.05) difference in the antioxidant capacity of the two extracts with the AQ-ME extract showing better capacity.

4.4 Hydroxyl radical scavenging capacity of Moringa oleifera leaf extracts

The hydroxyl radical scavenging potential of AQ and AQ-ME leaf extracts of *Moringa oleifera* were recorded in terms of percentage inhibition as shown in Figure 4.4 and compared with the scavenging capacity of butylated hydroxy toluene which is a standard synthetic antioxidant

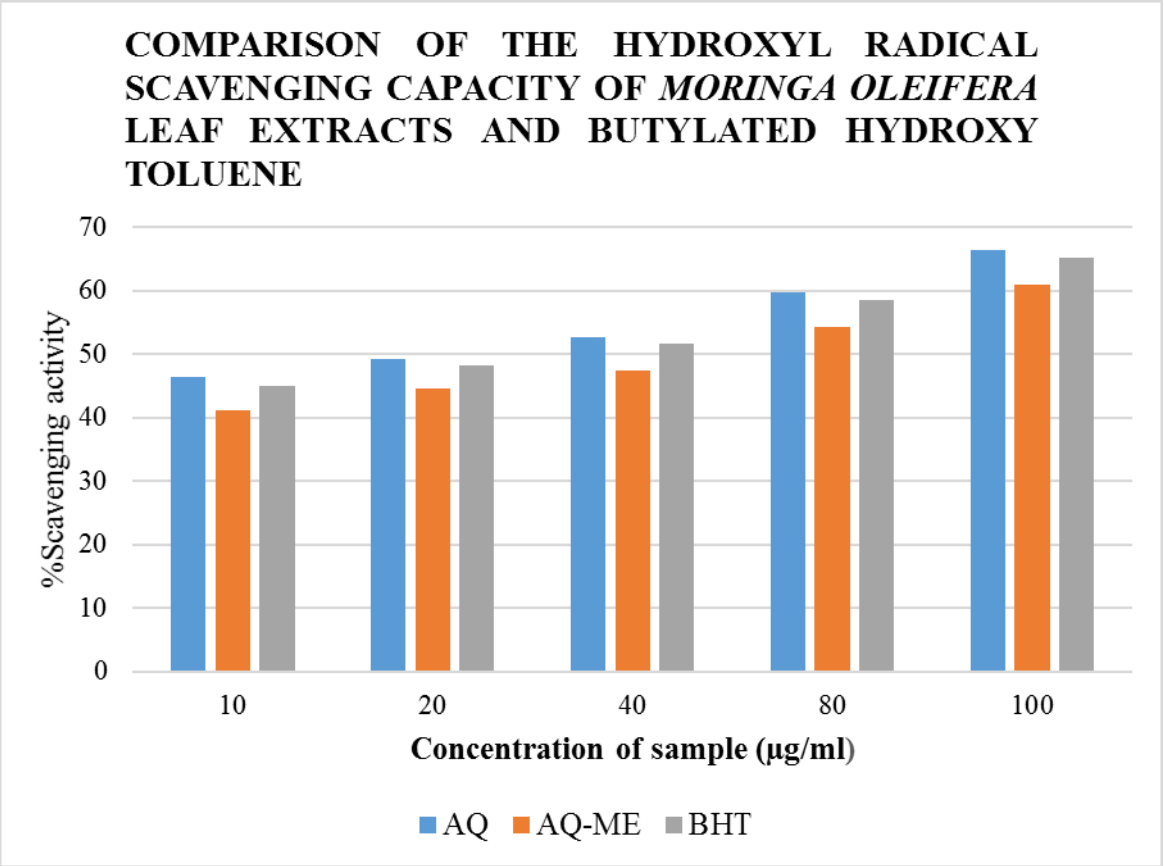
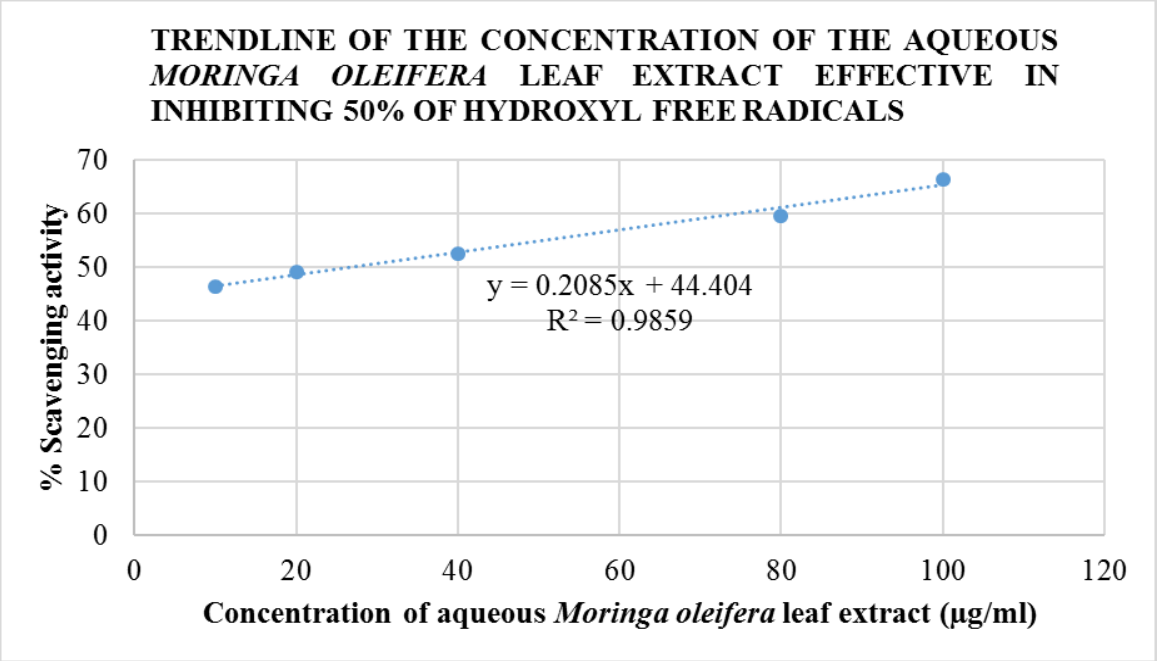


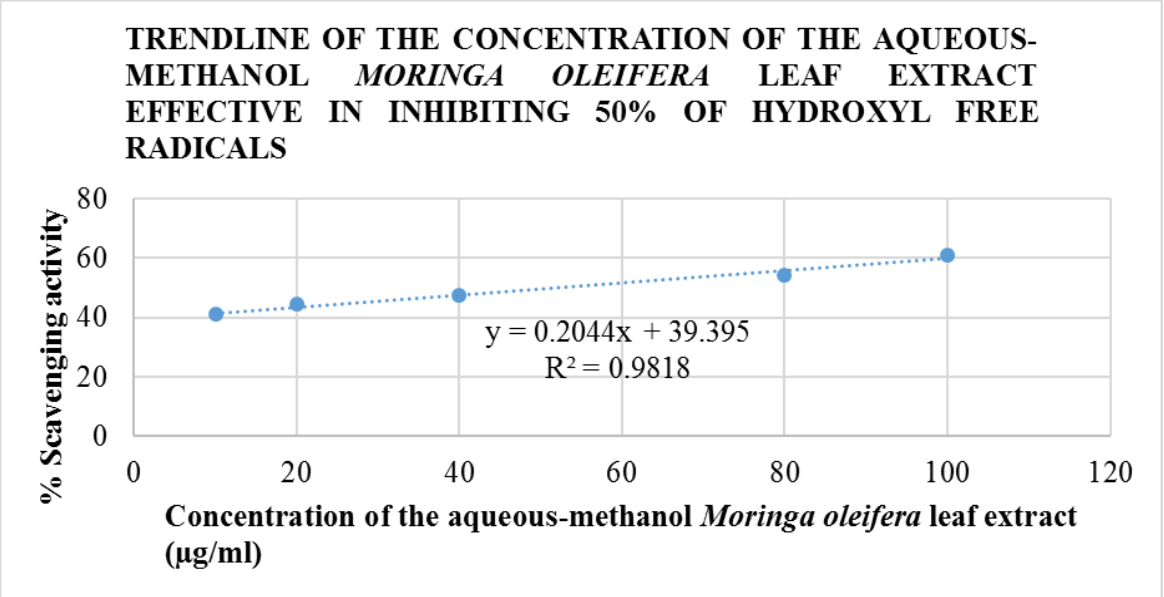
Figure 4.4; Comparative analysis of hydroxyl radical scavenging capacity of *Moringa oleifera* leaf extracts and butylated hydroxy toluene

It was observed from the Figure 4.4 that the *M. oleifera* extracts scavenged hydroxyl radicals in a concentration dependent manner. The scavenging activity of the AQ extract was in the range 46.36-66.36% compared to 41.04-60.95% of the AQ-ME extract and 44.93-65.23% of BHT. However, the ability of the AQ extract to scavenge these free radicals (66.36%) was found to be higher than both the AQ-ME extract (60.95%) and

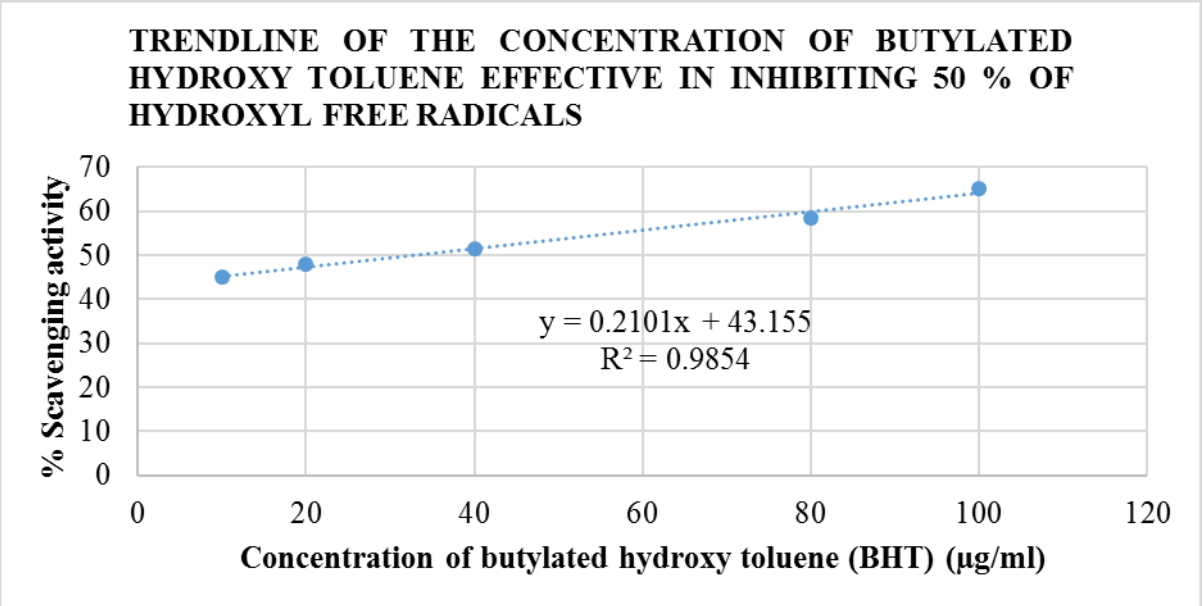
BHT (65.23%). Regression analysis in MS excel using trendlines (Figure 4.4.1) of the AQ, AQ-ME leaf extracts and BHT standard substance established the concentration of the extracts and standard necessary to inhibit 50% of the hydroxyl radicals (IC_{50}).



a) Aqueous extract



b) Aqueous-methanol



c) Butylated hydroxy toluene

Figure 4.5; Trendlines for the determination of the concentration of *Moringa oleifera* leaf extracts and butylated hydroxy toluene effective in scavenging 50% of hydroxyl free radicals

4.5 Comparative analysis of the concentration of Moringa oleifera leaf extracts and butylated hydroxy toluene effective in inhibiting 50% of the hydroxyl free radicals

The concentration of the extracts (aqueous and aqueous-methanol) and standard (butylated hydroxy toluene) responsible for inhibiting 50% of the hydroxyl free radicals (IC_{50}) was determined by substitution of the value of y with 50% in the respective trend line equations and calculating the value of x; AQ (y=0.2085x + 44.404), AQ-ME (y=0.2044x+39.395), BHT (y=0.2101x+43.155). The hydroxyl radical scavenging IC_{50} values of the AQ, AQ-ME and BHT were found to be 29.57, 68.24 and 26.00. The lower the IC_{50} value, the better the hydroxyl radical scavenging The hydroxyl radical scavenging capacity of the leaf extracts of *M. oleifera* and BHT was in the order BHT>AQ>AQ-ME.

4.6 Hydrogen peroxide free radical scavenging capacity of Moringa oleifera leaf extracts

The hydrogen peroxide radical scavenging potential of AQ and AQ-ME leaf extracts of *M. oleifera* was recorded in terms of percentage inhibition as shown in Figure 4.6 and compared with butylated hydroxy toluene (standard substance).

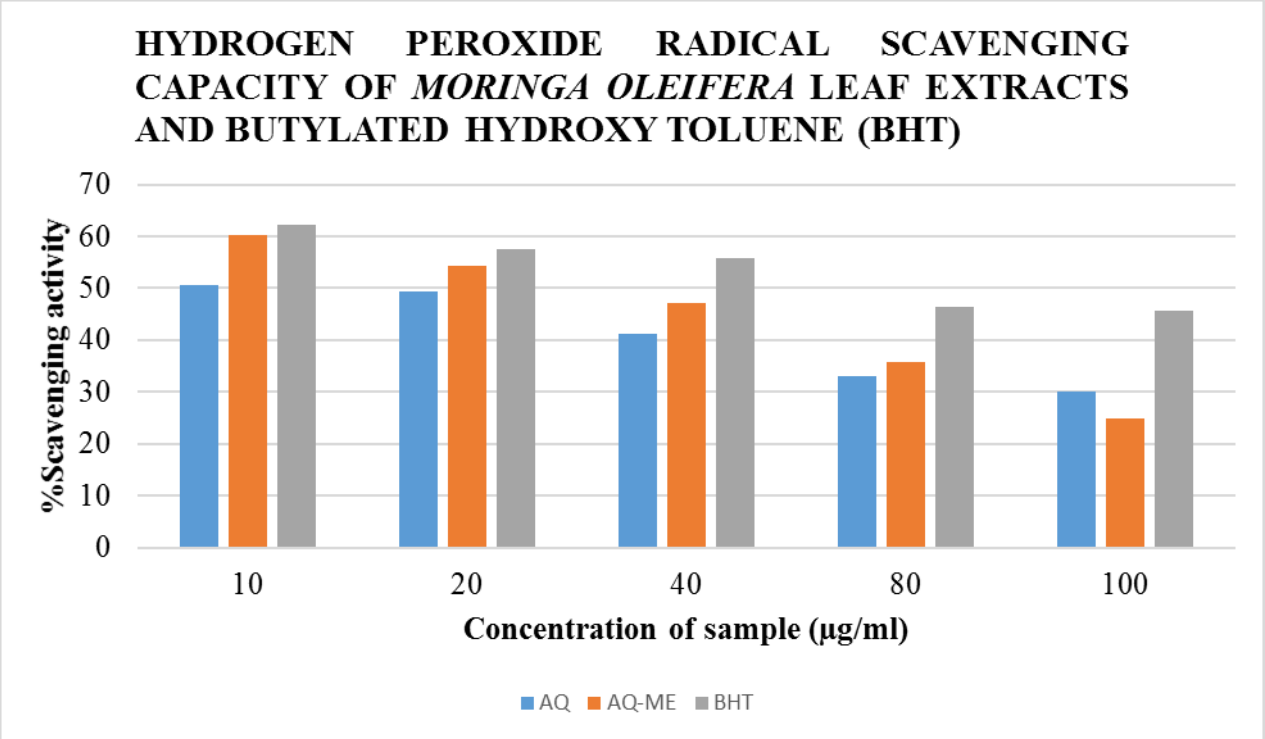
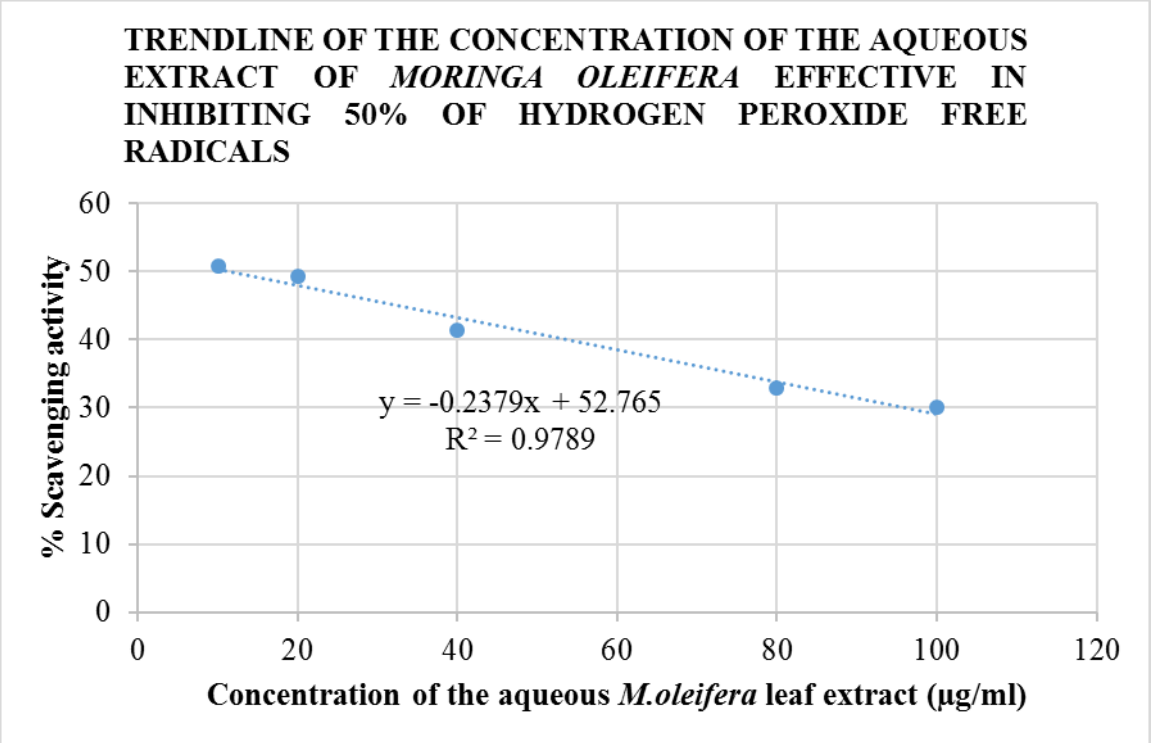


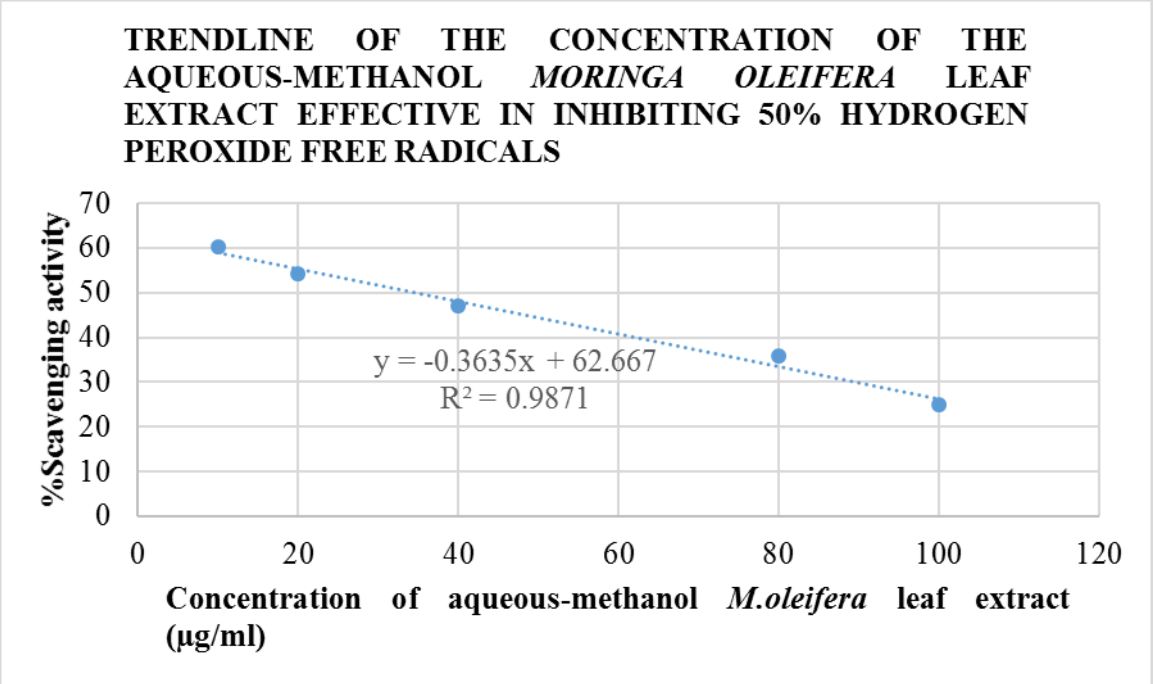
Figure 4.6; Comparative analysis of hydrogen peroxide radical scavenging capacity of leaf extracts of *M. oleifera* and BHT

It was observed from Figure 4.6 that the *M. oleifera* extracts scavenged hydrogen peroxide radicals in a concentration dependent manner. The hydrogen peroxide free radical scavenging activity of AQ extract was in the range 30.07-50.72% compared to 25-60% of the AQ-ME extract and 45.65-62.32% of BHT. In scavenging hydrogen peroxide free radicals, the ability of BHT (62.32%) was found to be higher than both the AQ-ME extract (60.14%) and the AQ extract (50.72%). Regression analysis in MS excel using trendlines of the AQ, AQ-ME and BHT standard substance (Figure 4.7) established

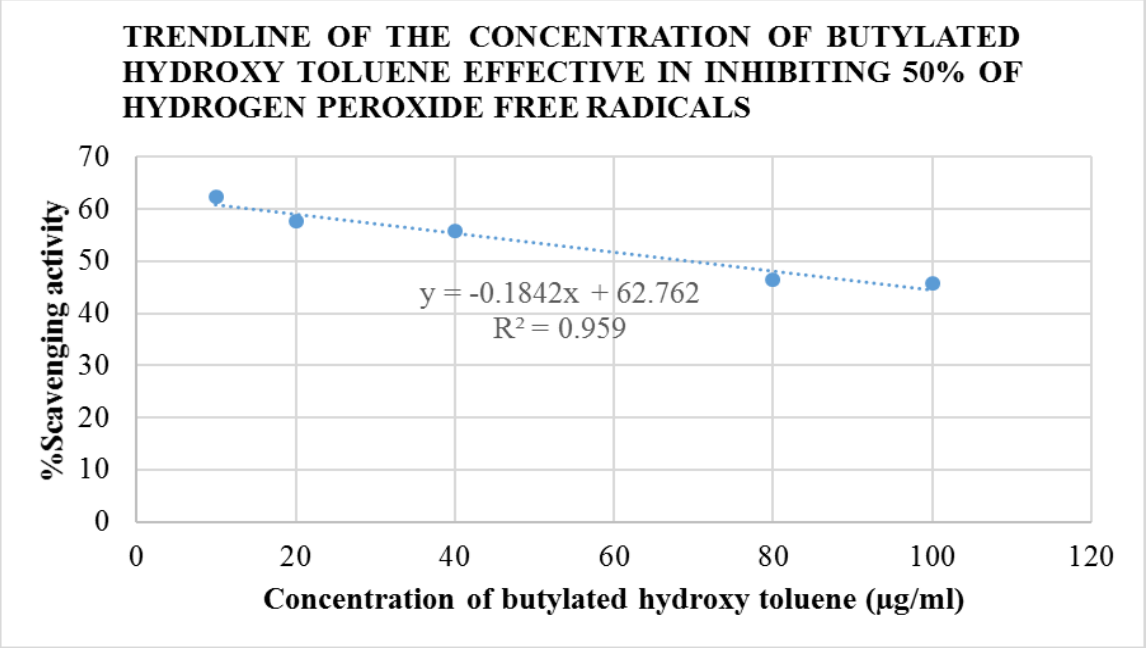
the concentration of the extracts and standard necessary to inhibit 50% of the hydrogen peroxide free radicals (IC_{50}).



a) Aqueous extract



b) Aqueous-methanol extract



c) Butylated hydroxy toluene

Figure 4.7; Trendlines for the determination of the concentration of *Moringa oleifera* leaf extracts and butylated hydroxy toluene effective in scavenging 50% of hydrogen peroxide free radicals

4.7 Comparative analysis of the concentration of Moringa oleifera leaf extracts and butylated hydroxy toluene effective in inhibiting 50% of the hydrogen peroxide free radicals

The concentration of the extracts (aqueous and aqueous-methanol) and standard (butylated hydroxy toluene) responsible for inhibiting 50% of the hydrogen peroxide free radicals (IC_{50}) was determined by substitution of the value of y with 50% in the respective trend line equations and calculating the value of x; AQ (y=-0.2379x + 52.765), AQ-ME (y=-0.3635x+62.667), BHT (y=-0.1842x+62.762). The hydrogen peroxide IC_{50} values of the AQ, AQ-ME and BHT were found to be 11.62, 34.85 and 69.28. The lower the IC_{50} value, the better the hydrogen peroxide free radical scavenging capacity. Thus, the hydrogen peroxide radical scavenging capacity of the leaf extracts of *M. oleifera* and BHT was in the order AQ>AQ-ME>BHT.

4.8 Iron II chelating capacity of Moringa oleifera leaf extracts

The iron II chelating capacity of AQ and AQ-ME leaf extracts of *M. oleifera* were recorded in terms of percentage inhibition as shown in Figure 4.8 and compared with ethylene diamine tetra acetic acid (EDTA) which is a standard chelating agent.

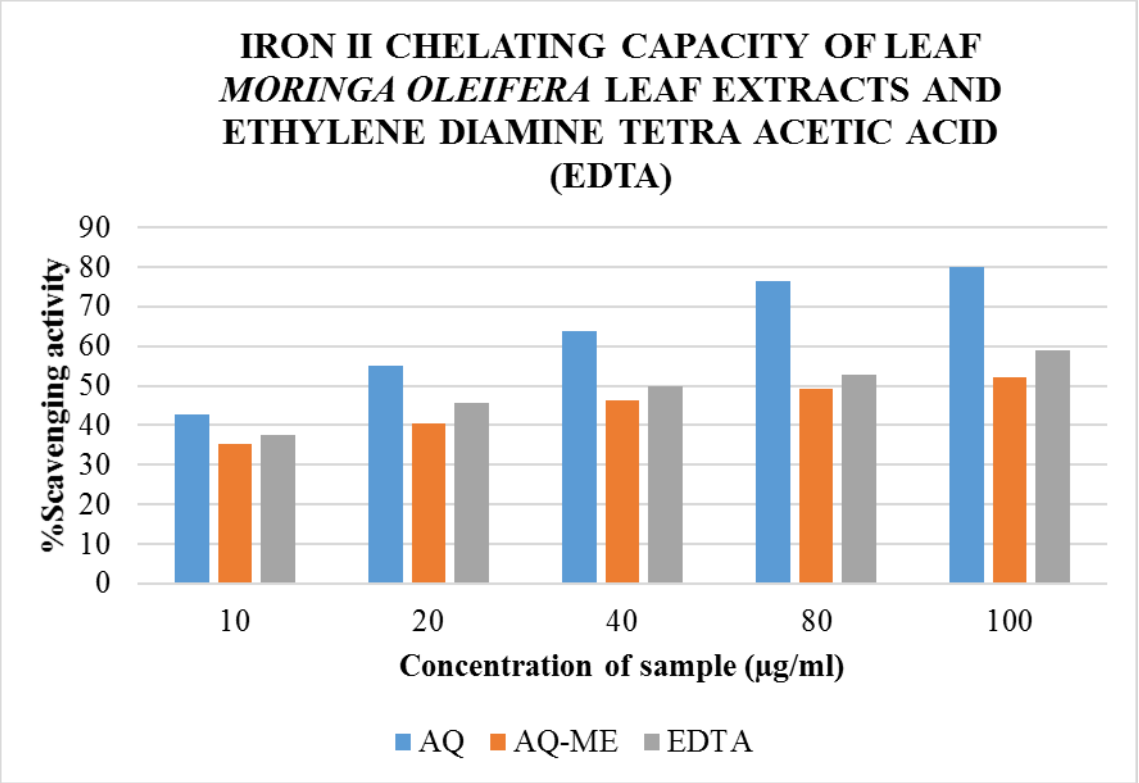
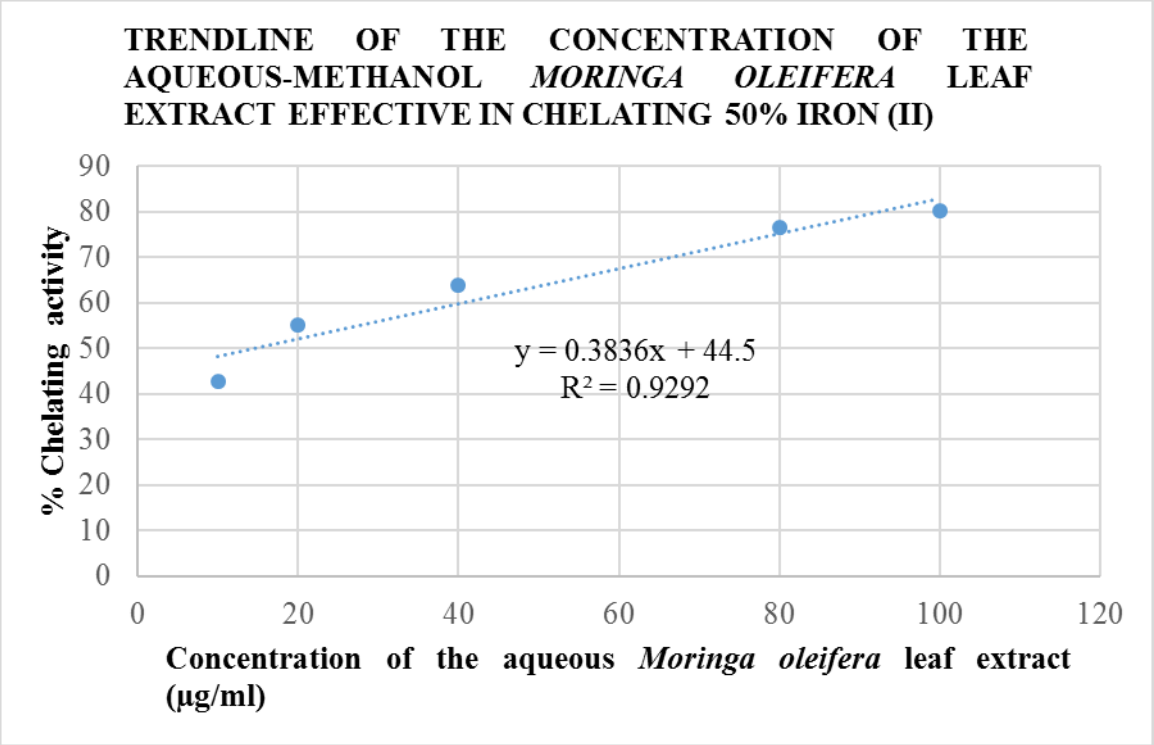
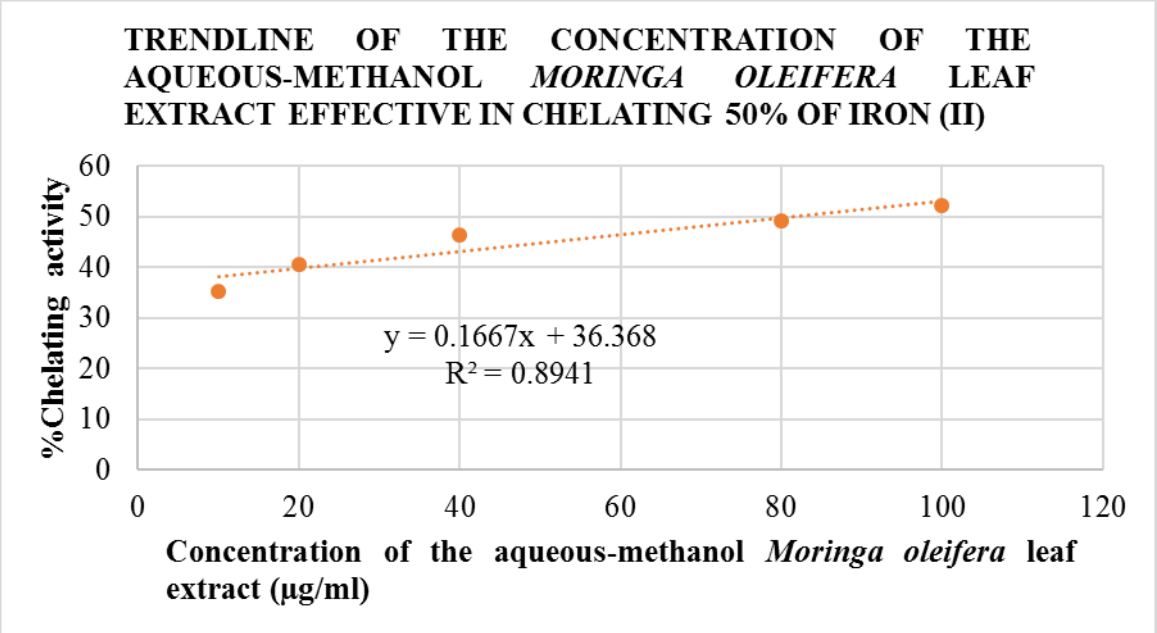


Figure 4.8; Comparative analysis of the Iron II chelating capacity of *Moringa oleifera* leaf extracts and EDTA

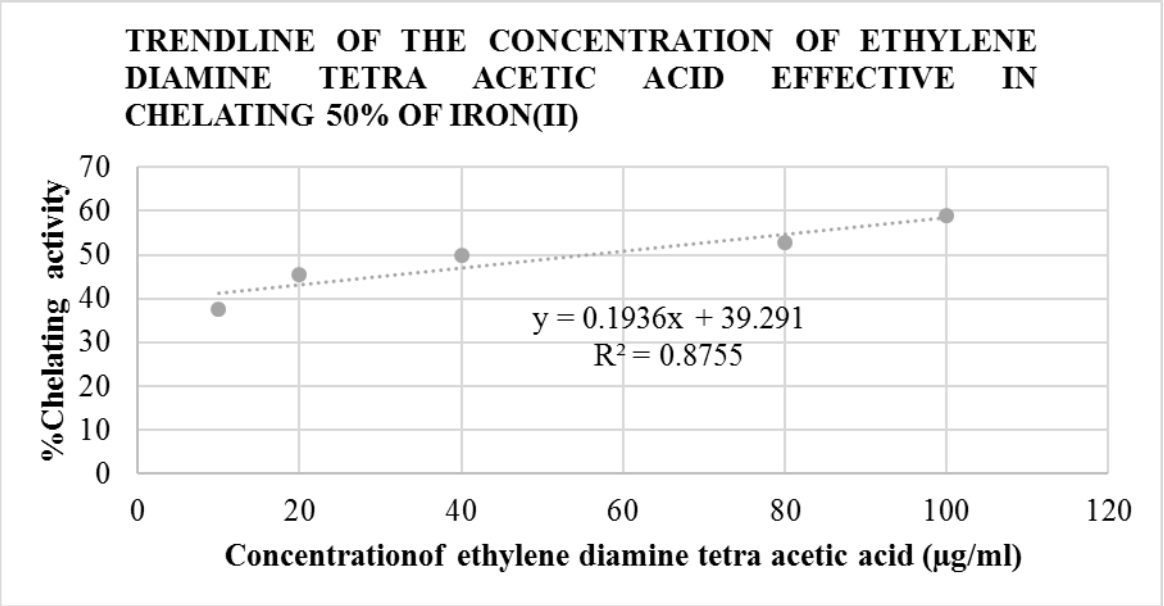
It was observed from Figure 4.8 that the *M. oleifera* extracts chelated Fe^{2+} in a concentration dependent manner. The iron (II) chelating capacity of AQ extract was in the range 42.65-80.15% compared to 35.59-52.21% of the AQ-ME extract and 37.5-58.82% of EDTA. In chelating iron (II), the ability of the AQ extract (80.15%) was found to be higher than both the AQ-ME extract (52.21%) and EDTA (58.82%). Regression analysis in MS excel using trendlines of the AQ, AQ-ME and BHT standard substance (Figure 4.9) established the concentration of the extracts and standard necessary to chelate 50% of iron II.



a) Aqueous extract



b) Aqueous-methanol



c) Ethylene diamine tetra acetic acid

Figure 4.9; Trendlines for the determination of the concentration Moringa oleifera leaf extracts and ethylene diamine tetra acetic acid effective in forming complexes with 50% of iron II

4.9 Comparative analysis of the concentration of Moringa oleifera leaf extracts and ethylene diamine tetra acetic acid effective in chelating 50% of Iron (II)

The concentration of the extracts (aqueous and aqueous-methanol) and standard (ethylene diamine tetra acetic) responsible for chelating 50% of iron (II) (Fe^{2+}-IC_{50}) was determined by substitution of the value of y with 50% in the respective trend line equations and calculating the value of x; AQ (y=0.3836x+44.5), AQ-ME (y=0.1667x+36.368), BHT (y=0.1936x+39.291). The IC_{50} of the AQ, AQ-ME and BHT was found to be 14.34, 81.78 and 55.32. The lower the IC_{50} value, the better the iron (II) chelating capacity. Thus, the iron (II) chelating capacity of the leaf extracts of *M. oleifera* and EDTA was in the order AQ>EDTA>AQ-ME.

4.10 Relationship between different antioxidant, radical scavenging and iron chelating activity assays

There was strong positive correlation between the different antioxidant assays. The correlation ranged from 0.8759-0.9999. Total phenolic content (TPC) and total flavonoid content (TFC) showed the strongest correlation while TFC and Fe^{2+} showed weakest correlation, Table 4.9

Table 4.9; Correlation between the different antioxidant, radical scavenging and iron chelating capacity assays of *M. oleifera* leaf extracts

Variables	TPC	TFC	VIT C	OH	H2O2	Fe^{2+}
TPC	_	0.9994	0.9064	0.9865	0.9600	0.8764
TFC	0.9994	_	0.9058	0.9858	0.9594	0.8759
VIT C	0.9063	0.9058	_	0.9188	0.9441	0.9669
OH	0.9864	0.9858	0.9188	_	0.9732	0.8884
H2O2	0.9600	0.9594	0.9441	0.9732	_	0.9129
Fe^{2+}	0.8764	0.8759	0.9669	0.8884	0.9129	_

Key; TFC: Total phenolic content, TPC: Total flavonoid content, VIT C: Vitamin C content, OH: Hydroxyl radical scavenging capacity, H_2O_2: Hydrogen peroxide radical scavenging capacity, Fe^{2+}: Iron II chelating capacity.

From the findings of this correlation analysis, it was established that total phenolic content (TPC) and total flavonoid content (TFC) had the highest correlation with each other (0.9994) than with any other parameter. Thus, owing to the fact that the AQ-ME *Moringa oleifera* leaf extract had significantly ($p<0.05$) higher contents of total phenolics and total flavonoids than the AQ extract, it was found to be the most suitable to be used in *in vivo* work involving female *wistar* albino rats.

4.11 Evaluation of the acute oral toxicity of the aqueous-methanol Moringa oleifera leaf extract

Parameters such as physico-clinical changes, mortality, biochemical and histopathological changes were used to evaluate the acute toxicity of the aqueous-methanol *Moringa oleifera* leaf extract on oral administration.

4.11.1 Physico-clinical changes and mortality

The oral administration of the aqueous-methanol *Moringa oleifera* leaf extract at a dose of 2000mg/kg did not produce any physical changes in the skin, fur, eyes and mucous membranes. Additionally, there were no significant changes in the clinical signs of respiration and heart rate, blood pressure, salivation, lacrimation, perspiration, urinary incontinence and defecation. The animals did not show any signs of aggression during handling. However, at the onset of extract administration (2000mg/kg), there was a decrease in the locomotor activity of three (3) out of five (5) animals which normalised after 10 minutes. Furthermore, post mortem analysis of the hepatic index (liver to body weight ratio) revealed that there was a non-significant increase ($p>0.05$) in the treatment group relative to the control, Table 4.10

Table 4.10; Effect of physiological buffer saline and a 2000mg/kg dose of the aqueous-methanol *M. oleifera* leaf extract on body and liver weight of *wistar* albino rats

Treatment (n=5)	Body weight (g)	Liver weight (g)	Hepatic index
Control	114.00 ± 15.47	5.96 ± 1.33	0.05 ± 0.01
2000mg/kg	125.90 ± 22.05	8.26 ± 2.47	0.07 ± 0.02

4.11.2 Biochemical changes

The levels of AST were significantly elevated in the treatment group relative to the control while there was a non-significant decrease in the levels of ALT in the treatment group relative to the control. However, there was no significant difference in the levels of bilirubin between the control and treatment groups. Furthermore, the AST/ALT ratio of control and treatment groups was in the range 1.22 ± 0.21 and 1.58 ± 0.14 respectively, Table 4.11.

Table 4.11; Biochemical profile of the *wistar* albino rats used acute oral toxicity testing of the aqueous-methanol *Moringa oleifera* leaf extract

Parameter	Control	2000mg/kg
AST (U/L)	127.46 ± 6.64	143.64 ± 9.22
ALT (U/L)	106.71± 16.01	91.18 ± 9.93
TB (mg/dl)	2.24 ± 1.24	3.00 ± 1.21
AST: ALT ratio	1.22 ± 0.21	1.58 ± 0.14

Key; AST: aspartate amino transferase, ALT: alanine amino transferase, TB: Total bilirubin, U/L: units per litre, mg/dL: milligrams per decilitre.

4.11.3 Histopathologic changes

Histopathology of the rat liver from the control group showed normal liver architecture characterised by normal looking hepatocytes separated by hepatic sinusoids and scanty congestion of the hepatic vein (Figure 4.10). However, histopathological investigations on the liver of an experimental rat treated with a 2000mg/kg dose of the AQ-ME *Moringa oleifera* leaf extract revealed a mild form of hepatic injury characterized by hepatocyte swelling and necrosis in areas around the central vein and hepatic veins as well as congestion of hepatic vessels (Figure 4.11).

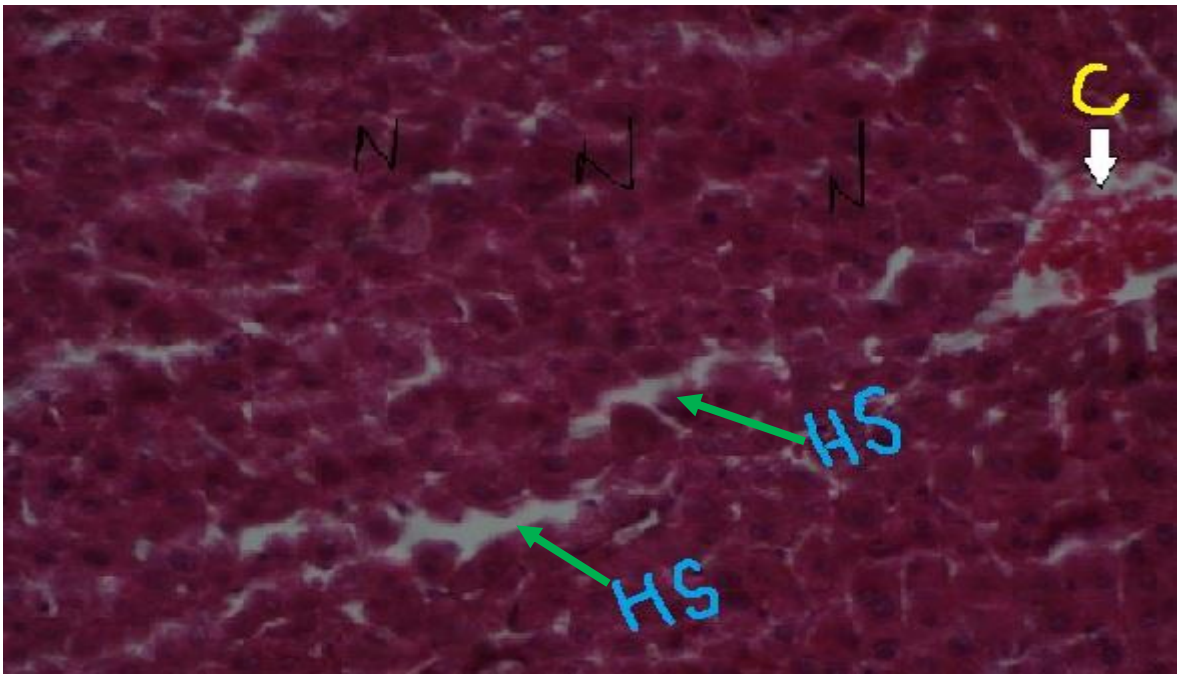


Figure 4.10: Photomicrograph of a liver section from a rat treated with physiological buffer saline (H&E×200)

Key; N- normal appearing hepatocytes, C-congestion of the hepatic vein, HS- Hepatic sinusoids

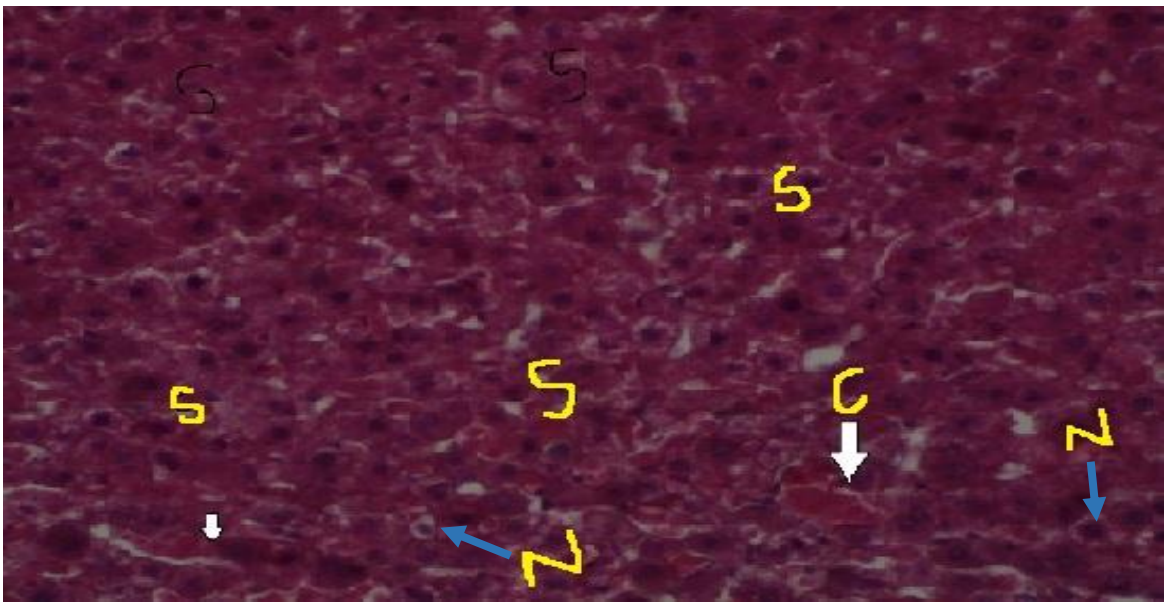


Figure 4.11: Photomicrograph of liver section from a rat treated with a 2000mg/kg dose of aqueous-methanol Moringa oleifera leaf extract (H&E×200)
Key; S-Cloudy swelling, C- congestion of hepatic vein, N- liver cell necrosis

Owing to the absence of any physico-clinical changes in the rats as well as non-significant changes in the biochemical parameters, it was observed that the (forty-eight) 48 hour LD_{50} of the aqueous-methanol *Moringa oleifera* leaf extract was above 2000mg/kg dose. However, further histological evaluation of liver sections of rats treated with 2000mg/kg of the aqueous-methanol *Moringa oleifera* extract revealed early stages of liver cell distortions in the form of hepatic necrosis. In light of this finding, the dose of the extract for the hepatoprotective study was tapered to a lower dose of 1000mg/kg as this study involved a much longer time frame than the 48-hour acute oral toxicity time period tested.

4.12 Evaluation of the protective effect of the aqueous-methanol M. oleifera against artesunate-amodiaquine induced toxicity

Animal body weight, hepatic index, biomarkers of liver injury (AST, ALT, TB) and liver histology were used as parameters for evaluating the protective effects of the aqueous methanol *Moringa oleifera* leaf extract. The standard hepatoprotectant, siliphos® was used for comparison.

4.12.1 Changes in body weight and hepatic index

There was no significant difference in the hepatic index between control and treatment group animals as represented on Table 4.12

Table 4.12; Analysis of the body weight and hepatic index of wistar albino rats used in the prophylactic study of M. oleifera and siliphos

Group	Treatment code (n=3)	Body weight (g)	Liver weight(g)	Hepatic index
1	PBS only	103.02 ± 34.01	7.11±2.01	0.06±0.005
2	SCG	148.72 ± 21.29	7.32 ±0.36	0.04±0.005
3	MCG	141.76 ± 5.98	5.15 ± 0.84	0.03±0.005
4	CD-ASAQ	132.46 ±11.46	7.02 ± 0.27	0.05±0.000
5	2×CD-ASAQ	116.27 ± 17.71	5.81±1.21	0.04±0.006
6	4×CD-ASAQ	133.80 ±10.11	6.63 ± 0.86	0.04±0.006
7	S+CD-ASAQ	122.61±10.31	6.59± 0.40	0.05±0.000
8	S+2CD-ASAQ	98.01±20.79	5.90±0.19	0.06±0.005
9	S+4CD-ASAQ	140.93±4.89	6.43 ± 0.54	0.04±0.000
10	M+CD-ASAQ	114.09±18.44	5.63±0.30	0.04±0.006
11	M+2CD-ASAQ	105.25±10.27	5.28 ± 1.22	0.04±0.006
12	M+4CD-ASAQ	112.91± 19.52	5.46 ± 0.98	0.04±0.000

Key; PBS: Physiological buffer saline, SCG: Siliphos control group, MCG: Moringa control group, CD-ASAQ: Clinical dose of artesunate-amodiaquine, 2×CD-AS-AQ: Double the clinical dose of artesunate-amodiaquine, 4×CD-ASAQ: Four times the clinical dose of artesunate-amodiaquine, S+CD-ASAQ: pre-treatment with siliphos first then after an hour treatment with a clinical dose of artesunate-amodiaquine, S+2* CD-ASAQ: pre-treatment with siliphos first then after an hour treatment with double the clinical dose of ASAQ, S+4×CD-ASAQ: pre-treatment with siliphos first then after an hour treatment with four times the clinical dose of ASAQ, M+CD-ASAQ: pre-treatment

with the aqueous-methanol *Moringa oleifera* leaf extract first then after an hour treatment with the clinical dose of AS-AQ: M+2×CD: ASAQ: pre-treatment with the aqueous-methanol *Moringa oleifera* leaf extract first then after an hour treatment with double the clinical dose of ASAQ : M+4×CD-ASAQ: pre-treatment with the aqueous-methanol *Moringa oleifera* leaf extract first then after an hour treatment with four times the clinical dose of AS-AQ.

4.12.2 Changes in serum liver markers

There was a dose-dependent increase in the levels of AST, ALT and TB upon treatment with the AS-AQ antimalarial combination. Pre-treatment with siliphos (200mg/kg body weight per oral) and AQ-ME leaf extract of *M. oleifera* at a dose of 1000mg/kg body weight per oral significantly reduced the levels of serum AST but non-significantly reduced the levels of ALT and TB when compared to the AS-AQ intoxicated groups, Table 4.13

Table 4.13; Biochemical evaluation of hepatospecific biomarkers of liver injury in wistar albino rats used in the prophylactic study of the aqueous-methanol M. oleifera and siliphos against artesunate-amodiaquine induced toxicity

Group	Treatment code (n=3)	AST(IU/L)	ALT(IU/L)	TB(mg/dl)
1	PBS only	122.37±13.73	65.18±20.08	0.83±0.67
2	SCG	127.67±25.07	78.95±24.11	3.08±1.30
3	MCG	113.93±5.46	80.73±21.65	1.69±0.38
4	CD-ASAQ	138.97±13.14	76.44±0.85	2.92±2.06
5	2×CD-ASAQ	158.8±20.08	85.70±17.55	1.19±0.94
6	4×CD-ASAQ	220.33±53.97	90.39±13.62	2.70±0.96
7	S+CD-ASAQ	130.97±16.61	58.01±20.16	1.15±0.57
8	S+2CD-ASAQ	125.74±45.13	64.47±23.65	2.00±2.07
9	S+4CD-ASAQ	184.00±23.60	68.00±28.83	2.93±2.32
10	M+CD-ASAQ	138.77±24.33	66.37±13.29	0.53±0.35
11	M+2CD-ASAQ	171.13±52.88	80.38±21.56	1.61±0.80
12	M+4CD-ASAQ	140.03±24.45	76.77±14.61	1.17±0.66

Key; PBS: Physiological buffer saline, SCG: Siliphos control group, MCG: Moringa control group, CD-ASAQ: Clinical dose of artesunate-amodiaquine, 2×CD-AS-AQ: Double the clinical dose of artesunate-amodiaquine, 4×CD-ASAQ: Four times the clinical dose of artesunate-amodiaquine, S+CD-ASAQ: Siliphos treatment first followed by treatment with clinical dose of artesunate-amodiaquine, S+2×CD-ASAQ: Siliphos treatment first followed by treatment with double the clinical dose of ASAQ, S+4×CD-ASAQ: Siliphos treatment first followed by treatment with four times the clinical dose of ASAQ, M+CD-ASAQ: Moringa treatment first followed by treatment

with the clinical dose of AS-AQ, M+2×CD-ASAQ: Moringa treatment first followed by treatment with double the clinical dose of ASAQ, M+4×CD-ASAQ: Moringa treatment first followed by treatment with four times the clinical dose of AS-AQ

4.12.3 Histological examination of rat liver sections

The transverse liver section of a rat treated with a 200mg/kg dose of siliphos (standard). The architecture of the hepatocytes appears normal and liver cells are separated by hepatic sinusoids (Figure 4.12).

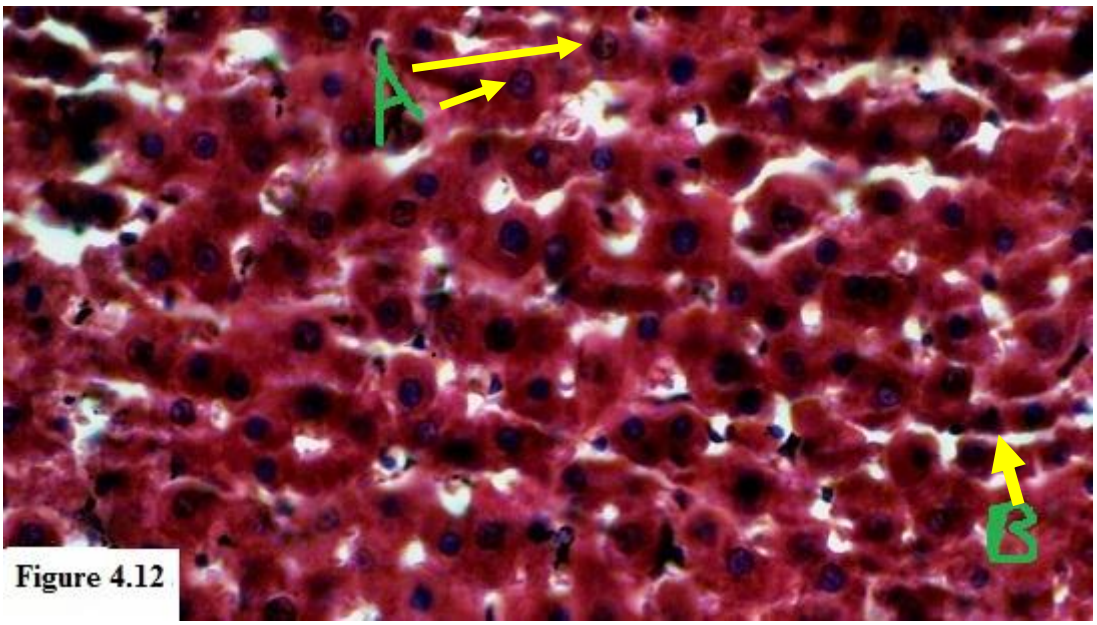


Figure 4.12: Photomicrograph of liver section from a rat treated with a 200mg/kg dose of siliphos. (H&E×200)
Key; A-Hepatic cells, B- Hepatic sinusoid

For a rat treated with four times the clinical dose of ASAQ, the transverse liver section was characterized by hepatocytic vacuolation and necrosis of the liver cells (Figure 4.13)

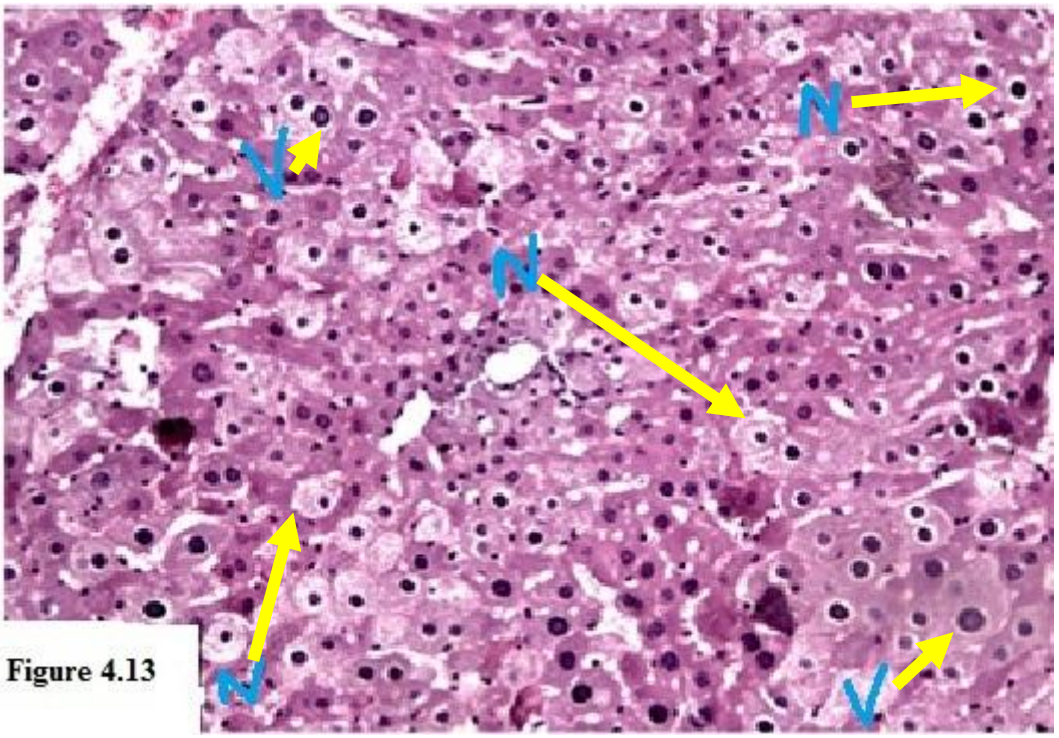


Figure 4.13. Photomicrograph of liver section from a rat treated with four times the clinical dose of AS-AQ (H&E ×400)
Key; V-Hepatic vacuolation, N- Hepatic cell necrosis

The transverse section of a rat pre-treated with a 1000mg/kg dose of *M. oleifera* followed by treatment with four times therapeutic dose showed an improvement in the degree of vacuolation. Mild hepatic vein congestion was also observed (Figure 4.14)

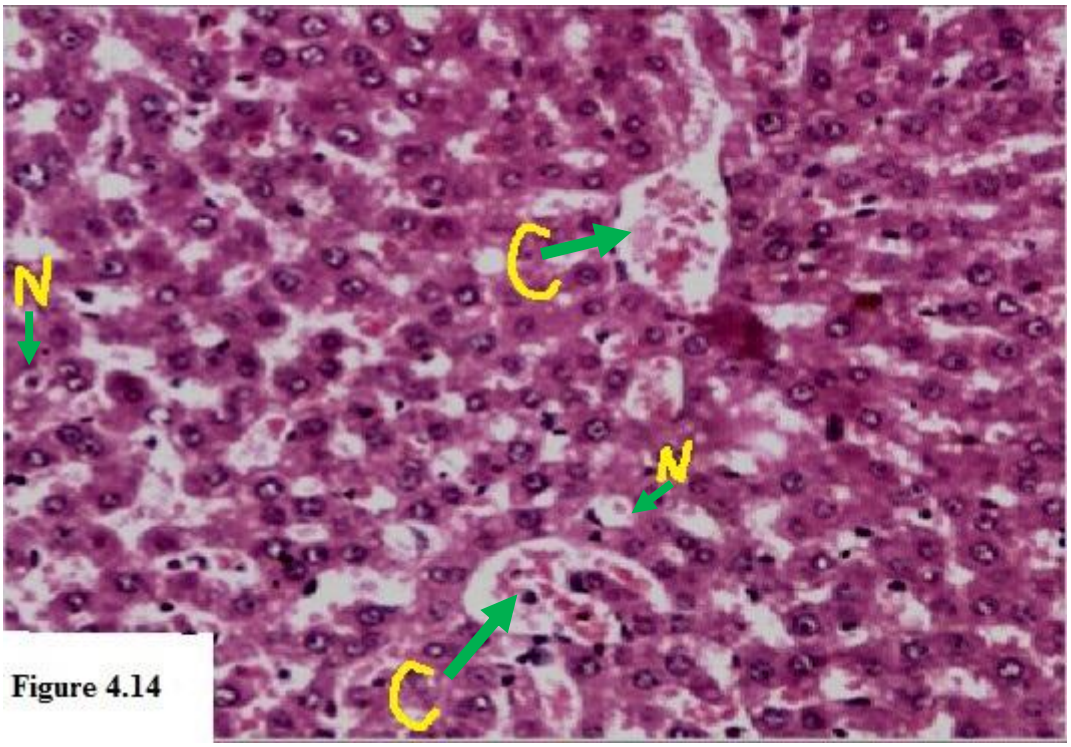


Figure 4.14: Photomicrograph of liver section from a rat treated with *Moringa oleifera* and four times the clinical dose of ASAQ (H&E×200)
Key; C- Hepatic vein congestion, N- Hepatic cell necrosis

CHAPTER FIVE

DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 DISCUSSION

Two extracting solvents, water and 80% methanol were comparatively evaluated for their efficacy in extracting compounds with antioxidant properties from *M. oleifera*. The aqueous methanol solvent (18g/100g) was more efficient than water (14g/100g) in extracting these compounds as observed by the extract yield. This observation compares well with the studies of Fatiha *et al.,* 2012 who reported that a co-solvent system comprising of water and organic solvents gave the best extraction rates of *Mentha spicata.*

In most studies on *Moringa oleifera*, single solvent systems using either water (Otulowa *et al.,* 2014) or ethanol (Singh *et al.,* 2014) have been used. Authors who have investigated the influence of extraction solvents on antioxidant capacity did not include aqueous-organic solvents in their studies (Kasolo *et al.,* 2010).

From the findings of this investigation, it could be suggested that a mixture of water and organic solvents has better potential in recovering antioxidant components from *Moringa oleifera* than a purely aqueous solvent. This co-solvent system may be given consideration in future studies involving extraction of antioxidants from this plant.

The present investigation used the methods of Harborne 1998, Kokate 2007, Trease and Evans 2009 to qualitatively screen the phytoactive chemicals in the plant extracts established the presence of alkaloids, cardiac glycosides, flavonoids, phenolics, saponins, tannins and ascorbic acid in both extracts.

No phytosterols were present in the aqueous-methanol extract which concurs with a report by Raghavendra *et al.,* 2013. Furthermore, both extracts tested negative for

anthraquinones, coumarins and triterpenes. This observation compares well with those of earlier investigations by other authors who established that not all phytochemicals may be present in plant parts (Ayinde, 2007).

The phytochemical compounds reported in the present study are known to have biological activities in tissues and cells. Furthermore, previous workers have shown the importance of total phenolics, total flavonoids and ascorbic acid contents as parameters of evaluating antioxidant properties of medicinal plants (Shah, 2010; Roy *et al.,* 2013)

The aqueous-methanolic extract of *Moringa oleifera* showed higher content of phenolics than the aqueous extract. This was consistent with the findings of other authors (Anwar *et al.,* 2013) who reported that aqueous organic solvents gave better phenolic contents than single solvents. However, the results of these calculated parameters were much lower than what was reported for *Malvaceae* species (De Oliveira *et al.,* 2012) and *Vitis vinifera* (Cassaza *et al.,* 2010).

Phenolics are one of the major secondary metabolites that are present in plant materials and have been associated with antioxidant properties (Holasova *et al.,* 2002).

The quantity of flavonoid compounds in the aqueous-methanol *Moringa oleifera l*eaf extract was found to be significantly more than that in the aqueous extract. This may be attributable to the differences in the polarities of the solvents as reported by other authors (Pourmorad *et al.,* 2006). However, the values obtained were significantly lower than what was reported on Bruckwheat groats (Hês *et al.,* 2014).

Literature on flavonoid content in the Moringa plant is scanty. Adisakwattana and Chananthong, 2011 reported flavonoid contents in Moringa that were lower than those reported presently.

The vitamin C (ascorbic acid) content of the aqueous-methanolic *Moringa oleifera* leaf extract was significantly higher than the vitamin C content of the aqueous extract. However, both the values obtained were much lower than what has been reported by other authors (Oboh and Omoregie 2011; Akomolafe *et al.,* 2012). The differences observed could be attributable to intrinsic or extrinsic factors such as agro-climate, method of cultivation and genetics (Fratianni *et al.,* 2007).

Ascorbic acid augments the fight response of the immune system against infections and tumour cells. It also protects tissues from free radical damage (Kronhausen *et al.,* 1989). Furthermore, vitamin C contents of leafy spices have been shown to contribute synergistically to antioxidant as well as other medicinal properties of leaves (Oboh and Omoregie, 2011).

The highest concentration of both extracts and the standard were most effective in their capacity to curtail the hydroxyl free radicals generated from the simulated Fenton reaction. This observation is supported by the work of other authors (Sowndhararajan and Kang, 2013) who identified a concentration dependent increase in the hydroxyl free radical scavenging ability of different solvent extracts of *Bauhinia Vahlii*.

The standard substance, butylated hydroxy toluene showed better scavenging capacity than the extracts. This may probably be because butylated hydroxy toluene is a known standard synthetic polyphenolic substance (Choi *et al.,* 2002).

The IC_{50} value is the concentration of the plant extract sufficient to inhibit 50% of free radicals (Choi *et al.,* 2002). The lower this value, the higher the antioxidant capacity of medicinal plants. The data from inhibitory concentration of free radicals at 50% (IC_{50}) calculation of the extracts and butylated hydroxy toluene suggests that the hydroxyl free

radical scavenging capacity of the aqueous extract is superior to that of the aqueous-methanolic extract but less than the standard substance. This observation compares well with previous studies by other researchers (Batool *et al.,* 2010).

In this study, there was a concentration dependent decrease in the capacity of the extracts and butylated hydroxy toluene to scavenge for hydrogen peroxide free radicals. This observation is supported by the work of others (Kumaran and Karunakaran, 2007) who suggested that the concentration of hydrogen peroxide free radicals drops significantly during the reaction due to its rapid decomposition to water. The polyphenolic compounds in the extracts may have accelerated this conversion since phenolics have been shown to be good electron donors (Pieroni *et al.,* 2010).

Following investigations on the ability of extracts and standard to bind iron II, this study established that the highest concentrations of both extracts and standard (EDTA) exhibited maximum capacity to bind to iron II. This observation is in agreement by the findings of Adjimani and Asare, 2015. Moreover, the values reported from this study are in close agreement with those reported by Khan *et al.,* 2012 on *Sonchus asper* extracts. According to Robak and Marankiewicz 1995; Li *et al.,* 2001, the chelating capacity of plant extracts may be an important strategy in avoiding free-radical induced oxidative stress as well as reduction in iron load.

According to Koleva *et al.,* 2000, it is important to use different methods in evaluating antioxidant activity in medicinal plants. This is because not only do these methods differ in their sensitivity and specificity but also the antioxidant power of various plants is influenced by several factors.

In the present investigation, correlations were tested in order to establish a relationship between the antioxidant, radical scavenging and iron chelating parameters of the leaf extracts of *M. oleifera.* There were strong positive correlations between the parameters ranging from 0.8759-0.9999. Moreover, this data suggests that the quantity of phenolic and flavonoid substances had strong correlations with the radical scavenging assays (hydroxyl and hydrogen peroxide) in tandem with earlier studies that have also reported strong positive correlations between antioxidant and radical scavenging capacity (Velioglu *et al.,* 1998 Jayaprakasha *et al.,* 2008). Moreover, the present study adds to the burden of proof that no single method of testing suffices in the estimation of antioxidant capacity of plant materials. The combination of the several methods applied in this study was a good choice in evaluating the antioxidant capacity of *M. oleifera* and could be recommended for other similar investigations. However, the results reported for the first three assays *vis-à-vis* total phenolic, flavonoid and ascorbic acid content assays were in contradiction to those reported by the last three assays *vis-à-vis* radical scavenging and iron (II) chelating capacity assays.

Previous investigations by Zhou *et al.,* 2004 and Prior *et al.,* 2005 have attributed this to phenomenon to be due to differences in the reaction kinetics of antioxidant substances. Furthermore, based on the work of Hider *et al.,* 2001, a sample with a high composition of polyphenolics may not necessarily form good complexes with Iron (II) or scavenge free radicals. This is because some polyphenolic compounds may be devoid of functional groups essential to Iron (II) complexing behavior and radical scavenging effects.

The lack of physical and clinical changes on oral administration of the aqueous-methanol *Moringa oleifera* leaf extract at a dose of 2000mg/kg may indicate that the extract has low oral toxicity. According to Husna *et al*., 2013 the absence of toxic manifestations and mortality in animals treated with a particular test dose implies that the LD_{50} is greater than the test dose. However, at the onset of extract administration (2000mg/kg), there was a decrease in the locomotor activity of three (3) out of five (5) animals which normalised after 10 minutes.

According to Mahendran *et al.,* 2014, the locomotor activity of experimental animals is a good indicator of the degree of alertness. Thus, a decrease in locomotor activity is suggestive of a sedative effect of the aqueous-methanol *M. oleifera* leaf extract. Investigations by Bakre *et al.,* 2013 established that phytochemicals such as flavonoids present in medicinal herbs may have some sedative and anti convulsant effects.

In the present investigation, preliminary phytochemical analysis of the leaf extracts of *M. oleifera* revealed the presence of flavonoids. It may thus be suggested that the observed decrease in locomotor activity in the experimental rats may be attributed to the presence of this phytochemical.

No mortality or morbidity was observed in any of the animals during the entire period of the study suggesting that the LD_{50} of the aqueous-methanol *Moringa oleifera* leaf extract was above 2000 mg/kg. Furthermore, post mortem analysis of the hepatic index (liver to body weight ratio) revealed that there was a non-significant increase *($p>0.05$*) in the hepatic index of the treatment group relative to the control. This observation is in agreement with the findings of previous authors (Elvin-Lewis, 2001; Roy *et al.,* 2013)

who reported alterations in the hepatic index in animal models treated with various plant extracts.

The levels of AST were significantly elevated in the treatment group relative to the control while there was a non-significant decrease in the levels of ALT in the treatment group relative to the control. This is suggestive of the occurrence of general cellular damage and is consistent with the observations of previous authors (Cavanaugh, 2003). Moreover, according to Singh *et al.,* 2011 the enzyme ALT is a more specific biomarker of liver injury, hence based on the observations from this study, it may be suggested that the aqueous-methanol *Moringa oleifera* leaf extract may not obviously cause toxic manifestations in the liver.

VanWagner and Green, 2015 reported that elevated levels of total bilirubin may point to the occurrence of haemolysis. Moreover, studies by Bailey *et al.,* 2004 suggested that the severity of acute liver injury can be diagnosed more accurately using total bilirubin than ALT.

In the present investigation, there was no significant difference in the levels of bilirubin between the control and treatment groups. This may further suggest that the aqueous-methanolic *Moringa oleifera* extract has low toxic potential. According to the findings of Greaves, 2007, the AST/ALT ratio may be useful in the diagnosis of some diseases of the liver. A value of less than 1 may point to the occurrence of leakage of ALT into systemic circulation while a ratio greater than 1 may be indicative of myocardial infarction. When the value is greater than 2 it may suggest alcoholic hepatitis or steatosis (Sacher and Mepherson, 1991).

From the observations of this study, the AST/ALT ratio of control and treatment groups was in the range 1.22 ± 0.21 and 1.58 ± 0.14 respectively. The slightly elevated levels in the treatment group relative to the control compare well with the work of other authors who studied the effects of these parameters post extract administration (Dufuor *et al*., 2000; Cavanaugh *et al.,* 2003).

Histopathology of the livers from the control group showed normal liver architecture characterised by normal polygonal shaped hepatocytes with scanty congestion of the hepatic veins. At a 2000mg/kg dose of the (AQ-ME) *MO* extract, histopathology of the liver revealed a mild form of hepatic injury characterized by focal hepatocyte swelling and necrosis in areas around the central vein and hepatic veins as well as congestion of hepatic vessels thus indicating that the extract may have some potentially deleterious effects on the liver at higher doses. This is in agreement with the work of other authors (Roy and Bhattacharya, 2006) who studied the effect of some medicinal plants on the livers of mice. These effects may possibly be due to the metabolic products of one or several of the phytochemicals including; alkaloids, cardiac glycosides, flavonoids, phenolics, saponins and tannins present in the extract.

The serum levels of AST, ALT and TB in the 4×clinical dose of AS-AQ were 101.54%, 18.75% and 144% higher than the physiological buffer saline control group. At 2×clinical dose of AS-AQ these parameters were 31.43%, 21.10% and 42% higher than those of the normal control group (physiological buffer saline).

In both the acute oral toxicity and hepatoprotective studies of the aqueous-methanol *Moringa oleifera* leaf extract, no comparison was made with normal control values for the biomarkers of liver injury (AST, ALT and TB) in *wistar* albino rats.

Instead of the normal/reference values, internal controls were incorporated in the study. This formed the basis for comparison of the serum liver enzyme activities between the respective treatment groups.

Based on the recommendations of the International Federation of Clinical Chemists (IFCC), internal controls are a better parameter of evaluating the effects of xenobiotic substances on various animal models than reference compounds (Schuman *et al.,* 2002). This is because variables such as species of animal used, atmospheric conditions, animal handling protocols affect the outcome of animal experiments (Schuman *et al.*, 2002).

The observation of high serum enzymatic activities was corroborated by dose dependent alterations in the physiology of the hepatocytes. This suggests that the antimalarial combination may be more toxic to the liver at higher doses. These findings were consistent with the observations of other workers (Obianime and Aprioku, 2011). Artesunate-amodiaquine (AS-AQ) is an ACT (artemisinin based combination therapy) antimalarial combination comprising artesunate and amodiaquine in a fixed ratio (1:2.7).

The mechanism of the observed effects due to the antimalarial combination may be attributable to the capacity of their active ingredients to induce oxidative stress in cells which in turn may be associated with their pharmacological mechanisms of action. Artesunate is an artemisinin derivative thus has an endoperoxide bridge as the pharmacophore (Wells *et al.,* 2009) while amodiaquine is an aryl methanol which forms an active metabolite (desmethylamodiaquine) which is responsible for its antimalarial action (Walter *et al.,* 2006). These pharmacophores are an indispensable part of the activity of the combination. Thus, the individual drugs in the combination have different

biochemical targets on the malarial parasite. However, the cleavage of the endoperoxide moiety by haem iron species results in free radical generation which includes reactive oxygen species (ROS) (Meshnik, 1994). These species induce oxidative stress in parasitic cells ultimately resulting in damage to their cell membranes (Little *et al.,* 2009) and leakage of cell contents. In addition to forming the toxic metabolite desmethylamodiaquine (Krishna and White, 1996) amodiaquine also works in a similar manner to its homologues, quinine and chloroquine by forming a complex with blood components that damage the cells of the parasite and probably that of the host (Slater, 1993) resulting in haemolysis.

In as much as there is selective distribution of the artemisinin derivatives such as artesunate to plasmodium infected erythrocytes, these drugs are also distributed to the liver, plasma and central nervous system and once here may become oxidized to reactive oxygen molecules that induce oxidative damage (Efferth *et al.,* 2010; Obianime and Aprioku, 2011). Furthermore, artemisinin derivatives such as artesunate are metabolized by the cytochrome P450 isoenzymes which then induce other enzymes notably ortho xanthine oxidase and superoxide dismutase (Robert *et al.,* 2000). The latter enzyme causes mobilization of iron from components of blood which subsequently cleaves the active pharmacophore of the artemisinin compound thereby generating reactive oxygen species which damage biological cells and tissues in the liver resulting in the leakage of cell contents including AST and ALT. This may account for the elevation in the hepatospecific biomarkers as well as the observations seen on histological examination of the rat liver sections.

This is the first study that compares the bio protective effects of siliphos (standard hepatoprotective) and the AQ-ME leaf extract of *M. oleifera* against AS-AQ induced liver injury. The levels of the hepatospecific biomarkers (AST, ALT, TB) obtained in the animal groups pre-treated with Moringa were nearer to the control values than the values obtained in the siliphos pre-treated groups. This could be because the dose of extract selected (1000mg/kg) may have higher composition of antioxidant compounds than the dose of siliphos used (200mg/kg).

Pre-treatment of the animals with AQ-ME leaf extract of *M. oleifera* at 1000mg/kg and siliphos (200mg/kg) orally in different groups prevented the adverse effects of artesunate and amodiaquine on the biochemical parameters (AST, ALT, TB). Moreover, the effect of lowering serum liver enzymes by *M. oleifera* was comparable and even better in certain groups than that of siliphos. This could possibly be explained by the work of other researchers (Tanaka *et al.,* 1997; Obianime and Aprioku 2011) who reported that the use of two or more antioxidants concurrently creates additional or synergistic activity.

The presence of significant amounts of phenolics, flavonoids and ascorbic acid strongly suggests that there may be a synergistic effect being produced in the tested extract of *Moringa oleifera*. Moreover, the effect produced by the occurrence of these phytochemicals together appears to be as efficacious as the effect produced by the standard hepatoprotectant (Siliphos ®). However, the effect produced on concurrent administration of artesunate amodiaquine antimalarial combination and the aqueous-methanol leaf extract of *M. oleifera* in the presence of malarial parasites has not been determined. Previous authors (Gbeassor *et al.,* 1990; Olsen, 1987) have reported on the effectiveness of *M. oleifera* seeds on *Plasmodium falciparum* and *Schistosoma*

cercariae, however, similar activity has not been reported on the leaves. Other authors have suggested that antioxidants and supplements of herbal origin (grape fruit juice, orange fruit juice, ascorbic acid) alter the efficacy of antimalarial drugs in clearing parasitaemia (Talman *et al.,* 2004; Bledsoe, 2005; Owira and Ojewole, 2010).

5.2 CONCLUSIONS

Based on the findings of these studies, the following conclusions were made;

1. Solvent variation has a significant influence on both the yield of extract and the phytochemical composition of the leaves of *Moringa oleifera.*

2. The aqueous-methanol leaf extract of *Moringa oleifera* has better antioxidant properties than the aqueous leaf extract of *Moringa oleifera*

3. The aqueous-methanol *Moringa oleifera* leaf extract has low toxicity on oral administration.

4. A 1000mg/kg dose of the aqueous-methanol *Moringa oleifera* leaf extract was effective in mitigating the effects of high doses of artesunate-amodiaquine antimalarial combination.

5.3 RECOMMENDATIONS

Based on the findings of the present study, the following recommendations may be drawn;

1) A mixture of water and methanol in a 20:80 volume by volume (v/v) ratio is more effective in extracting antioxidant compounds than water used on its own. Thus, this co-solvent system may be considered in future studies involving extraction of antioxidant substances from leaves.
2) There is a need to develop a single assay method that can comprehensively analyse the diverse antioxidant nature of medicinal plants.
3) Studies to generate data on the effect of sub-acute and chronic exposure of experimental animals to the aqueous-methanol *Moringa oleifera* leaf extract are needed.
4) Studies of the pharmacological effect of a combination of Siliphos® and *Moringa oleifera* should be performed.
5) The exact mechanism of activity of *Moringa oleifera* as an antioxidant is unknown. Thus, there is a need for further work to determine its mechanism of activity with an aim of classifying it as either a primary or secondary antioxidant.
6) The effect of concurrent administration of the aqueous-methanol *Moringa oleifera* leaf extract and artesunate-amodiaquine antimalarial combination in the presence of malarial parasites is unknown. Thus, there is a need for studies aimed at evaluating the effect of concurrent administration of this medicinal plant and artesunate amodiaquine antimalarial in the presence of malaria.

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ANNEXES

Annex 1; Ethical approval form

UNIVERSITY OF NAIROBI

FACULTY OF VETERINARY MEDICINE

DEPARTMENT OF VETERINARY ANATOMY AND PHYSIOLOGY

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Dr Okumu Mitchel Otieno
c/o Dept. of Public Heath, Pharmacology & Toxicology

20/01/2016

Dear Dr Okumu,

RE: Approval of Proposal by Biosafety, Animal use and Ethics committee

Prophylactic efficacy of Moringa oleifera leaf extracts against liver injury induced by artesunate-amodiaquine antimalarial combination
By Okumu Mitchel Otieno J56/76385/2014

We refer to our earlier communication to you of 10/12/2015 and the revised proposal that you submitted to this committee. We have noted that you have justified the types of experiments, number of animals to be used and that the animals will be treated humanely. Futhermore, we have noted the changes made in the proposal regarding occupational health and safety.

We however require that any animal treatments including anaesthesia and euthanasia, be done under the supervision of a qualified Veterinary Surgeon.

We hereby approve your work as per your revised proposal.

Rodi O. Ojoo BVM, M.Sc., Ph.D
Chairman
Biosafety, Animal Use and Ethics Committee
Faculty of Veterinary Medicine.

Annex 2; Output summary of the antioxidant capacity of *Moringa oleifera* leaf extracts

Anova: Two-Factor with replication

SUMMARY	TPC	TFC	VIT C	Total
AQ				
Count	3	3	3	9
Sum	106.2	236.1	6.1	348.4
Average	35.4	78.7	2.0	38.7
Variance	33.6	169.0	0.4	1159.4
AQME				
Count	3	3	3	9
Sum	156.1	1097.0	9.1	1262.2
Average	52.0	365.7	3.0	140.2
Variance	9.9	7560.3	4.2	30926.3
Total				
Count	6	6	6	
Sum	262.3	1333.1	15.2	
Average	43.7	222.2	2.5	
Variance	100.4	27796.7	2.2	

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Sample	46397.7	1.0	46397.7	35.8	0.000	4.7
Treatments	163586.8	2.0	81793.4	63.1	0.000	3.9
Interaction	77543.7	2.0	38771.8	29.9	0.000	3.9
Within	15555.1	12.0	1296.3			
Total	303083.2	17.0				

Annex 3; Output summary of the body weight of the rats used in the acute oral toxicity study

Two-sample t-test

Variates: Control, Treatment.

Summary

Sample	Size	Mean	Variance	Standard deviation	Standard error of mean
Control	5	5.97	1.67	1.292	0.578
Treatment	5	16.24	28.32	5.322	2.380

Difference of means: -10.276

Standard error of difference: 2.449

95% confidence interval for difference in means: (-16.80, -3.749)

Test of null hypothesis that mean of Control is equal to mean of Treatment

Test statistic t = -4.20 on approximately 4.47 d. f.
Probability = 0.011

Annex 4; Output summary of the liver to body weight ratio of the rats used in acute oral toxicity study

Two-sample t-test

Variates: Control, Treatment.

Summary

Sample	Size	Mean	Variance	Standard deviation	Standard error of mean
Control	5	0.07340	0.0005678	0.02383	0.010656
Treatment	5	0.04840	0.0001648	0.01284	0.005741

Difference of means: 0.0250

Standard error of difference: 0.0121

95% confidence interval for difference in means: (-0.002913, 0.05291)

Test of null hypothesis that mean of Control is equal to mean of Treatment

Test statistic t = 2.07 on 8 d. f.

Probability = 0.073

Annex 5; Output summary of serum liver parameters of rats used in the acute oral toxicity study of the aqueous-methanol leaf extract of Moringa oleifera

Analysis of variance

Variate: ALT

Source of variation	d. f.	s. s.	m. s.	v. r.	F pr.
Group	1	602.8	602.8	3.40	0.103
Residual	8	1420.3	177.5		
Total	9	2023.1			

Information summary

All terms orthogonal, none aliased.

Message: the following units have large residuals.

Group control *units* 3 24.7 s. e. 11.9

Tables of means

Variate: ALT

Grand mean 98.9

Group	control	Treatment
	106.7	91.2

Standard errors of differences of means

Table	Group
rep.	5
d. f.	8
s. e. d.	8.43

Analysis of variance

Variate: AST

Source of variation	d. f.	s. s.	m. s.	v. r.	F pr.
Group	1	654.48	654.48	10.14	0.013
Residual	8	516.24	64.53		
Total	9	1170.72			

Information summary

All terms orthogonal, none aliased.

Tables of means

Variate: AST

Grand mean 135.6

Group	control	Treatment
	127.5	143.6

Standard errors of differences of means

Table	Group
rep.	5
d. f.	8
s. e. d.	5.08

Least significant differences of means (5% level)

Table	Group
rep.	5
d. f.	8
l. s. d.	11.72

Analysis of variance

Variate: TB

Source of variation	d. f.	s. s.	m. s.	v. r.	F pr.
Group	1	1.459	1.459	0.97	0.353
Residual	8	12.031	1.504		
Total	9	13.490			

Information summary

All terms orthogonal, none aliased.

Tables of means

Variate: TB

Grand mean 2.63

Group	control	Treatment
	2.24	3.01

Standard errors of differences of means

Table	Group
rep.	5
d. f.	8
s. e. d.	0.776

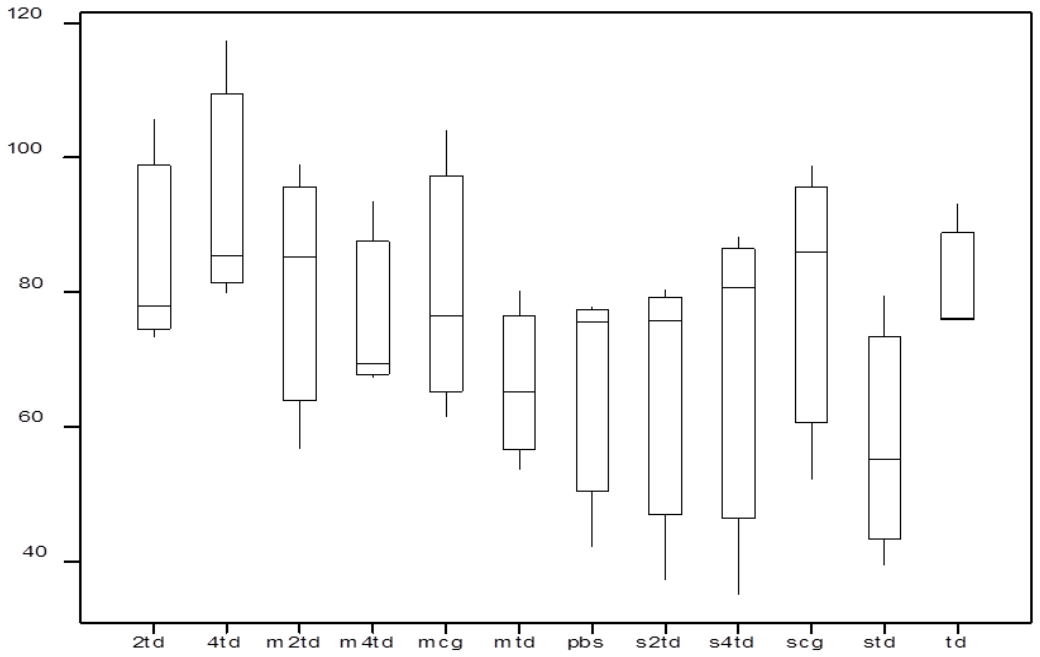
Least significant differences of means (5% level)

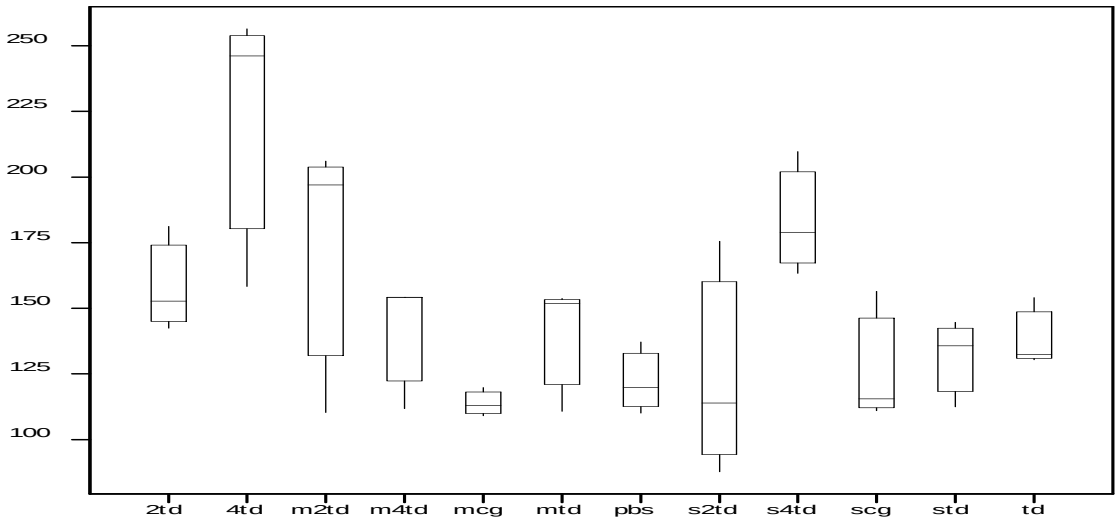
Table	Group
rep.	5
d. f.	8

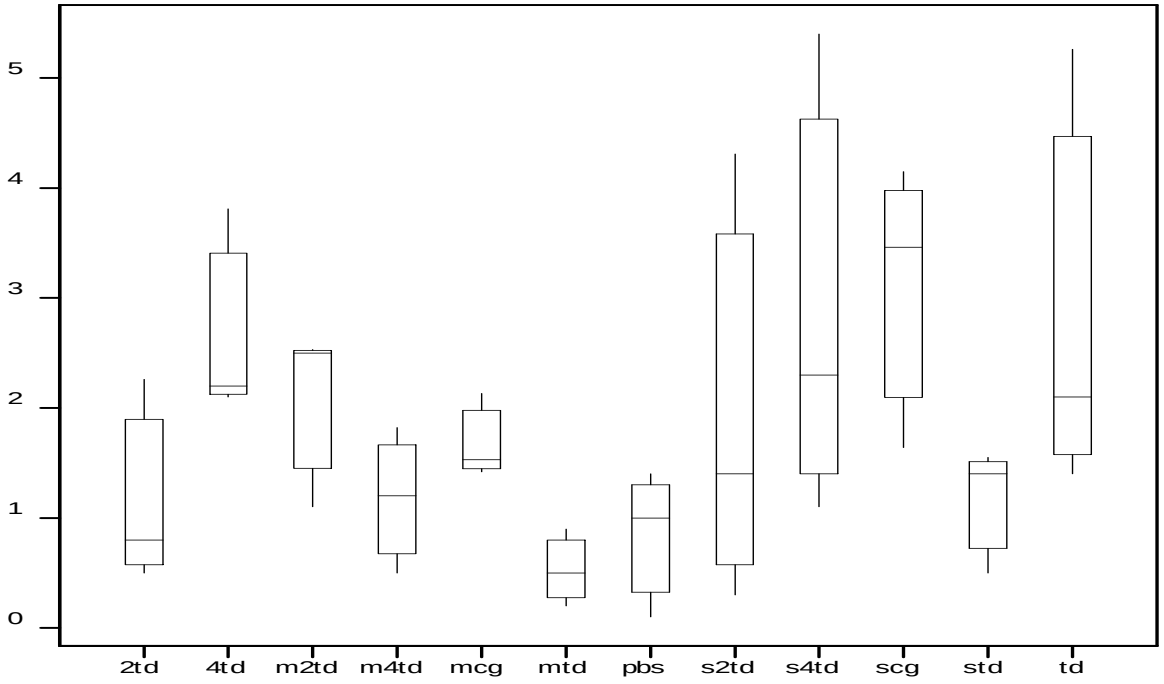
Annex 6; Descriptive statistics of the serum liver parameters used in the protective study of the AQ-ME MO leaf extract.

Box and whisker plot representation of the mean ALT values for the various treatment groups

Box and whisker plot representation of the mean ALT values for the various treatment groups







Box and whisker plot representation of the mean TB values for the various treatment groups

Annex 7; Output summary of the serum liver parameters of rats used in the protective study

Mean and standard deviation of the levels of alanine amino transferase (ALT) in the various treatment groups in the prophylactic study of the aqueous-methanol Moringa oleifera leaf extract

Group	Mean	s. d.
2td	85.71	17.55
4td	94.26	20.23
m2td	80.38	21.56
m4td	76.77	14.61
mcg	80.73	21.65
m t d	66.37	13.29
p b s	65.18	20.08
s2td	64.48	23.65
s4td	68.00	28.83
s cg	78.95	24.11
s td	58.01	20.16
td	81.67	9.90

Mean and standard deviation of the levels of aspartate amino transferase (AST) in the various treatment groups in the prophylactic study of the aqueous methanol *Moringa oleifera* leaf extract

Group	**Mean**	**s. d.**
2td	158.8	20.08
4td	220.3	53.97
m2td	171.1	52.88
m4td	140.0	24.45
mcg	113.9	5.46
m t d	138.8	24.33
p b s	122.4	13.73
s2td	125.7	45.13
s4td	184.0	23.60
s cg	127.7	25.07
s td	131.0	16.61
td	139.0	13.14

Mean and standard deviation of the levels of total bilirubin (TB) in the various treatment groups in the prophylactic study of the aqueous methanol *Moringa oleifera* leaf extract

Group	**Mean**	**s. d.**
2td	1.187	0.942
4td	2.703	0.960
m2td	2.043	0.817
m4td	1.173	0.660
mcg	1.693	0.382
m t d	0.533	0.351
p b s	0.833	0.666
s2td	2.003	2.072
s4td	2.933	2.219
s cg	3.083	1.297
s td	1.150	0.568
td	2.920	2.057

Analysis of variance

Variate: ALT

Source of variation	d. f.	s. s.	m. s.	v. r.	F pr.
Group	11	3689.9	335.4	0.82	0.623
Residual	24	9839.9	410.0		
Total	35	13529.7			

Information summary

All terms orthogonal, none aliased.

Tables of means

Variate: ALT

Grand mean 75.0

Group	2td	4td	m2td	m4td	mcg	m td	p b s
	85.7	94.3	80.4	76.8	80.7	66.4	65.2
Group	s2td	s4td	s cg	s td	t d		
	64.5	68.0	78.9	58.0	81.7		

Standard errors of differences of means

Table	Group
rep.	3
d. f.	24
s. e. d.	16.53

Analysis of variance

Variate: AST

Source of variation	d. f.	s. s.	m. s.	v. r.	F pr.
Group	11	31277.8	2843.4	3.05	0.011
Residual	24	22382.1	932.6		
Total	35	53659.9			

Information summary

All terms orthogonal, none aliased.

Tables of means

Variate: AST

Grand mean 147.7

Group	2td	4td	m2td	m4td	mcg	m td	p b s
	158.8	220.3	171.1	140.0	113.9	138.8	122.4

Group	s2td	s4td	s cg	s td	t d
	125.7	184.0	127.7	131.0	139.0

Standard errors of differences of means

Table Group

rep. 3

d. f. 24

s. e. d. 24.93

Analysis of variance

Variate: TB

Source of variation	d. f.	s. s.	m. s.	v. r.	F pr.
Group	11	26.424	2.402	1.51	0.192
Residual	24	38.147	1.589		
Total	35	64.571			

Information summary

All terms orthogonal, none aliased.

Tables of means

Variate: TB

Grand mean 1.85

Group	2td	4td	m2td	m4td	mcg	m td	p b s
	1.19	2.70	2.04	1.17	1.69	0.53	0.83

Group	s2td	s4td	s cg	s td	t d
	2.00	2.93	3.08	1.15	2.92

Standard errors of differences of means

Table Group

rep. 3

d. f. 24

s. e. d. 1.029