HISTOMORPHOLOGICAL EFFECTS OF WARBURGIA UGANDENSIS METHANOLIC EXTRACT ON ATHEROSCLEROTIC LESIONS IN AORTIC TUNICA INTIMA OF NEWZEALAND RABBITS UPON INDUCTION OF ATHEROSCLEROSIS

BY

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DECLARATION

This research project is my original work and has not been presented for award of a degree in any other University. I wish to declare that works from other authors used in the development of this thesis has been duly acknowledged in the reference section.

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DEDICATION

I dedicate this thesis to my wife Monica, and My children Bellamy Jabali, Brielle Adoyo and Brion Hawi for their prayers, support and encouragement.

ABSTRACT

Atherosclerosis is one of the leading causes of vascular disease worldwide. Its clinical manifestation includes ischemic heart disease, peripheral arterial disease and ischemic stroke. It affects aortic tunica intima manifested by endothelial damage, oxidative stress, inflammation and finally formation of a plaque which eventually harden and narrows the arteries. *Warburgia ugandensis* a common indigenous plant in East and Central Africa that has been used in treatment of various illnesses and it has been noted to have potential anti-atherosclerotic properties such as anti-inflammatory and anti-oxidant. There is scarcity of information and data in regards to histomorphological effects of *W. Ugandensis* on atherosclerotic lesions in aortic tunica intima. The current study therefore evaluated the histomorphological effects of *Warburgia ugandensis* on aortic tunica intima of New Zealand rabbits upon induction of atherosclerosis. Specifically, the study determined acute oral toxicity of *W. ugandensis* histoinhibitory effects and finally historestorative effects of *W. ugandensis* on atherosclerotic lesion. In this experimental study, 30 Male pure breed New Zealand rabbits (n=30) weighing 1.8-2kg aged 8-12 weeks sourced from University of Nairobi biology animal house were used as study models. The rabbits were divided into the following study groups: 12 for control ,6 for the experimental and 12 to determine acute oral toxicity. The experimental group was further divided into 2 sub-groups; 3 for histo-inhibitory group, 3 historestorative with *W. ugandensis.* Induction of atherosclerosis was done using high fat diet for 7 weeks, Later the rabbits were euthanized, tissues harvested and analyzed. One-way Analysis of Variance with post hoc Bonferroni test for continuous data was used to determine difference among and between groups. Flavonoids, Phyto-steroids, tannins, phenols, saponins, alkaloids and anthraquinones were present in methanolic extract while cardiac glycosides were absent. A dose of < 5000mg/kg of *W. ugandensis* did not have toxic effects. mean fraction of vehicle control significantly increased as compared to negative control group ((p=0.0001). The mean area fraction of histo-inhibitory and historestorative groups significantly reduced as compared to vehicle control group (0.49057,0.37335versus 0.52701). On histological findings; control group, there was a normal tunica intima, well distributed endothelial cells. Vehicle control group; had a fibro-atheroma. On histo-inhibitory groups; had a fatty streak while historestorative groups; had a pre-atheroma with lipid pools. The study concludes that flavonoids, tannins, phenols and Phyto-steroids are present in methanolic bark extract of *W. ugandensis* and are useful antioxidant and anti-inflammatory components. Safe dose of *W. ugandensis* in animal study is < 5000mg/kg. W. *ugandensis* has both histo-inhibitory and historestorative benefits on atherosclerosis. The research therefore, recommends that, quantitative and potency of phytochemical analysis should be carried out to determine the most active molecule in management of atherosclerosis. W. *ugandensis* bark extract, dose of <5000mg/kg is safe in management of atherosclerosis. Further studies to be conducted to ascertain the drug interactions and effects of W. *ugandensis* lipid profiles.

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LIST OF ABBREVIATIONS

EDTA	Ethylenediaminetetraacetic acid
EGL	Endothelial glycocalyx
ENOS	Endothelial Nitric Oxide Synthase
HMG CoA	Hydroxyl B-methylglutaryl coenzyme A
JKUAT	Jomo Kenyatta University Agriculture and Technology
LDLC	low density lipoprotein cholesterol
LDL	low density lipoprotein
MMP	Matrix Metalloproteinase
NO	Nitric Oxide
NOs	Nitric Oxide synthase
OXLDL	Oxidized Low density lipoprotein
SMCs	Smooth muscles cells
VLDL	Very low-density lipoprotein
VCAM	Vascular cell adhesion molecule

DEFINATIONS TERMS

ATHEROSCLEROSIS : Chronic inflammatory disease triggered by the sub endothelial accumulation of lipids that elicit a maladaptive, unremitting immune response

ANTIOXIDANT **:** Substance that protects cells from the damage caused by free radicals

ANTINFLAMMATORY: To reduce inflammation

HISTOMORPHOLOGY: Use of histology to study the morphology of cells and tissues

ISCHAEMIC HEART DISEASE : Term given to heart problems caused by narrowed heart arteries

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CHAPTER ONE

INTRODUCTION

1.1 Background of the Study

Atherosclerosis is characterized by accumulation of lipids within the sub endothelial layer usually maladaptive and unremitting immune response (Fernandez & Giannarelli, 2022) . It is a major cause of vascular disease worldwide, manifesting as peripheral arterial disease, ischemic heart disease and stroke (Barquera *et al*., 2015). According to Arun (2006), the primary risk factors for atherosclerosis are smoking, foreign substances, obesity, diabetes, hypertension, hyperlipidemia, bacterial and viral infections. The diagnosis is mainly dependent on imaging modalities such as MRI, CT angiograms, Cardiac Angiograms, Ultrasound and magnetic angiography. Lipid profile, cardio ankle vascular index and brachial pressure index are considered key physiological investigations (Teramoto *et al*., 1992). Lifestyle modification and pharmaceutical intervention with statins have been used widely in the management of this condition.

Warburgia ugandensis is an indigenous plant, commonly referred to as East African green-heart. local names Muthiga (Kikuyu), Moissot (Kipsigis), Sogo-maitha, Ol-sogunoi (Maa-Kenya), Muhiya (Haya-Tanzania), Olmsogoni (Maa- Tanzania), Msokonoi (Rangi-Tanzania), Mukuzanume/Muwiya (Luganda) (Dhar *et al.*, 2014). It's an evergreen tree at 25 m tall with a dense, rounded canopy. Bark rough, black-brown, fissured in rectangular scales. Leaves glossy, dark green above, up to 10 cm long. Flowers inconspicuous, greenish to cream-colored. Fruit round to ovoid, hard, 3 to 5 cm long, green, turns black-violet when ripe. All parts of the tree have a sharp pepper flavor. Widespread in low montane rainforests and drier upland forest areas. Also found in riverine forests and Acacia xanthophloea forests at altitudes of 1,000 to 2,000M. It is used as Shade, toothbrush, fruits are edible, Ornamental, Mulch, firewood, herbal medicine, Resin and Insecticide among other uses(Dhar *et al*., 2014).

This plant has a long history of extensive use in the treatment of a variety of human diseases in many African countries. In terms of medicinal value, it ranks second among the highly prioritized medicinal plants in Kenya (Chung *et al*., 2019). Studies have shown that *W. Ugandensis* extracts are effective against a range of pathogens that directly cause or are closely related to certain human diseases, including malaria, diarrhea, asthma, skin infections, opportunistic HIV infections, measles, candidiasis, cytotoxic, and pneumonia (Erizon & Karani, 2020; Maroyi, 2014; Okello *et al*., 2018).

The incidence of Ischemic heart Disease caused by atherosclerosis is rising in sub-Saharan Africa region (SSA) as a result of epidemiological in addition to demographic changes (Nkoke & Luchuo, 2016). Currently, the disease represents a major cause of morbidity and mortality in SSA (Ebireri *et al*., 2016; Ikem & Sumpio, 2011). Studies done by (Ogeng'o *et al*., 2010) revealed that in Kenya, acute myocardial infarction was the leading cause of cardiovascular deaths and the second most common cause of heart failure (Bloomfield *et al*., 2016). There are fears of an imminent epidemic of coronary heart disease (Dhar *et al.*, 2014.) unless conscious steps are taken to mitigate the impending surge (Önen *et al*., 2013).

Observations by (Kronzon & Tunick, 2006) demonstrated that aortic atherosclerosis was commonly associated with coronary artery disease and discovery of aortic atherosclerosis was projection of presence and severity of coronary artery disease. The epidemiology of ischemic heart disease (IHD) in sub-Saharan Africa (SSA) remains largely enigmatic and some of the Major obstacles to our understanding of the condition include inadequate diagnostic capabilities, shortage of physicians and cardiologists, and misguided opinions in terms of management and prevention (Önen *et al*., 2013).

Histologically atherosclerosis, is typified by accumulation of oxidized low density lipoprotein cholesterol (OxLDL-C) in the vascular tunica intima which follows endothelial dysfunction (Alfarisi *et al*., 2020). Through a variety of methods, antioxidants, both natural and artificial,

are essential for the prevention and treatment of atherosclerosis. These include: preventing the formation of atherosclerotic plaque and platelet aggregation; inhibiting the oxidation of low-density lipoprotein (LDL); reducing the production of reactive oxygen species (ROS); inhibiting the secretion of cytokines; preventing mononuclear cell infiltration; improving endothelial dysfunction and vasodilation; raising the bioavailability of nitric oxide (NO); and controlling and suppressing the expression of adhesion molecules on endothelial cells causing foam cell formation (Malekmohammad *et al*., 2019). A recent study by (Zhuang *et al*., 2019) revealed that *W. ugandensis*, an indigenous plant in Africa has more antioxidant and anti-inflammatory effects thus the current study postulates that it has anti-atherosclerotic properties making anatomical research on its histomorphological effects on atherosclerotic lesions worthwhile. Therefore, there was need to establish the phytochemicals present in *W ugandensis*, acute oral toxicity of *W ugandensis*, historestorative and finally histo-inhibitory action on the atherosclerotic lesions on aortic tunica intima upon administration *W ugandensis*.

1.2 Statement of the Problem

Atherosclerosis is the most common cause of IHD. Even though IHD is still not very common in SSA, its prevalence is expected to increase over the next 20 years due to an increase in risk factors, primarily diabetes, hypertension, obesity, overweight, and physical inactivity, as well as an increase in tobacco use and dyslipidemia. It was estimated that age- standardized mortality rates for IHD due to atherosclerosis was to rise by 27% in African men and 25% in women by 2015, and by 70 and 74%, respectively by 2030 (Önen *et al*., 2013).

Studies have been done on *W. ugandensis* extract in regards to treatment of respiratory conditions such as asthma, diarrhea, common cold, stomachache, malaria and toothache among other conditions. However, there is deficiency of information on anatomical histomorphological effects of this plant on atherosclerotic lesion on aortic tunica intima despite

the plant having potential antioxidant and anti-inflammatory effects, resulting in maintenance of aortic tunica intima homeostasis. Furthermore, data on its safety profiles is very crucial and may be helpful in patient management however, this information remains largely unknown. More of its phytochemicals are still yet to be discovered as well. A breakthrough in positive effects of this plant on atherosclerotic lesions will help reduce morbidity and mortality which stand at 11,972 in Kenya (Mortensen & Nordestgaard, 2020). Therefore, this study sought to bridge the gap on knowledge of anatomical histomorphological effects of *W. ugandensis* on aortic tunica intima protection against atherosclerosis lesion and the safety levels of the plant.

1.3 Justification

According to Barquera *et al.*(2015),the global cost of cardiovascular disease (CVD) was projected to be US$863 billion in 2010. Bloom *et al.* (2011) on the other hand noted the amount is expected to rise to US$1044billion by 2030. Atherosclerotic cardiovascular disease is a major cause of morbidity and mortality worldwide. The risk is higher among low-income families, the uninsured, and non-elderly individuals who lack access to universal healthcare through Medicare (Khera *et al*., 2020). As noted by Ramkumar *et al*. (2016), statins, which are the drugs of choice in the management of atherosclerosis, are used with caution due to their severe adverse effects, such as liver injury, muscle toxicity, and numerous drug interactions. These risks limit their use in patients with co-morbidities. Research on the anatomical, histoqualitative, and stereological effects of *W. ugandensis* methanolic bark extract in the treatment of atherosclerosis is currently insufficient. Therefore, the purpose of this study is to investigate the effects of *W. ugandensis* on atherosclerosis management, its mode of action, and its efficacy, with the aim of producing data that could support its use. The results will be shared with healthcare workers to provide useful information to the community about the medicinal value of *W. ugandensis* in the management of atherosclerosis. Additionally, the data

will be used to explore the potential development of patented medicine for the treatment of atherosclerosis, thereby contributing to the reduction of morbidity and mortality and the overall improvement of healthcare.

1.4 Research Objectives

1.4.1 Main objective

To evaluate the histomorphological effects of *Warbugia ugandensis* methanolic bark extract on aortic tunica intima of New Zealand rabbits upon induction of atherosclerosis.

1.4.2 Specific Objectives

I. To determine qualitative phytochemicals, present in crude bark extract of *W. ugandensis.*
II. To ascertain acute oral toxicity of *W. ugandensis* on white New Zealand rabbits.
III. To assess histo-inhibitory effects of *W. ugandensis* on an atherosclerotic lesion of white New Zealand rabbits.
IV. To find out historestorative effects of *W. ugandensis* on atherosclerotic lesion of white New Zealand rabbits.

1.5 Research Hypotheses

1.5.1 Null hypotheses (H_O)

i. *Warbugia ugandensis* has no histomorphological effects on aortic tunica intima of New Zealand rabbits upon induction of atherosclerosis.

1.6 Limitation and delimitation of the Study

The study limitations included failure of rats to attain desired weight at prescribed time, sickness or death of animals along experimental process due to the process of drug administration which may not have been tolerated by animals. These animals were replaced immediately. In order to reach the needed weight, the animals that would not have acquired it by the time of the experiment were fed individually.

CHAPTER TWO

LITERATURE REVIEW

2.1 What is atherosclerosis

Atherosclerosis is a chronic inflammatory vascular wall disease which has been known to target tunica intima, the innermost histological layer of blood vessels. This condition is featured by accumulation of oxidized low-density Lipid-C in macrophages located within tunica intima. Moreover, it presents with migration and proliferation of smooth muscle cells into tunica intima thus interfering with histomorphological organization of the aorta *(*Marchio *et al*., 2019).

2.2 Atherosclerosis and the aortic tunica intima

Atherosclerosis is characterized by damage of endothelial cells of aortic tunica intima. The LDL passes through endothelial cells by transcytosis. It then enters the body cell by receptor mediated endocytosis to be used in normal cell processes. The progression of atherosclerosis begins when endothelial cell of arterial wall becomes damaged exposing tunica intima, caused by hypertension, smoking, hyperglycemia and hypercholesterolemia. Endothelial damage increases permeability of arterial wall allowing LDL to enter the tunica intima. White blood cell such as monocyte usually moves freely in the blood vessel and do not attach to the endothelial cells, however when endothelial cells are exposed to irritating stimuli or damage, they express adhesion molecules that can capture nearby white blood cells (Alfarisi *et al*., 2020). The white blood cells undergo morphological changes that allow them to flatten and squeeze in between the endothelial cells a process called diapedeses.

White blood cells are capable of producing free radicals and when this free radical comes into contact with LDL oxidation occurs. Oxidized LDL, results in oxidative stress, inflammation and injury in the endothelium (Lian *et al*., 2018). OxLDL particles are effective in tracking and activating white blood cells. White blood cells engulf the OxLDL which stimulates to produce even more free oxygen radicals leading to accumulation and a positive feedback situation begin

to arise, bringing in even more immune cells. Macrophages in the tunica intima start to engulf free oxygen particles leading to production of a form cell which is saturated by lipid particles *(*Di Meo *et al*., 2016).

The foam cells will eventually die releasing its contents attracting more macrophages. Accumulation of dead cell leads to formation of plaque. Endothelial cells usually cover plaque and pilling up of calcium salts will lead to hardening of arteries. Nitric oxide (NO) and Nitric Oxide Synthase (NOS) system in vascular SMCs are important controls of local vascular injury associated with atherosclerosis. In normal blood vessels, NO is produced from endothelium by endothelial isoform of NOS (Adamova *et al*., 2017) and functions as an endothelium-derived relaxing factor critical for maintaining vascular homeostasis (Di Meo *et al*., 2016).

The vascular endothelium is a main regulator of vessel wall homeostasis through the production of vasoactive regulatory substances including nitric oxide. Studies indicate that endothelial dysfunction and chronic inflammation with high plasma LDL-C are key contributors to the formation, growth and rupture of atherosclerotic plaques (Marchio *et al*., 2019). This combination together with disturbed shear stress within the blood vessel leads to the activation of platelets and endothelium and thus to monocyte adhesion with eventual diapedesis into the tunica intima blood vessel (Förstermann *et al*., 2017; Pong & Huang, 2015; Shah & Lecis, 2019). Within the tunica intima, monocytes differentiate into pro-inflammatory macrophages and increase their affinity for oxidized LDL-C which later change into foam cells as they accumulate oxidized LDL-C in their cytoplasm (Malekmohammad *et al*., 2019).

The foam cells further drive the inflammatory process by producing inflammatory cytokines and growth factors, as well as the recruitment and migration of smooth muscle cells (SMCs) into the tunica intima, which also differentiate into foam cells (Bäck *et al*., 2019; Fernández-Friera *et al*., 2017; Herrington *et al*., 2016). The development of atherosclerotic lesions in the tunica intima proceeds through several stages, including initial fatty streak development, early

fibro atheroma, progressive atheroma, and advanced lesion development, with each stage representing a more advanced change in tunica intima. According to (Shibata *et al*., 2017) during early fatty streak lesions, the hallmark is intimal thickening and formation of form cells. Early atherosclerotic lesions follow endothelial dysfunction characterized by an imbalance between endothelial-dependent vasodilation and vasoconstriction, leading to accumulation of LDL in intimal macrophages that form fatty streaks. (Insull Jr, 2009) adds that LDL becomes oxidized or modified, further increasing the accumulation of pro-inflammatory molecules. According to (Mahmoudi, 2018), vasodilation is mainly mediated by nitric oxide, in addition to prostaglandin I2, bradykinin and endothelial hyperpolarizing factors.

The study further notes that in addition to its vasodilator properties, nitric oxide has been shown to have antiatherosclerotic properties by mediating inhibition of leukocyte adhesion, vascular smooth muscle cell proliferation, and inhibition of platelet aggregation. The results of (Malekmohammad *et al*., 2019) indicate that in chronic inflammatory conditions there is an increased production of superoxide species, which interact with nitric oxide to produce peroxynitrite, which decreases the bioavailability of nitric oxide.

Studies by (Mahmoudi, 2018) also show that decreased nitric oxide bioavailability causes expression of vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 and P selectins, which mark cell surface receptors for platelets, monocytes and T-lymphocytes beginning of the early phase of atherosclerotic lesion formation. Studies by (Di Meo *et al*., 2016; Ference et al., 2017) have also shown that platelets attach to endothelial cells via glycoprotein Ia and IIb/IIIa receptors, leading to further activation of the endothelium, resulting in a further attachment of monocytes and migration of vascular smooth muscle cells to the tunica intima are sustained. These monocytes in the sub endothelial space transform into macrophages, which also release other chemotactic factors, including interleukin-1, tumor necrosis factor alpha, transforming growth factor beta, proteolytic enzymes, and insulin-like

growth factor-1.

All of these factors play significant roles in maintaining a chemical gradient for cellular chemotaxis. Macrophages within the sub endothelial space express scavenger receptors for oxidized or modified LDL, causing oxidized LDL to accumulate and convert into foam cells. Other cells involved in the early phase of atherosclerosis pathogenesis include the T lymphocytes, whose entry into the tunica intima is mediated by vascular cell adhesion molecule 1. T lymphocyte activation occurs by oxidized LDL and once in the intima they further increase levels of inflammatory cytokines thus increasing the inflammatory milieu in the sub endothelial space. For example, the interaction of the CD40/CD40L receptor between macrophages and activated T lymphocytes upregulates production of tissue factor and matrix metalloproteinase, and according to (Insull Jr, 2009) the atherosclerotic changes during this early developmental phase of the fatty streak are reversible.

2.3 Histology of the aortic wall

The aortic wall comprises of three major layers namely; tunica intima, tunica media and tunica adventitia with the primary type of cells being smooth muscle cells, endothelial cells, and adventitial fibroblasts. Histology wise the integrity of the vessel wall is majorly dependent on the interaction between the three cells in terms of mechanical adaptation, collagen production and chemical stimuli as well as recovery from the damage. The entire process is linked to the extracellular matrix interactions which in general contribute to the mechanochemical integrity of the aortic wall.

2.3.1 Tunica intima

The tunica intima is made up of endothelial cells and sub endothelium is the innermost layer of aorta. The intima consists of a lining layer of longitudinally oriented endothelial cells covering sub endothelial layer of thin connective tissue with sparse cell matrix and inner elastic

membrane (Milutinović *et al*., 2020). The endothelium consists of simple squamous epithelial lining with the luminal feature being covered by the endothelial glycocalyx (Adamova *et al.*, 2017) bioactive substances which is entirely comprised of proteoglycans, glycosaminoglycan and cell adhesion molecules. The EGL modulate vascular tone, leukocyte adhesion and prevent platelet aggregation. It also prevents harmful substances from entering the arterial wall and act as a selective diffusion barrier between the blood and the other wall layers. Studies have shown that EGL is a crucial component in the pathophysiology of cardiovascular disease especially atherosclerosis in whose thickness has been demonstrated to increase at arterial bifurcation points in rat arteries.

Just Like any epithelium, endothelial cells of aorta likewise sit on basement membrane which offers support in addition to production of cytokines that influence cell multiplication, adhesion, migration and differentiation. The main collagen type in basement membrane is type IV collagen and laminin with trace amounts of type VIII collagen, entactin and sulfated proteoglycans. Other collagens found in basement membrane include type XV, which supports cells on the membrane and type XVIII which hinders angiogenesis and endothelial cell migration. Opposed to other elastic arteries, the intima of the aorta has a thin layer of fibrillar collagens, mainly type I, III, V collagen and occasionally vascular smooth muscle cells found between the basement membrane and the inner elastic lamina. This layer is the sub endothelium. The intimal layer is extremely thrombogenic layer due to the presence of collagen types I, III and VI which recruit and activate platelets and thus platelet plug formation with resultant occlusion of the vessel lumen.

2.3.2 Internal elastic lamina

This is the tissue interface between tunica intima and tunica media. It consists of fenestrated leaves and plays a crucial role in mechanical and transport properties of both the tunica intima and tunica media. The windows serve as transport units for water transport.

2.3.3 Tunica media

The tunica media, or middle coat, is made up principally of smooth (involuntary) muscle cells and elastic fibers arranged in roughly spiral layers. In addition to supporting the vessel, it modifies its diameter to control blood pressure and flow.

2.3.4 Tunica adventitia

Adventitial layer of the aorta is majorly composed of collagen and some elastin fibers with occasional fibroblasts. The collagen fibers shown an axial orientation and are organized into bundles or individual collagen fibrils connected to the bundles. Apart from offering structural support to the blood vessel, the adventitial layer is highly recognized to participate in inflammation and regulation of vascular homeostasis.

2.4 Management of Atherosclerosis

Statins, such as rosuvastatin, atorvastatin, fluvastatin, pravastatin, and lovastatin, are the preferred medications for managing atherosclerosis (Ramakumar., *et al* 2016). Among these, rosuvastatin is the most effective, followed by atorvastatin. However, statins are known to interact with various drugs, including macrolide antimicrobials and immunosuppressant like cyclosporine and tacrolimus, which inhibit CYP3A4 and OATPIBI, leading to increased plasma concentrations of statins. Protease inhibitors can also interact with statins, causing muscle toxicity, while azoles and calcium channel blockers can increase their toxicity, and antacids can reduce their plasma concentrations (Bansal, A. B., *et al* 2019, Ramkumar., *et al* 2016).

The adverse effects of statins include myalgias, rhabdomyolysis, acute kidney injury, hepatic and renal dysfunction, aggression, diabetes mellitus, neuropathies, cataracts, urinary tract infections, and others. However, these drugs are contraindicated during pregnancy due to teratogenicity, acute liver failure, and other reasons (Bansal, A. B., *et al* 2019).

Atorvastatin is a synthetic inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase. HMG-CoA is an intermediate molecule in the synthesis of cholesterol from acetyl-CoA and acetoacetyl-CoA by HMG-CoA synthase and according to (Sirtori *et al*., 2014) HMG-CoA is normally converted to mevalonic acid by HMG-CoA reductase. a rate-limiting step in cholesterol biosynthesis.

2.4.1 Mode of action of atorvastatin in atherosclerotic cardiovascular disease

Inhibition of HMG-CoA reductase

Atorvastatin is classified as statin and it is used in lowering blood cholesterol levels besides stabilizing plaque and inhibiting strokes through anti-inflammatory among other mechanism (Mathew *et al*., 2014; Sorrentino, 2011). According to (Ferri & Corsini, 2020) inhibition of HMG-CoA reductase leads to reduced intracellular cholesterol levels and thus upregulation of LDL receptors, which in turn leads to enhanced removal of LDL-cholesterol from plasma.

Inhibition of HMG-CoA reductase also causes inhibition of hepatic synthesis of very low-density lipoprotein cholesterol (VLDL), which is a progenitor to Low Density Lipoprotein-cholesterol, and according to Hyatt (1997), in addition to lowering plasma LDL, atorvastatin indirectly lowers the Plasma triglyceride levels, and therefore cholesterol levels, are required for the synthesis of VLDL, which is necessary for triglyceride transport, therefore reduced cholesterol synthesis causes reduced assembly and secretion of VLDL, which directly effects triglyceride transport.

2.5 Reduction of vascular inflammation

Studies have shown that statins up regulate the production of NO, a significant vasodilator that has anti-inflammatory and antioxidant effects on the vessel wall. Vascular resistance, inflammation, and increased antioxidant levels play critical roles in atherogenesis. (Liberale *et al*., 2020) demonstrate that the mechanisms by which statins increase NO levels include:

upregulation of endothelial nitric oxide synthase (Adamova *et al.*, 2017)gene expression achieved through inhibition of Rho-geranyl-geranyl phosphorylation, thus increased the expression of the Kruppel-like factor, a transcription factor. NO levels are also increased by activating the P13-Akt protein kinase signaling pathway and stabilizing eNOS transcription. Statins down regulate the negative modulator of NO synthesis caveolin-1, leading to increases in plasma NO levels and eventual activation of ROS scavenging systems such as catalase, superoxide dismutase and thioredoxin reductase (Ferri & Corsini, 2020).

2.6 Description and characteristics of Warbugia ugandensis

Warbugia ugandensis is a tree native to East Africa, commonly referred to as Kenyan greenheart tree, Ugandan greenheart tree, East African greenheart tree, and pepper bark tree. It has a variety of uses (Orwa *et al.*, 2009) . The genus *Warbugia*, is a member of cinnamon family Canellaceae. It has been described as Africa's panacea. It is an evergreen tree that can grow up to 30 m tall and 70 cm in diameter.

It has a variant bark which is sometimes smooth or scaly, light green and a rounded crown. The leaves grow to 3-15 cm by 1.4-5 cm and appear alternately on the stems and have dotted glands on their surface with no stipules (Orwa *et al.*, 2009). The flowers are kidney-shaped and appear either solitary or in small 3-4- flowered cymes, with flowering occurring in the early part of the wet season, fruiting occurring in the late part of the wet season, and the fruit may remain on the tree for quite a long time. In Kenya, *W.* ugandensis flowers in December-January with sown in May.

W. ugandensis does well in lowland rainforests at altitudes of 100-2200m with a mean annual rainfall of 1000-1500 mm (Orwa *et al.*, 2009).

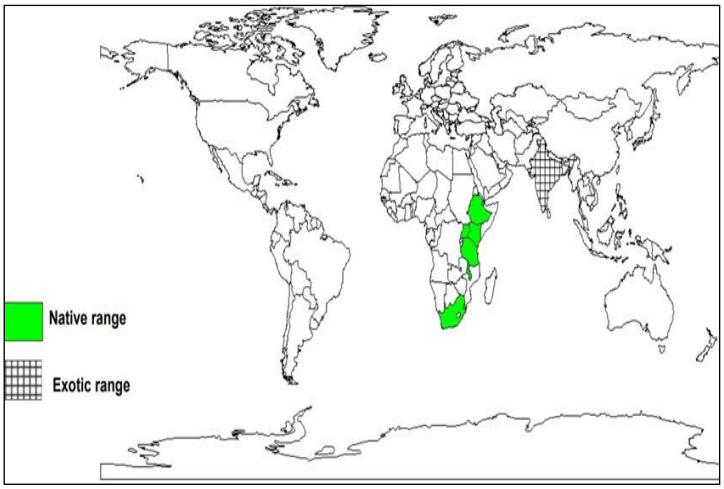

Figure 2. 1 : Species of *Warbugia ugandensis* distribution across Africa and other regions (Orwa *et al.*, 2009)

2.7 Phytochemicals Composition of Bark extract of W. ugandensis bark extract

These are natural products which are rich in chemical components produced by plants. These chemicals include; Tannins, flavonoids, triterpenes, steroids, alkaloids and saponins. They represent an important cache for the discovery of new drug compounds. Studies by (Khera *et al*., 2020) suggest that *W. ugandensis* has remarkable anti-inflammatory and antioxidant activities, particularly with discovery of a new aryl naphthalene lignan amide. Extracts of *W. ugandensis* have potent antioxidant and anti-inflammatory effects, mainly due to the presence of plenty terpenoids, drimane and coloratan type sesquiterpenoids, in addition to ugadensial, waburganal, mukaadial and other secondary metabolites such as tannins, flavonoids, saponins, steroids and mannitol (Chung *et al*., 2019) .

According to Maobe *et al*. (2012), W. *ugandensis* contains phytochemicals such as naringenin, flavanone, monin, kaempterol, and galangin, however many are still unknown. Furthermore Studies done by Otieno *et al*. (2016) revealed that W. *ugandensis* activity and phytochemicals is affected by plant portion examined, extraction solvents used and the site of collection. This calls for phytochemicals studies to be carried out before an experiment is done to determine the phytochemicals present.

2.8 Acute Oral Toxicity of the Methanolic Bark Extract of W. ugandensis

Studies done across the globe have shown that *W. ugandensis* has no toxicity. However, there is contradictory information about plant toxicity, (Mwitari *et al*., 2013) suggests that W. *ugandensis* was cytotoxic whereas (Anywar *et al*., 2021) indicated that its cellular assay was not toxic. Karani *et al*., (2013) reports that *W. ugandensis* at a dose of LD50 >5000 mg/kg showed no symptoms of toxicity or mortality. According to Ahmad *et al*. (2017), W. *ugandensis* was found to be non-toxic with acute exposure but exhibited toxicity with chronic exposure. No pharmacological interactions with other drugs components or plants have been documented and this calls for a general exploration of this plant in terms of toxicity. Due to this existing information the current study seeks to determine the safe doses of W. *ugandensis*.

2.9 The histo-inhibitory effects of *W. ugandensis* on an atherosclerotic lesion of white New Zealand rabbits.

Typically, a histo-inhibitory agent works to stop atherosclerosis from developing. This calls for the use of both protecting and harmful agents in combination. After then, the effect is assessed histologically. Atherosclerosis is initiated by sub endothelial injury that causes inflammation and infiltration of LDL which becomes oxidized and forms a fatty streak as initial stage. According to (Castro *et al*., 2009) studies reveal that oxygen reactive species contribute to atherogenesis and cardiovascular disease progression, while exposure to low-density lipoproteins and nucleic acids can lead to harmful oxidative changes. (Kollár & Hotolová, 2003) oxidative theory suggests that excessive LDL oxidation promotes atherosclerosis, as native LDL isn't pathogenic. Antioxidants reduce lesions formation, suggesting lipid oxidation plays a role in atherogenesis. Research conducted by Khera *et al*. (2020) indicates that *W. ugandensis* possesses exceptional anti-inflammatory and antioxidant properties, especially after the identification of a novel aryl naphthalene lignan amide.

In a study done by (Mandlik & Namdeo, 2021) on atherosclerosis of middle cerebral artery, it was discovered that the lesion area significantly reduced secondary to use of herbal plant with high antioxidative and anti-inflammatory benefits. However, Studies done by Poznyak *et al.* (2020) stipulates that more targeted antioxidant medications are required, as several well-known antioxidants, such vitamins E and C, have not shown effectiveness in treating the oxidative stress linked to atherosclerosis in clinical trials. Therefore, it is important to assess antioxidant and anti-inflammatory phytochemicals in W. *ugandensis* if at all it has histo-inhibitory effect.

2.10 Historestorative effect of W. ugandensis on atherosclerotic lesion of white New Zealand rabbits.

Historestorative is generally the ability of an organ to attain its histological make secondary to toxicity or damage. It's science that involves causing damage using a toxic substance and thereafter administering another substance that possess restorative abilities (Allan *et al*., 2023). Atherosclerosis is typical the development of a plaque within a lumen of a blood vessel after a high fat or cholesterol diet which initiates inflammation and oxidation. historestorative changes in atherosclerosis can be mapped by changes in histological make of blood vessel wall, and size of aortic intima. Plants with high phytochemical components have high antioxidative and anti-inflammatory benefits thus can highly reverse the changes caused. According to (Kollár & Hotolová, 2003), the presence of polyphenolic acid slowed the progression of an atherosclerotic lesion since it had a wide range of biological effects, including antiplatelets, antioxidants, endothelium protection qualities, and smooth muscle cell proliferation. Research done by (Wakabayashi, 1999) on the other side, demonstrated that red wine's phenols could lower LDL oxidation, making them cardio protective. Alkaloids, flavonoids, phenolics, sugar alcohols, and unsaturated fatty acids, particularly linoleic acid, were also found in *W ugandensis* active chemical components (Abuto *et al*., 2016) and hence this explains why it is

important to try the historestorative activity of *W ugandensis*. Indeed, it has been documented that active principles originating from plants, such as polyphenols, tannins, flavonoids, alkaloids, and so on, decrease the formation of cholesterol in the liver and impede the actions of enzymes that synthesize cholesterol (Ram *et al.*, 2014).They also reported that the histopathological changes were also reverted to normalcy and reports that the levels of lipid profile markers significantly reduced signifying restoration, **nevertheless studies done by** Poznyak *et al* (2020) concluded that not all compound with anti-oxidants are effective in reduction of oxidative stress process in atherosclerosis and that more specific antioxidant drugs are indeed required. Furthermore, there is paucity of data in regards to historestorative ability of Warbugia *ugandensis* extract and this explains why it is important to explore more about this plant if at all it has effects despite having shown to have antioxidants and anti-inflammatory phytochemicals.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study site

Animal experiment was carried out at University of Nairobi biology Animal House. The choice of this study site was based on availability of animals, a stand by veterinary doctor and the expertise in handling the rabbits. Tissue processing of specimens for microscopy was conducted in Kabarak University histology laboratory in the School of Medicine and health sciences. *W ugandensis* methanolic extract was carried out at the department of pharmacognosy school of pharmacy Kabarak university.

3.2 Study design

This study design was a true experimental post-test in which an intervention was implemented and the outcomes were compared between the experimental and control groups.

3.3 Experimental animals/study models

Experiment was conducted on 30 male New Zealand rabbits that attained the desired weight of 2.0 ± 0.2 kg and age (8-12 weeks). All New Zealand rabbits that portrayed signs of sickness and weakness and non-pure breed were excluded from the study. The use of this rabbits was based on their similarities in lipid metabolism with humans. Rabbits are known to be sensitive to high-cholesterol diets and accumulate large amounts of cholesterol in their plasma. The adoption of these animals as experimental models to assess development of atherosclerosis was highly relevant and provided information about factors that contribute to progression and regression of disease. Moreover, they are larger, suitable for sampling and ease to induce atherosclerosis (Lee *et al*., 2017). The rabbits were kept in a conducive environment such as free access to water, regular change of beddings and fed with normal rabbits' pellets. They were then allowed to acclimatize for one week with close monitoring of their health status

before and during the experiment. Daily weighing of animals was done during the same period. Figure 3.1 below shows male white New Zealand rabbit

Figure 3.1: **White New Zealand Rabbit**

3.4 Sample size determination

In this study Modified Resource equation method of sample size calculation was adopted as there was no previous research done to determine the standard deviation (Arifin & Zahiruddin, 2017).

n=DF/K+1

N= n* k

n- Number of animals per group

DF-Error of degree of freedom

K-Number of groups

N=Total number of subjects

DF range from 10 to 20 to obtain minimum and maximum number of each group

Sample size in each group was calculated as follows;

K=4

= (10/4) +1

=2+1

=3

n=3 rats in each group

=3x6

=**18**

3.5 Sampling of Animals

Simple random sampling method was used, whereby 18 rabbits were picked from the pure-bred New Zealand rabbits and ascribed in to experimental or control group. In addition to this another set of 12 rabbits was sampled to determine safe doses of *W ugandensis* by conducting acute oral toxicity hence making the total number of rabbits for whole study to be 30.

3.6 Grouping of animals

The 18 rabbits were randomly assigned to two groups; controls and experimental groups. The control group had 12 rabbits while experimental had 6 rabbits. The process was done by assigning numbers to the sample frame and then balloting was done to choose for each category. Figure 3.2 shows algorithm for animals grouping

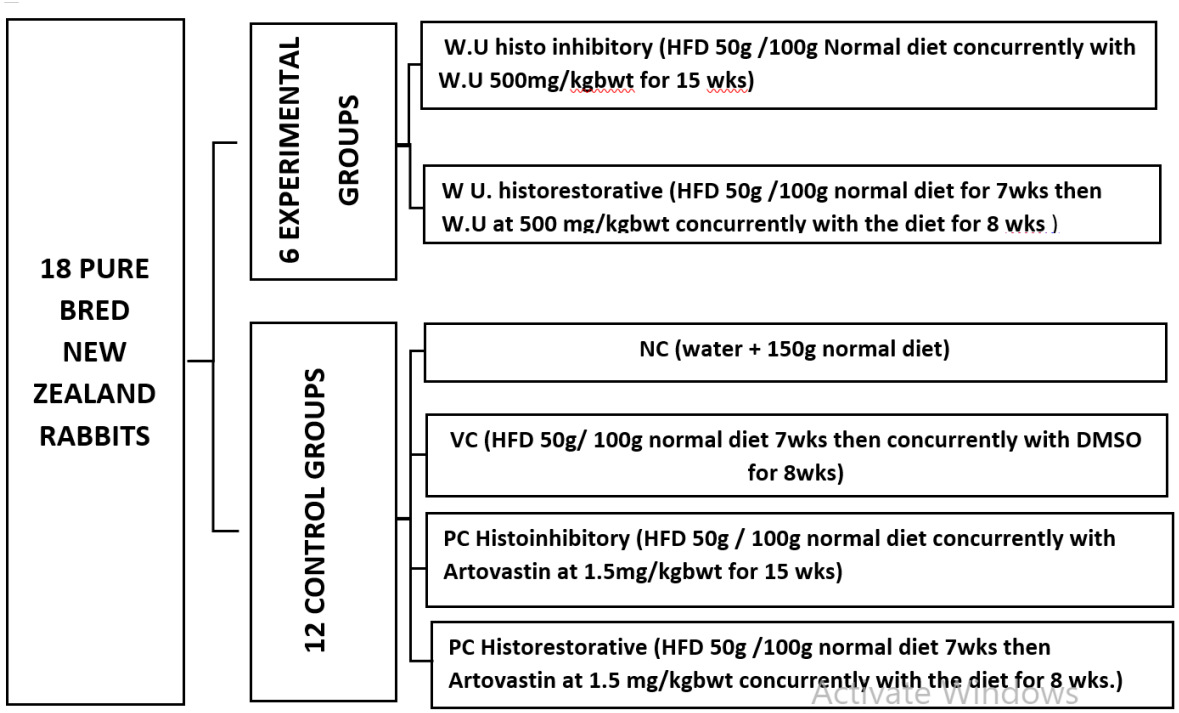

Figure 3. 2*:Shows the grouping of animals*

3.7 Preparation of W. ugandensis

3.7.1 Sample Collection

The Stem barks was collected randomly with the assistance of a plant taxonomist from Mount Kenya forest ensuring sustainable debarking (Khumalo, 2007). A voucher specimen of *W. ugandensis* bark, flower, and fruit was deposited at botanical herbarium, and a voucher number obtained for each plant. Figure 3.3 shows dried *Warbugia ugandensis* stem bark.

Figure 3. 3*:Shows Warbugia Ugandensis barks.*

3.7.2 Sample Preparation

In order to remove dirt and soil, stem barks were washed, then cut into smaller pieces which were air dried at room temperature (25°C) for 2-3 weeks out of direct sunlight. The air-dried pieces were then ground into powder with an electric grinder, weighed and stored at 4°C in watertight plastic bags until extraction. Figure 3.4 below shows W. *ugandensis* powder

Figure 3. 4:***W. ugandensis* coarse powder**

3.7.3 Extraction of *W. ugandensis*

1000 grams of each plant powder were soaked in 2 litres of 75% methanol at room temperature for one day (24 hours) and then filtered out with Whatman No. 1 filter paper, after which solvent evaporation was achieved by vacuum evaporation using a rotary evaporator (Rotavapor R100, Buchi) to get the bark extract. The respective extracts were stored in air tight bijou bottles at -20 °C until use. Calculation of percentage yield of bark extract.

$$Percentage\ yield = \frac{Weight\ of\ plant\ after\ extraction}{Weight\ of\ plant\ before\ extraction} \times 100$$

3.7.4 Qualitative determination of phytochemicals in *W. ugandensis*

1. Alkaloids

Crude powder extract was dissolved in 2 N HCl, filtered and filtrate divided into four parts to be used for the following alkaloid tests (Uma & Sekar, 2014)

a) Dragendroff's test: Dragendroff's reagent (solution of potassium bismuth iodide) was applied in 2ml of filtrate. There was formation of a red-orange precipitate indicating the absence of alkaloids.

b) Mayer's test: a few drops of Mayer's reagent (potassium mercuric iodide) was added to the 2ml filtrate and there was no formation of yellow precipitate hence no presence of alkaloids.

c) Wagner's test: Wagner's reagent (iodine in potassium iodide) was used for the 3rd filtrate. If yields no reddish or brownish precipitate, then absence of alkaloids was noted.

d) Hager's test: Hager's reagent (saturated picric acid solution) was employed for the 4th precipitate which did not yield yellow precipitate hence absence of alkaloids.

2. Flavonoids

a) Lead acetate test: the extract was treated with a few drops of lead acetate solution which formed a yellow precipitate to indicating presence of flavonoids.

b) Shinoda's test: The extracts were treated with magnesium and concentrated HCl to yield a red colour to indicates presence of flavanone orange-red colour to indicate presence of flavanols.

3. Tannins

Braymer's test: The aqueous extract of the crude dry powder was treated with 10% alcoholic FeCl3 to yield a blue-black color indicating presence of tannins.

4. Steroids and triterpenoids

Liebermann-Burchard test: The extract sample was dissolved in 2ml of chloroform in a dry test tube and 10 drops of acetic anhydride as well as 2 drops of concentrated sulphuric acid added. A sequence of color changes from red, to blue and finally bluish green was observed indicating presence of a steroidal nucleus while color change to red- purple indicated presence of a triterpenoid nucleus.

5. Saponins

Froth/Foam test: Crude dry powder of extract was dissolved in 2ml of distilled water then briskly shaken and allowed to stand for 10 minutes. Formation of froth which lasted 10 minutes indicates presence of moderate saponins.

6. Cardiac glycosides

0.5g of the extract was hydrolyzed with mineral acid (dilute sulphuric acid) of 20ml in a boiling bath for 3 minutes, and filtered. Three drops of a strong lead sub-acetate solution were added to the filtrate solution. The filtrate was shaken with 5ml of chloroform in a separating funnel. The lower organic layer was separated in two crucibles to test for lactones and deoxy sugars, the other two test tubes of extract was tested for Liebermann's and Baljet

A) Keller-Killian Test

Chloroform was evaporated to dryness, then 0.4ml Glacial acetic acid with a trace of Ferric chloride was added down the side of the tube, followed by 0.5ml concentrated Sulphuric acid. There was absence of red-brown color at the junction, upper acetic blue denoting absence of deoxy sugar.

B) Kedde Test

Chloroform was evaporated to dryness followed by the addition of 1 drop of 90% alcohol and 2 drops of 2% of 3.5-dinitrobenzoic acid. The solution was then made alkaline by addition of 20% sodium hydroxide (NaOH). there was absence of violet/purple color for lactones portraying absence of lactones.

7. Test for Anthraquinone

Borntrager test

0.5g of the extract was dissolved in 5ml of dilute hydrochloric acid and boiled for 2-3 minutes, filtered and transferred in a separating funnel and extracted with 5ml of chloroform. 5ml of the lower organic layer was transferred in a test tube and 4 drops of ammonia was added. Observation was made for the formation of rose pink to red color in the ammoniacal layer which demonstrated moderated amount.

3.8 Determination of acute oral toxicity of *W. ugandensis*

Acute oral toxicity was conducted so as to guide on safe doses of plant extracts as per Lorkes protocol (Chinedu *et al*., 2013). These animals were not part of the 18 animals considered for study however, they were used to evaluate safety levels of plant.

Phase I

Nine animals (n=9) were utilized in this phase, in which they were assigned into 3 groups of 3 animals each. Animals in each group got plant extract as follows; Group A1, 10 mg/kg, Group B1, 100 mg/kg and finally Group C1, 1000 mg/kg. Animals were observed for behavioral

changes and mortality for over 24 hours.

Phase II

Three animals (n=3) were used in this phase. Each group received different doses of plant extracts as follows; Group A2 1600 mg/kg, Group B2 2900 mg/kg and finally Group C2 5000 mg/kg. Animals were observed for behavioral changes and mortality for over 24 hours.

Rabbits were monitored for piloerection, respiratory distress, mucosal abnormalities, somatomotor activity, salivation, diarrhea, coma, convulsions, and mortality over 48 hours.

The adopted observation plan was as follows; immediately after plant extract administration, 30 minutes, 1 hour, 4 hours, 24 and 48 hours after plant extract administration.

Monitoring for signs of toxicity continued for the next 14 days, during which weighing was carried out daily. Final sacrificing of animals occurred on day 15 after an overnight fast. A necropsy was performed with help of the Vet surgeon.

3.8.2 Determination of LD50

The following formula was applied as per Lorke's method (Lorke, 1983)

Lethal dose (LD50).

LD 50 = $\sqrt{D0\ x\ D100}$

Where,

a) LD 50 - lethal dose at which 50% of the animals died

b) D0 – highest dose that didn't result in mortality

c) D100 - lowest dose that didn't result in mortality.

3.9 Acquisition of Atorvastatin

The drug was **Batch No. 0420155** acquired from Trans wide pharmaceutical company recognized by Kenya pharmacy and poison board in form of 10mg tablets and each tablet was dissolved in 6ml saline.

3.10 Feeding and induction of atherosclerosis

The rabbits in the negative control group were fed 150 g of rabbit pellets (FUGO), purchased from Unga feeds daily for a total of fifteen weeks. Positive control historestorative group, and vehicle control groups were fed on a high-fat diet (1% cholesterol, 10% lard, 10% egg yolk powder, 0.2% propylthiouracil and 67.8% normal diet) 50 g in morning and 100 g normal diet in afternoon daily for a total of seven weeks to induce atherosclerosis (Qiao *et al*., 2017) and thereafter Atorvastatin and DMSO for a period of 8 weeks respectively . The positive control histo-inhibitory group received high fat diet and Atorvastatin at 1.5mg/kgbwt concurrently for a period of eight weeks. Artovastin dosage was derived from previously used dosage in an experiment that was conducted in rabbits by (Zhou *et al*., 2010). The experimental rabbits were further grouped into histo-inhibitory group (n=3) and historestorative group (n=3), whereby histo-inhibitory group received high fat diet concomitantly with *W. ugandensis at 500mg/kg bwt* (n=3). Historestorative sub-groups were fed on a high fat diet for seven weeks to induce atherosclerosis and thereafter received *W. ugandensis* extract at 500mg/kg bwt for 8 weeks. The conversion of the dosage was calculated from previous safe dosage used in rat model using a dosecal (Janhavi *et al*., 2022). Figure 3.5 shows induction, development of atherosclerosis lesion and harvesting after administration of high fat diet.

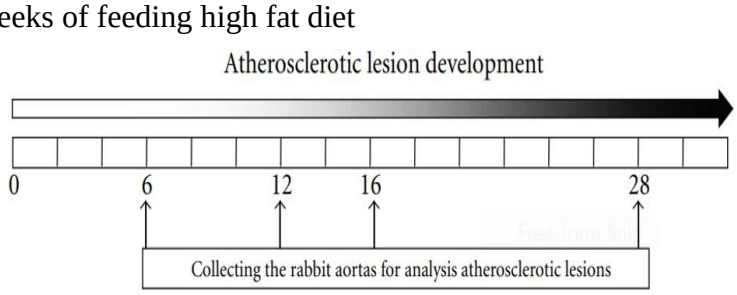

Figure 3. 5:*Schema for induction and development of atherosclerotic lesions and harvesting of aortas*

3.11 Sacrificing the animals, harvesting the aorta and fixation

Animals were sacrificed at the end of week 15 using concentrated CO2 and then placed in supine position. Thereafter, they were sprayed with 70% ethanol to prevent fur contamination of blood samples. A midline incision was made from jugular notch to pubic symphysis to expose thoracoabdominopelvic cavities. The heart was identified and right atria cardiac puncture made using a 23G needle to bleed animal, blood collected and stored in EDTA coated tubes as well as plain tubes for biochemical analysis. Through femoral vein, saline was pushed replacing the blood. Perfusion with 10% formalin done and its Adequacy indicated by an engorged liver (Centa, Jin, *et al*., 2019).

The heart and aorta dissected out from surrounding organs up to its bifurcation into common iliac arteries preserving 3mm of brachiocephalic trunk, left common carotid, left subclavian and renal arteries as anatomical landmarks. A circumferential piece of diaphragm with a diameter of 1cm was left as a landmark between the thoracic and abdominal aorta. The aorta then preserved in 4% formalin (Centa, Jin, *et al*., 2019). Figure 3.6 shows the aorta of New Zealand rabbit being harvested.

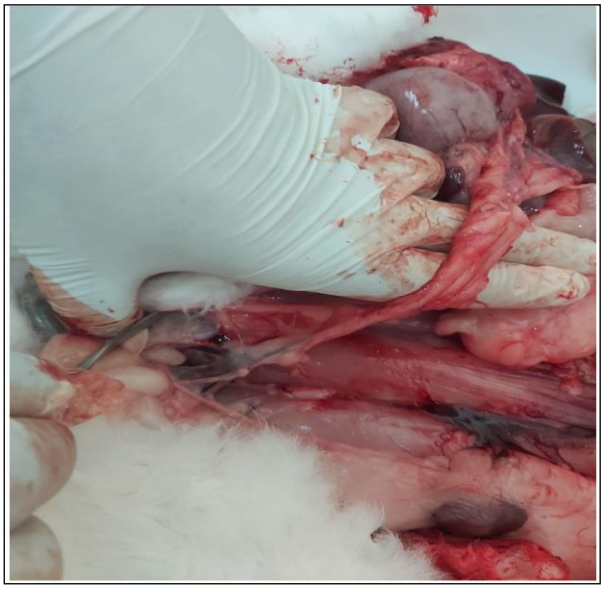

Figure 3. 6:*Shows the Aorta*

3.11.2 Enface analysis of the aorta

Aorta detached from the heart by making a cut through the basal 1/3rd of the heart through the left and right auricles taking care to preserve the left ventricular outflow tract. Staining of the atherosclerotic lesions was done using Oil Red O working solution (Lin *et al*., 2015).

3.11.3 Preparation of Oil Red O stock and working solution

1 g Oil Red O powder dissolved in 200 ml isopropyl alcohol Stock solution: double distilled water, filtered at room temperature was used within 2hours of preparation. The fixed aorta washed three times in double distilled water (1 min/wash) then immersed in 60% isopropyl alcohol for 5 min and rinsed in distilled water. The aorta was then incubated in the Oil Red O solution at 37˚C for 30 minutes. Atherosclerotic lesions-stained contrasting red color.

3.11.4 Tissue Processing for Light Microscopy

Aortic tissue was preserved in 10% formalin for 24 hours before dehydration in ascending concentrations of alcohol (50%, 60%, 70%, 80%, 90%, 95% and 100 % each for one hour). This was then cleared with xylene for 12 hours. The aortic sections were then infiltrated with paraffin wax for 12 hours after being embedded in paraffin wax. Leitz sledge hammer microtome was used to cut longitudinal sections 5-7µm thick, floated in water at 37^{0}C then fished onto glass slides using egg albumin, applied as thin film with a micro-dropper. To display the general characteristics and cellular elements of the aortic tunica intima, slides were dried at 37^{0}C for 24 hours and then stained with hematoxylin and eosin. Acquisition of micrographs was done using Leica M125 Stereomicroscope mounted with DFC450 camera at a magnification of X8. Calculation of aortic tunica intima area fraction was done using Image J image analysis software.

3.12 Data Management and analysis

On objective one, grading criteria was performed based on intensity of color produced by the reactions observed in test tubes and amount of foaming. The reactions was designated as follows; + for positive response and - for no observable response (Savithramma *et al*., 2011). Data for objectives two was entered in on Microsoft excel table and analyzed. Objective three and four data was entered into Microsoft Excel spreadsheet then to SPSS for analysis. One-way analysis of variance (ANOVA) was used to determine mean differences;' and intergroup significance was determined using post-hoc Bonferroni test. A significance level of ($p \leq 0.05$) was adopted.

3.13 Ethical Considerations

Ethical approval from the Animals Ethics and Research Committee for conducting the study was obtained from Jomo Kenyatta University Agriculture Technology Institutional Scientific and Ethics Review Committee **(JKU/ISERC/02316/0891**), **(**appendix III). National Commission for Science, Technology and Innovation, approval number **NACOSTI/P/23/28152** granted permission to conduct the research (appendix II). Animals were handled in accordance with established University of Nairobi Biology Animal House handling guidelines. Ethical considerations followed included: Reducing to the fewest number of animals possible that meet research goals and provide statistically robust data; Refinement where all animals were placed in standard polycarbonate cages measuring 30 x24 x 18 inches to reduce stress, fighting and injury (Hungu, 2011), beddings changed daily or when soiled, 12-hour day/light cycle, humane culling using concentrated CO_2 and sick animals cared for by an in-house veterinarian. The frail animals, were recalled from the experiments and immediately sacrificed by concentrated CO_2 euthanization. All animals used in the experiments were sacrificed using humane end points at the end of the study (Kirkwood *et al*., 2013). The protocol followed Guidelines for Care and Use of Laboratory rabbits in Biomedical Research, and the rabbit were only used once in the experiment (Leary *et al*., 2013).

CHAPTER FOUR

RESULTS

4.1 Introduction

The chapter relays data on qualitative phytochemical compounds of *W. ugandensis,* acute oral toxicity, histo-inhibitory and historestorative activities. It also presents data on both negative and positive control groups. Data on weight and surface area was uploaded on SPSS and analyzed. One Way ANOVA was used to compare group data and post hoc Bonferroni was embraced to find group significance levels. Data was presented based on the objectives as follows.

4.2 Qualitative phytochemicals, present in crude methanolic extract of W. *ugandensis*.

In order to determine qualitative phytochemicals, screening test as per the protocols for presence of antioxidants and anti-inflammatory components was done. It was observed that cardiac glycosides was absent while Anthraquinones and Alkaloid were of low amount in crude methanolic extracts of *W. ugandensis*. Flavonoids and Phyto-steroids were high although Tannins, phenols and saponins were moderate in crude methanolic extracts of *W. ugandensis*. The aqueous extract showed that phenols, anthraquinones, and cardiac glycosides were absent, while alkaloids, tannins, and phytosteroids were in low traces. Flavonoids and alkaloids were found to be moderately present in the aqueous extract (Table 4.1).

Table 4.1: Phytochemicals in methanolic and aqueous extracts.

Phytochemicals	Methanolic extract	Aqueous extract
Alkaloids	+	+
Flavonoids	+	+
Tannins	+	+
Phyto-steroids	+	+
Phenols	+	-
Saponins	+	+
Cardiac glycosides	-	-
Anthraquinones	+	-

KEY: += present, -=Absent,

4.3 Determination of acute oral toxicity of W. ugandensis on white New Zealand rabbits.

Acute oral toxicity of *W. ugandensis* on white New Zealand rabbits was done in two phases and in each phase different doses of *W. ugandensis* were given and its effects were monitored against time.

Phase 1

Control group: This group was subjected to 5% DMSO + distilled water for 48 hours and the effects were reported as follows;

Immediate: The rabbits displayed normal activities.

30 minutes – 48 hours: The rabbits displayed normal activities.

Mortality: after 48 hours of the experiment, no mortality was recorded therefore, mortality rate was zero.

Experimental groups

A1 group: this group had 3 rabbits which were subjected to 10 mg of *W. ugandensis* extract in 5% DMSO and effects were observed for 48 hours, during which activities and mortalities of rabbits were monitored and recorded.

Immediate: Normal activities of rabbits were recorded and no mortality was reported.

30 minutes – 48 hours: Normal activity of rabbits was noted and no mortality was reported.

B1 group: 3 rabbits were used, subjected to 100mg of *W. ugandensis* bark extract dissolved in 5% DMSO. The following was observed;

Immediate: rabbits had normal activities and no mortality was observed.

30 minutes – 48 hours: normal activity of rabbits was displayed and no mortality was recorded.

C1 group: this group comprised of 3 rabbits that were treated with 1000mg of *W. ugandensis* bark extract dissolved in 5% DMSO for 48 hours. Rabbit activities and mortality were observed and recorded.

Immediate: rabbits in this group displayed normal activities and no mortality was reported.

30 minutes – 48 hours: normal rabbit activity was observed and no mortality was reported.

All the rabbits (control and experimental) in phase 1 displayed normal activities and no mortality were reported. This therefore signifies that *W. ugandensis* bark extract of 10 mg to 1000mg has no toxicity.

Phase II

A2 group: here only one rabbit was used. It was subjected to 1600mg of *W. ugandensis* bark extract dissolved in 5% DMSO for 48 hours. The following results were obtained;

Immediate: rabbit had normal activity and no mortality was reported.

30 minutes – 48 hours: rabbit had normal activity and no mortality was recorded.

B2 group: a single rabbit was used which was treated with 2900mg of *W. ugandensis* bark extract for 48 hours.

Immediate: rabbit had normal activity and no mortality was reported.

30 minutes – 48 hours: normal rabbit activity and no mortality was recorded.

C2 group: one rabbit was used which was treated with 5000mg of *W. ugandensis* bark extract for 48 hours.

Immediate: rabbit displayed normal activity with no mortality.

30 minutes – 48 hours: normal rabbit activity with no mortality.

It was observed that rabbits subjected to 1600mg up to 5000mg of *W. ugandensis* bark extract had normal activity with no mortality therefore no acute oral toxicity (Table 4.2)*Table 4.2: Comparison of dose effect of W. ugandensis in relation to time and mortality for acute oral toxicity*

	OBSERVATIONS								
	Dosage (Centa, Jin, et al.)	**Immediate**	**30min**	**1hr**	**4hrs**	**24hrs**	**48hrs**	**Mortality (n=3)**	**Mortality Rate**
PHASE I									
WUBE IN 5%DMSO	A1 10MG	NA	NA	NA	NA	NA	NA	0	0
	B1 100MG	NA	NA	NA	NA	NA	NA	0	0
	C1 1000MG	NA	NA	NA	NA	NA	NA	0	0
CONTROL	5%DMSO+ DISTILLED WATER	NA	NA	NA	NA	NA	NA	0	0
PHASE II									
PHASE1 WUBE IN DMSO 5%	A2 1600	NA	NA	NA	NA	NA	NA	0/1	0
	B2 2900	NA	NA	NA	NA	NA	NA	0/1	0
	C2 5000	NA	NA	NA	NA	NA	NA	0/1	0

KEY: WUBE: Warbugia Ugandensis Bark Extract, NA: Normal Activity, DMSO: Dimethyl Sulfoxide.

4.3 histo-inhibitory effects of W. ugandensis on an atherosclerotic lesion of white New Zealand rabbits.

Table 4.3: Comparison of mean area fraction of different groups in histo inhibitory.

GROUPS	Negative control(water +food)	Vehicle control(DMSO)	*W ugandensis* histoinbitory(HFD 50g/day+*W ugandensis* 500mg /kg/day)	Positive control (Artovastin1.5mg/kg/ +HFD 50g/day)	Df	F	P value
Area Fraction(mm2)	0.15144±0.2	0.51622±0.36			5	86.50	0.0001*
Area Fraction(mm2)		0.51622±0.36	0.45584±0.06		5	86.50	0.0001*
Area Fraction(mm2)		0.51622±0.36		0.1682±0.35	5	86.50	0.0001*
Area Fraction(mm2)	0.15144±0.2		0.45584±0.06		5	86.50	1.000
Area Fraction(mm2)	0.15144±0.2			0.1682±0.35	5	86.50	1.000
			0.45584±0.06	0.1682±0.35			0.0001*

All values are expressed and presented as the mean± the standard error of the mean; n=3. Data analyzed by one-way analysis of variance followed by post-hoc Bonferroni. Asterisks represents significant ($p \leq 0.0001$).*

The mean area fraction of vehicle control group significantly (p = 0.0001) increased when compared to negative control group at 0.51622mm2 and 0.15144 mm2, respectively. The mean area fraction for W. ugandensis histo inhibitory group was significantly (p = 0.0001) reduced when compared to vehicle control group at 0.45584mm2 and 0.51622mm2 respectively.

The mean fraction area of atorvastatin inhibitory group was significantly (p = 0.0001) reduced to 0.16821 mm2 as compared vehicle group of 0. 51622mm2.There was no significant (p=1.000) difference in the mean area fraction of Warbugia inhibitory group when compared with the negative control group. There was no significant difference in the mean area fraction of negative control group when compared with Atorvastatin inhibitory group (p = 1.000). There was a significant increase in the area fraction in W ugandensis inhibitory group (high fat diet-50g/day + Wu-500mg/kg/day) compared to atorvastatin inhibitory group (high fat diet-50g/day

+ 1.5 mg/kg bwt atorvastatin), which recorded a mean of 0.45584mm2 and 0.16821 mm2, respectively. A noteworthy statistical distinction ($p<0.005$) was observed between the Warbugia ugandensis inhibitory group compared to atorvastatin inhibitory group (table 4.3).

4.3.1 Histological comparison of different groups in histo-inhibitory

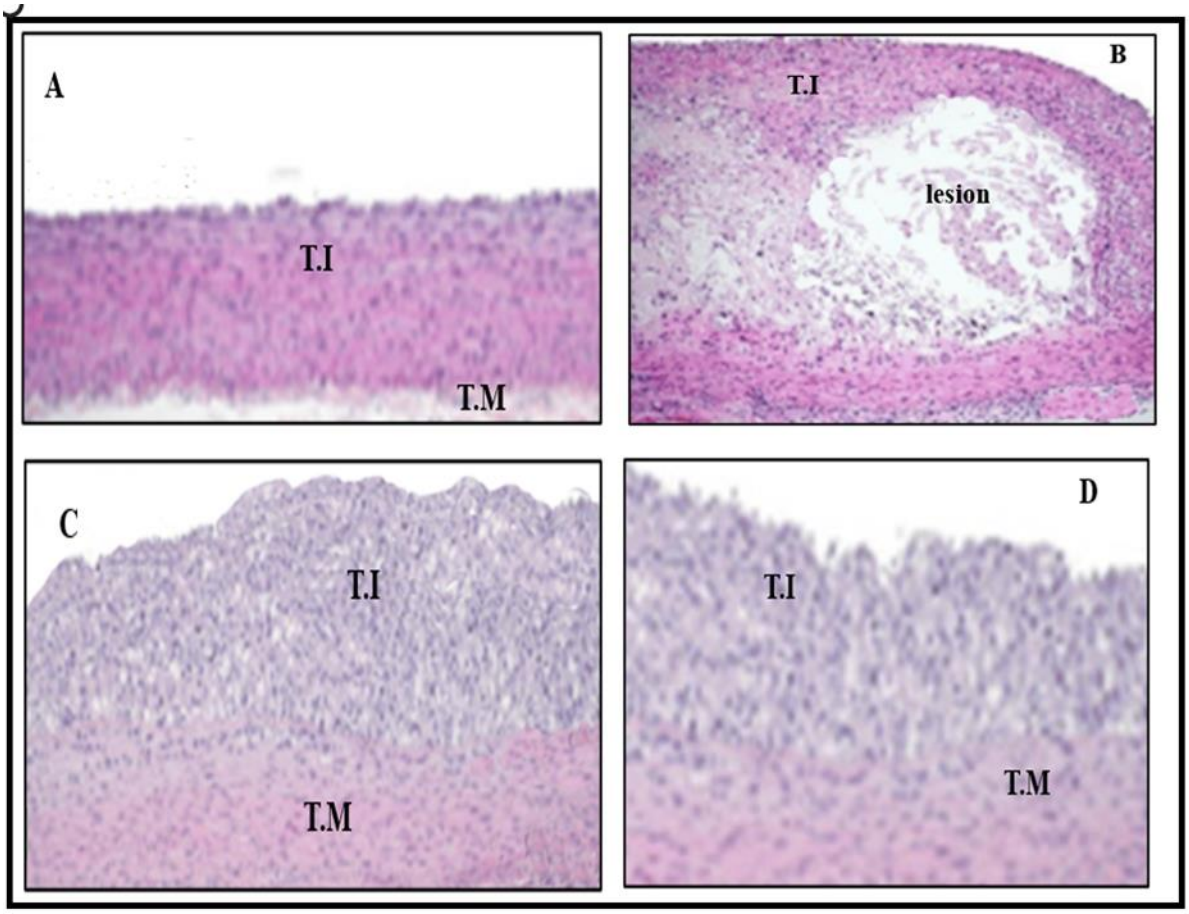

Figure 4. 1: ***Showing A= negative control group (feeds + water ad libitum) and B= vehicle control group (high fat diet + 5% DMSO), C= W. ugandensis histo-inhibitory group and D= Atorvastatin histo-inhibitory group.***
KEY: *TM=tunica media and TI= tunica intima*

It was observed that the negative control group had a well distributed simple squamous endothelial cells lining endothelium, with sub endothelium showing a sparse nucleus and matrix whereas vehicle control group had a large tunica intima, an evident lipid core lesion with poorly distributed cells, a necrotic area, and a fibrous cap.

It was also noted that *W. ugandensis* histo-inhibitory group had a fatty streak within the tunica intima characterised by foam cells that accumulated lipids in their cytoplasm. On the other hand, Atorvastatin histo-inhibitory group had a less pronounced fatty streak (Figure 4.1).

4.4 To assess the historestorative effect of *W. ugandensis* on atherosclerotic lesion of white New Zealand rabbits.

Table 4.4: Comparison of mean area fraction of different historestorative groups.

GROUPS	NC (water + 150g normal diet)	VC(DMSO+ Feeds)	*WUR* (HFD 50g/day+*WU* 500mg /kg/day)	PC (Artovastin1.5mg/kg/ +HFD 50g/day)	Df	F	P value
Area Fraction(mm2)	0.15144±0.2	0.51622±0.36			5	86.493	0.0001*
Area Fraction(mm2)		0.51622±0.36	0.35022±0.20		5	86.493	0.0001*
Area Fraction(mm2)		0.51622±0.36		0.20461±0.35	5	86.493	0.0001*
Area Fraction(mm2)	0.15144±0.2		0.35022±0.20		5	86.493	1.000
Area Fraction(mm2)	0.15144±0.2			0.20461±0.35	5	86.493	1.000
Area Fraction(mm)			0.35022±0.20	0.20461±0.35	5	86.493	0.0001*

KEY: All values are expressed and presented as the mean± the standard error of the mean; n=3. Data analyzed by one-way analysis of variance followed by post-hoc Bonferroni. Asterisks* represents significant ($p \leq 0.0001$).

The mean area fraction of vehicle control group significantly increased as compared to negative control group (p = 0.0001) at 0.51622mm^2 and 0.15144mm^2 respectively. The mean area fraction of *W. ugandensis* restorative group was significantly (p = 0.0001) reduced when compared to vehicle control group at 0.35022 mm2 and 0.51622mm^2 respectively. The mean fraction area of atorvastatin restorative group was significantly reduced (p = 0.0001) to 0.20461mm^2 as compared vehicle group of 0.51622mm^2 respectively. There was no significant difference of mean area fraction of *W. ugandensis* restorative group when compared with negative control group (p=1.000). There was no significant difference of mean area fraction of negative control group when compared with the atorvastatin restorative group (p = 1.000).

There was a significant increase in the area fraction in *W. ugandensis* restorative group (high fat diet 50 g/day/100mg + Wu 500 mg/kg/day) compared to the atorvastatin restorative group (high fat diet 50 g/day/100mg normal diet + atorvastatin), which recorded a mean of 0.35022mm and 0.20461 mm, respectively. A statistically significant difference ($p<0.005$) was observed between the *W. ugandensis* restorative group compared to atorvastatin restorative group (Table 2).

4.4.1 Histological comparison between control and historestorative groups.

W. ugandensis historestorative group had smaller lesion as compared to vehicle control but not smaller than Atorvastatin restorative group. The lipid core was smaller in size with a large fibrous cap around it. Atorvastatin historestorative had a small lesion with endothelial cells covering the lesion as opposed to vehicle control and *Warbugia ugandensis* historestorative group.

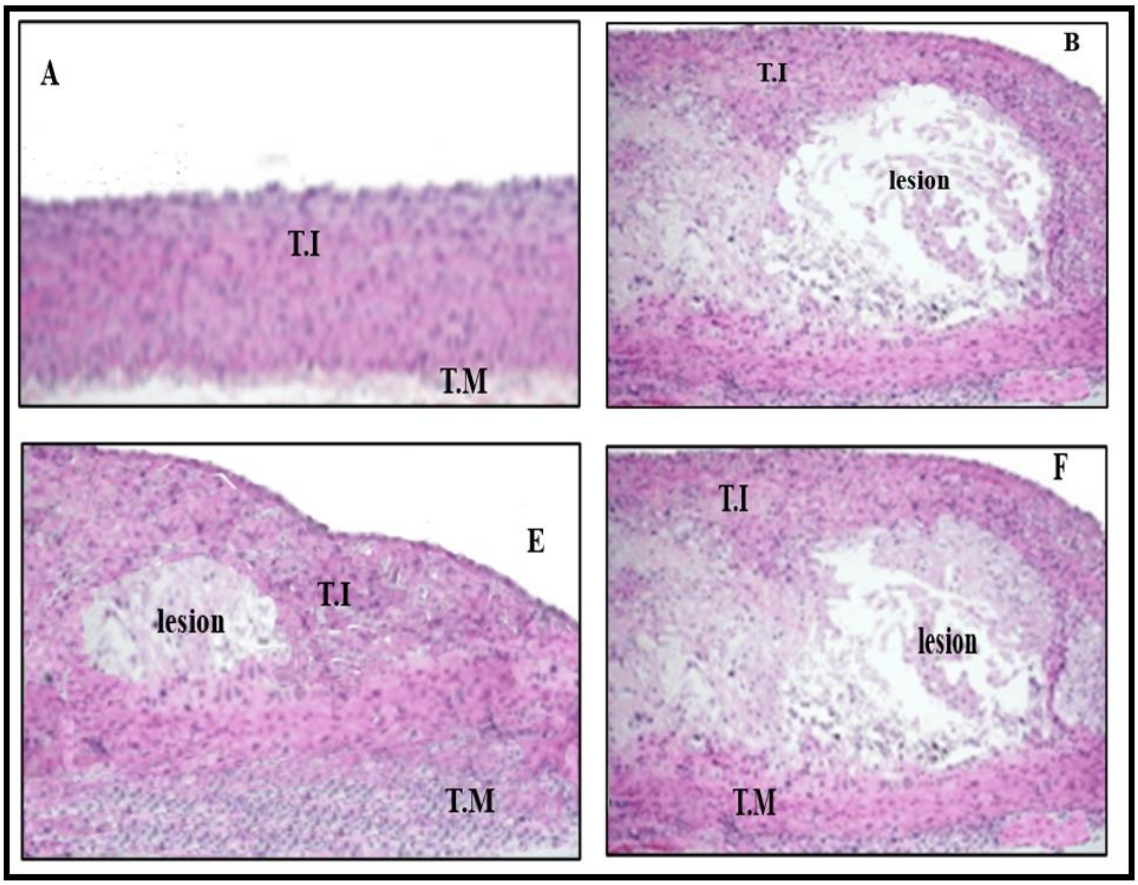

Figure 4.2: ***Showing A= negative control group, B=vehicle control group, E= Atorvastatin restorative group and F= W. ugandensis restorative group.***
KEY: ***T. I= tunica intima and T.M= tunica media.***

Table 4.5: Post hoc Bonferroni multiple comparison analysis of the groups

(I) GROUP	(j) GROUP	Mean Difference (I-J)	Std.Error	P-Value
Negative control	Vehicle control	-364776*	.030054	.000
	artovastatin histoinhibitory	-.016767	.029900	1.000
	artovastatin historestorative	-.053169	.030591	1.000
	Warbugia histoinhibitory	-304396	.029900	.000
	Warbugia historestorative	-198773*	030397	.000
Vehicle control	PC Atorvastatin histoinhibitory	.348008*	.021656	.000
	PC Atorvastatin historestorative	.311606*	.022600	.000
	Warbugia historestorative	.166003*	.022337	.000
PC Atorvastatin histoinhibitory	Warbugia histoinhibitory	-.287628	.021656	.000
	Warbugia historestorative	-.182006*	.022131	.000
	Negative control	.304396*	.030054	.000
	artovastatin historestorative	-.036402	.022396	1.000
Warbugia histoinhibitory	PC Atorvastatin historestorative	.251226*	.022600	.000
	Warbugia historestorative	.105623*	.022337	.000
PC Atorvastatin historestorative	Warbugia historestorative	.145604*	.023056	.000
	PC artovastatin histoinhibitory	.036402	.022396	1.000
Warbugia historestorative	artovastatin histoinhibitory	.182006*	.022131	.000
	Warbugia histoinhibitory	-.105623	.022337	.000
	artovastatin historestorative	.145604*	.023056	.000

***Statistically significant**

The table shows post hoc Bonferroni analysis where by it indicates that majority of comparison were significantly different however these parameters were not statically different between negative control and histoinhibitory groups and historestorative groups of positive control. The same observation was made between the two positive control.

CHAPTER FIVE

DISCUSSION

5.1 Qualitative phytochemicals present in crude methanolic extract of *W. ugandensis*.

Phytochemicals are natural products rich in chemical components produced by plants and represent an important cache for discovery of new drug compounds (Khera *et al*., 2020). Discovery of a new aryl naphthalene lignan amide suggest that *W. ugandensis* has remarkable antioxidant and anti-inflammatory effects (Khera *et al*., 2020). Anti-inflammatory and antioxidants chemicals are vital in prevention of lesions and enhancing human body infection defense systems by reactivating the ability to counter infections (Halliwell, 2011).

In the present study, flavonoids, tannins, saponins, phenols and Phyto-steroids were present and this concurred with studies done by Ngugi *et al.,* (2020) on effects of *W. ugandensis* in treatment of Asthma. It was observed that levels of flavonoids were high and moderate in both methanolic and aqueous extracts respectively. This observation is similar to that of (Okello *et al*., 2023) which demonstrated that the levels of flavonoids and phenols were high .

It was also noted that Phyto-steroids were present in methanolic extract of *W. ugandensis* which concurs with the findings of a previous study (Das *et al*., 2018) in which the researchers showed that Phyto-steroids defend inflammation by pulling down the pro-inflammatory mediators. Again, it was observed that Tannins were moderate denoted by results from brammers test which yielded a blue black colour in methanolic extracts of *W. ugandensis.* These findings are similar to (Carlson *et al*., 2008) concluded that tannins have the potential for various health-promoting activities, particularly antioxidant, antitumor, cardio protective, anti-inflammatory and antimicrobial activity. phenols and saponins were moderately present while anthraquinones were in very low in amounts as indicated in the phytochemical testing under methodology which concurs with (Bayram *et al*., 2012).

In the present study it was observed that cardiac glycosides were absent which is in tandem with findings of (Morsy, 2014) who points out that cardiac glycosides are typically found in small levels in plants, which may have an impact on their isolation. However, these findings are contrary to the works of **(Bakir *et al*., 2022**; Oyewole & Akingbal, 2011; Prabasheela *et al*., 2015) who found out that cardiac glycosides were present in most plants as they played a role both antimicrobial and anti-heart failure role when administered to individuals or used in diet. it can be postulated that the levels in cardiac glycosides were absent in *W. Ugandensis* as compared to other studies and plants of similar family due to time differences in conducting the studies and other studies were conducted on leaves as compared to present study that was done on barks. Part of plant where phytochemicals were done plays have a variant presence. According to the findings, saponin, anthraquinone, alkaloids were present and cardiac glycoside phytochemicals was absent in *W. ugandensis* bark extract made from both aqueous and Methanol. This was in line with a study conducted by (Abuto *et al*., 2018), in which the bark of *W. ugandensis* was extracted using methanol and examined using GC-MS (gas chromatography-mass spectrometry) to identify changes in the profiles of plant chemicals from various communities in the Kenyan Rift Valley, they deduced from this research that *W. ugandensis* had high concentrations sesquiterpenoids with Low amounts of other types of chemicals, like and Phytosterols, phenolics, and tocopherols.

5.2 Acute oral toxicity of *W. ugandensis* on white New Zealand rabbits.

The LD_{50} value was used as a statistical derived dose (Walum, 1998). It is important to note that animals were subjected to different doses of *W. ugandensis* bark extract and their behaviors were monitored at different times and recorded.

It was observed that in phase1; both control and experimental groups, normal activities were observed at immediate and between 30 minutes to 48 hours. No mortality was observed during this period. These findings are similar to (Anywar *et al*., 2021) and (Ngugi *et al*., 2020) who

established that *W. ugandensis* had no toxicity at this doses. This therefore signifies that acute oral toxicity test is prudent when evaluating safe doses of plant extract. In the present study evidence of no mortality and normal activity shown by the rabbits up to a dose of 1000mg/kgbwt of *W. ugandensis* signifies that at this dose *W. ugandensis* is safe for use as it cannot induce any effect on animals under study.

In phase II of the study, it was observed that animals subjected to 1600, 2900 and 5000mg/kg bwt of *W. ugandensis* demonstrated normal activity and no mortality was reported. These findings are in agreement with (Karani *et al*., 2013) who found out that the oral acute toxicity of bark extract of *W ugandensis* at a dose of LD_{50} <5000mg/kg had no signs of toxicity or mortality. Moreover, (Ngugi, 2020) found similar results when assessing the effects of *W. ugandensis* on asthma. According to (Kathare et al., 2021) *W. ugandensis* was safe for use at certain dose conducted via acute oral toxicity. Based on the present study, it can be established that any dose between 1600 to 5000mg/kgbwt of *W. ugandensis* is safe and therefore does not pause any health effect to the rabbits under the study. The LD_{50} of *W. ugandensis* in the current study was discovered to be > 5000 mg/kg body weight, making it a generally safe species according to classification by (Lorke, 1983).This concurs with a cytotoxicity test of *W. ugandensis* performed on Vero E6 by (Karani *et al*., 2013) MTT experiment on cells which demonstrated that it was harmless for use because its CC_{50} was >250ug/ml in relation to the LD_{50} and Rukunga and Simon categorization of cytotoxicity exceeded 5000mg/kg.

Based on the two phases of drug evaluation, it can be noted that *W. ugandensis* is safe for use at any dose up to 5000mg/kgbwt. These could be due to the fact that the levels of phytochemical components present at this dose or within these doses are not sufficient enough to cause both histological and physiological harm to animals. It is prudent to note that any dose that exceeds confirmed acute oral toxicity is not safe as this could potentially be harmful to important organs that are involved in drug pharmacokinetics. However, Bark extracts, leaves, and young shoots

of *W. ugandensis* have been utilized for years without experiencing any negative effects (Kirkwood *et al*., 2013; Kokwaro, 2009).

5.3 Histo-inhibitory effects of *W. ugandensis* on an atherosclerotic lesion of white New Zealand rabbits.

Histo-inhibition is simply the prevention of changes in histological make up of tissues as a result of use of drug or any chemical substance. This can be evaluated based on physical, physiological and histological evidence. Physiological evidence is more complex in the present set up as it involves study of complex tissue processes and chemical elements. The physical and histological changes are rather easy as it only composes evaluation of rabbit weight in response to treatment, surface area of aortic intima lesion and evident histological changes on the endothelium of aorta. Any evidence of deviation of named parameters might simply indicate histo-inhibitory processes.

It was noted that mean area fraction of aortic intima in vehicle control group significantly increased as compared to negative control group ($p=0.0001$). These findings are similar to observations of (Centa *et al*., 2019)when quantifying the atherosclerotic lesion on mice. The researcher noted that aortic arch was more prone to development of lesions and lesions alone was not adequate enough to cause harm unless its components were evaluated. In the present study, the rabbits were exposed to high fat diet of 50g for 7 weeks and it was observed that lesions developed within the aortic intima. The mean area fraction of lesion was $0.51622mm^2$ and this was due to exposure high fat diet which led to development of atherosclerosis. It is usually characterized by damage to endothelial cells of aortic tunica intima which accumulates LDL. Such damage increases permeability of arterial wall of aorta which increases collection of LDL in these vessels (Alfarisi *et al*., 2020) . Damaged endothelial cells adhere to nearby white blood cells causing morphological changes, release of free radicals that interact with LDL causing oxidation (Alfarisi *et al*., 2020) This leads to increased oxidative stress, inflammation and damage to endothelium causing endothelial dysfunction (Lian et al., 2018).

Endothelial dysfunction and chronic inflammation contribute to formation, growth and rapture of atherosclerotic plaque which cause coronary artery heart attack (Marchio *et al*., 2019). Therefore, the present study agrees that presence of high fat diet caused endothelial damage, leading to permeation of LDL into aorta and interacted with white blood cells causing oxidation that led to formation and growth of atherosclerosis lesion.

It was observed that the mean area fraction of W. *ugandensis* histo-inhibitory group significantly (P=0.0001) reduced in comparison to vehicle control group that was subjected to high fatty diet. This finding is consistent with research by Wen Hua Ling *et al*. (2002), which showed that oxidative stress is a major factor in the development of atherosclerosis and that antioxidant supplements reduce oxidative stress-induced damage to smooth muscle or endothelial cells in arteries, thereby preventing the formation of atherosclerotic plaque in the aorta.

This observation is also similar to that of a previous study (Mandlik & Namdeo, 2021) which found out that Ashwagandha *Somnifera* demonstrated a protective effect against middle cerebral artery occlusion. The researcher argues that this was achieved as the active compounds of the plant have ability to reduce lesion area by reducing the oxidative stress induced during inflammation process and ensuring that there is balance of anti-inflammatory mediators. This plant has been adopted as the two are from a similar family therefore may have common phytochemical compounds. Similar results were observed in a study of (Omara *et al*., 2022) on Clausena *anisata* in ethanolic and aqueous extracts that significantly reduced atherosclerotic plaque. According to (Kimondo, 2020), who made the same observations about a Maasai plant called ilkisonko that had a high phenolic content and, as a result, had beneficial antioxidant and anti-inflammatory properties. This indicated that *W ugandensis* decreased oxidative stress. Because oxidative stress plays a large role in the development atherosclerosis, the decreased peroxidation processes and DNA oxidative damage by supplementation of antioxidant reduced

the damage of oxidative stress to artery endothelial or smooth muscle cells, contributing to the inhibition of atherosclerotic plaque formation in aorta.(Wen Hua Ling *et al*., 2002)

This study therefore, attributes the significant reduction of area fraction of atherosclerotic lesion to high levels of flavonoids, phenol and Phyto-steroids that were present on phytochemical analysis.

On histological observation, it was observed that the negative control group had a normal tunica intima with well disturbed endothelial cells covering endothelial layer of thin connective tissue. The endothelium had a simple squamous epithelial lining which concurs with the findings of (Milutinović *et al*., 2020). On vehicle control, the tunica intima appeared to have increased in size, endothelial cells and nucleus sparsely distributed and had a lipid core lesion that was capped with a thin fibrous cap which makes the lesion unstable. This observation is similar to (Shibata *et al*., 2017) who noted that atherosclerotic lesion in tunica intima undergoes several complex stages including; early fatty streak, fibroatheroma and atheroma which invades tunica intima. This study notes that the obvious changes on tunica intima and development of lesion might have undergone a similar developmental process before inscribing within the tunica intima.

On histo-inhibitory, it was observed that there were fatty streaks evidenced by white spicules which did not stain in the tunica intima in comparison between Atorvastatin and *Ugandensis* groups. There were few whitish spicules in histo-inhibitory with Atorvastatin as compared to *W. ugandensis* however, there was no development of atherosclerotic lesion but fatty streak. This could be due to the fact that atorvastatin up regulates the production of nitric oxide which is a significant vasodilator with both anti-inflammatory and antioxidative effects (Mathew, 2014,Sorrentino, 2011) This increases vascular resistance, reduces vascular inflammation and increases antioxidant levels which play a critical role in reducing atherogenesis (Liberale *et al*., 2020). On the other hand, *W. ugandensis,* presence of high levels of flavonoids and tannins

which are secondary metabolites with both antioxidant and anti-inflammatory properties might have played a critical role in preventing development of atherosclerotic lesion. This was achieved due to the physiological and histological activities of reducing oxidation of low lipoproteins thereby reducing atherosclerotic lesions (Ciumărnean *et al*., 2020).

5.4 Historestorative effect of W. *ugandensis* on atherosclerotic lesion of white New Zealand rabbits.

Historestoration is the ability of a tissue or cell to return to its normal status after a pathological process has occurred. It is evaluated through many parameters however; in present study it was evaluated through measuring the area fraction of lesion within the tunica intima and correlating with the different histological findings. Any deviation from the normal findings as evidenced by area fraction of lesion in tunica intima and histological observations of different group denoted restoration.

It was observed that there was significant reduction (p=0.0001) in area fraction of *W. ugandensis* historestorative group as compared to vehicle control group at 0.35022mm^2 and 0.51622mm^2 respectively. This significant reduction in area fraction of lesion might have been due to high Phyto-steroids in methanolic extract of *W. ugandensis* which concurs with the findings of (Das *et al*., 2018) in which the researchers clearly state that Phyto-steroids defend inflammation by pulling down the pro-inflammatory mediators. According to Tiwari and colleagues, Phyto-steroids have similar structure to glucosteroids therefore are of high value in disease healing (Tiwari *et a*l., 2020) They also play a role in gene expression as they might inhibit gene expression which plays a role in treatment of malignancies(Sandeshkrishna *et al*., 2022) On the same note the restorative effects is also attributed to presence of flavonoids which has both anti-inflammatory and antioxidant properties which contributes to slowing down the development of atherosclerosis hence cardio protective (Fernández-Friera *et al*., 2017). Giugliano, D. (2000) notes that rates of heart disease are inversely connected with the consumption of fruits, vegetables, red wine, and tea due to the fact that they are rich in natural

antioxidants. An adequate intake of antioxidant-rich diets may help prevent or postpone the onset of pathological alterations brought on by oxidative stress. Similar outcomes were also noted by Yang et al. (2005), who concluded that Polygonum multiflorum Thunb.'s anti-oxidant and anti-free radical properties may be responsible for the plant's ability to prevent atherosclerosis.

On historestorative effects, it was observed that lipid core was smaller as compared to vehicle control with numerous cells, a small area of lipid spicules and thick fibrous cap. This is synonymous to reducing the progression of atherosclerotic lesion. On Atorvastatin restorative group, the lesion was even smaller, with no lipid spicules and thicker fibrous cap because of the effects of antioxidant and anti-inflammatory properties of Atorvastatin. The same was replicated in *W. ugandensis* historestorative however; it was not as much as compared to Atorvastatin hence making the drug more effective compared to *W. ugandensis.* The histological changes were attributed to presence of high amounts of flavonoids, phytosteroids, tannins and saponins in the present study which gives both physiological and histological picture of the use of *W. ugandensis* in historestoration and histo-inhibition of tissue especially when rabbits were subjected to high fat diet. This therefore improves normal cardiac function. According to Wiseman, H. (1999) Dietary flavonoid consumption has been found to be negatively correlated with CHD mortality in epidemiologic studies. Strong antioxidants, such as flavonoids can guard against LDL oxidation, which is linked to atherogenesis. Flavonoids also exhibit anti-inflammatory and antilipoperoxidant characteristics as well.

(Singh & Chaudhuri, 2018) demystifies that saponins play an important role in cardio protection due to its structural characteristics and pharmacological effects. Some of its properties are Ca^{2+} ion regulation, antiapoptotic, antiatherosclerotic, antihyperlipidemic and vasodilatory while pharmacologically its high permeability through cell membrane makes it a better cardio protective agent.

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

6.1 CONCLUSIONS

a) Flavonoids, tannins, phenols, saponins, Phyto-steroids, anthraquinones and alkaloids are present in methanolic bark extract of *W. ugandensis* and therefore, are useful antioxidant and anti-inflammatory components.

b) The safe dose of *W. ugandensis* for use in animal study is ≤ 5000mg/kgbwt as at this dose there is normal animal activity and no mortality was reported.

c) *W. ugandensis* has positive histo-inhibitory effects characterized by failure of formation of atherosclerotic lesion.

d) *W. ugandensis* has historestorative benefits portrayed by reduction of atherosclerotic lesion with a lipid core covered by a large fibrous cap.

6.2 STUDY RECOMMENDATIONS

This study therefore recommends the following

a) *Warbugia ugandensis* has inhibitory and restorative effect that can be used in the management of atherosclerosis.

6.3 RECOMMENDATIONS FOR FUTURE STUDIES

a) A study on quantitative and potency of phytochemicals should be carried out to determine the amounts of flavonoids, tannins and saponins to help in management of other conditions.

b) Further studies to be conducted to ascertain any drug interaction of W. *ugandensis***.**

c) Further studies to be conducted on the most effective doses of W. *ugandensis* in management of atherosclerosis**.**

d) Biochemical studies should be conducted to ascertain the effects of W. *ugandensis* on lipid profiles.

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APPENDICES

APPENDIX I: DATA COLLECTION FORM

EXPERIMENTAL GROUP											
HISTOINHIBITORY EFFECTS OF WARBUGIA UGANDESIS METHANOLIC EXTRACT											
		ATHEROSCLEROTIC LESION									
		OIL RED O/SUDAN III BLACK/CONGO RED				Plaque vulnerability index		OTHER FEATURES			
CODE	WT	NO	SITE	AREA/volume FRACTION LIPID CORE	AREA FRACTION FIBROUS CAP	RATIO AFLC/AFFC	NECROCTIC	LDL	HDL	LFTS	HEPATIC STEATOSIS
RABBIT 1HIW											
RABBIT 2HIW											
RABBIT 3HIW											
RABBIT 4HIW											
RABBIT 5HIW											
RABBIT 6HIW											
HISTOPROCTECTIVE EFFECTS OF WARBUGIA UGANDENSIS METHANOLIC EXTRACT											
		ATHEROSCLEROTIC LESION									
		OIL RED O/SUDAN III BLACK/CONGO RED				Plaque vulnerability index					
CODE	WT	NO	SITE	AREA/VOLUME FRACTION LIPID CORE	AREA FRACTION FIBROUS CAP	RATIO AFLC/AFFC	NECROCTIC	LDL	HDL	LFTS	HEPATIC STEATOSIS
RABBIT 1HPW											
RABBIT 2HPW											
RABBIT 3HPW											
RABBIT 4HPW											
RABBIT 5HPW											
RABBIT 6HPW											
HISTOINHIBITORY EFFECTS OF ATORVASTIN NORMAL DOSAGE											
		ATHEROSCLEROTIC LESION									
		OIL RED O/SUDAN III BLACK/CONGO RED				Plaque vulnerability index					
CODE	WT	NO	SITE	AREA FRACTION LIPID CORE	AREA FRACTION FIBROUS CAP	RATIO AFLC/AFFC	NECROCTIC	LDL	HDL	LFTS	HEPATIC STEATOSIS
RABBIT 1HIA											
RABBIT 2HIA											
RABBIT 3HIA											
RABBIT 4HIA											
RABBIT 5HIA											
RABBIT 6HIA											

CONTROL GROUP 1+A4:MA4:M31												
P +ve CONTROL GROUP ATHEROSCLEROTIC LESION INDUCED WITH NO INTERVENTION												
		ATHEROSCLEROTIC LESION										
		OIL RED O/SUDAN III BLACK/CONGO RED					OTHER FEATURES/ADJUNCT DATA					
CODE	WT	NO	SITE	AREA FRACTION LIPID CORE	AREA FRACTION FIBROUS CAP	RATIO AFLC/AFFC	NECROCTIC	LDL	HDL	LFTS	HEPATIC STEATOSIS	
RABBIT 1PC												
RABBIT 2PC												
CONTROL GROUP 2												
NEG -ve CONTROL GROUP RABBIT ON NORMAL DIET AND NO INDUCTION OF ATHEROSCLEROSIS												
		ATHEROSCLEROTIC LESION IF POSITIVE										
		OIL RED O/SUDAN III BLACK/CONGO RED					OTHER FEATURES/ADJUNCT DATA					
CODE	WT	NO	SITE	AREA FRACTION LIPID CORE	AREA FRACTION FIBROUS CAP	RATIO AFLC/AFFC	NECROCTIC	LDL	HDL	LFTS	HEPATIC STEATOSIS	
RABBIT 1NC												
RABBIT 2NC												
CONTROL GROUP 3												
VEHICLE CONTROL GROUP ATHEROSCLEROTIC LESION INDUCED THEN DMSO ADMINISTERED												
		ATHEROSCLEROTIC LESION										
		OIL RED O/SUDAN III BLACK/CONGO RED					OTHER FEATURES/ADJUNCT DATA					
CODE	WT	NO	SITE	AREA FRACTION LIPID CORE	AREA FRACTION FIBROUS CAP	RATIO AFLC/AFFC	NECROCTIC	LDL	HDL	LFTS	HEPATIC STEATOSIS	
RABBIT 1DMSO												

APPENDIX II: NACOSTI LICENSE

REPUBLIC OF KENYA

NATIONAL COMMISSION FOR SCIENCE,TECHNOLOGY & INNOVATION

Ref No: **462090**

Date of Issue: **28/July/2023**

RESEARCH LICENSE

This is to Certify that Mr.. SPENCER OPIYO OYUGI of Maseno University, has been licensed to conduct research as per the provision of the Science, Technology and Innovation Act, 2013 (Rev.2014) in Nairobi on the topic: HISTOMORPHOLOGICAL EFFECTS OF WARBURGIA UGANDENSIS EXTRACT ON ATHEROSCLEROTIC LESIONS IN AORTIC TUNICA INTIMA OF NEWZEALAND RABBITS UPON INDUCTION OF ATHEROSCLEROSIS for the period ending : 28/July/2024.

License No: **NACOSTI/P/23/28152**

462090

Applicant Identification Number

Director General
NATIONAL COMMISSION FOR SCIENCE,TECHNOLOGY & INNOVATION

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See overleaf for conditions

APPENDIX III: ETHICAL APPROVAL LETTER

JOMO KENYATTA UNIVERSITY OF AGRICULTURE AND TECHNOLOGY
P.O BOX 62000(00200) NAIROBI, Tel:(067) 58700001-4
(Office of the Deputy Vice Chancellor, Research Production and Extension Division)

JKUAT INSTITUTIONAL SCIENTIFIC AND ETHICS REVIEW COMMITTEE

REF: JKU/2/4/896B

Date: 15th June, 2023

SPENCER OPIYO OYUGI
DEPARTMENT OF HUMAN ANATOMY, SCHOOL OF MEDICINE
MASENO UNIVERSITY
P.O. Box 3275-40100, KISUMU, KENYA

Dear Mr. Oyugi,

RE: HISTOMORPHOLOGICAL EFFECTS OF WARBURGIA UGANDENSIS EXTRACT ON ATHEROSCLEROTIC LESIONS IN AORTIC TUNICA INTIMA OF NEWZEALAND RABBITS UPON INDUCTION OF ATHEROSCLEROSIS

This is to inform you that JKUAT Institutional Scientific and Ethical Review Committee has reviewed and approved your above research proposal. Your application approval number is **JKU/ISERC/02316/0891.** The approval period is **15th June 2023 to 14th June 2024.**

i. Only approved documents including (informed consents, study instruments, MTA) will be used
ii. All changes including (amendments, deviations, and violations) are submitted for review and approval by JKUAT ISERC.
iii. Death and life threatening problems and serious adverse events or unexpected adverse events whether related or unrelated to the study must be reported to JKUAT ISERC within 72 hours of notification
iv. Any changes, anticipated or otherwise that may increase the risks or affected safety or welfare of study participants and others or affect the integrity of the research must be reported to JKUAT ISERC within 72 hours
v. Clearance for export of biological specimens must be obtained from relevant institutions.
vi. Submission of a request for renewal of approval at least 60 days prior to expiry of the approval period. Attach a comprehensive progress report to support the renewal.
vii. Submission of an executive summary report within 90 days upon completion of the study to JKUAT ISERC.

Prior to commencing your study, you will be expected to obtain a research license from National Commission for Science, Technology and Innovation (NACOSTI) https://oris.nacosti.go.ke and also obtain other clearances needed.

Yours sincerely

Dr Patrick Mburugu

INSTITUTIONAL ETHICS REVIEW COMMITTEE
15 JUN 2023
APPROVED
P.O. Box 62000-00200 NAIROBI

APPENDIX IV: SGS APPROVAL LETTER

MASENO UNIVERSITY
SCHOOL OF GRADUATE STUDIES

Office of the Dean

Our Ref: MSC/SM/00015/020

Private Bag, MASENO, KENYA
Tel:(057)351 22/351008/351011
FAX: 254-057-351153/351221
Email: sgs@maseno.ac.ke

Date: 09th May, 2023

TO WHOM IT MAY CONCERN

RE: PROPOSAL APPROVAL FOR SPENCER OPIYO OYUGI — MSC/SM/00015/020

The above named is registered in the programme of Master of Science in Human Anatomy in the School of Medicine, Maseno University. This is to confirm that his research proposal titled **"Histomorphological Effects of *Warburgia Ugandensis* Extract on Atherosclerotic Lesions in Aortic Tunica Intima of Newzealand Rabbits upon Induction of Atherosclerosis"** has been approved for conduct of research subject to obtaining all other permissions/clearances that may be required beforehand.

Prof. J.O. Agure
DEAN, SCHOOL OF GRADUATE STUDIES

APPENDIX V APPROVAL FROM UNIVERSITY OF NAIROBI

UNIVERSITY OF NAIROBI
Department of Biology, P.O. Box 30197-001, Nairobi, Kenya.

16th June, 2023

Mr. Spencer Opiyo Oyugi
0721827327

REF: REQUEST TO CONDUCT EXPERIMENTS IN ANIMAL HOUSE

I have approved your request to conduct experiments in our animal house for a period of 10 weeks. This is in line with the need to foster interuniversity cooperation.

I note that your study is scientifically sound.

Liase with the animal house technologists, Mr. Joshua Ogat and Paul Ambugo.

Please note that you can only work in the presence of the animal house technologists for reasons of accountability, administration and **security.**

In addition, you will be expected to cater for your consumables, including animal feeds and bedding.

Regards,

Prof. Jacques M. Kabaru
Head, Biological Natural Products thematic area,
Coordinator, Animal House and Insectary,
Department of Biology
jkabaru@uonbi.ac.ke, Tel: 0722 582800

APPENDIX VI APPROVAL FROM KABARAK UNIVERSITY

SCHOOL OF MEDICINE AND HEALTH SCIENCES

DEPARTMENT OF BIOMEDICAL SCIENCES

P.O BRIVATE BAG 20157 KABARAK.

15TH APRIL, 2023

Mr. Spencer Opiyo Oyugi

0721827327

Cpenyugi@gmail.com

REF: REQUEST TO CONDUCT EXPERIMENTS IN OUR LABORATORIES

This is to certify that I have approved your request to conduct your research experiments in our laboratories for a period of one year. This is in line with the interuniversity collaboration.

We have looked at your study and it is scientifically sound in tandem with laboratory procedures.

On the date of commencing your studied liase with the in charge laboratory technologist Mr. Hadan Baiwo

NOTE: Please note that you can only work in our laboratories in the presence of technologist for reasons of security and safety. In addition, you are expected to cater for your consumables, including reagents and any other requirement that will not be available in our laboratories.

Regards,

Fred kipsang (Laboratory coordinator)

Msc Infectious diseases

Department of Biological Sciences

Kabarak university.

APPENDIX VII : DATA OUTPUT

```
SAVE OUTFILE='C:\Users\renegade\Desktop\spencer\spencer data.sav'
  /COMPRESSED.
DATASET ACTIVATE DataSet2.
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DATASET ACTIVATE DataSet2.
SAVE OUTFILE='C:\Users\renegade\Desktop\spencer\spencer data.sav'
 /COMPRESSED.
ONEWAY FRACTION BY GROUP
  /STATISTICS DESCRIPTIVES HOMOGENEITY
  /MISSING ANALYSIS
  /POSTHOC=BONFERRONI ALPHA (0.05).
```

Oneway

Notes

Output Created		03-SEP-2023 23:02:29
Comments		
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	Active Dataset	DataSet2
	Filter	<none>
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	N of Rows in Working Data File	261
Missing Value Handling	Definition of Missing	User-defined missing values are treated as missing.
	Cases Used	Statistics for each analysis are based on cases with no missing data for any variable in the analysis.
Syntax		ONEWAY FRACTION BY GROUP /STATISTICS DESCRIPTIVES HOMOGENEITY /MISSING ANALYSIS /POSTHOC=BONFERRONI ALPHA(0.05).
Resources	Processor Time	00:00:00.03
	Elapsed Time	00:00:00.83

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Descriptives

AREA FRACTION

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean
					Lower Bound
negative control	18	.15144	.010456	.002464	.14624
vehicle control	50	.51622	.037978	.005371	.50543
artovastatin histoinhibitory	52	.16821	.014631	.002029	.16414
warbugia histoinhibitory	50	.45584	.122204	.017282	.42111
artovastatin historestorative	44	.20461	.212845	.032088	.13990
warbugia historestorative	46	.35022	.077914	.011488	.32708
Total	260	.32765	.178001	.011039	.30591

Descriptives

AREA FRACTION

	95% Confidence Interval for Mean	Minimum	Maximum
	Upper Bound		
negative control	.15664	.140	.170
vehicle control	.52701	.460	.572
artovastatin histoinhibitory	.17228	.150	.195
warbugia histoinhibitory	.49057	.290	.632
artovastatin historestorative	.26932	.152	1.580
warbugia historestorative	.37335	.250	.487
Total	.34939	.140	1.580

Test of Homogeneity of Variances

AREA FRACTION

Levene Statistic	df1	df2	Sig.
7.851	5	254	.000

ANOVA

AREA FRACTION

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	5.170	5	1.034	86.493	.000
Within Groups	3.036	254	.012		
Total	8.206	259			

Post Hoc Tests

Multiple Comparisons

Dependent Variable: AREA FRACTION

Bonferroni

(I) GROUP	(J) GROUP	Mean Difference (I-J)	Std. Error	Sig.
negative control	vehicle control	-.364776*	.030054	.000
	artovastatin histoinhibitory	-.016767	.029900	1.000
	warbugia histoinhibitory	-.304396*	.030054	.000
	artovastatin historestorative	-.053169	.030591	1.000
	warbugia historestorative	-.198773*	.030397	.000
vehicle control	negative control	.364776*	.030054	.000
	artovastatin histoinhibitory	.348008*	.021656	.000
	warbugia histoinhibitory	.060380	.021867	.093
	artovastatin historestorative	.311606*	.022600	.000
	warbugia historestorative	.166003*	.022337	.000
artovastatin histoinhibitory	negative control	.016767	.029900	1.000
	vehicle control	-.348008*	.021656	.000
	warbugia histoinhibitory	-.287628*	.021656	.000
	artovastatin historestorative	-.036402	.022396	1.000
	warbugia historestorative	-.182006*	.022131	.000
warbugia histoinhibitory	negative control	.304396*	.030054	.000
	vehicle control	-.060380	.021867	.093
	artovastatin histoinhibitory	.287628*	.021656	.000
	artovastatin historestorative	.251226*	.022600	.000
	warbugia historestorative	.105623*	.022337	.000
atorvastatin historestorative	negative control	.053169	.030591	1.000
	vehicle control	-.311606*	.022600	.000
	artovastatin histoinhibitory	.036402	.022396	1.000
	warbugia histoinhibitory	-.251226*	.022600	.000
	warbugia historestorative	-.145604*	.023056	.000
warbugia historestorative	negative control	.198773*	.030397	.000
	vehicle control	-.166003*	.022337	.000

Multiple Comparisons

Dependent Variable: AREA FRACTION
Bonferroni

(I) GROUP	(J) GROUP	95% Confidence Interval	
		Lower Bound	Upper Bound
negative control	vehicle control	-.45383*	-.27572
	artovastatin histoinhibitory	-.10537	.07183
	warbugia histoinhibitory	-.39345*	-.21534
	artovastatin historestorative	-.14382	.03748
	warbugia historestorative	-.28885*	-.10870
vehicle control	negative control	.27572*	.45383
	artovastatin histoinhibitory	.28384*	.41218
	warbugia histoinhibitory	-.00442	.12518
	artovastatin historestorative	.24464*	.37858
	warbugia historestorative	.09981*	.23219
artovastatin histoinhibitory	negative control	-.07183	.10537
	vehicle control	-.41218*	-.28384
	warbugia histoinhibitory	-.35180*	-.22346
	artovastatin historestorative	-.10277	.02996
	warbugia historestorative	-.24758*	-.11643
warbugia histoinhibitory	negative control	.21534*	.39345
	vehicle control	-.12518	.00442
	artovastatin histoinhibitory	.22346*	.35180
	artovastatin historestorative	.18426*	.31820
	warbugia historestorative	.03943*	.17181
artovastatin historestorative	negative control	-.03748	.14382
	vehicle control	-.37858*	-.24464
	artovastatin histoinhibitory	-.02996	.10277
	warbugia histoinhibitory	-.31820*	-.18426
	warbugia historestorative	-.21392*	-.07728
warbugia historestorative	negative control	.10870*	.28885
	vehicle control	-.23219*	-.09981

Multiple Comparisons

Dependent Variable: AREA FRACTION

Bonferroni

(I) GROUP	(J) GROUP	Mean Difference (I-J)	Std. Error	Sig.
warbugia historestorative	artovastatin histoinhibitory	.182006*	.022131	.000
	warbugia histoinhibitory	-.105623	.022337	.000
	artovastatin historestorative	.145604*	.023056	.000

Multiple Comparisons

Dependent Variable: AREA FRACTION

Bonferroni

(I) GROUP	(J) GROUP	95% Confidence Interval	
		Lower Bound	Upper Bound
warbugia historestorative	artovastatin histoinhibitory	.11643*	.24758
	warbugia histoinhibitory	-.17181	-.03943
	artovastatin historestorative	.07728*	.21392

*. The mean difference is significant at the 0.05 level.