Evaluation of the *in vivo* anti-hyperlipidemic activity of the triterpene from the stem bark of *Protorhus longifolia* (Benrh.) Engl.

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2014

Evaluation of the *in vivo* anti-hyperlipidemic activity of the triterpene from the stem bark of *Protorhus longifolia* (Benrh.) Engl.

By

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A dissertation submitted to the Faculty of Science and Agriculture, University of Zululand, KwaDlangezwa, South Africa, in fulfillment of the requirements for the degree of Master of Science in the Department of Biochemistry and Microbiology.

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January 2014

DECLARATION

The work described in this dissertation was conducted in the Department of Biochemistry and Microbiology, Faculty of Science and Agriculture, University of Zululand, under the supervision of Prof. A.R. Opoku and Prof. T.G Djarova.

This study represents the original work by the author. Where use was made of the work of others, it has been duly acknowledged in the text.

I declare the above statement to be true.

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Kgothatso Eugene Machaba

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Prof. A.R. Opoku

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Prof. T.G Djarova

DEDICATION

This work is dedicated to my mother

Chabompya Elisa Machaba

ACKNOWLEDGEMENTS

I wish to acknowledge the support and advice received from Prof. A.R Opoku and Prof. T.G Djarova the supervisor and co-supervisor of my research project. Their guidance and support through times of trial kept me going, even when I was unsure of the outcome. The useful criticisms they gave during the process of writing this dissertation are highly appreciated. I thank you so much.

I appreciate the work done by Cobongela S.Z.Z and Mosa R.A. Their work has made my research easier. This research was supported by the MRC and the University of Zululand Research Committee.

Finally, I would like to thank my family and friends for putting up with me while I have been antisocial for the better part of the last few years. Specifically, thank you for your support to: Mr. & Mrs. Machaba (Mom and Dad), Oscar Machaba, Eunice Machaba, Joshua Machaba, Tendani Maemu, Austin, Ofentse, Papi, Gladwin and Stanley.

I give all the glory and honor to the Lord God Almighty, who was, and is, and is to become for giving me wisdom and understanding.

ABSTRACT

Hyperlipidemia is a condition characterized by elevated levels of lipids (fats), including cholesterol and triacylglycerol, circulating in the blood. Hypercholesterolemia is well known as a risk factor for cardiovascular diseases (CVD) such as atherosclerosis. This suggests a need for the discovery of effective and safe anti-hyperlipidemic agents, preferably of natural origin. Triterpenes are now targets for drug development due to their diverse, potential significant pharmacological activities. In this study, the *in vivo* lipid-lowering activity of the triterpene (Methyl-3β-hydroxylanosta-9,24-dienoate), isolated from crude chloroform extract from the stem bark of *Protorhus longifolia,* in high fat diet (HFD)-induced hyperlipidemic rats was investigated.

The pure compound isolated from crude chloroform extract (through column chromatography) was identified using nuclear magnetic resonance (NMR), mass spectrometry (MS), and infrared (IR) techniques. Rats were divided into two groups: normal diet (ND) and HFD. After 21 days of experiment period on their respective diets, rats were sub-divided into a total of nine (9) groups of four rats per group and treated with (50, 100, 200 mg/kg bw) the triterpene (KEM) for 15 days. At the end of the experimental periods, the rats were sacrificed and blood samples were collected for biochemical assays.

The results show that there were significant increases in total serum cholesterol (TC) and low-density lipoprotein cholesterol (LDL-c) with a reduction in high-density lipoprotein cholesterol (HDL-c) in HFD-induced hyperlipidemic rats after 21 days. Oral administration of the triterpene (50mg/kg, 100mg/kg and 200mg/kg) for a period of 15 days resulted in significant lowering of the levels of TC and LDL-C with an increase in HDL-c in HFD-induced hyperlipidemic rats. Significant decrease in atherogenic index and coronary risk index by the KEM was observed in HFD-induced hyperlipidemic rats.

It is concluded that KEM could contribute to new formulation with significant hyperlipidemic effects.

LIST OF ABBREVIATIONS USED

AI	Atherogenic index
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
ANOVA	One-way analysis of variance
Apo	Apolipoproteins
AST	Aspartate aminotransferase
CRI	Coronary risk index
CVD	Cardiovascular diseases
EtOA	Ethyl acetate
FC	Food conversion
FER	Food efficiency ratio
GIT	Gastrointestinal tract
GLU	Glucose
H_2SO_4	Sulphuric acid
HDL	High density lipoproteins
HFD	High fat diet
HX	Hexane

IDL	Intermediate density lipoproteins
IR	Infrared
LDL	Low density lipoproteins
LIP	Lipase
LP	Lipoprotein lipases
MS	Mass spectrometry
Na_2HPO_4	di-sodium hydrogen orthophosphate
NaH_2PO_4	Sodium di-hydrogen orthophosphate
ND	Normal group
NMR	Nuclear magnetic resonance
TG	Triacylglycerol
THA	Trihydroxyacetophenone
TLC	Thin layer chromatography
TP	Total protein
UREAL	Urea
US	United States of America
VLDL	Very low density lipoprotein
WHO	World Health Organisation

CONTRIBUTION TO KNOWLEDGE

(See appendix E for details)

Machaba K.E, Cobongela S.Z.Z, Mosa R.A, Djarova T.G, Opoku A.R. *In vivo* hypo-lipidemic activity of methyl-3β-hydroxylanosta-9,24 dienoate from the stem *bark of Protorhus longifolia* (Benrh) Engl. Indigenous plant use forum (IPUF), 1-4 July 2013. Nelspruit, Mpumalanga, South Africa (*was awarded the 3rd prize for best presentation*)

K. Eugene Machaba, A.R Opoku, T.G Djarova. Evaluation of the *in vivo* anti-hyperlipidemic activity of the triterpene from the stem bark of *Protorhus longifolia* (Benrh.) Engl. 8th Annual faculty of science and agriculture research symposium 2013, 1st November 2013. University of Zululand, South Africa (*was awarded the 1st prize for best presentation*)

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CHAPTER 1

INTRODUCTION

Lipids; cholesterol and triacylglycerol, are one of the macronutrients that provide the body with energy. Cholesterol in the blood is mainly from diet even though some is usually created by the body in the liver**.** Triacylglycerol is normally from the breakdown of fat in the diet.

Consumption of a high-lipid diet may create diverse patterns of hyperlipidemia. Hyperlipidemia refers to abnormally elevated levels of any or all lipids in the blood. It is classified according to which types of lipids are elevated, hypercholesterolemia and/or hypertriglycerolemia. Hyperlipidemia is a serious public health problem both in developed and developing countries. It is considered as one of the five leading causes of death in the world (Thayyil et al., 2011).

Treatment of hyperlipidemia involves changes in lifestyle (diet control and exercise) as initial treatment but if lifestyle modifications are not improving lipid levels then medication management may be appropriate. Statins, one of the most commonly prescribed medications for treating hyperlipidemia, lower cholesterol levels. However, these drugs reportedly have some adverse effects. This suggests a need for the discovery of more effective and safer anti-hyperlipidemic agents, preferably of natural origin.

For many years, medicinal plants have been used to treat infections and diseases. Plants are rich sources of biologically active compounds, such as triterpenes, vital to human health. Medicinal plants contribute more than 50% of all drugs in clinical use today (Van Wyk *et al.,* 2009). Triterpenes isolated from *Protorhus longifolia* have been reported to exhibit anti-inflammatory, anti-platelet aggregation, and *in vitro* anti hyperlipidemic activities in our laboratories (Mosa, 2011). This present study aims to investigate the *in vivo* hypolipidemic activity of the triterpene from *Protorhus longifolia* in rats.

1.1 STRUCTURE OF THE DISSERTATION

Chapter 1: This chapter gives a brief introduction and the motivation for such a study.

Chapter 2: This chapter presents a detailed background and the literature review of hyperlipidemia.

Chapter 3: This chapter gives a brief description of the various materials and methods used in this study.

Chapter 4: This chapter presents the results obtained when the triterpene was screened for hypolipidemic activity

Chapter 5: This chapter gives a discussion of the results obtained in this study.

Chapter 6: This chapter delivers a brief conclusion about the study.

CHAPTER 2

LITERATURE REVIEW

Cholesterol and triacylglycerol (fig. 2.1) are the two major types of lipids in the blood and are important building blocks in the structure of cells and are also used in making hormones and producing energy. Both or either of these may be raised, creating diverse patterns of hyperlipidemia.

2.1 LIPID METABOLISM

Cholesterol is classified as animal sterol and triacylglycerol is an ester derived from three fatty acids and glycerol. Cholesterol in the blood is mainly from diet even though some is usually synthesised by the body in the liver**.** Triacylglycerol is normally from the breakdown of fat in the diet.

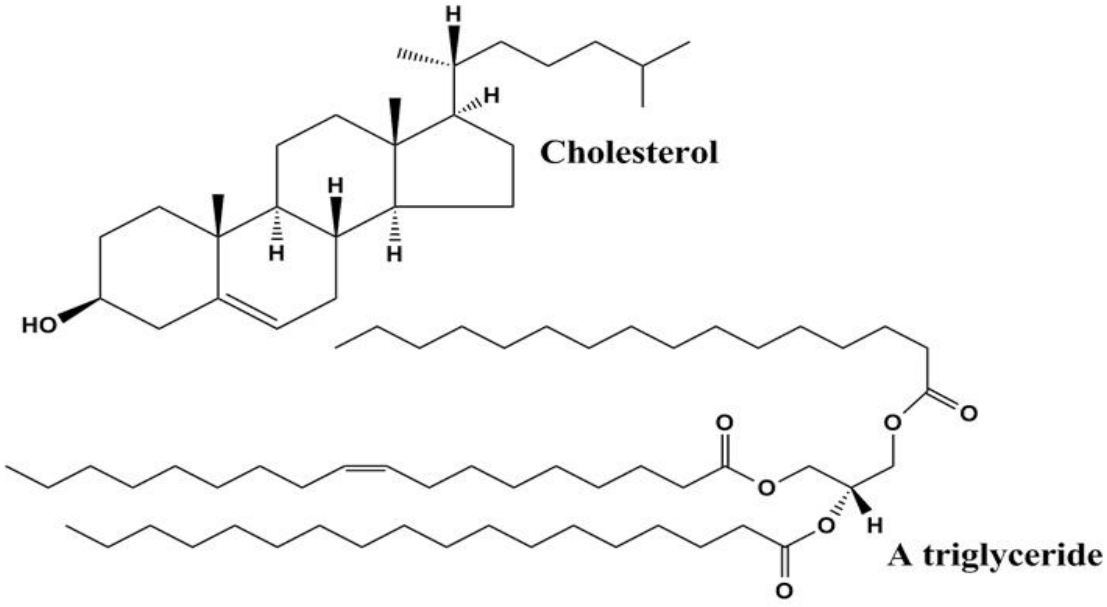


Figure 2.1: Chemical structural difference between cholesterol and triglyceride (MacManus, 2008).

Cholesterol is one of the major components of cellular membranes; it regulates and stabilizes the fluidity of the outer cell membrane, thus determines the fluidity of lipid bilayers (Brown and Goldstein 1986). In addition, this sterol is an essential precursor of vitamin D, bile salts and steroid hormones such as cortisol and testosterone (Rodriguez-Agudo *et al.,* 2008).

Triacylglycerol accumulated in the cellular cytoplasm, particularly in adipocytes, provides an essential reserve of energy for tissue. In addition, triacylglycerol plays a central role as energy supply for skeletal and cardiac muscles, according to cellular demand.

2.2 LIPOPROTEIN METABOLISM: Lipids Digestion & Transport

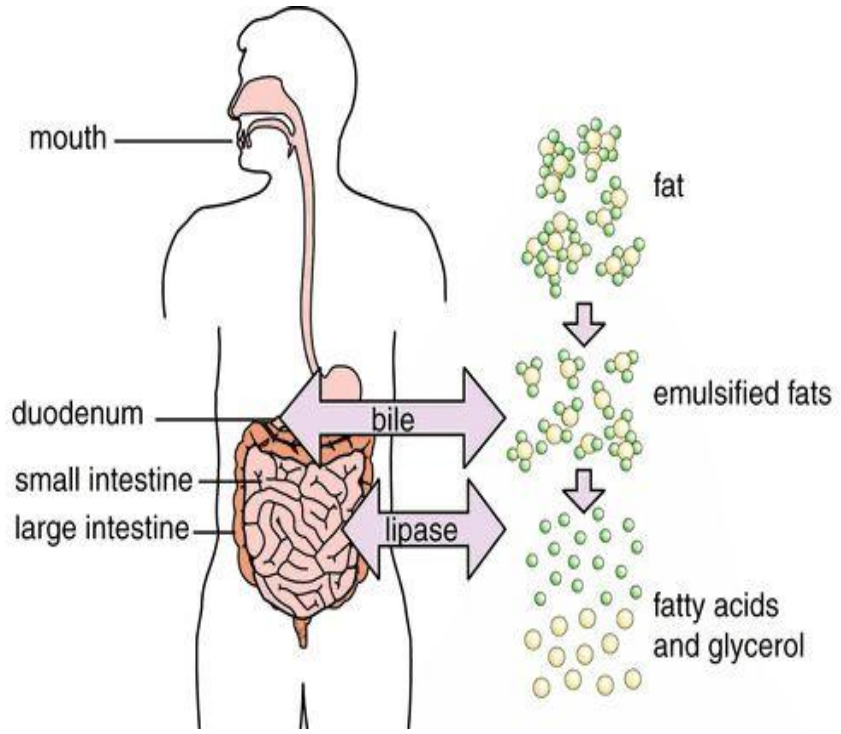
Dietary lipids in the form of triacylglycerol (TG), require digestion and absorption during the passage through the gastrointestinal tract (GIT) (Bauer *et al.,* 2004).

Gastrointestinal lipid digestion consists of several sequential steps that include physicochemical and enzymatic events: **(I)** the dispersion of bulk fat globules into divided emulsion-water particles, **(II)** the enzymatic hydrolysis of fatty acid esters, starts in the stomach with the action of gastric lipase, at the emulsion-water interface, **(III)** digestion continues in the duodenum with the synergetic action of gastric and colipase-dependent pancreatic lipase and **(IV)** the desorption and dispersion of insoluble lipid products into an absorbable form (Bauer *et al.,* 2004; Carey *et al.,* 1983).

After digestion, dietary fats are converted into more polar derivatives with a higher degree of interaction with water i.e. triacylglycerols are broken into fatty acids and monoglycerides, and the esters of cholesterol are broken into fatty acids and non-esterified cholesterol associate with bile salts and phopholipids to form micelles.

Micelles transport the poorly soluble monoacylglycerols and fatty acids to the surface of the enterocyte where they are absorbed. Before the absorption, breaking down and re-forming of micelles takes place because of their nonpolar nature. Only freely dissolved

monoacylglycerols and fatty acids from triacylglycerol are absorbed by diffusion across the plasma membrane of the enterocyte (fig. 2.2).

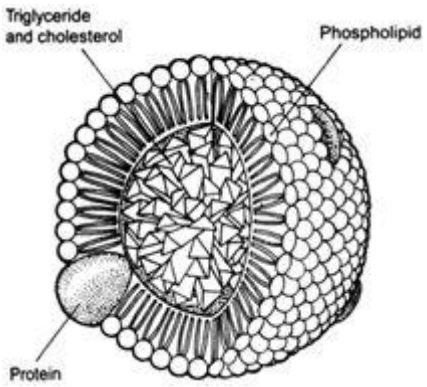


(http://www.talktalk.co.uk/reference/encyclopaedia/hutchinson/m0007992.html)-- (2013)

Figure 2.2: The digestion of fat. Fat is broken down into fatty acids and glycerol by bile and the enzyme lipase.

At the end point of digestion, triacylglycerol and cholesterol are transported through the body via blood and lymph to their site of utilization or storage. This is best achieved by the formation of lipid-protein complexes called lipoproteins.

Lipoproteins are soluble complexes of apolipoproteins (Apo), major components of lipoproteins, and lipids (phospholipid, cholesterol and triacylglycerol) that transport lipids through the body via blood (fig.2.3). As part of lipoproteins, the cholesterol and triglycerides can be carried around in the bloodstream throughout the body.

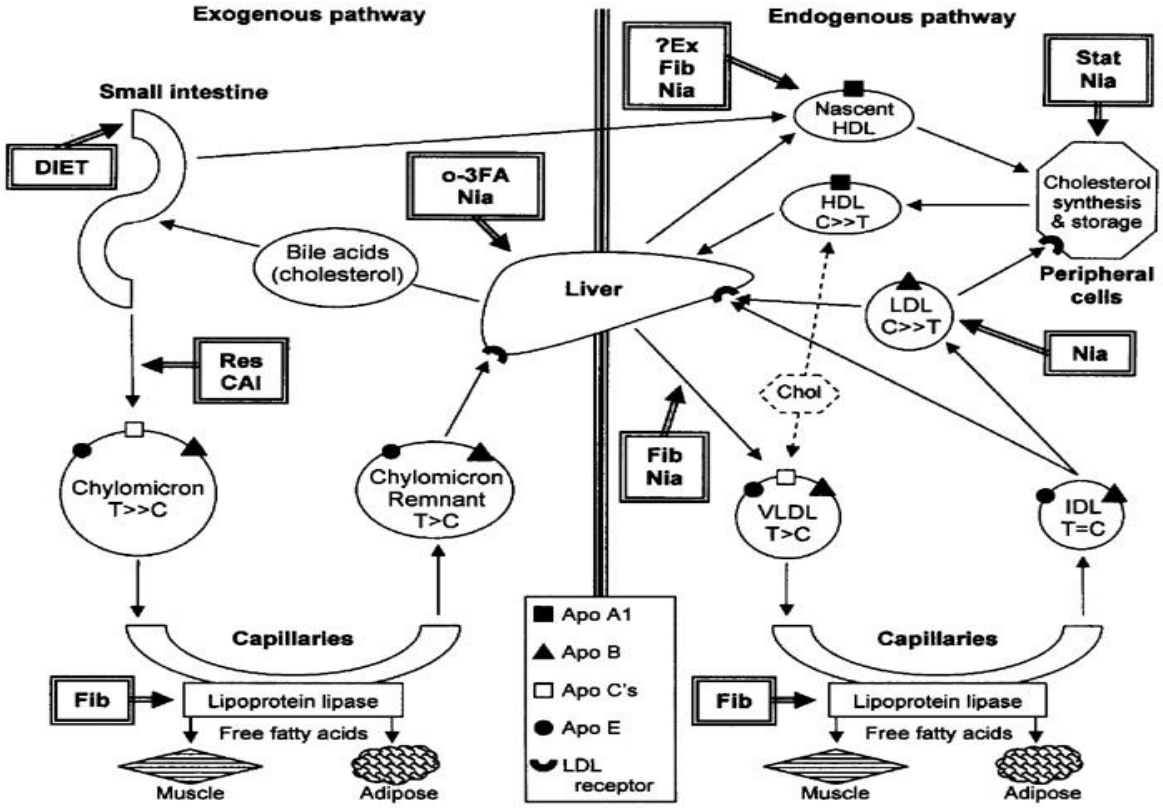


(http://www.answers.com/topic/lipoprotein) – (2013)

Figure 2.3: A lipoprotein

Lipoproteins are classified based on their density. The lowest density lipoproteins are the chylomicrons followed by very low density lipoproteins (VLDLs), intermediate density lipoproteins (IDLs), low density lipoproteins (LDLs), and high density lipoproteins (HDLs). The higher the protein content the higher the density of the lipoprotein. Chylomicrons have only 1-2% protein while HDL have about 50% protein by weight i.e. the greater the quantity of apoproteins, the greater the density, and the greater the quantity of TG, the lower the density (Vance and Vance, 2002; Alves and Lima, 2008).

The transport of lipid by lipoproteins in plasma has been described as involving two pathways: Exogenous and endogenous pathways (fig.2.4) (Walker *et al.,* 1990).



(http://www.montana.edu/wwwrjf/dyslipidemia/dyslipidemia1.html) – (2013)

Figure 2.4: Normal Lipoprotein Biochemistry and Physiology

(a) Exogenous Pathway is the transport of cholesterol and triglycerides absorbed from dietary fat in the intestine. Dietary cholesterol and triacylglycerol are absorbed through the wall of the intestine and packaged into chylomicrons.

In the blood vessels, chylomicrons bind to the membrane of adipose and muscle tissues, where the triacylglycerols are hydrolyzed by lipoprotein (LP) lipases into fatty acids. These fatty acids then pass through the membrane and reach the adipocytes where they are resynthesized into triacylglycerols and stored. In the muscle, the fatty acids are oxidized to provide energy.

As the tissues absorb the fatty acids, the chylomicrons progressively shrink to chylomicrons remnants, smaller and richer in cholesterol. The chylomicrons remnants reach the liver, and are rapidly absorbed.

(b) Endogenous Pathway is the transport of cholesterol and triglycerides from the liver. In the liver, cholesterol either is secreted into the intestine, mostly as bile acids, or is packaged with triacylglycerols in very-low-density lipoprotein (VLDL) and secreted into the blood circulation.

Like chylomicrons, VLDL in blood vessels bind to the membrane of adipose and muscle tissues, where the triacylglycerols are hydrolyzed by LP lipases into fatty acids. These fatty acids then pass through the membrane and reach the adipocytes where they are once again resynthesized into triacylglycerols and stored. In the muscle the fatty acids are oxidized to provide energy.

The VLDL shrink, as the tissues absorb the fatty acids, to cholesterol enriched remnants forming intermediate-density lipoprotein (IDL), which contain a relatively high cholesterol content. Some of IDL are absorbed by the liver or further catabolized by LP lipases and the remnants stay in the circulation and are converted into LDL by hepatic lipase (Miles, 2003; Walker *et al.,* 1990; Rader and Hobbs, 2008).

LDL is the main plasma cholesterol carrier, delivering cholesterol to the liver and peripheral tissues; it contains relatively high cholesterol content (Miles, 2003). Absorption of LDL occurs through endocytosis. LDL tends to deposit part of the cholesterol into artery walls and other body tissues such as tendons; LDL is mainly responsible for hypercholesterolemia (Krieger, 1998). LDL is commonly called the bad cholesterol.

The liver and intestinal cells synthesize HDL, active in the reverse transport of cholesterol; they remove excess cholesterol and carry the excess to the liver to

be metabolized into bile salts. HDL are associated with lower levels of heart disease, therefore higher levels of HDL promote vascular health by extracting cholesterol from tissues and delivering it back to the liver (Daniels *et al.,* 2009).

2.3 LIPIDS DISORDER: Hyperlipidemia

Hyperlipidemia (also called hyperlipoproteinemia) is a broad term that refers to abnormally elevated levels of any or all lipids and/or lipoproteins in the blood (Hassan, 2013). It is also identified as dyslipidemia, any abnormal lipid levels, elevated levels of TC or TG, or low levels of HDL.

Hyperlipidemia is classified according to which types of lipids are elevated, hypercholesterolemia, in particular LDL-c, and/or hypertriglycerolemia (Hor et al., 2011). Hyperlipidemia can also be classified according to elevated levels of lipoprotein such as VLDL and/or LDL.

Hyperlipidemia is basically divided into two subtypes which are: primary hyperlipidemia and secondary hyperlipidemia. **Primary hyperlipidemia** is caused by specific genetic problems i.e., mutation within receptor protein, while **Secondary hyperlipidemia** (also called acquired) will arise as a result from another underlying disorder like diabetes (Hassan, 2013).

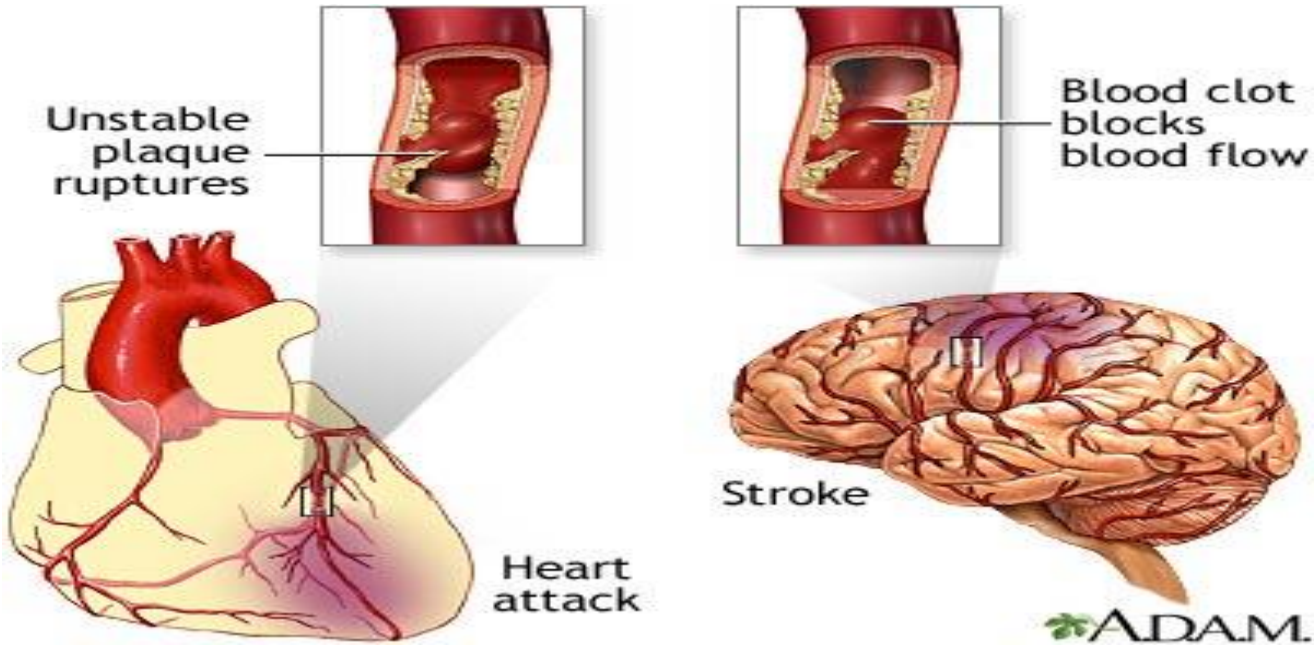
There are critical factors that play a role in hyperlipidemia incidence such as family history, diabetes mellitus, renal failure, hypothyroidism, alcoholism and smoking, hypertension, obesity and unhealthy diet intake (Omae *et al.,* 2006; O'Brien *et al.,* 1993; Hassan, 2013). Several factors including consumption of a high-fat diet, modern lifestyle and hypertension, have been reported to cause heart failure (Hor *et al.,* 2011)

Because of a modern lifestyle and an increase in consumption of a high-fat diet, hyperlipidemia is a serious public health problem both in developed and developing countries (Jacobson *et al.,* 2007). It is considered as one of the five leading causes of death in the world (Thayyil *et al.,* 2011).

Chronic hyperlipidemia is crucial to the progression of cardiovascular, cerebrovascular and metabolic syndrome diseases (Adisakwattana *et al.,* 2010). It is estimated that by 2030 almost 23.6 million people will die from cardiovascular diseases (WHO, 2011).

Atherosclerosis is the formation of plaque which builds up inside the arteries, as a result of the accumulation of fatty materials such as cholesterol and triglyceride. Arteries are blood vessels that carry oxygen-rich blood to the brain, heart and other parts of the body.

Over time, formation of plaque narrows the arteries. This limits the flow of oxygen-rich blood to the brain, heart and other parts of the body. A heart attack or stroke may occur when an area of plaque ruptures and a clot forms over the location (fig.2.5). The formation of plaque in arteries, atherosclerosis, can lead to serious problems such as heart attack, stroke, or even death (Jasmin, 2012).



(http://www.nlm.nih.gov/medlineplus/ency/imagepages/19314.htm) – (2013)

Figure 2.5: Cause of heart attack or stroke as a result of narrowing of the arteries by atherosclerosis.

The incidence of atherosclerotic disease such as coronary heart disease and stroke can be lower in younger women than in men of the same age but increases after menopause (Akishita, 2004).

It has been reported that atherosclerotic cardiovascular diseases affect millions of adults in the United States of America (U.S.), leading to morbidity and mortality (Lim *et al.,* 2011). The burden of cardiovascular diseases was predicted to increase rapidly in developing countries (Schamroth, 2012). The known risk factors for a heart attack are present in South Africans. In South Africa the highest death rates for heart and blood vessel diseases are found in Indian people, followed by the coloured people, while the white and black African people have the lowest rates (Norman *et al.,* 2006). About 195 people died per day, between 1997 and 2004, because of some form of heart and blood vessel disease (CVD) in South Africa. In addition, about 60 die per day because of stroke, while 33 people die per day because of a heart attack (Krisela, 2007).

2.4 TREATMENT OF *LIPIDS DISORDER*: Hyperlipidemia

Lifestyle, such as diet control and exercise, is the initial treatment of hyperlipidemia. However, if lifestyle modifications are not improving lipid levels then medication management may be appropriate.

The inhibition of digestion of dietary lipids, therefore limiting their intestinal absorption, could be an ideal therapeutic intervention. Antihyperlipidemic agents have been available for several decades (Miller, 2001). Medication management, such as drugs, that are used for treating hyperlipidemia are statins, fibrates, niacin, and bile acid sequestrants.

Statins, one of the most commonly prescribed medications for treating hyperlipidemia, lower cholesterol levels; Statins are a class of drugs such as lovastatin, Simvastatin, Atorvastatin and Rosuvastatin that inhibit the enzyme HMG-CoA reductase, which plays a central role in the production of cholesterol in the liver.

Statins slow down the body's ability to make cholesterol by targeting hepatocytes and inhibit HMG-CoA reductase, the enzyme that converts HMG-CoA into mevalonic acid, a cholesterol precursor. Since statins are similar to HMG-CoA on a molecular level they take the place of HMG-CoA in the enzyme and reduce the rate by which it is able to produce mevalonate. This is significant because most circulating cholesterol is synthesized from the liver. When the liver can no longer produce cholesterol, levels of cholesterol decrease. (Suresh Pichandi *et al.,* 2011; Stancu and Sima, 2001)

However, these drugs reportedly have some adverse effects: Certain statins are associated with liver function abnormalities and skeletal muscle complaints, including clinically important myositis and rhabdomyolysis, elevated CK levels, muscle weakness, and muscle cramps (Thompson *et al.,* 2003; Hor *et al.,* 2011).

This suggests a need for the discovery of more effective and safe anti-hyperlipidemic agents, preferably of natural origin

2.5 INDIGENOUS USE OF PLANTS

Medicinal plants have played an essential role in the development of human culture. For many years, medicinal plants have been used to treat infections and diseases. These plants are rich sources of biologically active compounds (phytochemicals) vital to human health. These compounds have no nutritive value in plants but are responsible for the various medicinal activities attributed to medicinal plants (Motlhanka *et al.,* 2010).

Medicinal plants contribute more than 50% of all drugs in clinical use today and higher plants contribute no less than 25% of the total (Van Wyk *et al.,* 2009). Phytochemicals can be developed as drugs directly or provide novel structural templates (Kee *et al*., 2008). Some of the modern medicines such as antimicrobial, anticancer, anti-inflammatory, anti-hyperlipidemic etc. agents are produced indirectly from medicinal plants.

It is estimated that 80 percent of the world population presently uses herbal medicine for health care (Mishra *et al.,* 2010). In developing countries most people use traditional medicine; in South Africa an estimated 250 000 traditional healers supply healthcare to around 80% of the black population and in China, traditional herbal preparations account for 30–50% of the total medicinal consumption. (Edinburg, 1998).

A number of plants and natural products have been screened for their anti-lipid activity. Such studies include Grape Seed Extract (GSE) that significantly inhibited pancreatic lipase and cholesterol esterase in a dose-dependent manner (Adisakwattana *et al.,* 2010). *Coriolus versicolor* (CV) significantly reduced serum TC and LDL-C levels in a dose-dependent manner in rats (Hor *et al.,* 2011). A compound (2-phenylethyl 2,6 dihydroxybenzoate) isolated from the ethanolic extract of *Geophila herbacea*, exhibited *in vitro* lipid-lowering bioactivity (Luo *et al.,* 2011). 2, 4, 6-Trihydroxyacetophenone (THA) isolated from *Myrcia multiflora* has anti-obesity and mixed hypolipidemic effects with the reduction of lipid intestinal absorption. In addition, THA cause greater total cholesterol (37%) and triglyceride (46%) serum reduction than lovastatin (Ferreira *et al.,* 2011). A bioactive phytochemical, protodioscin, isolated from the rhizomes of *Dioscorea nipponica* was identified for its antihyperlipidemic affects (Wang *et al.,* 2010). Medicinal plants, like *P. longifolia*, are now new targets for drug development due to their diverse potentially significant pharmacological activities.

2.5.1 *Protorhus longifolia* (Benrh.) Engl.

Protorhus longifolia is the only species in the genus Protorhus of the family anacardiaceae (Mango family). *P longifolia* is found in Southern Africa while about 20 of the other species are found in Madagascar (Archer, 2000).

P. longifolia is an evergreen, indigenous tall tree (up to 15 m), which usually has some yellow-red coloured leaves (fig.2.6). The plant is also known as red beech (English), unhlangothi (Zulu) and Uzintlwa (Xhosa). It grows in the forest, on river banks, and woodland. In South Africa, the plant is distributed in KwaZulu Natal, Eastern Cape, Limpopo Province and Mpumalanga.

Figure 2.6: A picture of Protorhus longifolia (Naidoo, 2012)

P. longifolia is one of the commonly used plants by Zulu traditional healers in the management of blood-clotting related diseases (Mosa *et al.,* 2011).

Some scientific work has been done to evaluate the biological activities of *P. longifolia*. Antimicrobial activity of the leaf extracts has been reported (Suleiman *et al.,* 2009; 2010). Antioxidant, cytotoxic and anti-platelet aggregation activities of the crude extracts of the plants have recently been investigated (Mosa, 2011). Two lanosteryl triterpenes (fig 2.7) with anti-platelet aggregation and anti-inflammatory activities have been isolated and characterized from extracts of the stem bark of the plant (Mosa *et al.,* 2011).

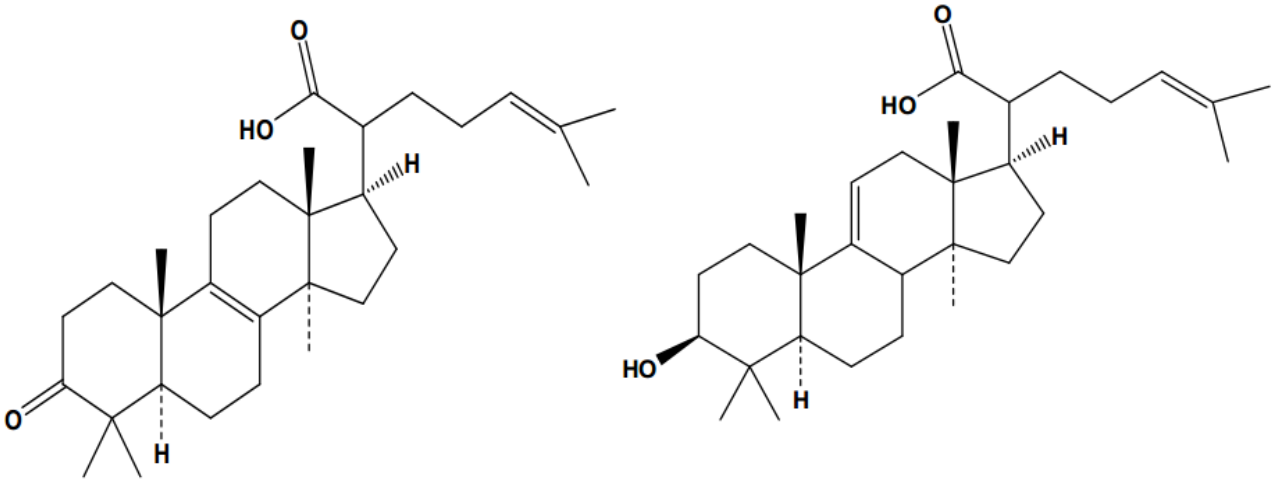


Figure 2.7: Chemical structures of lanosteryl triterpenes 3-oxo-5α-lanosta-8,24-dien-21-oic acid and 3β-hydroxylanosta-9,24-dien-24-oic acid (Mosa *et al.,* 2011)

2.5.2 Triterpenes

Triterpenes are a group of active natural compounds (widely distributed in the plant kingdom) consisting of six isoprene units; they have the molecular formula $C_{30}H_{48}$, (Melanie, 2009). They are typically in white crystalline form. As with most beneficial substances in plants, it protects the plant from microbial infection.

It is well-recognized that triterpenes have long been used as glues, pigments, waxes, fibers, flavors and polymers. Herbal products containing triterpenes are widely prescribed to prevent or treat a variety of diseases by traditional healers in many Asian countries (Parmar *et al.,* 2013).

Over 20,000 triterpenes are known to occur in nature. Among them, tetracyclic and pentacyclic triterpenes (fig 2.8) are the most abundant (Michaudel, 2013). Tetracyclic triterpenes such as oleandrin and euphol are methylated steroids. Pentacyclic triterpenes are divided into many subgroups: ursane, lupanes, etc. based on their carbon skeleton (Michaudel, 2013; Parmar *et al.,* 2013).

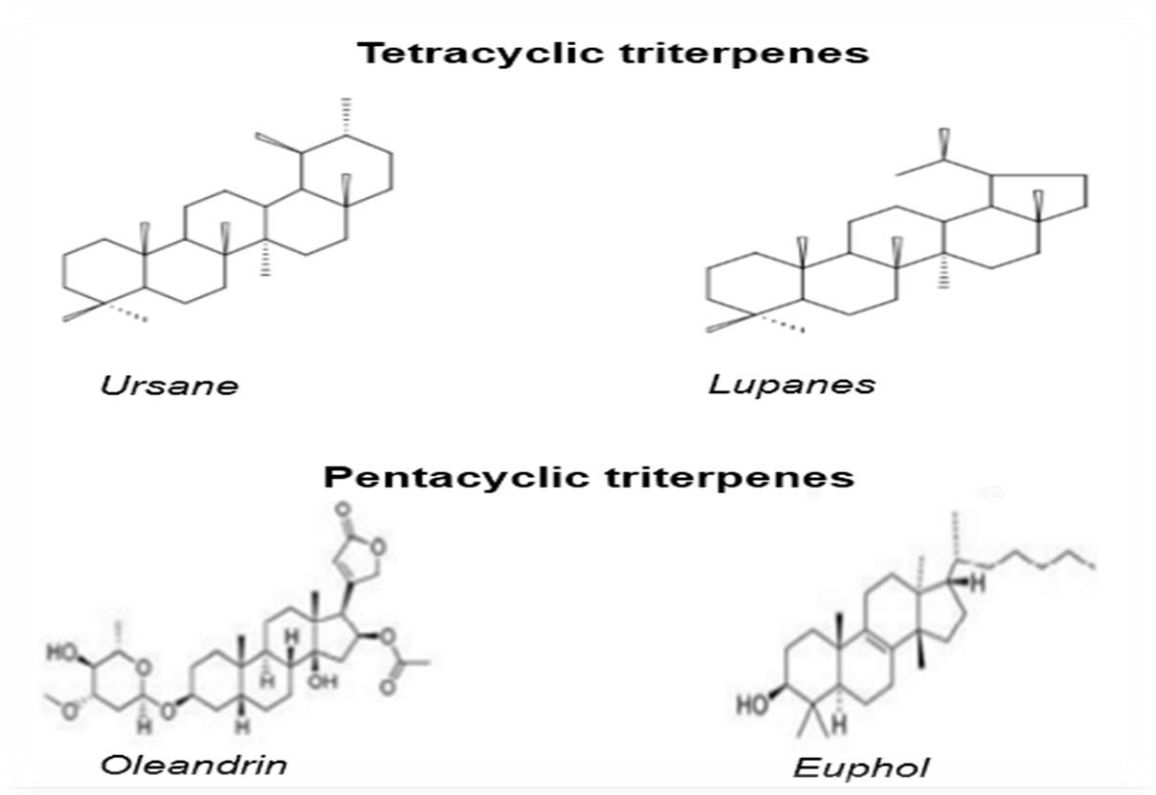


Figure 2.8: Chemical structures of tetracyclic and pentacyclic triterpenes (Patocka, 2003; Parmar *et al.,* 2013)

Triterpenes are now new targets for drug development due to their diverse potentially significant pharmacological activities such as anti-hyperglycaemic (Ghosh *et al.,* 2010), antihyperlipidemic (Liu *et al.,* 2007), anticancer (Zhou *et al*., 2008), and antimicrobial activity (Mallavadhani *et al.,* 2004).

The *in vitro* anti-hyerlipidemic activity of the triterpenes from *P. longifolia* has been established in our laboratories (unpublished data). Thus, this study aims at evaluating the *in vivo* anti-hyperlipidemic activity of the triterpenes from the stem bark of *P. longifolia.*

2.6 OBJECTIVE AND SCOPE OF THE WORK

Chronic hyperlipidemia is crucial to the progression of cardiovascular, cerebrovascular and metabolic syndrome diseases. The currently available anti-hyperlipidemic agents have limited efficacy and are associated with undesirable side effects. Thus, there is a need to discover and develop new and more effective anti-hyperlipidemic agents of natural origin.

Screening of such plants, as used by the traditional healers, will give scientific validation to this traditional practice. This could also give a lead to the discovery of new pharmacologically active drugs.

2.6.1 Aim

The aim of this study was to investigate the *in vivo* hypolipidemic activities of the triterpene from *Protorhus longifolia* in rats.

2.6.2 Objective

- Collection and identification of the plant material
- Isolation, identification and characterization of the triterpene from the plant
- Administer the triterpene to various rat groups
    - Determine the activity of the compound on liver function.
    - Determine the cholesterol, triacylglycerol and other serum lipids.
    - Histology of the liver of the rats.

CHAPTER 3

MATERIALS AND METHODS

This chapter gives a brief description of materials and methods used to extract, isolate, identify and characterize the compound from the plant. It also describes the administration of the triterpene to various rat groups and the various biochemical tests carried out. The details of the preparation of reagents and the methods are presented in Appendix A and B, respectively.

3.1 MATERIALS

3.1.1 Equipment

- ✓ Rotary evaporator - Heidolph Instruments.
- ✓ Grinding mill (IKA) - Polychem supplies.
- ✓ Platform shaker (Labcon) - Polychem supplies.
- ✓ Eppendorf centrifuge 5804 R, - Merck.
- ✓ TLC plates (silica gel 60 TLC aluminium sheets 20cm x 20 cm, F254) - Merck.
- ✓ Stuart smp11 (melting point) – Shalom laboratory supplies.
- ✓ Tensor 27 FT-IR – Bruker
- ✓ Kern pls 3100-2f (Precision Balance scale) - Polychem supplies.
- ✓ Cobas c111 analyzer - Roche.

3.1.2 Chemicals and reagents

I. *Reagents supplied by Merck:*

Silica gel (60 0.063-02 mm), purified sea sand, hexane, chloroform, ethyl acetate, Methanol, Sodium di-hydrogen orthophosphate and di-sodium hydrogen orthophosphate.

II. *Reagents supplied by Sigma-Aldrich:*

Cholesterol, pig bile salt, 4-Hdroxy-2-mercapto-6-methylpyrimidine (thimecil), lovastatin and tween-20.

3.2 METHODS

3.2.1 Plant material

Fresh stem bark of *Protorhus longifolia* (Benrh.) Engl. were obtained from Kwa-Hlabisa, Kwa-Zulu Natal, South Africa. The botanical identification of the plant was done by the staff of the Botany Department of the University of Zululand. Voucher specimens (RA01UZ) were prepared and stored at the University herbarium. The plant material was thoroughly washed with tap water and then air dried. The air dried plant material was ground into powder (2mm mesh) and stored in sterile brown bottles until use.

3.2.2 Extraction, isolation and purification

The powdered plant material was defatted with hexane and then extracted (1:5 w/v) with chloroform (24h, orbital shaker, 150rpm). The extract was concentrated to give a dry extract. The crude chloroform extract was subjected to silica gel column chromatography (20mm x 500mm; silica gel 60; 0.063 – 0.2mm; 70-230 mesh ASTM), eluted with hexane: ethyl acetate gradient (9:1 - 3:7). The eluted fractions were collected at intervals of 20 ml each. Thin layer chromatography (TLC) was used to analyze the fractions. The TLC plates were first developed using a 10% H_2SO_4 spray reagent and then heated. The fractions with similar profile were combined and concentrated *in vacuo* (see figure 3.1). The pure compound, white crystals (sugar-like), from the plant material was identified using nuclear magnetic resonance (NMR), mass spectrometry (MS), infrared (IR) techniques and melting point to be similar to the compound extracted by Mosa *et al* (2011).

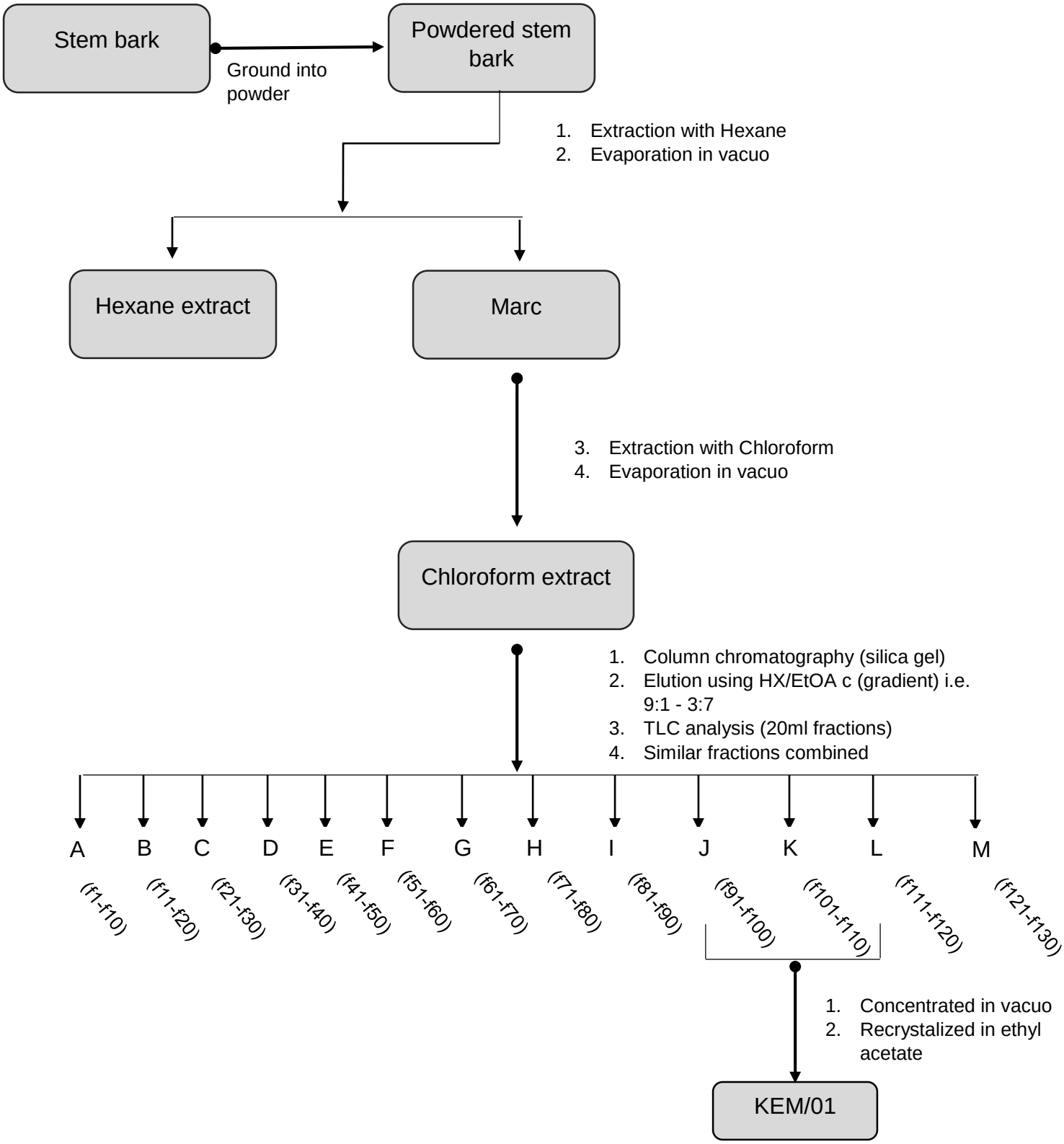


Figure 3.1: Schematic presentation of the extraction, isolation and purification of KEM/01.

3.2.3 Animals

This study was approved (S504/13) by the University of Zululand Research Animal Ethics Committee (see Appendix C). Sprague-Dawley rats were collected from the animal house in the Department of Biochemistry, University of Zululand, South Africa.

a) Experimental design

Experimental procedures were conducted following the guideline for care and supervision of experiments on animals. The animals were housed in standard cages and maintained at room temperature with 12:12-h light: dark cycle. All the rats had free access to water and normal pellet diet in the experimental environment for 1 week, before the experiments were conducted.

Once the animals had adapted to the environment, Forty four (44) Sprague-Dawley rats (140 – 200 g) were divided into two groups; normal group (ND) consisting of 20 rats and high fat diet (HFD) group consisting of 24 rats. After 21 days on their respective diet, 4 rats per group were sacrificed and the remaining rats were sub-divided into a total of nine (9) groups of four rats per group (Figure 3.2). Different amounts (50, 100, 200 mg/kg bw) of the compound were incorporated into the normal and HFD diets and the rats were further fed their respective diets for a further 15 days.

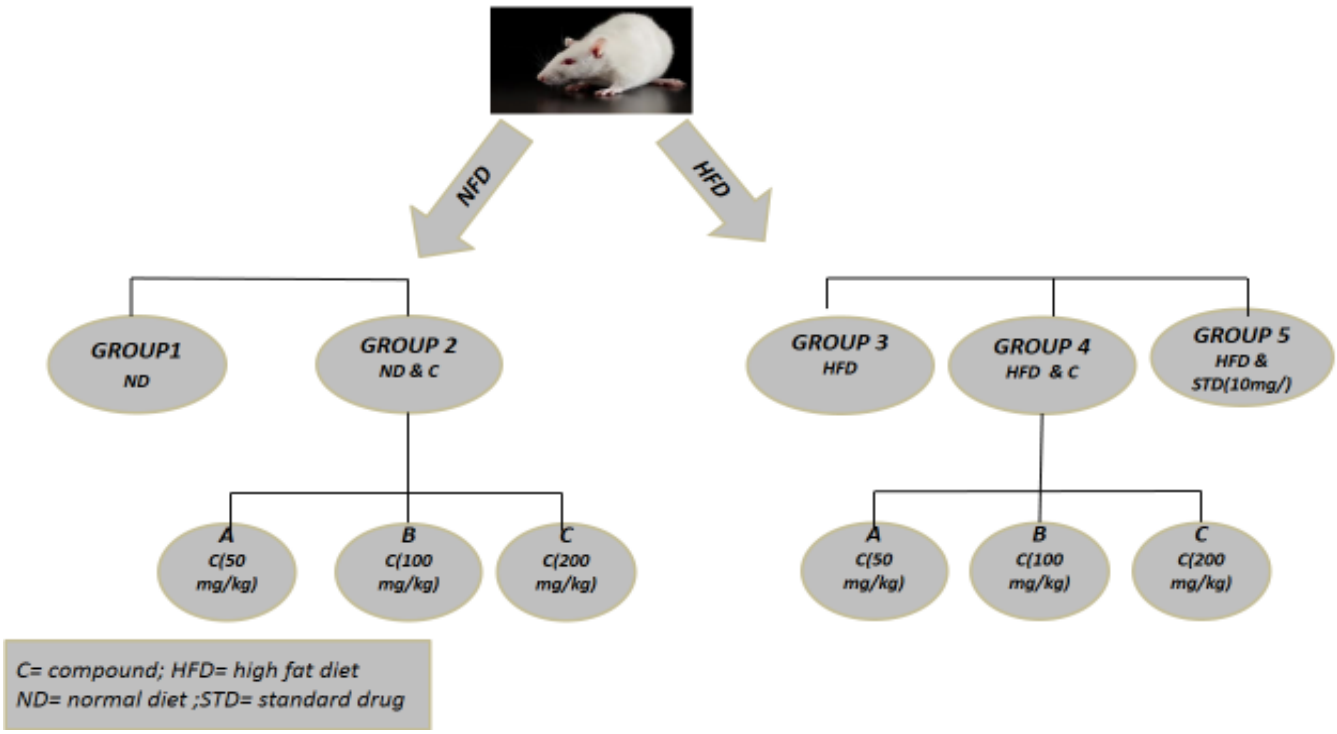


Figure 3.2: Schematic presentation of the anti-hyperlipidemic activity`s experimental design.

Hyperlipidemia was induced by feeding rats a high fat diet (HFD). The composition of the HFD is presented in table 3.1

Table 3.1: Composition of high-fat diet (HFD); ND-normal rat diet obtained from Doghouse, Empangeni, SA

Ingredients	Quantity %
Ground ND	79.3
Oil (Sunflower)	15
Bile salt	0.5
Cholesterol	5
Thirmecil	0.2
Total	100

3.2.4 Measurement of body weight and food intake

Body weight and food intake were recorded every other day over the study period of 21 or 36 days. Percentage weight change, food conversion (FC), and food efficiency ratio (FER) were then calculated.

$$\% \text{ wt. change on day 21 or 36} = \frac{body\ wt\ on\ day\ 21\ or\ 36 - body\ wt\ on\ day\ 1}{body\ wt\ on\ day\ 1} \times 100$$

$$\text{Food conversion (FC)} = \frac{Food\ intake\ (g)}{wt.gain\ (g)}$$

$$\text{Food efficiency ratio (FER)} = \frac{wt.gain\ (g)}{Food\ intake\ (g)}$$

$$\text{Relative liver wt. per 100g body wt. of rat} = \frac{wt.\ of\ rat\ liver(g)}{body\ wt.\ on\ day 21\ or\ 36} \times 100$$

3.2.5 Collection of blood samples and liver for lipid profile determination

At the end of the experimental periods, the rats were fasted for 8 hours, and then sacrificed by a blow to the head and blood samples were collected by cardiac puncture. The collected blood samples were centrifuged at 3500 rpm for 10 minutes and the serum collected for biochemical studies. The liver was excised, weighed and stored in formalin for histological studies.

3.2.6 Biochemical assays

The serum samples were used for the estimation of total cholesterol (TC), total triglyceride (TG), HDL-cholesterol (HDL-c), Blood glucose, Blood urea nitrogen, AST,

ALT, Alkaline phosphatase, total protein and lipase (colorimetric). Analysis was done using the Cobas c 111 analyzer.

LDL-cholesterol (LDL-c) was estimated using Friedwald`s equation (Friedwald *et al.,* 1972)

LDL-c = [TC-(HDL-c + (TG/5)]

Other lipid parameters such as VLDL- cholesterol, coronary risk index and atherogenic index (AI) were calculated (Chaudhari et al., 2012) as follows:

LDL-c= [TC-(HDL-c + (TG/5)]

VLDL-c= [TG – (HDL-c + LDL-C)]

Atherogenic index (AI) = LDL-c/HDL-c (mg/dl)

Coronary risk index (CRI) = TC/HDL-c (mg/dl)

Adiposity level = $\left[\sqrt[3]{\frac{Body\ wt\ (g)}{naso-anal\ length\ (mm)}} \right] \times 10^4$

3.3 STATISTICAL ANALYSIS

Data was analyzed using one-way analysis of variance (ANOVA) followed my Tukey-Kramer multiple comparison test using GraphPad InStat® version3. The results are presented as mean ± standard error of the mean (SEM). Values of $p<0.05$ were considered significant.

CHAPTER 4

RESULTS

This chapter presents the results obtained when the triterpene isolated from *P longifolia* was screened for hypolipidemic activities in normal fed diet (ND) and high fat diet (HFD) rats. Serum lipids and some liver function enzymes activity were monitored.

4.1 ISOLATION AND CHARACTERISATION

From the chloroform crude plant extract (run through column chromatography), a white crystalline material (KEM) with a melting point of 205 ^{0}C was confirmed (through IR, NMR and/or MS spectral techniques (see appendix D)) to be similar to the triterpene (figure 4.1) of Mosa *et al* (2011).

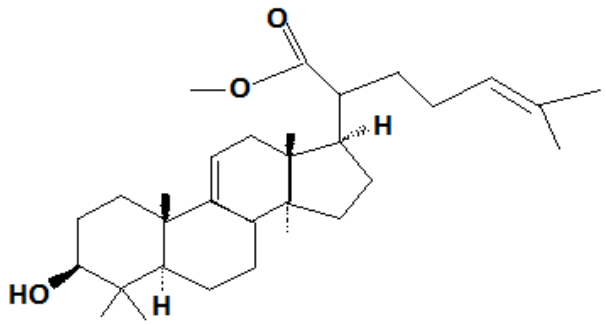


Figure 4.1: Chemical structure of Methyl-3β-hydroxylanosta-9,24-dienoate (KEM).

4.2 ANIMAL STUDIES

4.2.1 Effect on body weight, food conversion and food efficiency ratio

The effects of the normal diet and the diet with different concentrations of KEM, on the body weight of the rats over the period of study are shown in **table 4.1.** The control group of rats subjected to ND for 21 days showed an increase ($p<0.05$) in the body weight by 12.12 %. The group with ND for 36 days have shown an increase ($p<0.05$) by 41.93 %. It is apparent that the rats were gaining weight over the 36 days of study.

Table 4.1 Effect of KEM on body weight in NFD Sprague-Dawley rats

Group	Initial body weight (g)	Final body weight (g)	% Weight change (g)
Normal Controls			
ND (after 21days)	198.74±7.02	242.49±17.66	12.12±0.44$^{\#\#\# @}$
(after 36days)	184.92±10.26	244.34±18.48	41.93±6.29$^{@}$
Experiment group 1			
ND+KEM(50mg/kg.bw) after 36days	144.8±2.79	169.5±2.85	40.75±7.15
Experiment group 2			
ND+KEM(100mg/kg.bw) after 36days	191.22±6.07	197.76±1.64	5.37±0.98$^{\#\#\#}$
Experiment group 3			
ND+KEM(200mg/kg.bw) after 36days	159.83±6.71	199.53±9.89	17.46±0.84$^{\#\#\# @@}$

All values are expressed as mean ± SEM, (n=4); $^{\#\#\#}$p<0.001; % Weight change in normal control group after 21 days and all experiment group versus normal control group after 36 days; $^{@@}$p<0.01, $^{@}$p<0.05 initial body weight versus final body weight in all groups.

The effects of ND and ND fortified with different KEM concentrations (50-200 mg/kg.bw) administrated to rats of the three experiment groups indicated a reduction in the body weight. Rats treated with 100mg/kg.bw KEM showed a statistically significant reduction (p<0.001) but a small reduction (p<0.001) in rats treated with 200mg/kg.bw KEM was observed.

Table 4.2 shows the effects of the high fat diet, high fat diet separately fortified with different concentrations of KEM and lovastatin. The hyperlipidemia control group rats subjected to HFD for 21 days increased in the body weight by 6.18 %. The group with hyperlipidemia control for 36 days increased by 13.66 %.

Table 4.2 Effect of KEM on body weight in HFD-induced in Sprague-Dawley rats

Group	Initial body weight (g)	Final body weight (g)	% Weight change (g)
Normal Controls			
ND (after 21days)	198.74±7.02	242.49±17.66	12.12±0.44$^{###}$
(after 36days)	184.92±10.26	244.34±18.48	41.93±6.29
Hyperlipidemia Controls			
HFD (after 21days)	159.01±3.36	166.42±10.14	6.18±1.09
(after 36days)	159.21±8.85	182.99±9.74	13.66±1.77$^{###}$
Experiment group 1			
HFD+KEM(50/kg.bw) after 36days	196.6±8.98	203.3±7.94	12.95±2.98
Experiment group 2			
HFD+KEM(100/kg.bw) after 36days	166.09±8.15	174.79±7.41	6.01±1.57
Experiment group 3			
HFD+KEM(200/kg.bw) after 36days	174.80±7.41	173.25±7.16	10.04±2.72
Experiment group 4			
HFD+ lovastatin (10mg/kg.bw) after 36days	148.72±8.22	153.08±6.71	3.74±1.20**

All values are expressed as mean ± SEM, (n=4); *p<0.05, **p<0.01 % Weight change in all experiment group versus hyperlipidemia controls group after 36 days; $^{#}$p<0.05, $^{##}$p<0.01, $^{###}$p<0.001 % Weight change in normal control group after 21 days versus normal control group after 36 days.

The effects of HFD, different KEM concentrations (50-200 mg/kg.bw) and lovastatin (10mg/kg.bw) administrated to rats of the four experiment groups have shown a reduction in body weight. Rats treated with 10mg/kg.bw lovastatin show a statistically significant reduction (p<0.01) but no statistically significant changes were observed in rats treated with different KEM concentrations, but 100mg/kg.bw indicated a potential reduction.

The effects of the normal diet and the diet with different concentrations of KEM, on food conversion and food efficiency ratio are presented in **table 4.3**. The control group of rats subjected to ND for 36 days showed a reduction in food conversion and an increase ($p<0.001$) in food efficiency ratio, compared to the control group of rats subjected to ND for 21 days.

Table 4.3 Food Conversion and Food Efficiency Ratio in NFD- Sprague-Dawley rats

Group	Food Conversion	Food Efficiency Ratio
Normal Controls		
ND (after 21days)	0.82±0.07	1.23±0.14$^{###}$
(after 36days)	0.21±0.02	4.91±0.47
Experiment group 1		
ND+KEM(50mg/kg.bw) after 36days	0.59±0.12	1.91±0.37$^{###}$
Experiment group 2		
ND+KEM(100mg/kg.bw) after 36days	1.42±0.18$^{#}$	0.79±0.12$^{###}$
Experiment group 3		
ND+KEM(200mg/kg.bw) after 36days	0.31±0.03	3.31±0.28

All values are expressed as mean ± SEM, (n=4); $p<0.05$, $^{##}p<0.01$, $^{###}p<0.001$ compared to the ND 36days.

There was a significant increase ($p<0.05$) in the food conversion and a reduction in food efficiency ratio in rats treated with 100mg/kg.bw KEM. A statistically significant reduction ($p<0.001$) in food efficiency ratio was observed in rats treated with 100mg/kg.bw KEM but a small decrease ($p<0.001$) in rats treated with 50mg/kg.bw KEM.

Table 4.4 provides the effects of the high fat diet, high fat diet fortified with different concentrations of KEM or lovastatin on food conversion and food efficiency ratio. The control group of rats subjected to HFD for 36 days indicated an increase in food conversion and a reduction ($p<0.001$) in food efficiency ratio compared to the control group of rats subjected to ND for 36 days.

Table 4.4 Food Conversion and Food Efficiency Ratio in HFD-induced in Sprague-Dawley rats

Group	Food Conversion	Food Efficiency Ratio
Normal Controls		
ND (after 21days)	0.82±0.07	1.23±0.14$^{###}$
(after 36days)	0.21±0.02	4.91±0.47
Hyperlipidemia Controls		
HFD (after 21days)	1.30±0.35	0.96±0.25
(after 36days)	0.62±0.05	1.53±0.13$^{###}$
Experiment group 1		
HFD+KEM(50mg/kg.bw) after 36days	0.84±0.08	1.22±0.12
Experiment group 2		
HFD+KEM(100mg/kg.bw) after 36days	0.75±0.14	1.12±0.40
Experiment group 3		
HFD+KEM(200mg/kg.bw) after 36days	0.19±0.02	4.43±1.03***
Experiment group 4		
HFD+ lovastatin (10mg/kg.bw) after 36days	2.17±0.59**	0.69±0.14

All values are expressed as mean ± SEM, (n=4); *p<0.05, **p<0.01, ***p<0.001 compared to the HFD 36days; $^{###}$p<0.001 compared to the ND 36 days

The effects of HFD, different KEM concentrations (50-200 mg/kg.bw) and lovastatin (10mg/kg.bw) administrated to rats of the four experiment groups indicated an increase in the food conversion. A statistically significant increase ($p<0.01$) was observed in rats treated with 10mg/kg.bw lovastatin.

The effects of HFD, different KEM concentrations (50 and 100 mg/kg.bw) and lovastatin (10mg/kg.bw) administrated to rats of the four experiment groups showed a reduction in the food efficiency ratio while the rats treated with 200mg/kg.bw KEM indicate a statistically significant increase ($p<0.001$) in food efficiency ratio.

4.2.2 Effect on liver weight and adiposity level

The effects of the normal diet and the diet with different concentration of KEM, on liver weights and adiposity level are presented in **table 4.5**. The control group of rats subjected to ND for 36 days indicated a reduction in relative liver weight and no change was observed in adiposity level, compared to the control group of rats subjected to ND for 21 days.

Table 4.5 Effect of KEM on liver weights and adiposity level in NFD- Sprague-Dawley rats

Group	Liver (g)	Adiposity level x^{04}
Normal Controls		
ND (after 21days)	3.37±0.17	1.17±0.03
(after 36days)	2.97±0.03	1.17±0.04
Experiment group 1		
ND+KEM(50mg/kg.bw) after 36days	4.93±0.14	1.07±0.01$^{\#}$
Experiment group 2		
ND+KEM(100mg/kg.bw) after 36days	3.19±0.10	1.07±0.01$^{\#}$
Experiment group 3		
ND+KEM(200mg/kg.bw) after 36days	3.19±0.13	1.07±0.02$^{\#}$

All values are expressed as mean ± SEM, (n=4); $^{\#}p<0.05$, compared to the ND 36 days

Rats administrated with different KEM concentrations (50-200 mg/kg.bw) showed an increase in relative liver weight and a statistically significant reduction ($p<0.05$) in adiposity level.

Table 4.6 reports the effects of the high fat diet, high fat diet with different concentrations of KEM and lovastatin on liver weights and adiposity level. The control group of rats subjected to HFD for 36 days presented an increase ($p<0.001$) in relative liver weight and a decrease ($p<0.05$) in adiposity level, compared with rats subjected to ND for 36 days.

Table 4.6 Effect of KEM on liver weights and adiposity level in HFD-induced in Sprague-Dawley rats

Group	Liver (g)	Adiposity level x^{04}
Normal Controls		
ND (after 21days)	3.37±0.17	1.17±0.03
(after 36days)	2.97±0.03	1.17±0.04
Hyperlipidemia Controls		
HFD (after 21days)	4.67±0.06	1.02±0.01
(after 36days)	5.21±0.29$^{###}$	1.06±0.01$^{\#}$
Experiment group 1		
HFD+KEM(50mg/kg.bw) after 36days	3.38±0.04***	1.09±0.01
Experiment group 2		
HFD+KEM(100mg/kg.bw) after 36days	4.68±0.21	1.04±0.01
Experiment group 3		
HFD+KEM(200mg/kg.bw) after 36days	5.73±0.18	1.08±0.01
Experiment group 4		
HFD+ lovastatin (10mg/kg.bw) after 36days	4.75±0.14	1.03±0.01

All values are expressed as mean ± SEM, (n=4); *p<0.05, **p<0.01 compared to the HFD 36days; $^{\#}$p<0.05, $^{\#\#}$p<0.01, $^{\#\#\#}$p<0.001 compared to the ND 36 days

The effects of HFD, different KEM concentrations (50-200 mg/kg.bw) and lovastatin (10mg/kg.bw) administrated to rats of the four experiment groups showed a reduction in relative liver weight. Rats treated with 50mg/kg.bw KEM indicate a statistically significant reduction ($p<0.001$) in liver weight and 100mg/kg.bw KEM indicate a potential reduction in adiposity level.

4.2.3 Effect on serum lipids and lipoproteins

The effects of the normal diet and the diet with different concentrations of KEM on total cholesterol and triacylglyceride levels are given on **figure 4.2**. The control group of rats subjected to ND for 36 days presented an increase in total cholesterol and triacylglyceride compared to control group of rats subjected to ND for 21 days.

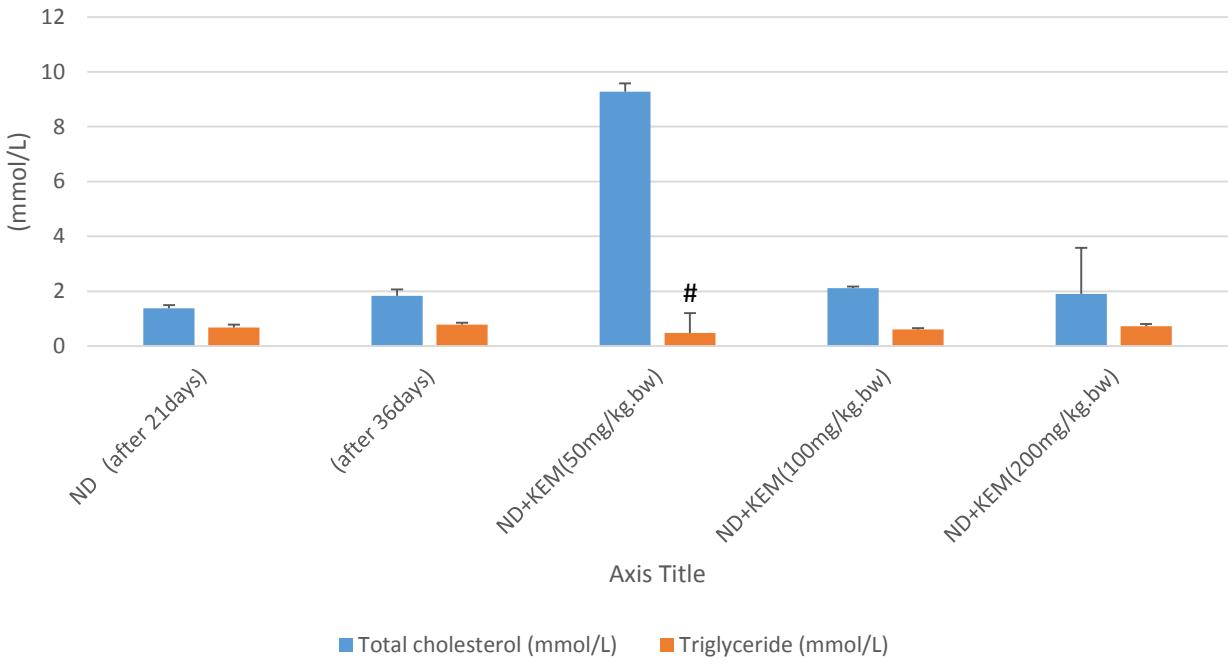


Figure 4.2: Effect of KEM on total cholesterol and triacylglyceride levels in NFD Sprague-Dawley rats. All values are expressed as mean ± SEM, (n=4); $^{\#}p<0.05$, compared to the ND 36 days.

The effects of ND and different KEM concentrations (50-200 mg/kg.bw) administrated to rats of the three experiment groups showed a reduction in triacylglyceride to rats of the three experiment groups. Rats treated with 50mg/kg.bw KEM indicate a statistically significant reduction ($p<0.01$) in triacylglyceride. No significant differences in total cholesterol were observed under the influence of the different KEM concentrations.

Table 4.7 presents the effects of the normal diet and the diet with different concentrations of KEM on serum lipoproteins level. The control groups of rats subjected to ND for 36 days have shown an increase in VLDL, LDL and HDL, compared with control groups of rats subjected to ND for 21 days.

Table 4.7: Effect of KEM on serum lipoproteins level in NFD Sprague-Dawley rats

Group	VLDL-c (mmol/L)	LDL-c (mmol/L)	HDL-c (mmol/L)
Normal Controls			
ND (after 21days)	0.14±0.04	0.11±0.04	1.14±0.9
(after 36days)	0.26±0.18	0.10±0.02	1.86±0.07
Experiment group 1			
ND+KEM(50mg/kg.bw) after 36days	0.12±0.02	0.26±0.03	2.26±0.16
Experiment group 2			
ND+KEM(100mg/kg.bw) after 36days	0.12±0.02	0.06±0.02	2.43±0.24
Experiment group 3			
ND+KEM(200mg/kg.bw) after 36days	0.15±0.02	0.15±0.03	2.89±0.07

Rats administrated with different KEM concentrations (50-200 mg/kg.bw) showed a reduction in VLDL and LDL but continued increase in HDL, to rats of the three experiment groups. Rats treated with 100mg/kg.bw KEM established a potential reduction in LDL level.

The effects of the high fat diet and high fat diet with different concentrations of KEM and lovastatin are presented in **figure 4.3**. The hyperlipidemia groups of rats subjected to HFD for 36 days indicated an increase ($p<0.001$) in total cholesterol but a small increase in triacylglyceride, compared to the normal group of rats subjected to ND for 36 days.

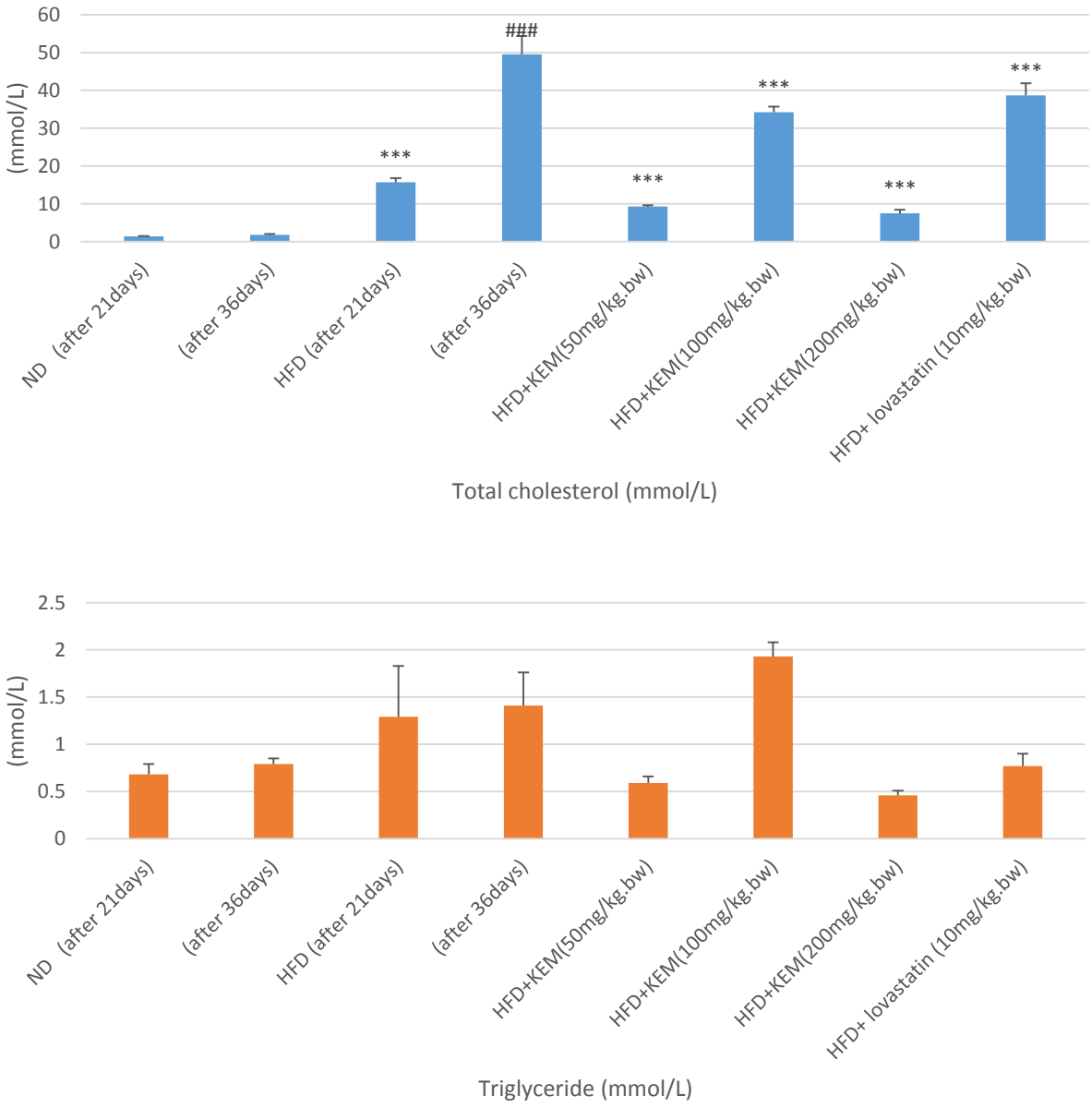


Figure 4.3. Effect of KEM on total cholesterol and triacylglyceride levels in HFD Sprague-Dawley rats. All values are expressed as mean ± SEM, (n=4); ***$p<0.001$ compared to the HFD 36days; $^{###}p<0.001$ compared to the ND 36 days.

The effects of HFD, different KEM concentrations (50-200 mg/kg.bw) and lovastatin (10mg/kg.bw) administrated to rats of the four experiment groups indicated a statistically significant reduction ($p<0.001$) in total cholesterol to rats of the four experiment groups. There was no statistically significant changes observed in triacylglyceride.

Table 4.8 presents the effects of the high fat diet, high fat diet with different concentrations of KEM and lovastatin on lipoproteins level. The hyperlipidemia groups of rats subjected to HFD for 36 days have shown an increase in lipoproteins level, compared to the normal group of rats subjected to ND for 36 days.

Table 4.8: Effect of KEM on serum lipoproteins levels in HFD-induced in Sprague-Dawley rats

Group	VLDL-c (mmol/L)	LDL-c (mmol/L)	HDL-c (mmol/L)
Normal Controls			
ND (after 21days)	0.14±0.04	0.11±0.04	1.14±0.9
(after 36days)	0.26±0.18	0.10±0.02	1.86±0.07
Hyperlipidemia Controls			
HFD (after 21days)	0.28±0.22	7.41±0.56**	14.75±0.19***
(after 36days)	0.28±0.14	10.72±0.94$^{###}$	6.17±0.16$^{###}$
Experiment group 1			
HFD+KEM(50mg/kg.bw) after 36days	0.10±0.01	5.39±0.25***	16.24±0.16***
Experiment group 2			
HFD+KEM(100mg/kg.bw) after 36days	0.39±0.05	5.85±0.81***	30.39±0.92***
Experiment group 3			
HFD+KEM(200mg/kg.bw) after 36days	0.09±0.01	4.46±0.82***	47.30±0.9***
Experiment group 4			
HFD+ lovastatin (10mg/kg.bw) after 36days	0.15±0.03	12.97±0.59	29.13±0.68***

All values are expressed as mean ± SEM, (n=4); *p<0.05, **p<0.01 compared to the HFD 36days; $^{#}$p<0.05, $^{##}$p<0.01, $^{###}$p<0.001 compared to the ND 36 days

The effects of HFD, different KEM concentrations (50-200 mg/kg.bw) and lovastatin (10mg/kg.bw) administrated to rats of the four experiment groups showed a reduction in VLDL and LDL but an increase in HDL. Rats administrated with different KEM concentrations (50-200 mg/kg.bw) indicated a reduction ($p<0.001$) in LDL while it affects the increase ($p<0.001$) of HDL.

4.2.4 Effect on atherogenic index (AI) and coronary risk index (CRI)

The effects of the normal diet and the diet with different concentrations of KEM on atherogenic index and coronary risk index are presented in **figure 4.4**. The control group of rats subjected to ND for 36 days indicated a reduction in atherogenic index and an increase in coronary risk index, compared to the control group of rats subjected to ND for 21 days.

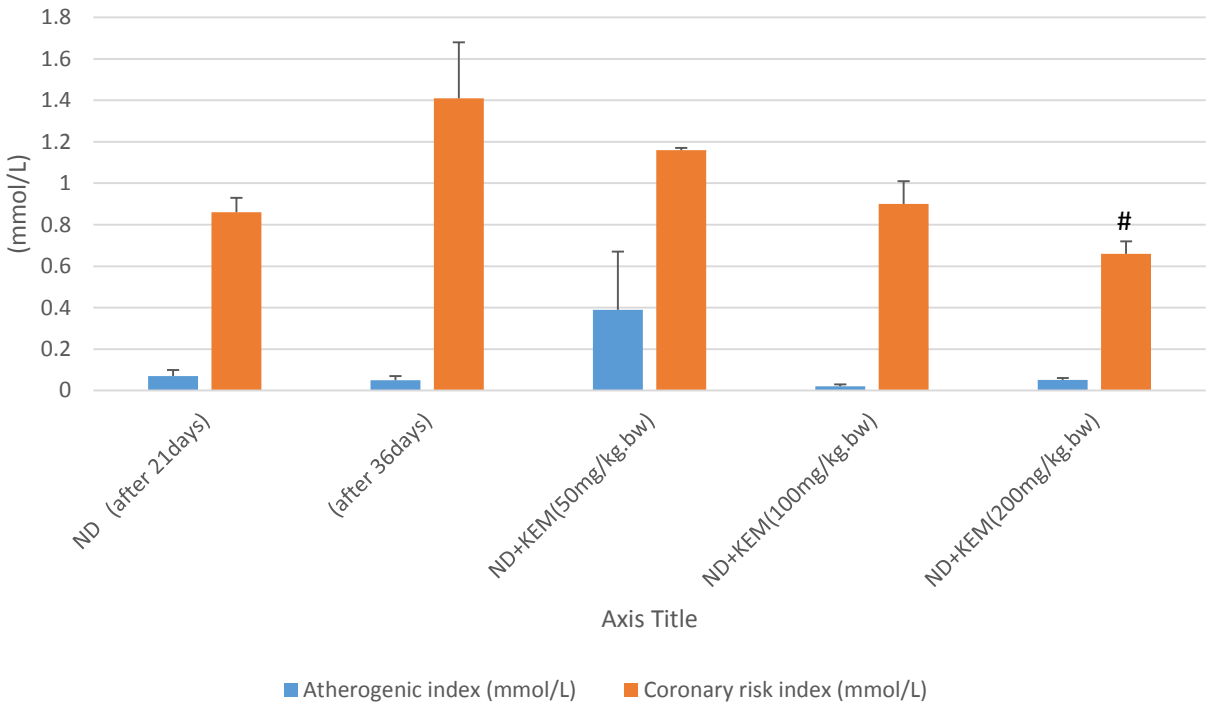


Figure 4.4: Effect of KEM on atherogenic index (AI) and coronary risk index (CRI) in NFD Sprague-Dawley rats. All values are expressed as mean ± SEM, (n=4); $^{\#}p<0.05$, compared to the ND 36 days.

Concentration of KEM 100mg/kg.bw showed a reduction in atherogenic index to rats of the experiment group. Rats treated with 10mg/kg.bw lovastatin showed a statistically significant reduction ($p<0.05$) and 100mg/kg.bw KEM indicated a potential reduction in coronary risk index.

Figure 4.5 presents the effects of the high fat diet, high fat diet with different concentrations of KEM and lovastatin on atherogenic index and coronary risk index. The control group of rats subjected to HFD for 36 days showed an increase ($p<0.001$) in both atherogenic index and coronary risk index, compared with rats subjected to ND for 36 days and HFD for 21 days.

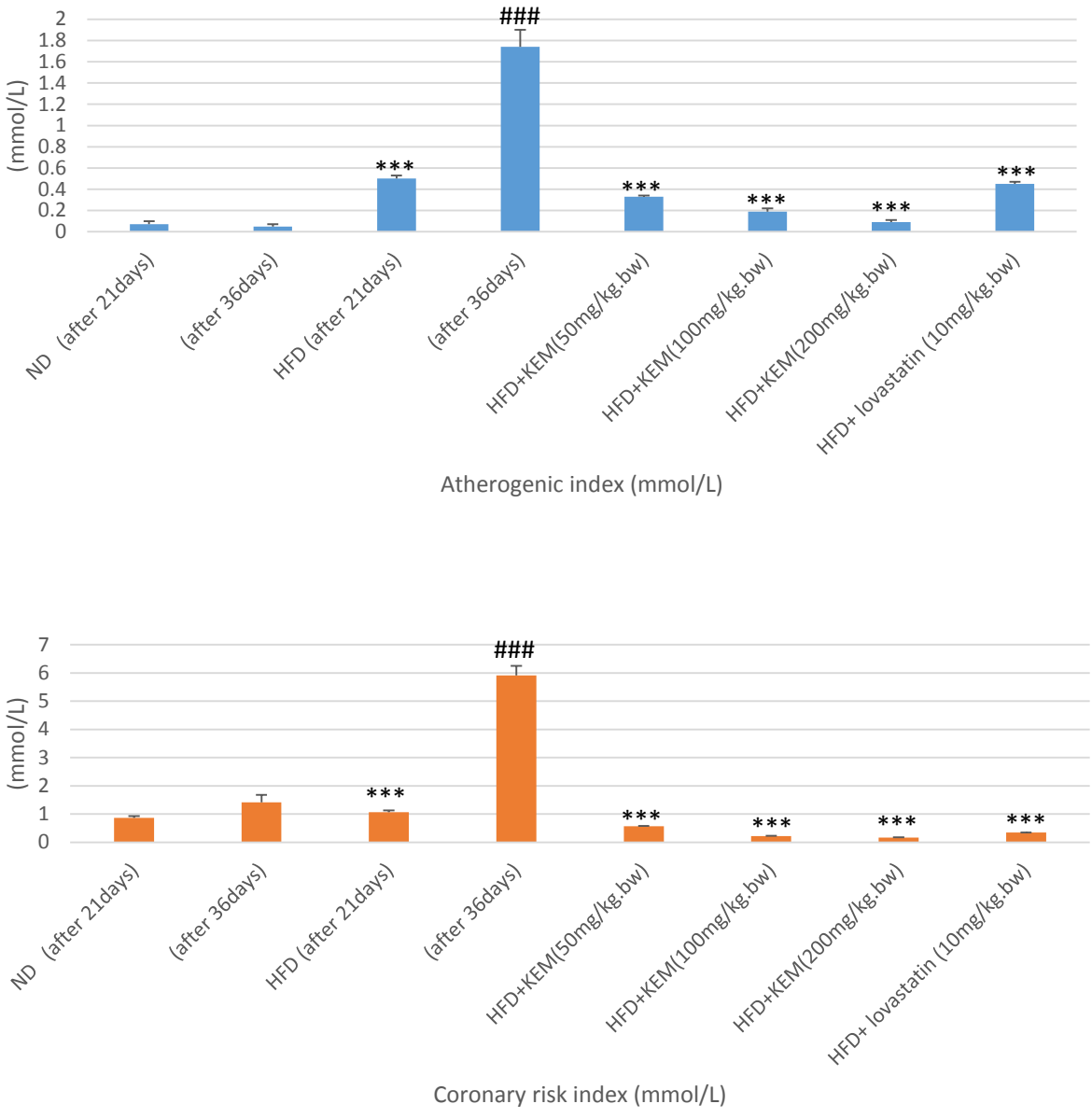


Figure 4.5: Effect of KEM on atherogenic index (AI) and coronary risk index (CRI) in HFD-induced in Sprague-Dawley rats. All values are expressed as mean ± SEM, (n=4); ***$p<0.001$ compared to the HFD 36days; ###$p<0.001$ compared to the ND 36 days.

The effects of HFD, different KEM concentrations (50-200 mg/kg.bw) and lovastatin (10mg/kg.bw) administrated to rats of the four experiment groups have shown a statistically significant reduction in atherogenic index and coronary risk index, 100mg/kg.bw showed a potentially statistically significant reduction compared with other concentrations (50 and 200mg/kg.bw) and lovastatin.

4.2.5 Effect on some liver function enzymes and some biochemical parameters

The effects of the normal diet and the diet with different concentrations of KEM on liver enzymes are furnished in **table 4.9**. The control group of rats subjected to ND for 36 days indicated a relative increase in two liver enzymes (ALT and AST) and a reduction in ALP, when compared to the control group of rats subjected to ND for 21 days.

Table 4.9: Effect of KEM on liver enzymes in NFD-induced in Sprague-Dawley rats

Group	ALP (U/L)	ALT (U/L)	AST (U/L)
Normal Controls			
ND (after 21days)	73.00±24.85	45.90±13.45	226.00±95.57
(after 36days)	46.10±11.94	51.90±10.32	289.20±68.95
Experiment group 1			
ND+KEM(50mg/kg.bw) after 36days	*N/A*	31.33±2.03	95.40±15.12
Experiment group 2			
ND+KEM(100mg/kg.bw) after 36days	35.20±5.44	32.10±4.68	102.96±30.64
Experiment group 3			
ND+KEM(200mg/kg.bw) after 36days	27.30±8.39	63.30±5.14	447.00±43.75

N/A= not determine.

Rats administrated with different KEM concentrations (50 and 100 mg/kg.bw) showed a reduction in liver enzymes (ALT and AST); while rats treated with KEM 200mg/kg.bw indicated a potential reduction in ALP.

The effects of the high fat diet, high fat diet with different concentrations of KEM and lovastatin are presented in **table 4.10**. The control group of rats subjected to HFD for 36 days indicated an increase in ALP ($p<0.01$), ALT and AST, compared with rats subjected to ND for 36 days.

Table 4.10. Effect of KEM on liver enzymes in HFD-induced in Sprague-Dawley rats

Group	ALP (U/L)	ALT (U/L)	AST (U/L)
Normal Controls			
ND (after 21days)	73.00±24.85	45.90±13.45	226.00±95.57
(after 36days)	46.10±11.94	51.90±10.32	289.20±68.95
Hyperlipidemia Controls			
HFD (after 21days)	179.9±34.38	78.20±30.09	339.30±138.06
(after 36days)	249.00±60.40##	83.80±18.67	298.80±55.34
Experiment group 1			
HFD+KEM(50mg/kg.bw) after 36days	*N/A*	60.95±5.21	214.6±37.20
Experiment group 2			
HFD+KEM(100mg/kg.bw) after 36days	181.10±25.53	42.30±6.90	161.90±52.22
Experiment group 3			
HFD+KEM(200mg/kg.bw) after 36days	363.30±53.23	48.80±8.28	205.50±37.12
Experiment group 4			
HFD+ lovastatin (10mg/kg.bw) after 36days	108.80±35.03	44.11±9.82	188.80±52.76

All values are expressed as mean ± SEM, (n=4); ##$p<0.01$, compared to the ND 36 days. N/A= not determine

The effects of HFD, KEM concentrations (200 mg/kg.bw) and lovastatin (10mg/kg.bw) administrated to rats of the four experiment groups indicated a reduction in all the liver enzymes.

The effects of the normal diet and the diet with different concentrations of KEM on some biochemical parameters are presented in **table 4.11**. Total protein (TP) and glucose (GLU) indicated a decrease while urea (UREAL) and lipase (LIP) indicated an increase in rats subjected to ND for 36 day when compared to rats subjected to ND for 21 days. Experimental groups indicated an increase in TP, GLU and LIP, but a reduction in UREAL.

Table 4.11: Effect of KEM on some biochemical parameters in NFD-induced in Sprague-Dawley rats

Group	TP (g/L)	GLU (mmol/L)	UREAL (mmol/L)	LIP (U/L)
Normal Controls				
ND (after 21days)	53.50±2.94	7.34±0.59	5.83±0.47	8.25±0.41
(after 36days)	52.90±3.05	4.99±0.46	7.21±0.88	11.90±1.17
Experiment group 1				
ND+KEM(50mg/kg.bw) after 36days	*N/A*	12.64±0.18	7.73±0.69	*N/A*
Experiment group 2				
ND+KEM(100mg/kg.bw) after 36days	61.60±0.86	5.88±0.75	6.82±0.90	15.20±1.79
Experiment group 3				
ND+KEM(200mg/kg.bw) after 36days	62.00±1.43	5.59±0.45	6.99±0.41	14.65±0.84

N/A= not determine; TP= Total protein; GLU= Glucose; UREAL= Urea; LIP= Lipase

Table 4.12 presents the effects of HFD, HFD with different concentrations of KEM and lovastatin. The control group of rats subjected to HFD for 36 days indicated an increase in TP ($p<0.05$), GLU and LIP but a reduction in UREAL, compared with the control group of rats subjected to HFD for 36 days.

Table 4.12: Effect of KEM on some biochemical parameters in HFD-induced in Sprague-Dawley rats

Group	TP (g/L)	GLU (mmol/L)	UREAL (mmol/L)	LIP (U/L)
Normal Controls				
ND (after 21days)	53.50±2.94	7.34±0.59	5.83±0.47	8.25±0.41
(after 36days)	52.90±3.05	4.99±0.46	7.21±0.88	11.90±1.17
Hyperlipidemia Controls				
HFD (after 21days)	61.60±15.97	6.69±1.82	7.07±1.51	15.95±3.07
(after 36days)	159.00±57.62#	6.35±0.23	5.49±0.56	12.00±1.34
Experiment group 1				
HFD+KEM(50mg/kg.bw) after 36days	*N/A*	8.21±0.72	4.82±0.32	*N/A*
Experiment group 2				
HFD+KEM(100mg/kg.bw) after 36days	164.00±20.27	2.93±0.63	6.26±0.72	9.75±0.91
Experiment group 3				
HFD+KEM(200mg/kg.bw) after 36days	53.90±0.44*	8.37±0.89	4.81±0.30	11.15±0.67
Experiment group 4				
HFD+ lovastatin (10mg/kg.bw) after 36days	56.60±6.85*	5.49±0.62	7.22±0.22	12.95±1.34

All values are expressed as mean ± SEM, (n=4); *$p<0.05$, compared to the HFD 36days; #$p<0.05$, compared to the ND 36 days. N/A= not determine; TP= Total protein; GLU= Glucose; UREAL= Urea; LIP= Lipase

Total protein (TP) was significantly increased ($p<0.05$) in rats subjected to HFD fortified with different concentrations of KEM or lovastatin while only slight increases in serum glucose (GLU) and lipase (LIP) levels were observed compared to rats subjected to ND.

4.2.6 Liver histology

The effects of ND, HFD or the diet with KEM on the histology of the liver are presented in **figure 4.6.** The most significant changes were those of vacuolar changes. The specific form of vacuolar change in this case is lipidosis or steatosis. High fat diet histologically appeared to have severe vascular changes with displaced nuclei because of the fat accumulation and increased inflammation. The KEM appeared to ameliorate the effect of the HFD.

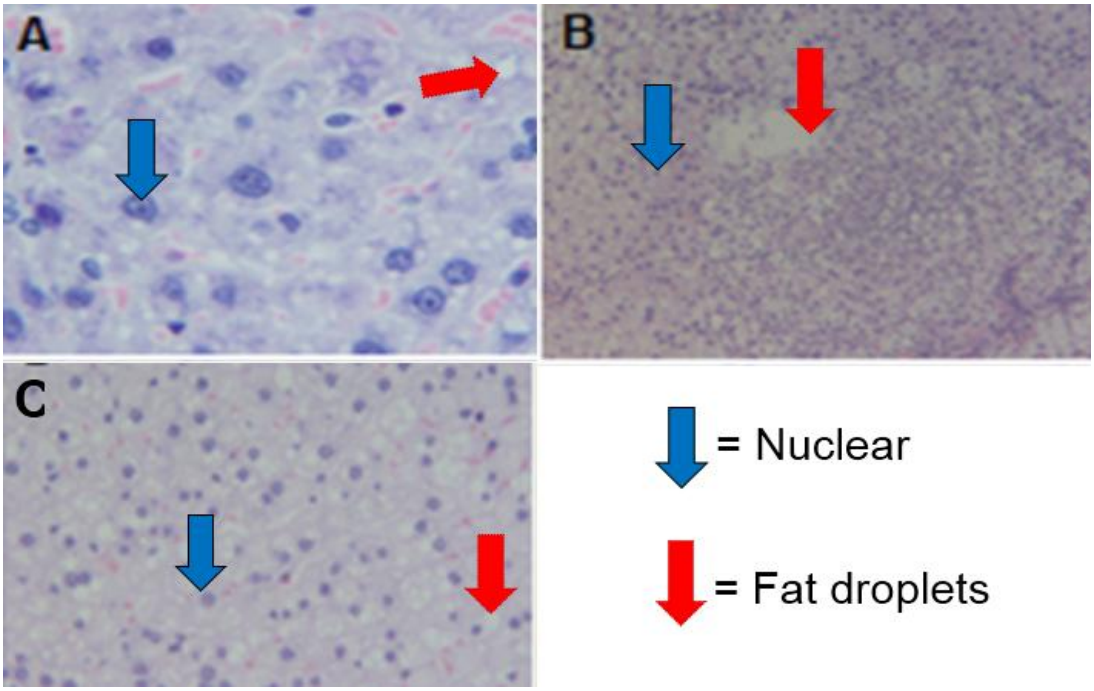


Figure 4.6: Histopathological changes in the liver of high fat diet induced hyperlipidemia [viewed at 3x20 magnification]. (A), Normal diet showing normal liver (B), liver of rats fed with HFD. (C), KEM (100/200mg/kg.bw) treated HFD fed rats.

The features include:

(A) Few fat droplets were observed in the liver of normal control diet.

(B) Many large fat droplets were indicated by red arrowheads in the liver of rats fed a high-fat diet (HFD); severe vascular changes with displaced nuclei is evident

(C) Pictures of liver tissue from KEM depicted a lower accumulation of lipid droplets.

CHAPTER 5

DISCUSSION

5.1 BODY WEIGHT, FOOD CONVERSION AND FOOD EFFICIENCY RATIO

Literature (Choi *et al.,* 2013; Kris, 2012) abounds with the fact that food conversion and food efficiency ratios play an important role in determining the increase of body weight i.e. the amount of food that is converted to body mass. Prolonged administration of a high fat diet (HFD) is known to result in inducing lipid metabolism and to increase body weight (Brown *et al.,* 2002). Thus a food efficiency ratio can be applied as a scale of obesity or increase in body weight. It is important to consider that a small value for the food efficiency ratio is also an effective parameter to predict the deliberate avoidance of obesity (Choi *et al.,* 2013).

The results of this study indicate that KEM (100 mg/kg.bw) had the potential to reduce the body weight of normal (12.12%) and HFD (6.01%) fed rats. A reduction in body weight gain in both ND and HFD groups were accompanied by changes in food conversion and food efficiency ratio (Table 4.3). Rats treated with KEM converted more food to body mass but had fewer efficacies. However, the groups treated with 100mg/kg.bw of KEM demonstrated a decrease in the level of body weight. It can be suggested that the middle concentration 100mg/kg.bw of KEM is effective in reducing body weight.

5.2 LIVER WEIGHT AND ADIPOSITY LEVEL

Long intake of a high-fat diet is known to cause an increase in the weight of liver and adipose tissues in rats (Chaudhari *et al.,* 2012; Brenesel *et al.,* 2013). In addition, long intake of high-fat diet in rats will accelerate the synthesis of TG, inhibit the metabolism of fatty acids and diminish the secretion of TG from the liver to blood by decreasing the

β-oxidation of fatty acids. This leads to the accumulation of excess TG in the liver (Luo *et al.,* 2009; Chaudhari *et al.,* 2012)

It is apparent from this study that rats subjected to HFD for 36 days had a significant increase in relative liver weight but the adiposity level was significantly lower, compared with rats subjected to ND for 36 days. It was supposed that the weights of the liver in the HFD groups were elevated by the high-fat diet. Rats treated with HFD and 50mg/kg.bw KEM showed a statistically significant reduction in liver weight, reaching levels similar to those in the ND group while rats treated with 100mg/kg.bw KEM and lovastatin showed a potential reduction in adiposity level in HFD rats. These findings suggest that KEM can minimize the accumulation of lipid droplets in liver.

5.3 LIPIDS METABOLISM

Cholesterol and triacylglycerol are the major lipids that play an important role in the body i.e. the body needs cholesterol and triacylglycerol. However, excess lipids circulating in the body creates diverse patterns of hyperlipidemia. Abnormalities of lipid metabolism are associated with cardiac diseases, obesity and their associated disorders (Chaudhari *et al.,* 2012). Currently, a number of antihyperlipidemic agents have been introduced for the treatment of hyperlipidemia. One of the most widely used antihyperlipidemic agents at present is lovastatin, which reportedly slows down the body's ability to make cholesterol by targeting hepatocytes and inhibiting HMG-CoA reductase; the enzyme that converts HMG-CoA into mevalonic acid, a cholesterol precursor (Stancu and Sima, 2001). However, this drug is associated with undesirable side effects in humans, such as myositis and rhabdomyolysis, elevated CK levels, muscle weakness, and muscle cramps (Thompson *et al.,* 2003; Hor *et al.,* 2011).

In the present study, prolonged (36 days) administration of HFD in rats resulted in significantly increasing the serum TC and LDL-c but lowering the HDL-c (Figs 4.2, 4.3, Tables 4.7 & 4.8). However, rats treated with 50mg/kg.bw KEM showed a statistically significant reduction in triacylglycerol and no significant differences in total cholesterol was observed under the influence of the different KEM concentrations in rats subjected

to ND for 36 days. In the hyperlipidemia groups of rats subjected to HFD for 36 days, a significant increase in total cholesterol but a small increase in triacylglyceride, compared to normal groups of rats subjected to ND was observed. Rats subjected to HFD with different KEM concentrations; KEM (50-200 mg/kg.bw) and lovastatin (10mg/kg.bw) groups showed a statistically significant reduction in total cholesterol but there was no statistically significant changes observed in triacylglycerol levels.

Lipoproteins, such as VLDL, LDL and HDL play an important role in transporting lipids within the body and because of their clinical importance, a very high proportion of research on lipoproteins deals with their functions in humans in relation to health (Mora, 2009). VLDL is known to be a transport of TG, while LDL and HDL are the main transporters of cholesterol. Dyslipidemia is caused by elevated amounts of VLD and/or LDL and low levels of HDL. It has been observed in this study that different concentrations of KEM have a beneficial effect on lipoprotein's level in rats subjected to ND.

LDL-c is known as the "bad" cholesterol because when elevated, it can contribute to a coronary artery disease. HDL-c is known as the "good" cholesterol, it protects against coronary artery disease. Controlling of LDL–c levels has been shown to substantially reduce cardiovascular disease morbidity and mortality (CTT *et al.,* 2012). Long term intake of HFD or deficiencies of the LDL receptor results in increased concentration of LDL. The primary function of HDLs is to remove excess cholesterol and carry the excess to the liver to be metabolized. KEM in rats treated with ND display a beneficial effect on lipoprotein's levels. Rats treated with different concentrations of KEM showed a statistically significant increase in HDL-c, the "good" cholesterol, and a statistically significant reduction of LDL-c, bad cholesterol, in HFD rats. Lovastatin is a well-known drug for treating hyperlipidemia but KEM shows more activity in reducing LDL-c than lovastatin.

These results indicate that prolonged administration of HFD results in dyslipidemia. KEM does not affect plasma lipids' metabolism levels when rats are subjected to ND. Although the mechanism of the antihyperlipidemic effect of KEM has not been revealed yet, its action appears to be similar to that of lovastatin which is currently of use for

treatment. KEM could effectively reduce or control the amount of serum cholesterol and LDL.

5.4 ATHEROGENIC INDEX (AI) AND CORONARY RISK INDEX (CRI)

Atherosclerosis is the formation of plaque which builds up inside the arteries, as a result of the accumulation of fatty materials such as cholesterol and triglyceride. LDL plays a role in the buildup of plaque inside the arteries while HDL plays a role in reversing or reducing the formation of plaque.

LDL receptors are easily damaged or oxidized by free radicals and consumed by macrophages, becoming foam cells. The foam cells accumulate inside the arteries, causing atherosclerosis. The formation of plaque in arteries, atherosclerosis, can lead to serious problems such as stroke, heart attack or even death (Jasmin, 2012). HDL possesses many features that contribute to the protection from atherosclerosis. HDLs have the ability to inhibit, or paradoxically, to enhance vascular inflammation, lipid oxidation, plaque growth, thrombosis and may reflect changes in specific enzyme and protein components (Benjamin *et al.,* 2005). In addition, it is well known that antioxidants such as vitamins A, C and E can prevent the generation of oxidized LDL that contribute to the formation of atherosclerosis. In our laboratory (unpublished data), KEM does exhibit antioxidant activity in hyperlipidemic rats.

Rats subjected to ND with 200mg/kg.bw KEM showed a statistically significant reduction in elevated coronary risk index. In rats treated with HFD and KEM (50-200mg/kg.bw) a statistically significant reduction of AI and CRI, similar to that of lovastatin, was observed. Significant reduction in AI and CRI were reported by Chaudhari *et al.,* (2012) using embelin extracted from *Embelia ribes* and it was considered beneficial in patients with atherosclerosis and obesity.

5.5 LIVER ENZYMES

Liver enzymes are the proper indicator of normal function of the liver. Elevated liver enzymes may indicate inflammation or damage to cells in the liver (Imafidon and Okunrobo, 2012). Liver enzymes levels are usually raised in acute hepatoxicity, but tend to decrease with prolonged intoxication leading to end-stage liver failure (Obi *et al.,* 2004). More common causes of elevated liver enzymes include certain prescription medications, including statin drugs used to control cholesterol (Mayor Clinic., 2011). In addition, a long term intake of HFD causes significant liver damage, as a result of liver-fat deposition (do Santos *et al.,* 2012).

The specific elevated liver enzymes most commonly found are Alanine transaminase (ALT), Aspartate transaminase (AST) and alkaline phosphatase (ALP).

The results obtained in this study indicate that HDF caused an elevated level of ALP, ALT and AST compared to ND rats. Slightly reduced levels of ALP, ALT and AST were observed in rats treated with HFD, KEM and lovastatin, reaching levels similar to those in the ND group. KEM also exhibited a reduction in elevated liver enzymes in rats subjected to ND. Reduced liver enzymes in both ND and HFD were accompanied by histopathological changes in the liver (fig 4.5). Many large fat droplets were observed in the liver of rats fed a high-fat diet and few fat droplets in the liver of rats on a normal control diet. Experimental groups treated with KEM showed a lower accumulation of lipid droplets reaching levels similar to those in the ND group. From the results it is apparent that KEM possesses a protective effect with lower toxicity. A low cytotoxicity on hepatic and kidney cells of the triterpene has been reported (Mosa et al 2011).

5.6 SOME BIOCHEMICAL BLOOD PARAMETERS: Total protein (TP), glucose (GLU), urea (UREAL) and lipase (LIP).

Elevated amounts of some biochemical parameters such as Total protein (TP), glucose (GLU), urea (UREAL) and lipase (LIP) can be a result of organ damage. High blood protein may be a warning sign of chronic inflammation or infection, particularly of the liver. A high amount of lipase is found in the blood when the pancreas is damaged or

when the pancreatic duct is blocked (Case-Lo, 2013). Urea is a product of amino acid metabolism excreted in urine. High blood levels of urea indicate kidney damage, increased degeneration of proteins and dehydration.

High blood glucose levels occur in insulin deficiency; found in diabetics which if untreated results in ketoacidosis and diabetic coma as well as insulin resistance and down regulation or dysfunction of insulin receptors in the peripheral tissues and organs (Liu *et al.,* 2011; Umpierrez *et al.,* 2002).

Some hypolipidemic drugs (e.g. Atrovastatin) have been reported to have a profound effect on the liver function test markers where they lead to an increase in TP (Pichandi *et al.,* 2011).

In this study, total protein (TP) was significantly increased (while only slight increases in serum glucose (GLU) and lipase (LIP) levels) were observed in rats subjected to HFD compared to rats subjected to ND. The reason might be the liver cell damage and changes in the hormonal regulation of the lipase. The level of urea has shown no significant changes suggesting no disruption in amino acid metabolism and production of ammonia. KEM has shown to have no adverse effects on the synthesis of TP and urea and lipase in the liver and glucose levels were within the normal range in rats subjected to ND. Rats treated with 200mg/kg.bw and lovastatin in HFD have shown TP levels similar to rats in ND. In addition, KEM and lovastatin have a positive effect on normalizing the levels of TP.

The currently available anti-hyperlipidemic agents have limited efficacy and are associated with undesirable side effects (3 *et al.,* 2011). The results from this study indicate that KEM could effectively control the amount of serum lipids and liver enzymes with lower toxicity.

CHAPTER 6

CONCLUSION

Hyperlipidemia is crucial to the progression of cardiovascular, cerebrovascular and metabolic syndrome diseases. The results presented in this study show that KEM (Methyl-3β-hydroxylanosta-9,24-dienoate) isolated from *Protorhus longifolia* has a significant hypolipidemic activity in high fat diet fed rats. This is evidenced by the reduction of serum TC and LDL-c, with an increased HDL-c concentration in treated groups, 100mg/kg.bw showed a good lipid lowering potential compared to other two concentrations. Significant reduction in AI and CRI by KEM was observed, it can thus be considered beneficial in reducing the risk of atherosclerosis. KEM has exhibited the effect of ameliorating the levels of elevated liver enzymes and accumulated lipid droplets in the livers of HFD fed rats.

Lovastatin is a known drug that lowers cholesterol, but other treatments should be put into consideration. Further development of various drugs resulting in the prevention of hyperlipidemia is of importance and KEM could contribute to a new formulation with significant pharmacological effects.

6.1 SUGGESTIONS FOR FUTURE STUDIES

The *in vivo* hypolipidemic activity of the triterpene (KEM) from *Protorhus longifolia* in rats has been established in this study. It is suggested that the following studies be carried out to increase the understanding of the activities presented in this dissertation:

- Synthesizing derivatives for structural activity relationship studies.
- The mechanism of the antihyperlipidemic effect of KEM.
- Evaluate other biological activities such as anti-cancer and anti-diabetic activity.

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Appendix A

Details of reagents preparation.

A1 0.1M phosphate buffer, pH 7.2

1.42g of di-sodium hydrogen orthophosphate (Na_2HPO_4) and 1.2g of Sodium di-hydrogen orthophosphate (NaH_2PO_4) were dissolved in 200ml of distilled water at pH 7.2. The solution was stored at 4 ^{0}C.

A2 10% Phosphate Buffered Formalin

50 ml 37% Formaldehyde; 1.75g of Sodium di-hydrogen orthophosphate (NaH_2PO_4); and 3.25g of di-sodium hydrogen orthophosphate (Na_2HPO_4) were dissolved in 450 ml Distilled Water. The solution was stored at room temperature

A3 Composition of high-fat diet (HFD)

All the ingredients (79.3g of ground normal basal diet; 15ml of sunflower Oil; 0.5g of Bile salt; 5g of Cholesterol; and 0.2g of 4-Hdroxy-2-mercapto-6-methylpyrimidine) were combined and mixed well. The dough was then molded in the shape of pellets of about 3g each.

Appendix B

Details of methodology.

B1 Extraction

Stem bark of *Protorhus longifolia* was washed, air-dried and ground to powder (2mm mesh). The powdered plant material was extracted sequentially with hexane and chloroform in a platform shaker machine (150 rpm) for 24 h per each solvent, at room temperature. The ratio of the plant material to the solvent was 1:5. The extracts were separately filtered through Whatman no.1 filter paper. All the extracts were concentrated in vacuo at 37^{o}C ± 2^{o}C. The extracts were stored in sterile glass bottles and stored at room temperature until used.

B2 Isolation and purification

Wet packing method was done for the column chromatography. A thin sloppy mud was prepared by mixing silica gel (250g) with an initial distilled solvent system (hexane:ethyl acetate; 9:1). The sample, chloroform extract, to be analyzed was mix with a small amount of silica gel (1g) and dissolved in a very small amount of initial distilled solvent system (5ml). A small amount of cotton was placed at the bottom of the column to prevent escape of silica gel.

The column was packed by adding the thin sloppy mud to the column and allowed to reach a constant level and it was sealed with a little amount of sand (about 0.1-0.3mm; 50-150 mesh) to protect silica gel surface. Crude extract (8g) was loaded on to the column and it was again sealed with a little amount of the sand.

The initial solvent system was run through the column several times to ensure a tight packing as well as to remove any air bubbles. After about of the eluent was collected, series of collection of 20ml fractions were collected into beakers. Elution was done using hexane:ethyl acetate solvent system starting with 9:1 to 3:7.

Thin layer chromatography (TLC) was used to analyse the collected fractions. The TLC plates were developed using a 10% H_2SO_4 spray reagent and then heated. The

fractions with similar profile were combined. The combined fractions were separately concentrated in vacuo.

When the samples were completely dry, a brownish yellow crystals were formed. The crystals were further purified by dissolving it in ethyl acetate after which the yellow part dissolved in ethyl acetate and the white crystals were left and a TLC analysis was carried out to ensure the purity, a single brown-orange spot was observed.

The white crystals were obtained. Melting point measurements were done and the compound was taken for further analyses with IR, mass spectrometry (MS) and NMR.

B3 *In vivo* anti-hyperlipidemic activity

The animals were housed in standard cages and maintained at room temperature with 12:12-h light: dark cycle. All rats have access to water and normal pellet diet in the experimental environment, for 1 week, before the experiments were conducted. Body weight and food intake was measured after two days, from day one to the last day of the experiment.

The Sprague-Dawley rats were allocated into two dietary regimens consisting of 20 and 24 rats by feeding either ND or HFD (79.3g of ground normal basal diet, 15ml sunflower Oil, 0.5g Bile salt, 5g Cholesterol and 0.2g 4-Hdroxy-2-mercapto-6-methylpyrimidine (thimecil) for the initial period of 21 days.

After 21 days 4 rats per group was sacrificed and the remaining rats were randomly divided into groups, ND (4 groups) and HFD (5 groups), of four rats per group for the period of 15 days.

1) Group 1: normal diet and vehicle throughout the study.

2) Group 2: was subdivided into three groups (A, B, C) and received normal diet and compound (50, 100, and 200mg/kg body weight, respectively), dissolved in 2 % Tween 20.

3) Group 3: was subdivided into three groups (A, B, C) and received high fat diet and compound (50, 100, and 200mg/kg body weight, respectively), dissolved in 2 % Tween 20.

4) Group 4: high fat diet and vehicle throughout the study.

5) Group 5: high fat diet and simvastatin, the standard drug (10 mg/kg body weight), dissolved in 2 % Tween 20.

B4 Collection of blood samples and Liver

At the end of the experimental period, the rats were fasted for 8 hours and then sacrificed by a blow to the head, blood and liver were collected.

For the collection of blood, the rat was places on its back (dorsal recumbency) on top of a paper towel. 70% alcohol was applied on the abdomen of the rat. Makes a V-cut through the skin and abdominal wall ~1cm posterior to the last rib. Internal organs were moved to the side. A needle was inserted through the diaphragm and into the vena cava or heart. A negative pressure was gently applied on syringe plunger. Needle was withdraw after collection of blood and the liver was excised and stored in formalin for histological studies. The collected blood samples was centrifuged, at 3500 rpm for 10 minutes at 4^0C, the serum samples were collected for biochemical analysis.

Appendix C
(Ethic clearance)

Ethics Committee
Faculty of Science and Agriculture
University of Zululand
C/O Dr L Vivier
Department of Zoology
University of Zululand
Private Bag 1001
KwaDlangezwa
3886
Tel: 035 – 902 6741
Email: lvivier@pan.uzulu.ac.za

20 June 2012

To whom it may concern

ETHICS EVALUATION OF RESEARCH PROJECT PROPOSAL

This letter serves to confirm that **KE Machaba (Student no. 200706386)** are registered for an **MSc Degree** in the **Department of Biochemistry and Microbiology**, Faculty of Science and Agriculture, at the University of Zululand, and in accordance with appropriate rules, submitted a research project proposal to the Ethics Committee of the Faculty of Science and Agriculture. The research project these researchers will investigate is titled: **Evaluation of the in vivo anti-hyperlipidemic activity of the triterpenes from the stem bark of *Protorhus longifolia* (Benrh.) Engl.** Based on the research protocol stipulated, the Ethics Committee of the Faculty of Science and Agriculture could find no reason from an ethical standpoint to reject the proposed research. Provisional ethical clearance is granted pending approval by the UZREC.

Yours sincerely

Dr L Vivier
Chairperson
Ethics Committee
Faculty of Science and Agriculture
University of Zululand

Appendix D
(SPECTRA)

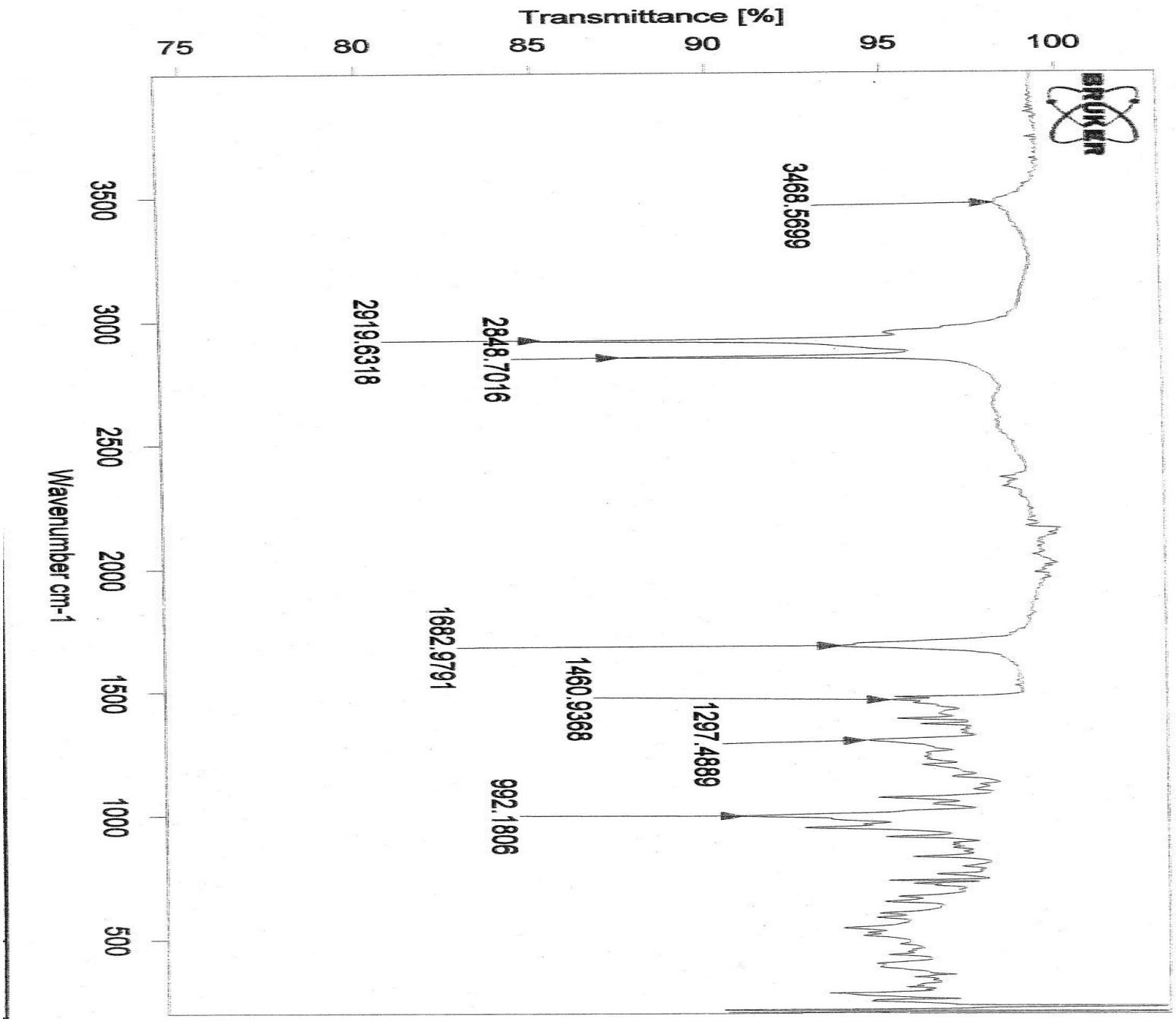

Figure D2: IR spectrum of compound KEM

Peak	ν(F1) [ppm]	ν(F1) [Hz]	Intensity [rel]	Annotation
1	5.2222	3133.8422	1.09	
2	5.2180	3131.3218	1.11	
3	5.1434	3086.5543	0.47	
4	5.1313	3079.2931	0.98	
5	5.1193	3072.0919	0.49	
6	4.2405	2544.7241	0.75	
7	3.8678	2321.0668	4.22	
8	3.3759	2025.8776	15.00	
9	3.2370	1942.5237	1.27	
10	2.5132	1508.1713	4.88	
11	2.5102	1506.3710	6.68	
12	2.5079	1504.9908	5.69	
13	2.2307	1338.6431	0.42	
14	2.1031	1262.0703	0.50	
15	2.0913	1254.9891	0.51	
16	1.9947	1197.0195	0.36	
17	1.9599	1176.1360	0.49	
18	1.9344	1160.8334	1.13	
19	1.9263	1155.9726	1.00	
20	1.9111	1146.8511	1.13	
21	1.9043	1142.7704	1.54	
22	1.8906	1134.5491	1.73	
23	1.8768	1126.2677	1.01	
24	1.8541	1112.6454	0.59	
25	1.8196	1091.9420	0.35	
26	1.7898	1074.0590	0.59	
27	1.7669	1060.3167	0.37	
28	1.7131	1028.0313	0.76	
29	1.7037	1022.3904	0.71	
30	1.6930	1015.9693	0.68	
31	1.6744	1004.8074	7.05	
32	1.6584	995.2058	0.79	
33	1.4844	890.7884	0.83	
34	1.4623	877.5262	1.53	
35	1.4495	869.8450	1.51	
36	1.4374	862.5837	2.01	
37	1.4249	855.0825	2.68	
38	1.4120	847.3412	1.83	
39	1.3970	838.3397	1.13	
40	1.3751	825.1975	0.53	
41	1.2645	758.8264	0.41	
42	1.2587	755.3459	0.45	
43	1.2198	732.0020	0.70	
44	1.2129	727.8613	0.46	
45	1.1974	718.5597	0.63	

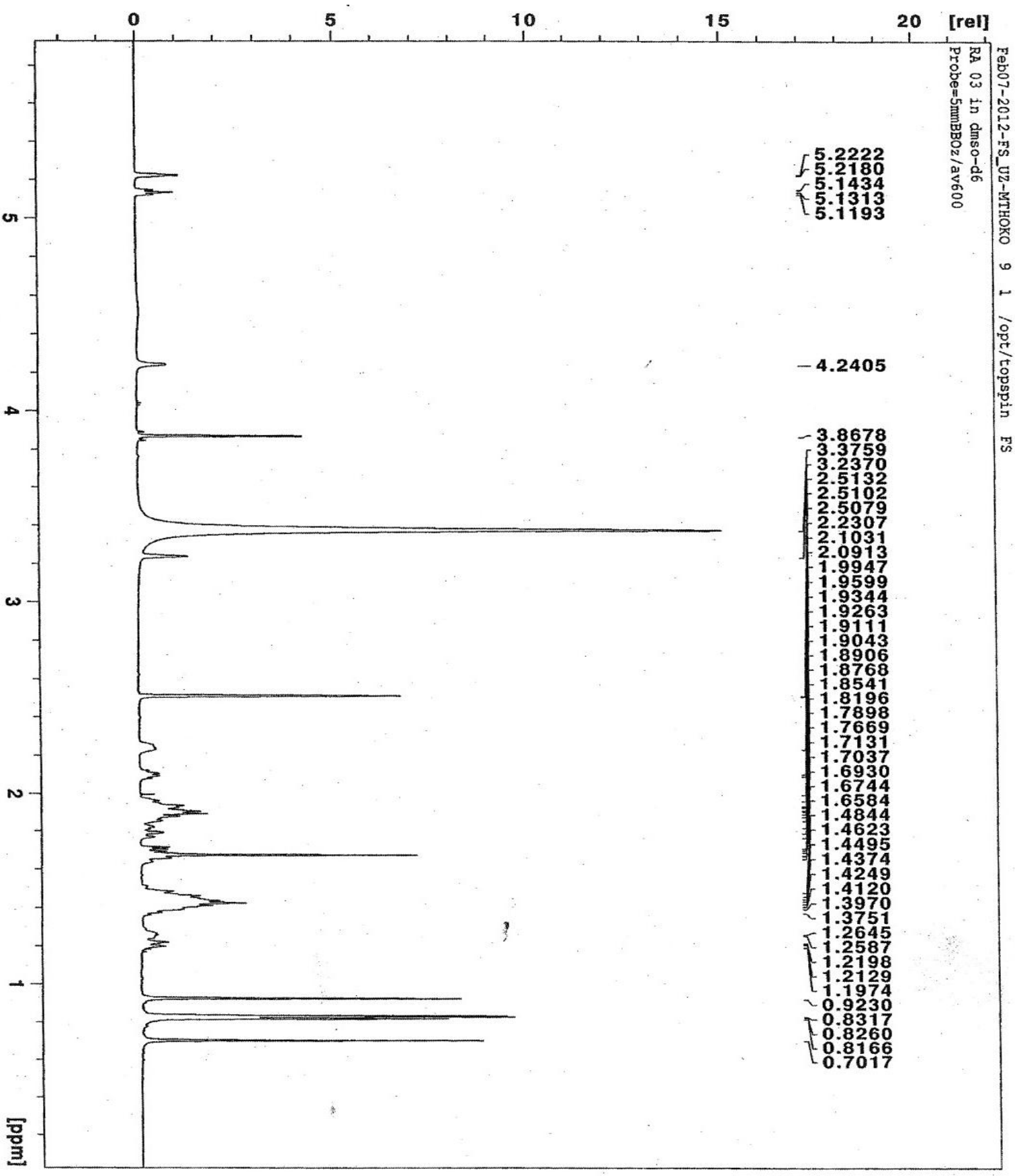

Figure D2: 1H-NMR spectrum of compound KEM

Peak	ν(F1) [ppm]	ν(F1) [Hz]	Intensity [rel]	Annotation
1	177.3261	26757.6689	0.21	
2	145.9439	22022.2435	0.19	
3	136.3578	20575.7464	0.32	
4	125.6167	18954.9652	0.36	
5	118.3428	17857.3682	0.21	
6	74.3543	11219.7118	0.23	
7	59.7785	9020.2926	0.55	
8	51.0279	7699.8685	0.48	
9	49.5895	7482.8207	0.31	
10	48.4481	7310.5889	0.31	
11	47.7044	7198.3681	0.31	
12	44.3752	6696.0076	0.33	
13	43.3265	6537.7637	0.46	
14	40.4923	6110.0963	0.20	
15	37.4822	5655.8865	0.39	
16	34.7427	5242.5089	0.43	
17	33.5960	5069.4773	0.22	
18	32.8466	4956.3964	0.23	
19	31.3390	4728.9067	0.25	
20	30.4389	4593.0859	0.24	
21	28.5857	4313.4468	0.40	
22	27.4363	4140.0078	0.51	
23	27.0013	4074.3683	0.22	
24	25.8420	3899.4354	0.22	
25	25.5188	3850.6661	0.33	
26	23.9132	3608.3887	0.23	
27	22.1463	3341.7718	0.43	
28	21.8613	3298.7667	0.42	
29	21.6753	3270.7001	0.58	
30	17.2798	2607.4400	0.22	
31	13.3042	2007.5408	0.52	

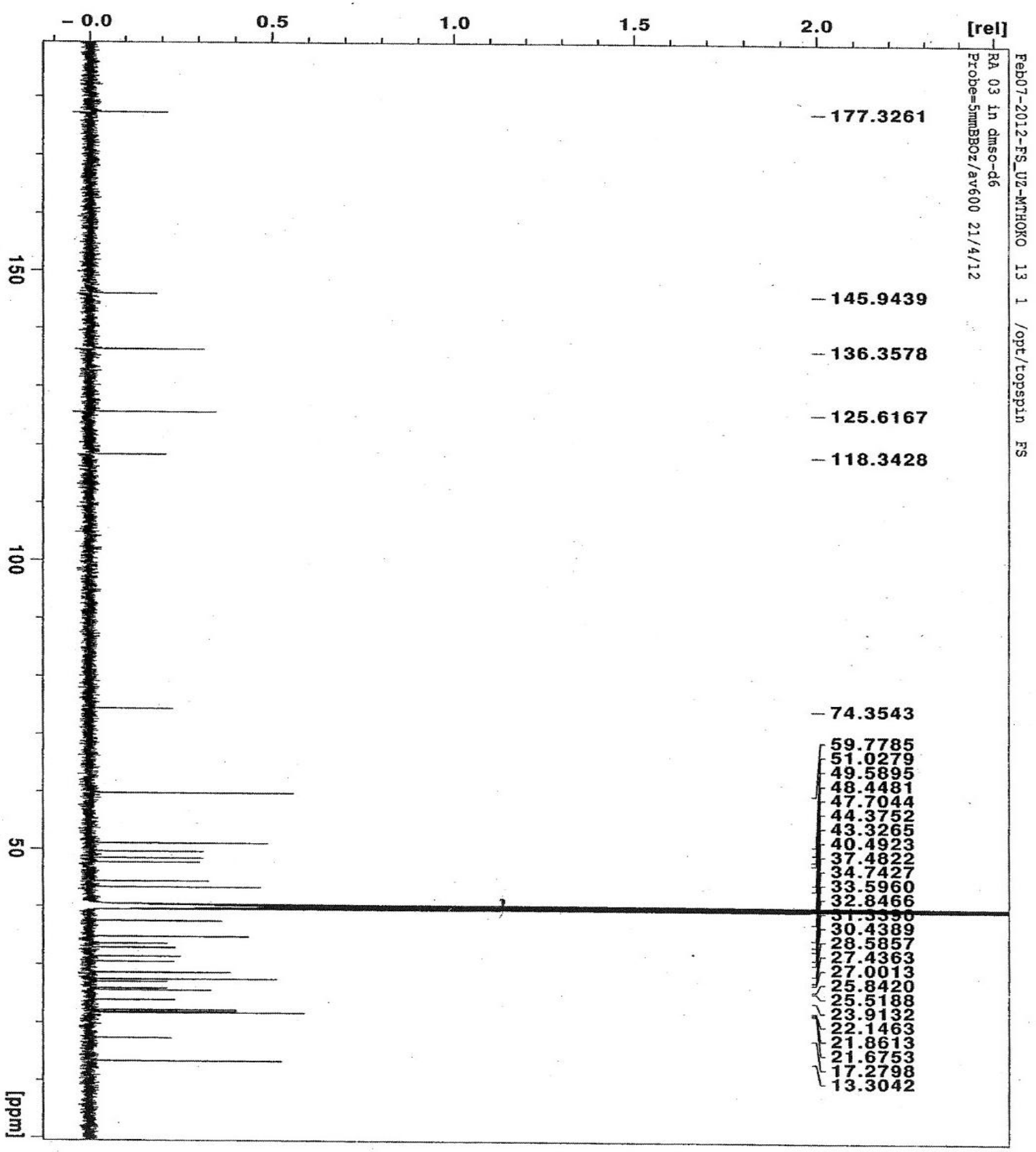

Figure D3: 13C-NMR spectrum of compound KEM

Appendix E
(CONTRIBUTION TO KNOWLEDGE)

In vivo hypo-lipidemic activity of methyl-3β-hydroxylanosta-9,24 dienoate from the stem *bark of Protorhus longifolia* (Benrh) Engl

Machaba K.E, Cobongela S.Z.Z, Mosa R.A, Djarova T.G, Opoku A.R
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Background

Hyperlipidemia is considered as one of the five leading causes of death in the world [1].The currently available anti-hyperlipidemic agents might have limited efficacy and are associated with undesirable side effects [2]. Thus, there is a need to discover and develop new effective anti-hyperlipidemic agents of natural origin. *In vitro* of hypo-lipidemic activity of methyl-3β-hydroxylanosta-9,24 dienoate have been established [3].The aim of the present study was to investigate the *in vivo* hypolipidemic activity of the methyl-3β-hydroxylanosta-9,24 dienoate from Protorhus longifolia in High Fat Diet-induced hyperlipidemic rats.

Methods

- The powdered plant material was extracted sequentially with hexane and chloroform.
- The crude chloroform extract was subjected to silica gel column chromatography, eluted with (gradient) hexane:ethyl acetate solvent system. The eluted fractions were collected at intervals of 20 ml each.
- The pure compound (figure 1) from the plant material was identified using, thin layer chromatography and nuclear magnetic resonance (NMR) techniques, mass spectrometry (MS) and infrared (IR).

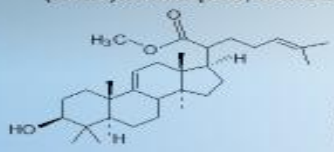

Figure.1 Chemical structure of methyl-3β-hydroxylanosta-9,24-dienoate (KEM)

Hypolipidemic study:

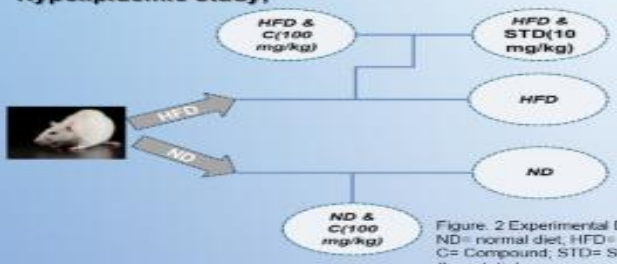

Figure. 2 Experimental Design. ND= normal diet; HFD= high fat diet C= Compound; STD= Standard drug (lovastatin)

Group	Atherogenic index (mmol/L)	Coronary risk index (mmol/L)
ND (after 21days)	0.09 ± 0.04	1.21 ± 0.03
(after 36days)	0.05 ± 0.01	1.22 ± 0.06
HFD (after 21days)	0.98 ± 0.16***	2.01 ± 0.15***
(after 36days)	0.32 ± 0.06	1.31 ± 0.06
ND+KEM(100mg/kg.bw)	0.03 ± 0.01	1.11 ± 0.01
HFD+KEM(100mg/kg.bw)	0.22 ± 0.04	1.23 ± 0.04

Table 1 The effect of KEM on atherogenic index (AI) and coronary risk index (CRI) on high fat diet induced hyperlipidemia in rats. Values are expressed as mean ± SEM, (n=4); ****p<0.0001 compared to the HFD 36days.

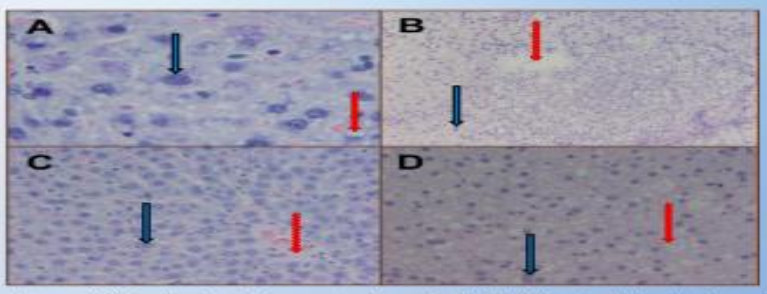

Figure. 4 Histopathological changes in liver of high fat diet induced hyperlipidemia. (A), Normal group showing normal liver. (B), Control group showing liver of rats fed with HFD. (C), KEM (100mg/kg/day)-treated normal diet fed rats. (D), KEM (100mg/kg/day)-treated HFD fed rats. = Nuclear, = Fat droplets

Results

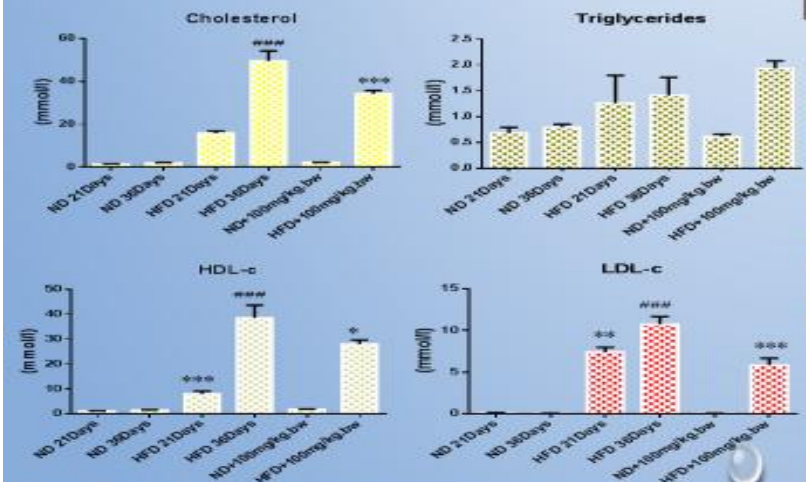

Figure. 3 The effect of KEM on cholesterol, triglycerides, high density lipoprotein (HDL-c) and low density lipoprotein (LDL-c) in HFD-induced in rats. Values are expressed as mean ± SEM, (n=4); *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 compared to the HFD 36days; #p<0.05, ##p<0.01, ###p<0.001, ####p<0.0001 compared to the ND 36 days

Discussion and Conclusion

The results of this study indicate that treatment with the triterpene for a period of 15 days caused reduction in the atherogenic and coronary indices in HFD fed rats. The triterpene also prevented accumulation of fats in the liver.

Acknowledgements

This study was supported by Medical Research council and University of Zululand, South Africa.

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