ADDIS ABABA UNIVERSITY

SCHOOL OF GRADUATE STUDIES

DEPARTMENT OF MEDICAL BIOCHEMISTRY

Antilipidemic Properties of *Calpurnia aurea* leaf extract on high fat diet induced hyperlipidemia male albino wistar rats

BY: Mengistu Welde

A THESIS SUBMITTED TO ADDIS ABABA UNIVERSITY SCHOOL OF GRADUATE STUDIES DEPARTMENT OF BIOCHEMISTRY IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN MEDICAL BIOCHEMISTRY

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This is to certify that the thesis prepared by Mengistu Welde entitled: Antilipidemic properties of *C. aurea* leaf extract on high fat diet induced Hyperlipidemia male Albino Wistar Rats and submitted in partial Fulfillment of the requirements for the Degree of Master of Science in Biochemistry.

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January, 2017
ADDIS ABABA, ETHIOPIA

Approval sheet

This is to certify that the thesis prepared by Mengistu Welde entitled: Antilipidemic properties of *C. aurea* leaf extract on high fat diet induced Hyperlipidemia male Albino Wistar Rats submitted in partial fulfillment of the requirements for the Degree of Master of Science in Biochemistry complies with regulation of the University and meets the accepted standards with respect to originality and quality**.**

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Abstract

Introduction: Hyperlipidemia is an elevation of the plasma lipids such as triglycerides, cholesterols, cholesterol esters, phospholipids and/or plasma lipoproteins including very low density lipoprotein and low density lipoprotein, and reduced in the circulating high-density lipoprotein levels. It is associated with an increased risk of coronary heart disease such as atherosclerosis and myocardial infarction.

Objective: To investigate the antilipidemic properties of hydromethanolic extract of *Calpurnia aurea* leafs against high fat diet induced hyperlipidemic male albino wistar rats.

Methods: Thirty albino Wistar rats' 60-75days old and weight of 150- 200 grams were divided randomly in to six groups of five each. Group I served as normal control, group II received high fat diet (48.8% fat w/w) containing lard made from beef fat and mixed with hydrogenated vegetable oil, group III received high fat diet supplement with 3.5 mg/kg/day atorvastatin as standard control and the remaining three groups IV, V and VI received high fat diet along with hydromethanolc extract of Calpurnia aurea at (200,300 and 400) mg/kg/day respectively for 60 days. Food intake, Body weight, body mass index, serum lipid profiles and liver histopathology were assessed.

Results: The result of this study revealed hydromethanolic extract of *Calpurnia aurea* /HMECA have dose dependent antilipidemic activities. *Calpurnia aurea* treatment of 400mg/kg caused a significant lowering of ($p < 0.05$) of serum LDL from 28.53±12.2 mg/dl to 9.70 ± 5.77 mg/dL; the serum Cholesterol level from 92.00± 13.0 mg/dl to 60.33± 8.60 mg/dl; and the serum triglyceride leve1from 71.83 ± 13.0 mg/dl to 84.73±19.4 mg/dl and on the other hand for the same dose it had significant elevated serum HDL levels from 11.66 ±1.23 mg/dl to 29.66± 1.52 mg/dl. At the medium dose of 300mg /kg it was not effective as 400mg /kg and at the minimal dose of 200mg/kg brought numerical difference not statisticaly significant among the serum lipid profile.

Conclusion: These observations suggest that HMECA has a dose dependent antihyperlipidemic effect against high fat diet induced hyperlipidemia. The molecular mechanism of antilipidemic activities of this drug need to be studied.

KEY WORDS: *Calpurnia aurea*, High fat diet, Hyperlipidemia, Lipid profile, Atorovastatin.

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Acronyms and abbreviations

ADP	Adenosine diphosphate
ApoB100	Apoprotein B hundred
BMI	Body mass index
CAD	Coronary artery diseases
CETP	Cholesterol ester transfer protein
CO	Cholesterol oxidase
CVD	Cardiovascular diseases
DHAP	Dihydroxyacetone phosphate
DPPH	1, 1-Diphenyl-2- picryl hydrazine
FFA	Free fatty acid
G-3-P	Glycerol-3- phosphate
HDL	High density lipoprotein
HED	Human equivalent dose
HFD	High fatty diet
HL	Hepatic lipase
HMG-COA	Hydroxymethyl glutareyl coenzyme A
LCAT	Lecithin cholesterol acyltransferase
LDH	Lactate dehydrogenase
LDL	Low density lipoprotein
LD_{50}	Lethal dose at fifty percent
LPL	Lipoprotein lipase
HMECA	Hydromethanolic extract of *calapurnia aurea*
MI	Myocardial Infarction
IC_{50}	Inhibitor concentration at fifty percent
IDL	Intermediate density lipoprotein
NAD^+	Nicontinamide Adenine Dinucleotide
ROS	reactive oxygen species
OECD	Organization for Economic Cooperation and Development
TC	Total cholesterol
TG	Triglyceride
TLC	Thin layer chromatography
VLDL	Very Low Density Lipoproteins

1. INTRODUCTION

1.1. General Features of hyperlipidemia

Hyperlipidemia is characterized by elevated in the plasma lipids including triglycerides, cholesterols, cholesterol esters, phospholipids and/or plasma lipoproteins such as very low density lipoprotein, low density lipoprotein and reduced in the circulating high density lipoprotein levels (Shattat, 2014). The high fatty substances travel and remain dissolved in the circulation attached to proteins make hyperlipoproteinemia (Harikumar *et al.,* 2013). It is also identified as dyslipidemia, to describe the manifestations of different disorders of lipoprotein metabolisms like atherosclerosis which causes cardiovascular diseases that accounts for one third of total deaths around the world. And it is believed that cardiovascular diseases will turn out to be the main cause of death and disability worldwide by the year 2020 (Jorgensen *et al*., 2013).

Hyperlipidemia causes significant production of free radicals, which may lead to increased oxidative stress. Oxidative stress occur when imbalance between antioxidant and reactive oxygen species (ROS) formation during biochemical reaction in cells. ROS can modify to low density lipoproteins, which play a significant function in the initiation and progression of atherosclerosis and associated cardiovascular diseases. For patients with a first myocardial infarction (MI) condition, hyperlipidemia has been reported to have a detrimental effect on ventricular function with a more pronounced deterioration. It involves not only heart and vessels, but also liver, kidneys and even the nervous system. Other common co-morbidities of CVDs are non-alcoholic fatty liver disease, chronic kidney disease (Mishra *et al.,* 2011).

The prevalence of hyperlipidemia has dramatically increased worldwide due to modern lifestyles which bring about increase in the consumption of high-fat diets (Ogbuehi *et al*., 2013). Many current studies reveal that there are significant differences in blood lipid levels and the prevalence of hyperlipidemia between ethnic groups, different dietary habits, life style and level of physical activity, as well as their genetic background (Ruixing *et al.,* 2006).

Another modifying factors in the development and progression of hyperlipidemia are age and gender. It has been shown that cholesterol levels rise as the person gets older (Grauvogel *et al.,* 2010).

The prevalence of hyperlipidemia is in the range of 39% , 51% and 26% worldwide, developed and developing countries, respectively .Overall, raised cholesterol is estimated to cause 2.6 million deaths and 29.7million disability adjusted life years (DALY) (Kanakavalli *et al.,* 2016).

1.1.1. Lipid metabolism

Lipoproteins consist of lipids and proteins (known as apolipoproteins [apo]), with the main function of transporting water-insoluble lipids such as cholesterol or triglycerides in plasma from the sites of absorption (gut) and/or synthesis(liver) to the sites of utilization (peripheral tissues) or processing. Besides contributing to the structure and the stability of the macromolecule, Apo-lipoproteins control the metabolism of the lipoproteins by activation or inhibition of enzymes and interaction with lipoprotein receptors (Bonnie *et al*., 2007).

Almost all the dietary fats are absorbed from the intestinal lumen into the intestinal lymph that are packed into chylomicrons. These lipoproteins move into the blood stream where they get hydrolyzed by endothelial lipoprotein lipase which splits the triglyceride into glycerol and free fatty acids. Then, chylomicron remnants are absorbed in the liver and packaged with cholesterol, cholesteryl esters and Apoprotien B100 to form VLDL. When VLDL are released into the blood stream, they are modified into IDL by the action of lipoprotein lipase and hepatic lipase where phospholipids and apolipoproteins are transferred back to HDL. Furthermore, after the hydrolysis by hepatic lipase, IDL will be converted to LDL (Mclaren *et al.,* 2011).

The peripheral cholesterol is returned to the liver by reverse cholesterol transport pathway using HDLs which are originally synthesized by the liver and intestine and released into the blood and relieves the peripheral cells from cholesterol burden .In the blood, HDL cholesterol is esterified by lecithin: cholesterol acyltransferase (LCAT) to cholesteryl ester and transferred to VLDL and the chylomicrons to return to the liver through LDL receptor. Cholesteryl ester are transferred to LDL particles by cholesteryl ester transfer protein; (CETP) and then subjected to LDL-receptors mediated endocytosis. Finally, cholesteryl esters are hydrolyzed to cholesterol and extracted from the liver as bile acid (Hegele, 2009).

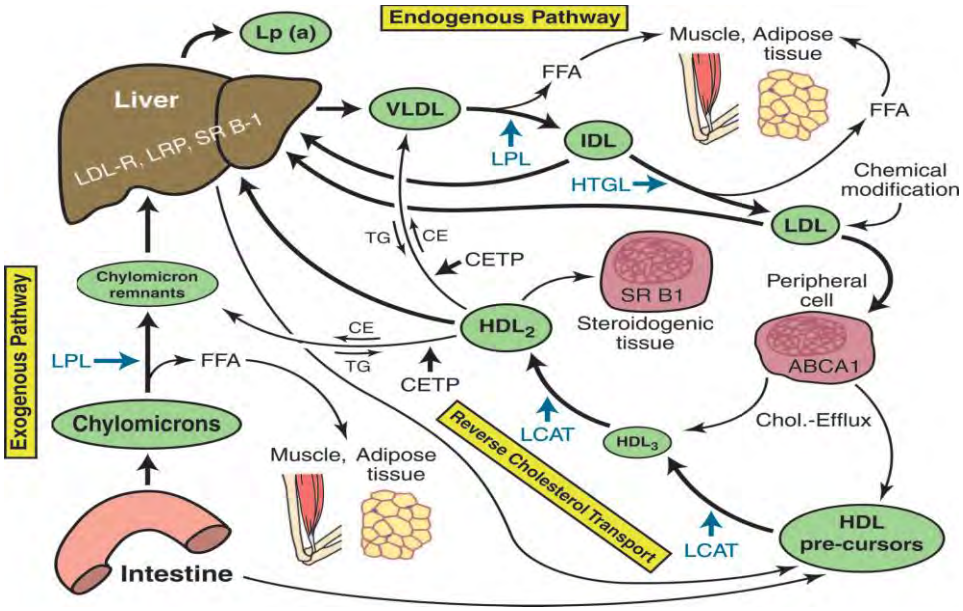

Figure: 1. Lipoproteins metabolism in exogenous and endogenous pathways (taken from Bonnie *et al*., 2007). Blue arrows refer to points of action of the respective enzymes. ABCA1, ATP binding cassette transporter 1 ; CE, cholesterol ester; CETP, cholesteryl ester transfer protein; FFA, free fatty acid; HTGL, hepatic triglyceride lipase; IDL, intermediate-density lipoprotein; LCAT, lecithin: cholesterol acyltransferase; LDL-R, LDL receptor; Lp(a), lipoprotein(a); LPL, lipoprotein lipase; LRP, LDL-R–related protein; SRB1, scavenger receptor B1; TG, triglyceride.

1.2. The pathophysiology of hyperlipidemia

1.2.1. Pathophysiology of primary hyperlipidemia

Primary hyperlipidemia is mostly associated with genetic disorders and the disease may be mostly treated using antilipidemic drugs. Most hereditary lipid metabolism disorders are common among families with obesity problems. It involves that the idiopathic hyperchylomicronemia defect in lipid metabolism leads to hypertriglyceridemia which is caused by a defect in lipoprotein lipase activity or the absence of the surface apoprotein CII. Familial Combined Hyperlipidemia (FCHL) can be caused due to decreased LDL receptor which leads to elevated plasma lipoprotein such as LDL and VLDL. Another hereditary condition called Familial Defective Apo-lipoprotein B-100 can cause the LDL cholesterol to increase in plasma and even raise the total cholesterol levels in blood. Some familial lipid metabolism disorders can directly result in over production of cholesterol by the body (Tripathi, 2008).

1.2.2. Pathophysiology of Secondary hyperlipidemia

The secondary type of hyperlipidemia originating from other diseases like diabetes, renal lipid nephritis or hypothyroidism, during these diseases the demands are for the treatment of the original disease rather than hyperlipidemia. The diabetes mellitus patients have been noted to possess low LPL activity which further causes higher synthesis of VLDL cholesterol by the liver ultimately leading to hyperlipidemia and diseases such as, hypothyroidism-induced low LPL activity and lipolytic activity has been noted to reduce hepatic degradation of cholesterol to bile acids (Baron, 2005).

Exogenous factors, lifestyle habits, mainly poor diet when fat intake from saturated fat and cholesterol exceeds 40 percent of the total calories uptake, alcohol consumption, use of contraceptives and other pharmacologic agents, diuretics, beta-blockers and medicines used to treat depression have also been reported to raise cholesterol levels and are the main secondary causes of hyperlipidemia in adults (Amuamuta *et al.,* 2014).

1.3. Complications of hyperlipidemia

1.3.1. Atherosclerosis

Atherosclerosis (hardening of the arteries) is a progressive disease caused when fat, cholesterol, and other substances, build up in the inner walls of arteries and form hard structures called plaques. Sometimes this plaque can break open. When this happens, a blood clot forms and blocks the artery causing heart attacks and stroke (World Health Organization, 2011).

1.3.2. Coronary Artery Disease

Atherosclerosis, the major cause of coronary artery disease, characterized by the accumulation of lipid and cholesterol that result in the formation of fibrous plaques within the wall of the arteries causes narrowing of the arteries that supply blood to the myocardium. Elevated lipid profile has been connected to the development of coronary atherosclerosis (Gao *et al.,* 2012).

1.3.3. Myocardial Infarction (MI)

Myocardial Infarction (MI) is characterized by necrosis of a portion of the heart muscle. It is one of the most frequent causes of death in developed countries. Coronary atherosclerosis is frequently an underlying factor in the pathogenesis of MI. The acute event is often

triggered by rupture of an atherosclerotic plaque, leading to the formation of an occlusive thrombus and vasospasm, which interrupt the delivery of oxygen to the myocardial tissue supplied by that artery (Ateş *et al*., 2012).

1.3.4. Ischemic stroke

Stroke is the fourth leading cause of death and it occurs due to blockage of an artery by a blood clot or a piece of atherosclerotic plaque that damage a small vessel within the brain. Many clinical trials revealed that lowering of low-density lipoprotein and total cholesterol by 15% significantly reduced the risk of the stroke (Osipov *et al.,* 2009).

1.4. Treatment of hyperlipidemia

The main aim of treatment in patients with hyperlipidemia is to reduce the risk of developing ischemic heart disease or the occurrence of further cardiovascular or cerebrovascular disease and its treatment depends on the patient's lipid profile (Anbu et al., 2011).The increase of lipid profile has been shown to be a strong risk factor for coronary heart diseases in many populations. The high cholesterol level in tissue and serum may be due to increased uptake of either exogenous fat or cholesterol or both and which was subsequently deposited and decreased cholesterol catabolism as evidenced by a reduction in bile acid production and turnover of bile acids (Venu *et al.,* 2010) and even high caloric diet increased significantly in the body mass index (BMI) of animals (Novelli *et al.,* 2007).

1.4.1. Mechanism of action of antilipidemic drugs

The serum lipids level can be conventionally improved, through the group of statins drugs, which are currently in use as lipid lowering agents. Atorvastatin is one of the major lipid lowering drugs used in hyperlipidemic conditions. Their primary site of action is in the liver where they inhibit HMG-CoA reductase, the metabolic pathway that produces cholesterol (Senecha *et al*., 2012).

They competitively inhibit the enzyme HMG-CoA reductase in the liver that converts HMG-CoA to mevalonate an early precursor for cholesterol synthesis. More over statins increase the expression of LDL receptors in liver. These LDL receptors increases uptake and Subsequent removal of LDL, VLDL, IDL and also reduce triglyceride levels by increasing LDL clearance.

On the other hand it increases circulating HDL cholesterol levels in the plasma, which finally restores cholesterol homeostasis (Ray and Rege, 2000).Although many efficacious lipid-lowering synthetic drugs exist, none is effective for all lipoprotein disorders, and all such agents are associated with some adverse effects. Therefore there is a need to search for traditional medicinal plants in the locality which are often cheaper, have less adverse effect and are easily consumable to provide better safety and efficacy on a long term usage (Anyanwu *et al.,* 2013).

1.5. Medicinal plants

They have a significant role in maintaining human health and had improved the quality of human life for thousands of years and served humans as valuable components of medicine. In general the medicinal value of plants lies in some chemical substances that produce a definite physiological action on the human body. The most important of these bioactive constituents of plants are saponins, flavonoids, phenolics and alkaloids. Many of the indigenous medicinal plants are used as spices and food plants **(**Birhanu, 2013).In developing countries, a large number of people lives in extreme poverty and some are suffering and dying for want of safe water and medicine, they have no alternative for primary health care, therefore who need to look inwards to search for herbal medicinal plants with the aim of validating the ethno-medicinal use and subsequently the isolation and characterization of compounds which will be added to the potential list of drugs (Ferreira *et al.,*2012)

1.5.1. *Calpurnia* aurea

Calpurnia aurea (Ait.) Benth, a member of the subfamily Papilionoideae belongs to the family of Fabaceae . This grows as shrub or a slender tree of up to 15 m tall, widespread along the east coast of Africa. *Calpurnia aurea* is known by several local names, chekata in Afaan Oromo and digita in Amharic. *C. aurea* has not been scientifically proven to have antilipidemic potential, it have been found to be medicinally useful in many ailments. Its widespread application for diverse ethno-medicinal uses has made it a subject for pharmacological and phytochemical studies. It is used by the Shinasha people to treat amoebiasis and giardiasis while the Amhara people from the same region use the leaves to treat malaria and the seeds to treat hypertension while a combination of the leaves and seeds is used to treat

diarrhea and diabetes (Kanakavalli *et al.,* 2016). The plant has also been used to induce uterine contractions, and to treat coughs, amoebic dysentery, syphilis, leishmaniasis, tapeworm, trachoma, ringworm, scabies, elephantiasis, abscesses and wounds as well as stomach ache, vomiting, headache and eye diseases (Mebrahtu *et al* ., 2013).

Fig 2: Calpurnia aurea *plant leaf image collected on September 2015 from Hora Lake bishoftu , Eastern Showa zone.*

1.5.2. Chemical component of *Calpurnia aurea* plant and its effect against various ailments

Pharmacological studies have shown that methanol extracts of the leaves and stems of *C.aurea* have a good antibacterial and antioxidant properties (Tadeg *et al.,* 2005; Adedapo *et al.,* 2008), validating its traditional use for a range of microbial infection. Quinolizidine alkaloids, butin (7,3',4'-trihydroxyflavanone), the flavonoids vicenin-2 (6,8-di-β-D-glucopyranosyl-5,7,4' trihydroxy flavone) and 3'-hydroxydaidzein (isoflavone) were isolated from the seeds of C.*aurea,* in keeping with flavonoids being the other major class of compounds consistently found in the Fabaceae. There is report on the isolation of five isoflavonoids, a pterocarpan and a quinolizidine alkaloid from stem and bark of *C.aurea* as well as the anticancer activity of the isolated isoflavonoids (Korir *et al*., 2014).

Calpurnia aurea plant leaves and seeds, both contain flavones and polyphenols though the levels were found more in leaves than the in seeds. The extracts of both leaves and seeds of the plant indicated strong antioxidant activities. Several studies have validated the use of anti-diarrheal medicinal plants by investigating the biological activity of extracts of such plants, which have antispasmodic effects, delay intestinal transit; suppress gut motility, stimulate water absorption, or reduce the intra luminal fluid accumulation (Atta and Mouneir, 2005). *Calpurnia aurea* seed extract obsessed heptoprotective activity against HAART induced liver injury in rats (Mulata *et al.,* 2015).

Chávez-Santoscoy *et al*., 2013 reported that some of the chemical constituents, such as flavonoids and saponins lowers cholesterol absorption by the inhibition of cholesterol micellar solubility, from other studies reported that plants secondary metabolite like flavonoids have antihypercholesterolemia activity and they also inhibited 3-hydroxy-3-methylglutaryl coenzyme A reductase, the enzyme crucial for cholesterol biosynthesis (Fajaryanti *et al*., 2016) and Varied biological activities of plants phenolics acids were also indicate in an increases bile secretion, reduces blood cholesterol and lipid levels are some of biological activities of phenolic acids (Ghasemzadeh,2011). It was reported that the presence of plants flavonoids, saponins and phenolics compound of plant has potential to increase HDL-C concentration and decrease in LDL and VLDL levels in hypercholesteremic rats (Patel *et al*., 2009; Yanping *et al*., 2005).
Furthermore saponins fraction isolated from leaf extract have an in vivo anti-obesity activity which show significantly decreased in weight in visceral organs and lipid profile such as triglycerides, total cholesterol, low-density lipoproteins and very low-density lipoproteins (Marrelli *et al.,*2016). Since there have no reports on the leaves of *C. aurea*, on the metabolic effect/ hypolipidemic activities, therefore having these various information of plants secondary metabolities of earlier reported, the study was started an investigation the effect of hydromethanolic leaves extract of *C.aurea* on food intake, body weight, body mass index and the lipid profile have been done using male wistar albino rats placed on high fat diet.

1.6. Statement of the problem

Hyperlipidemia and its metabolic syndrome are a growing world health burden in developed countries and it is an emerging as a problem in developing countries. The number of individuals with Hyperlipidemia and its metabolic syndrome as one of the greatest risk factors contributing to the prevalence and severity of Coronary heart disease, stroke, and atherosclerosis and myocardial infarction are the primary cause of death. Hyperlipidemia increases with chronological age. Responsibility of elevated total cholesterol and especially LDL-cholesterol is now well established in the occurrence of cardiovascular disease (Priya *et al.,* 2013). 10% reduction in serum cholesterol in men aged 40 has been reported to result in a 50% reduction in a heart disease within 5 years and the same serum cholesterol reduction for men aged 70 years can result in an average 20% reduction in heart disease occurrence in the next 5 years (WHO , 2013).

There is limited information available about the prevalence of chronic metabolic diseases (diabetes, obesity, hypertension and hyperlipidemia) in many developing countries. The prevalence of these four diseases in the population aged 20 years and above indicated; diabetes mellitus was 6% to 8%, obesity was close to 26% and hypertension and hyperlipidemia almost affected one third of the population respectively (Amuamuta , 2014). Hyperlipidemia, the risk factors for the development of cardiovascular diseases has been increased in developing countries, including Ethiopia. There is a knowledge gap on the epidemiology of the disease around the continent, including the lack of data regarding the distribution and occurrence of the diseases among adults.

Traditional medicine has remained as the most affordable and easily accessible source of treatment in the primary health care system of resource poor communities in Ethiopia. About 80% of the population in developing countries use traditional medicines because they cannot afford the high cost of western pharmaceuticals and for health care, though the drugs have adverse effects like hyperuricemia, diarrhea, nausea, myositis, gastric irritation, flushing ,dry skin and abnormal liver function (Harikumar *et al* ., 2014).

Medicinal plants are used for various research purposes. More than thirteen thousand plants have been studied for various pharmacological properties. Medicinal plants play a major role in hypolipidemic activity and suggest that the lipid lowering action is mediated through inhibition of hepatic cholesterol biosynthesis and reduction of lipid absorption in the intestine (Jegadeesha *et al.* ,2014). According to the study recorded south-central Zimbabwe ninety three plant species useful in traditionally managing various human diseases of these ,79 species are indigenous to Zimbabwe (84.9%),while 14 species are exotic (15.1%) of which are either naturalized as weeds or cultivated in home gardens as ornamentals or food plants (Maroyi,2013).

In Ethiopia, 70% of human and 90% of livestock population depend on traditional medicine (Teklehaymanot and Giday, 2007). Ethiopian plants have shown very effective medicinal value for some ailments of both human and domestic animals, thus medicinal plants and knowledge of their use provide a vital contribution to human and livestock health care needs in the country. There are 6,500 species of higher plants in Ethiopia, making the country one of the most diverse floristic regions in the world **(**Birhanu, 2013). Ethiopian traditional life is painted with the hallmark of widespread use of medicinal plants with various levels of sophistication within the indigenous medicinal lore, and the vast knowledge on traditional use of medicinal plants is not fully documented; most of the knowledge is conveyed from generation to generation through word of mouth. Like many other Ethiopians, people in Gondar Zuria district use plants for their primary health care. As is happening elsewhere in Ethiopia, the traditional knowledge as well as the plants used by these people is under threat mainly due to deforestation, degradation and cultural shift. With this background, a survey was conducted to document the indigenous knowledge of traditional healers in Gondar Zuria district **(**Birhanu, 2013).

1.7. Significance of the study

Hyperlipidemic factors are now markedly influence our health by causing hyperlipidemia and other cardiovascular related diseases as reveled by many studies. Due to these, the commercialy available synthetic antihyperlipidemic drug therapy is mandatory to save the patient, though side effects associated, like gastro-intestinal disturbance and immune suppression are associated with such drugs. So *Calpurnia aurea* plant which has widespread application and diverse ethno-medicinal uses, has no any claim for its hypolipidemic properties. Thus appreciating its widespread applications and diverse ethno-medicinal uses. Hence this study is aimed to find out antilipidemic properties of hydromethanolic extract of *Calpurnia aurea* on HFD fed albino wistar rats , that would help to better understand the therapeutic activity and other profile of the plant. Besides this the data could be baseline for other scientific investigation and to do further research on it.

2. Objective

2.1. General objective

To investigate antilipidemic effects of hydromethanolic leaves extract of *Calpurnia aurea* on high fatty diet induced hyperlipidemia Albino wistar rats.

2.2. Specific objectives

- ✓ To determine the effects of different doses of *Calpurnia aurea* leaves extract in food intake, body weight and body mass index
- ✓ To examine lipid profiles of high fat diet induced hyperlipidemic rats treated with leaves extract.
- ✓ To evaluate *in vitro* anti oxidant properties of *leaves* extract (IC_{50}) against DPPH radical
- ✓ To assess the histopathology of HFD induced hyperlipidemia and remedial effect of the leaves extract of *C.aurea*

3. Materials and methods

3.1 Study design

The study design was carried out by randomized experimental animals.

3.2. Study area

The study was conducted from December, 2015 to February, 2017 at the Department of Medical Biochemistry, School of Medicine, College of Health Sciences, Addis Ababa University.

3.3. Reagents and equipments

3.3.1. Reagents

During the study the following reagents and chemicals were used: Atorvastatin , methanol 80% (research lab fine chem. Industry, India), distilled water, kits for lipid profile, 1,1Diphenyl-2-Picrylhydrazil (DPPH), 10% formalin , Paraffin wax, xylem solution, diethyl ether, ascorbic acid (BDH chemicals Ltd Poole, England).

3.3.2. Equipments

In this study, volumetric flask (2000ml), Buchner funnels, filtering cloth, Whatman filter paper No 1, lyophilizer (OPR-FDU-5012,Korea), Triple beam balance, rotary evaporator (Heizbad Hei-VAP, Germany), plastic cage, full chemistry analyzer (BS-200, China), light microscope (LEICA DM750), centrifuge (PLC 012E ,Taiwan), desiccators, high definition colored camera (PC1732,China), oral gavages, test tubes, Serum vials, beaker (1000ml), metallic spatula, deep freezer(- 80^{o} C), Micropipettes (20µm, 20-200µm, 1000µm), TLC plate, Aluminum foil, Incubator, shaker (G25,USA) , centrifuge tubes (15ml,50ml), Scissors, mortar and pestle, electrical analytical balance (KERN ALJ220-4,Germany), vortex mixer (XH-D), Spectrophotometer (UV-1600 PC, Germany) and tissue cassettes were used

3.4. Plant material collection and authentication

The fresh leaves of *Calpurnia aurea* was collected from its natural habitat Debrezeyt district of eastern showa zone which is 45 km from Addis Ababa. The plant leaves were available mostly in early September and collected with proper care, then subsequently authenticated by Taxonomist of Ethiopian National herbarium of Addis Ababa University and

voucher specimen Collection number 001/mw/2015 of the plant was deposited in the Herbarium for further reference.

3.5. Preparation of plant extract

The plant leaves were cut into small fragments, air dried under shade area at room temperature and then ground to fine powder using sterile mortar and pistil. The powder was passed through sieve number 30 and stored in a glass container. The coarse powder of *Calpurnia aurea* leaves were 627 gram and it was macerated in 80% methanol (V/V) for three consecutive days (72 hours) with mechanical shaking three times a day. This process was repeated until the residue of the plant material gave no coloration to maximize the percentage yield. Then the extract was filtered first using filtering cloth to separate coarse powder from the filtrate and then by what man No.1 filter paper to separate fine powders from the filtrate and then the filtrate was evaporated to be dried by rotary evaporator and further the filtrate was concentrated under reduced pressure at 40^{O}C.Then the filtrate was taken to thermostatic oven at 40^{o}C and kept overnight to evaporate the remaining methanol. After that, the final extract which is almost free of methanol was taken to deep freezer minus (80) to have a solid consistency. This frozen and solidified extract was lyophilized repeatedly using freeze dryer (lyophilizer) until the water was completely removed and the extract obtained was weighed to determine percentage yield. Then the final extract was packed in glass which was not transparent to light.

$$\% \text{ yield} = \text{Weight of the dried extract/Weight of coarse powder} \times 100\%$$

3.6. Preliminary phytochemical screening

The phytochemical examination of the *C.aurea* leaves extract was performed by the standard methods.

3.7. Determination of *in-vitro* antioxidant activity hydromethanolic extracts of *C.aurea* leafs /HMECA

3.7.1. Thin Layer Chromatography technique

Antioxidant is a reducing agent which acts against DPPH assay is one of the most commonly used methods for determining the antioxidant activity of various extracts of medicinal plants (Blois, 1958).

In this procedure, a stable free radical DPPH (deep violet), after accepting electrons or hydrogen atom (protons), is converted into stable DPPH-H (pale yellow) (Ebrahimzadeh *et al.,* 2009; Muthusamy *et al.,* 2009).

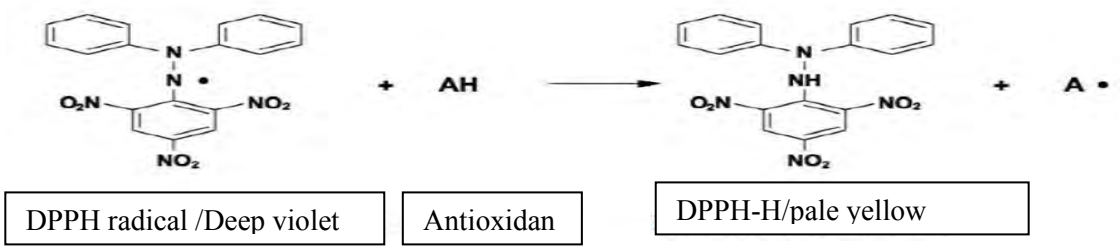

Figure 3: Principle of reaction of DPPH reaction antioxidants (Aurelia et al., 2009).

The free radical scavenging activities of the plant extracts against 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical .The antioxidant activity of hydromethanolic extract *C.aurea* leaves was determined by using DPPH as free radical and free radical scavenging activity of the extract was determined according to the method described by Yamaguchi *et al*.,1998. Hydromethanolic extract of *C.aurea* leaf / HMECA was dissolved in 80% methanol and the preparation was 200mg/100mL, 300mg /100mL and 400mg/100ml and 0.1mM of DPPH solution was prepared in methanol. Then, this adequate amount of DPPH solution was poured to a wide Petri dish for easy immersion on the TLC plates. After these preparations, 2µL of MECA from different concentration were placed on individual TLC plates and waited for 30 minutes until TLC plates completely dried. Then the TLC plates were stained with 0.1mM DPPH solution for few seconds. Then the mixture was allowed to stand at room temperature for 30 minutes. After that, the antioxidant activity of HMECA at various doses was analyzed, interpreted and presented in pictures.

3.7.2. *In vitro* antioxidant properties hydromethanollic extracts of *C.aurea* leaf and determination of IC_{50} (Spectrophotometric method)

The antioxidant activity of hydromethanolic extract of *C.aurea* leaf was also determined quantitatively by using DPPH (1, 1-diphenyl-2-picrylhydrazyl) as free radical which has an absorption band at 517 nm.Hence, the free radical scavenging activity of HMECA was assessed according to the method developed by Tekao and his colleagues (Tekao *et al .,* 1994). According to this method DPPH was prepared with a concentration of 0.008% and the extract of HMECA

was prepared with a concentration of 1mg/ml, 500µg/ml, 250µg/ml, 125 µg/ml and 62.5µg/ml in methanol. Ascorbic acid was also prepared in the same concentration with that of HMECA (1mg/ml, 500 µg /ml, 250µg/ml, 125 µg/ml and 62.5µg/ml) in methanol serially as a Standard. Then 1ml of the extract from each concentration was mixed with 1ml of 0.008% DPPH, and then incubated for 30 minutes at room temperature in darkness.

Finally, the Absorbance was measured at 517nm by using DPPH and methanol as a control and methanol as a blank .The free radical scavenging activity of extracts was calculated using the formula: DPPH radical scavenging activity or % inhibition (I) = [(Ab-Aa) /Ab] x 100, Where Ab is the absorbance of DPPH radical plus methanol (control sample) and *Aa* is absorbance DPPH radical plus sample extract/standard (Usmani, 2013).The percentage reduction in absorbance was calculated from initial (control sample) and final absorbance at each level.

The Concentration of the HMECA required for 50% reduction in absorbance (IC_{50}) was calculated from the calibration curve (Concentration of extract in µg/ml Vs % reduction in absorbance).Median inhibitory concentration (IC_{50}) is the concentration of the extract that decreases the absorbance of DPPH by half (50%).The DPPH scavenging capacity of the plant extracts may be related to the phenolic, flavanoid and other compounds present in extract. The result of DPPH assay was expressed in IC_{50} value. IC_{50} values are negatively related to the antioxidant activity of extract , as it express the amount of antioxidant concentration needed to decrease DPPH absorbance by 50%.The lower IC_{50} value represents the higher antioxidant activity of the tested sample (Williams *et al.,* 2004).

3.8. Acute oral toxicity test

Acute oral toxicity refers to those adverse effects occurring following oral administration of a single dose of a substance, or the dose may be given in smaller fractions over a period not exceeding 24 hours, and then Animals were individually observed for changes in skin, general behavioral pattern, tremors, convulsions, salivation, diarrhea, sleep, coma and mortality for a period of 14 days. In the acute oral toxicity study of methanol extract of *C.aurea leaf,* a limit dose of each 2000 mg/kg body weight of the animal was administered on a single test animal orally by gavages. The limit test was repeated three times on a single test animal as a part of an oral acute toxicity assay. No toxicity was observed at administered dose 0f plant extract.

3.9. Experimental animals

Six females and three male Albino wistar rats obtain from my colleagues and allowed to breed them and wait for three months. After three month stay male sex rats of weight 150-190gm with age of 60-75 days for in this study was used .All the male rats were derived from single cross isolation in one cage. The animals acclimatized to laboratory condition for one week prior to the experiments. They were kept in plastic cages at 22 ± 2°C, in relative humidity of 55% and on a 12 h light dark cycle with free access to pellet food and tap water ad libitum at the same animal house. Then rats were randomly divided into six groups of each contained five in number.

3.10. Composition of experimental diet

3.10.1. High fat diet (HFD) preparation

Many experiments have been used rats fed with commercial lard (animal fat) as hyperlipdemia model (Brainard *et al*., 2013). However, commercial lard is not easily available in Ethiopia. Due to that, a system was developed to prepare lard from bovine fat (choma) from local butchers and hydrogenated vegetable oil, a solid form of fat at room temperature from the market in Addis Ababa, Ethiopia.

During high fat food preparation, animal fat was melted, liquefied, and then non-fat solid material, including connective tissue and meat were removed, this was followed by solidifying animal fat for measuring the weight (gm) at room temperature. Although the process of melting and liquefying animal fat, was repeated during procedure till hot warm homogeneous mixture of lard produced, that was ready to be easily mixed with warm hydrogenated vegetable oil (liquid).This were done before it solidified and mixed with powdered standard pellet and allowed to cool to produce a solid homogeneous mixture of lard and hydrogenated vegetable oil / pellet (36% / 64% w/w) that could be fed to the rats (Figure: 4)

.The powdered pellet was prepared by grinding standard pellet food in a pestle and mortar. This homogeneous lard-oil containing pellet provided the high fat diet that induces hyperlipidemia. Standard rats' food pellets contained 20% fat, 60 % carbohydrates and 20 % proteins. Therefore, with the addition of 30 % lard and 6% hydrogenated vegetable oil (essentially 100% fat) to the pellets which contains 20 % fat, produced a food mixture containing 48.80% fat. Therefore, the term, "high fat diet" in this study refers to a diet containing 48.80% fat by weight (30% from animal lard, 6% from

hydrogenated vegetable oil and 12.8% fat from the standard pellet), since 20% of the fat obtained from the standard pellet is equal to 12.80%.Terfore a high fat diet (lard-oil) which was 48.8% were fed to rats for 60 days consecutively to induce hyperlipidimea during experiments.

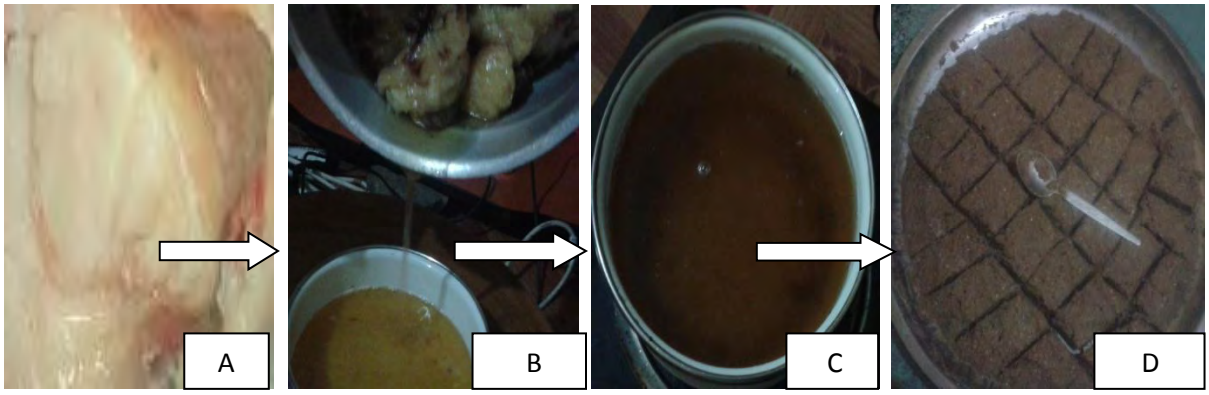

Figure: 4. Preparation of lard**.** The choma (A), liquefied by heating in a pan on a stove (B), then non-fat material including connective tissue and meat were removed and the liquefied fat was cooled to allow it to solidify into lard which was supplemented with few hydrogenated vegetable oil (C) and finally placed in powdered pellet while mixing properly and baking (D).

3.11. Extrapolation of atorvastatin dose

The humans doses of atorvastatin drug were extrapolated to animals dose by the formula; Human Equivalent Dose (HED) mg /kg = Animals Dose in (mg /kg) X (Animal Km ÷ Human Km),

Where Km is a correction factor reflecting the relationship between body weight and body surface area (Natural Health research institute, 2008).

Table: 1. shows the average Km value of most frequently used laboratory animals and human adults.

Average Km values of laboratory animals and human adult	
Mouse	3
Rat	6
Guinea pig	8
Rabbit	12
Dog	20
Human Adult	37

3.12. Experimental Protocol

The animals were randomly selected, weighed then marked for individual identification. The thirty rats were randomly assigned into six groups of five rats per cage**.**

Table: 2. Animal grouping during the experiment

Group	Category	Treatment dose
I	Normal control	Regular pellet +Distil water
II	Positive control	HFD +Distil water
III	Atorvastatin as control	HFD +3.5mg/kg
IV	Experimental	200mg/kg + HFD
V	Experimental	300mg/kg + HFD
VI	Experimental	400mg/kg + HFD

3.13. The food intake

The daily food intake of the rats was measured in the morning using a weighing balance. Food intake was calculated by subtracting the amount of food left over in each cage from the measured amount of food provided at the previous day (gm/day/cage). The mean of food intake was represented in gm/day/cage.

3.14. Anthropometrical determinations

The body weight of the rat was measured weekly in grams (g) and the body length (nose-to-anus length) was determined in centimeter in all rats. The measurements were made in anaesthetized (di-ethyl-ether) rats as inhalation exposure. The body weight and body length was used to determine the body mass index (BMI) as described by (Noveli *et al.* 2007). Body mass index of the rats were made to estimate, the body fat deposition around the abdominal and pelvic regions of rats fed a high fat diet, as there was an increasing of fat storage at these site.

Body mass index (BMI) = body weight (g)/length^2 (cm^2).

3.15. Blood collection and serum preparation from rats

At the end of the study, the rats were fasted overnight and anaesthetized with diethyl ether. The blood was collected by cardiac puncture using a 10 cc syringe. blood collected (3-4 ml) were

placed in serum separating test tube, left for 30 minutes at room temperature to clot and centrifuged at 4500 r. p .m for 5 minutes. Using a micropipette (1000µl), serum was isolated and stored at minus 80°C until serum tests like triglycerides (TG), total cholesterol (TC) serum high density lipoprotein (HDL) and low density lipoprotein (LDL) levels were done, using an Auto Lab 18 fully automated clinical chemistry analyzer following the manufacturer instrument.

3.15. 1. Determination of serum triglyceride (TG) concentration

The method is based on the enzymatic hydrolysis of triglycerides to glycerol and free fatty acids (FFA) by lipoprotein lipase (LPL). The glycerol is phosphorylated by glycerol kinase (GK) to glycerol-3- phosphate (G-3-P).G-3-P is oxidized by glycerol phosphate oxidase to form dihydroxyacetone phosphate (DHAP) and hydrogen peroxide (H_2O_2). The H_2O_2 then react with 4-aminoantipyrine and 3,5 DHBS (3,5-di chloro- 2 –hydroxybenzen) in the reaction catalyzed to yield a red colored quinoneimine dye. The intensity of the colored produced is directly proportional to the concentration triglyceride in the sample (Siedel *et al .,*1993).

Triglyceride + $3H_2O$ → Glycerol + 3FFA (catalyzed by LPL)

Glycerol + ATP → G-3-P + ADP (catalyzed by glycerol kinase)

G-3-P + O_2 → DHAP + H_2O_2 (catalyzed by glycerol-3-phosphate oxidase)

$2H_2O_2$ + 4-aminoantipyrine + phenol → Quininoid dye + $4H_2O$ (catalyzed by peroxidase)

The triglyceride test comes in the form of a commercial kit containing the reagents, reactants and enzymes needed. Serum samples were incubated with the kit reagents and enzymes for 5 minutes at 37^0C and absorbance measured at 500 nm against the reagent blank and against known concentrations of standard triglyceride concentrations. The change in absorbance is proportional to the concentration of triglyceride in the serum sample.

3.15.2. Determination of total cholesterol

Cholesterol esters are hydrolyzed to free cholesterol by cholesterol ester hydrolase (CE). The free cholesterol produced is oxidized by cholesterol oxidase (CO) to cholestinone with the simultaneous production of hydrogen peroxide, which oxidatively couples with 4-aminoantipyrine and phenol in the presence of peroxidase to yield a quinoneimine dye with maximum absorption at 538 nm.

Reactions:

Cholesterol ester + H_2O →Cholesterol + FFA (cholesterol esterase)

Cholesterol + O_2 →4-Cholestinone + H_2O_2 (cholesterol oxidase)

$2H_2O_2$ + 4-aminoantipyrine + phenol → Quinoneimine dye + $4H_2O$ (peroxidase)

The test comes in the form of a commercial kit in which serum sample is incubated with enzymes and reagents from the kit and the change in absorption at 538 nm is measured spectrophotometrically. This change in absorption is proportional to the concentration of total cholesterol in the serum sample and can be calculated by comparison with absorption changes that occur with standard solutions containing known cholesterol concentrations.

3.15.3. Serum HDL Determinaination

The method employs an immune inhibition reagent method which measures HDL directly in a two reagent format .The first reagent contains Anti-human beta lipoprotein antibody which binds to lipoprotein LDL,VLDL and chylomicrons other than HDL. The antibody inhibits the ability of LDL, VLDL and chylomicrons to react with the pegylated enzyme in the system. The second regent contain pegylated enzyme (`identical to those in the methods for the total cholesterol determination except that they are pegylated) .Which then selectively react with the cholesterol present in HDL particle. Consequently, only HDL cholesterol is subjected to cholesterol measurement (linsel-Nitschke *et al.,* 2005)

3.15.4. Serum LDL (low density lipoprotein) cholesterol

Serum low-density lipoprotein-cholesterol (LDL-C) fraction was determined according to the Friedewald equations (Friedewald *et al.,* 1972).

LDL = Total cholesterol – (triacylglycerol/5 +HDL-C), Where all concentrations are given in mg/dl.

3.16. Tissue Histopathological Studies

To see the effect of rats treated with high fat diet and counter effect of *C. aurea* leaves extract fed rats histopathological examination of liver was performed; Small pieces of Liver tissue was taken from each rat carefully after the rats were sacrificed and dissection was done from the neck to the pubis and the peritoneum was opened by using sterile scissor and part of the liver was excised and taken by blunt forceps to preserving in tissue can which contain 10% of formalin.

Then the tissue was taken out from the preservative and washed using standard procedure. By dehydrating sequential in different concentration of ethanol starting from 70%, 80 % 95% and 100% for one hour in each steps.

Tissue section was treated with xylene solution to remove ethanol from the tissue and replace this ethanol with fluid that readily miscible with paraffin wax which enhance the tissue to embed easily with the paraffin wax to form tissue blocks. Then Sections (5-6 μm) were cut and stained with haematoxylin and eosin and examined for Histopathological changes (Cardiff *et al*., 2014) under the microscope (Motic AE 21, Germany).The microphotographs were taken using Moticam1000 camera at original magnification of 100 xs.

3.17. Statistical analysis

Collected quantitative data was coded, entered to computer processed edited and analyzed using Microsoft excel and expressed to SPSS version 20 statistical software for analysis .

All results are expressed as mean ± SD. The difference among group were evaluated by independent sample t-test and one-way analysis of variance (ANOVA) .Values were considered statistically significant when ($p < 0.05$) and very strong significant when ($p < 0.01$)

3.18. Ethical consideration

Ethical approval was obtained from Addis Ababa University Department of Biochemistry Research and Ethics review committee (DRERC) by the protocol number MSc. Thesis 03 /15 on September, 2015. The experiment takes into account an ethical philosophy that experimental animals have the capacity to experience a range of physical sensation and emotion and were therefore subjects of moral concerns. Therefore, this experiment had been conducted in laboratory animal houses and was designed to minimize suffering of the kept animals from hunger, thirst, injury, discomfort, fear, distress, deprivation and/or pain as much as possible or to the best minimal level.

4. Results

To find out the antilipidemic effect of hyderomethanolic leaves extract of *Calpurnia aurea* against high fat diet induced hyperlipidemia in male wistar albino rats, the histopathological and biochemical change results were:

4.1. Percentage Yield of C. urea leaf Extracts

The amount of crude extracts which was obtained from 627 gram coarse Powder of *Calpurnia aurea* leaf was 68.4 gram. Therefore, the percentage yield of this extracts by using Hydro-methanol (80/20 v/v) was calculated and given as:

Percent yield = Actual yield/Theoretical yield X 100

= (68.4 /627) x100 = 15.9 %(w/w)**.**

4.2. Phytochemical screening of plant

The preliminary Phytochemical analysis of hydromethanolic leaves extraction of *C.aurea* revealed the presence of phytochemical constituents such as alkaloid, saponin, Flavanoid, phenolic compound, tannin and cardiac glycosides (Table:3)

Table: 3. Preliminary qualitative phytochemical screening of hydromethanolic leaf extract of *C.aurea*

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Phytochemical constituent	Status
Flavanoid	++
Saponins	++
Phenolic compounds	++
Glycosides	+
Alkaloid	+
Tannins	+
Anthraquinones	_

4.4. *In-vitro* Antioxidant Activity of HMECA

4.4.1. *In-vitro* Antioxidant Activity of HMECA leaves against DPPH

As shown in Figure: 5, the diameter of reduced zone increased in direct proportion to the dose of plant extract. That was the higher the concentration of extract, the larger is the diameter of inhibition. The yellowish pale color observed shows a reduced zone that masks the color of

DPPH radical and the unreduced region were where the antioxidant can't react and remains violet which is the color of DPPH.

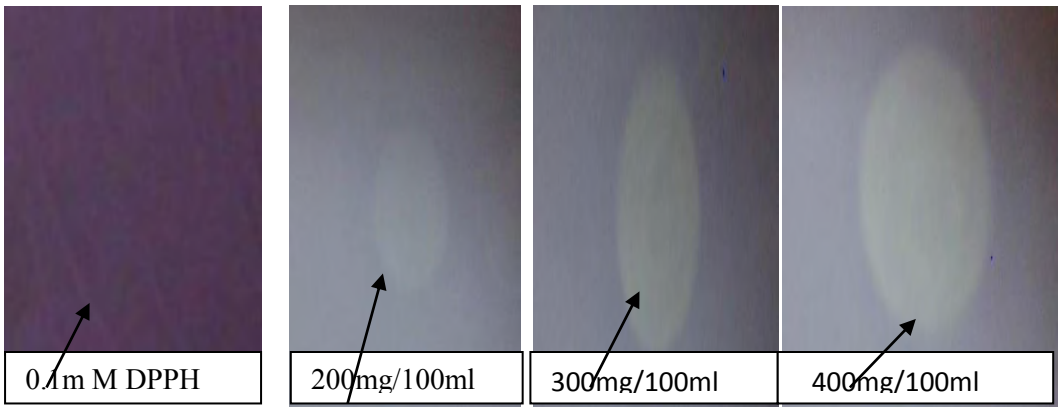

Figure: 5. *In-vitro* anti-oxidant properties of various concentration of 80% HMECA on TLC plate which is immersed in 0.1m M DPPH.

4.4.2. *In-vitro anti*-oxidant properties of HMECA and determination of their IC_{50}.

4.4.2.1. DPPH Radical Scavenging Activity

DPPH is a free radical and accepts an electron or hydrogen atom to become a stable molecule. Antioxidant are interact with DPPH radical and transfer electron or hydrogen atom to DPPH and then neutralizing its free radical character and convert it to 1,1-diphenyl-2- picryl hydrazine and the degree of discoloration indicates the scavenging activity of the extract. The reduction of DPPH radical was determined by the decrease in its absorbance at 517 nm that was induced by plant extracts

As shown in Figure 6, the IC_{50} of the MECA is 230µg/ml (0.23mg/ml) whereas that of Ascorbic acid is 160µg /ml (0.16mg/ml).This result shows that the crude *extract C. aurea leaves has* a closer antioxidant activity as compared with ascorbic acid and both MECA and ascorbic acid were shown that a decrease in the absorbance of DPPH, and the inhibitory effect of the plant extracts increase in dose dependent manner.The absorbance of 0.008% DPPH was 0.298 which was used as a control and the absorbance of methanol was 0.007 which was taken as a blank at 517 nm (figure - 6)

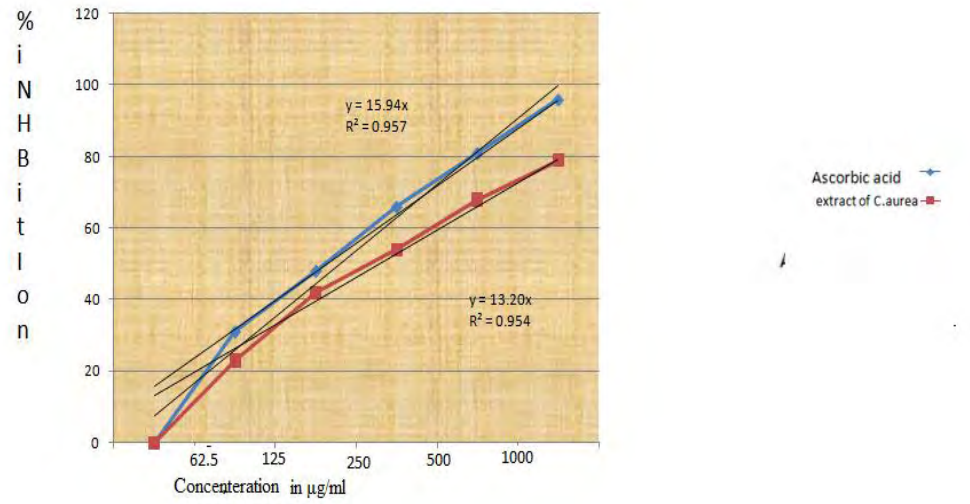

Figure 6: Antioxidant activities of methanolic extract C.aurea vs % inhibition of DPPH free radical

4.5. Effect of *C. aurea* leaf extracts on a high fat diet fed rats

4.5.1. Effect of *C. aurea* leaf extracts on food intake

The food intake (g) of the HFD fed control was increased though not significantly ($p > 0.05$) as compared to the normal control during 8th week treatment period. The food intake of rats fed on the HFD treated with atorvastatin, which was as standard positive control was increasing till the forth week as that of HFD fed control but later on the fifth week significantly decreased ($p < 0.05$) and even strong significant reduction ($p < 0.01$) on seventh and eighth week as compared to the HFD fed control throughout the treatment period.

On the other hand the food intake of rats fed on HFD supplemented with *C.aurea* leaves extract at 200 mg/kg ,300mg/kg and 400mg/kg dose co administered with HFD diet were increasing till fifth week in similar way as that of HFD fed control group but not significant ($p >0.05$). While on subsequent sixth and seventh week at the highest dose of 400mg/kg of the plant extracts co administered with HFD diet significantly decreased ($p < 0.05$) in food intake as compared to that HFD fed control group, even on the eighth week there was strong significant change ($p < 0.01$) in food intake as compared to the HFD fed alone .

Further analysis at lower dose of 300mg/kg extracts co administered with HFD diet on seventh week showed there was significant decrease ($p < 0.05$) on food intake of the rats as compared to the HFD fed control, and on the proceeding eighth week there was strong significant Change ($p < 0.01$) in food intake as compared to the HFD fed which were un treated with the plant extract. At the end, the lowest dose of 200mg/kg extract when co administered with HFD particularly on eighth week showed, there was significant decrease ($p < 0.05$) on food intake as compared to the HFD fed alone albino rats. Therefore the result of the present studies indicates hydromethanolic extract of *C.aurea* leaves co administered with HFD was effective in reduction of food intake in albino rats in a dose dependent manner when compared with atorvastatin (3.5mg/kg), which was standard drug as positive control had been supplemented with HFD during the treatment period (Table 4).

Table: 4. Experimental result of food intake in gram in oral gavages during hyperlipidemia treatment with different doses of *C.aurea leaf extract*

Group	1st week	2nd week	3rd week	4th week	5th week	6th week	7th week	8th week
Normal Control	35.0 ± 1.90	39.50 ± 2.25	45.14±2.03	53.14±3.33	63.85±2.79	72.14±3.13	80.57±2.76	86.71±4.85
HFD. control	34.14±1.49	39.00±1.41	48.00±1.84	56.00±2.00	67.00±3.82	76.85±1.88	85.15±1.86	91.0±7.83
Atorvastatin 3.5mg/kg+ HFD	33.42±2.00	38.07±1.48	45.14±1.86	53.85±3.28	62.57±3.8[a]	70.42±4.7[a]	74.60±6.2[b]	71.28±5.7[b]
HFD+200 mg /kg extract	34.85±1.37	41.42±3.49	48.21±1.99	57.71±1.49	66.14±1.21	74.4±2.07	80..01±1.0	82.28±3.4[a]
HFD+300 mg/kg extract	34.78±1.82	41.43±2.90	48.00±4.12	54.00±4.50	65.00±2.51	73.6 ±2.41	78.00±2.6[a]	77.3±2.50 [b]
HFD+400 mg /kg extract	33.71±1.49	38.59±2.50	46.70±2.63	54.65±3.31	64.73±3.23	71.0±3.43[a]	76.04±4.5[a]	74.25±8.0[b]

Values are mean ± SD (n=6).Values are statistically significant at $P<0.05$ and statistically strong significant at $p < 0.01$ using one way ANOVA followed by Post Hoc Tukey test.

[a] indicates $p < 0.05$ compared with hyperlpidemc control group .

[b] indicates $p < 0.01$ compared with high fat fed group.

4.5.2. Effect of *C.aurea* leaf extracts on body weight

All rats fed a high fat diet alone and rats fed a high fat diet supplemented with 200mg/kg, 300mg /kg and 400mg/kg /day extract suspended in drinking water orally showed a

significant increase ($p < 0.05$) in their final body weights when compared with their initial value(fig:7).

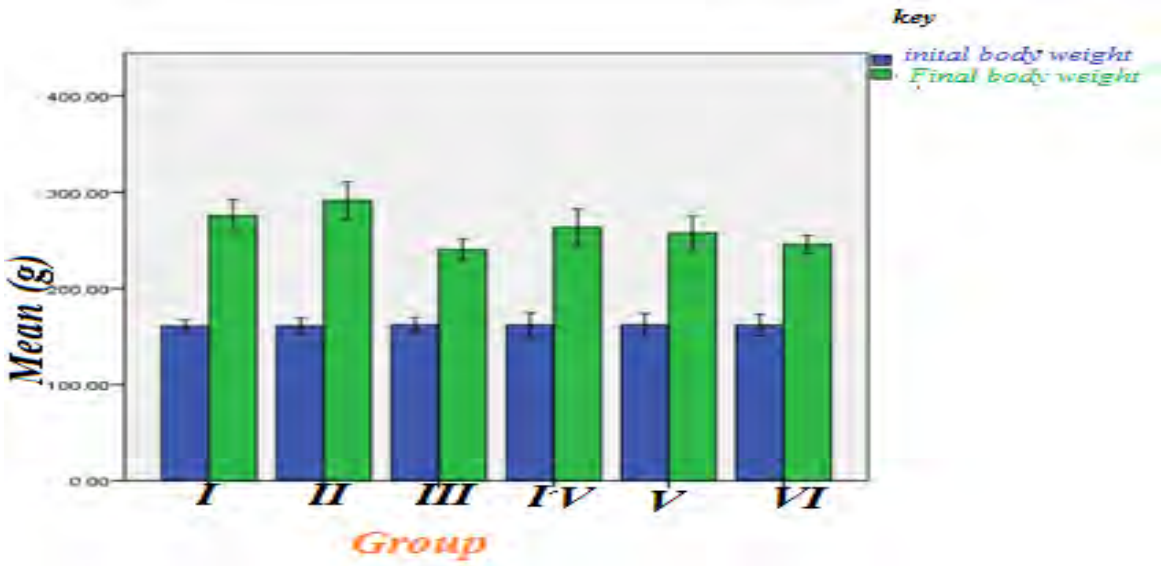

Fig: 7. Initial body weight of male albino rats when compared with final body weight during 60 days treatment period

Atorvastatin treatment ,which was used as standard positive control in study (3.5 mg/kg/ po) orally with HFD until 5 weeks did not cause significant effect ($p > 0.05$) on body weight of rats fed a high fat diet alone. Though there were significant reduction ($p < 0.05$) in their final body weights after the fifth week of rats fed a high fat diet treated with 3.5 mg /kg atorvastatin suspended in drinking water orally as compared with rats fed a high fat diet alone.

On the other hand there was no significant difference ($p > 0.05$) in weight between non-treated rats fed a high fat diet and rats fed a high fat diet treated with extract 200mg/kg, 300mg/kg and 400mg/kg until the sixth week, though on subsequent treatment of the seventh week in rats fed a high fat diet with 400mg/kg extract in drinking water revealed a significantly reduction on body weight of rats ($p < 0.05$) in their body weights (248.4 ± 11.8) when compared with non-treated rats fed a high fat diet alone (279.2 ±17.12) and on the last eighth week in rats fed a high fat diet treated with 400mg extract showed a significant reduction ($p < 0.05$) in their final body weights (246.7 ± 9.3b) when compared with rats fed a high fat diet alone (291.6 ±19.0). Even on the eighth week in rats fed a high fat diet treated with lower dose at 300mg extract in drinking water showed a significant change ($p < 0.05$) in their final body weights (257.6 ± 17.7) when compared with rats fed a

high fat diet alone (291.6 ±19.0) which were seen in the preceding value of reduction in weight.

Therefore there were only slight differences in the weight reduction pattern of the extract treated at 400mg/ml animals on HFD diet and those on standard drug treatment at 3.5mg/ kg. The data showed that the HFD fed with extract treated animals did not gain weight as much as HFD fed-untreated group, while the standard drug treated group with HFD gained weight initially but lost it as the week of treatment increases especial after the fifth week. Thus the result of the studies indicates methanolic extract of *C.aurea* leaves which was co administered with HFD was effective in reduction of weight in a dose dependent manner as compared with atorvastatin which was standard drug as control during the treatment period (Table:5).

Table: 5. Average body weights measurement in gram during the 8th weeks of male albino rates during high fat diet treatment with different dose *C.aurea* extract

Group	Initial weight	1st week weight	2nd week weight	3rd week weight	4th week weight	5thweek weight	6th week weight	7thwek weight	8thweek weight
Normal Control	160.6±6.65	172.6±7.90	186.7± 8.64	201.2 ± 10.8	216.4 ±12.28	230.1 ± 14.17	247.4 ±14.4	263.**8**± 14.28	275.6 ± 16.3
HFD control	160.8 ± 7.72	172.4 ± 10.43	189.2±13	207.90 ± 13.6	223.2 ±15.0	246.2 ± 15.7	264.8 ±16.8	279.2 ± 17.12	291.6 ± 19.0
Atorvastatin 3.5mg/kg+HFD	161.6 ± 5.85	176.0± 7.3	190.4 ± 8.41	205.4 ±9.31	219.0. ± 10.0	230.4±102	236.2.± 10.2 [a]	243.4 ± 11.8 [a]	240.4 ± 10.8 [a]
HFD+200mg extract	162.2 ± 12.2	176.8 ± 14.7	192.8±**16**	208.0 ± 16.4	222.4± 16.6	236.0±17.0	248.4 ±17.4	258.2 ± 18.7	263.6 ± 18.76
HFD+300mg extract	162.4 ± 11.6	177.6 ± 12.0	190.2 ± 12.4	205.0± 12.5	219.4±12.9	232.2 ± 12.3	244.2 ±16.0	250.1 ± 16.26	257.6 ± 17.7 [a]
HFD+400mg extract	162.0 ± 10.7	174.2 ± 11.34	189.6 ± 10.9	202.8 ±11.0	217.4± 12.2	231.8 ± 11.7	248± 11.3	248.4 ± 11.8 [a]	246.7± 9.3b [a]

Values are mean ± SD (n=6).Values are statistically significant at *P<0.05 using one way ANOVA followed by Post Hoc –Tukey test.

[a] indicates $p < 0.05$ compared with hyperlpidemc control group

4.5.3. Effect of C.aurea leaves extract on body mass index (BMI)

The measurements were made in anaesthetized (di-ethylether) as inhalation exposure to rats. The BMI of the rats fed a high fat diet fed group was marked increased as compared to the normal control at the end of the treatment period. But in the case of rats fed HFD treated with different dose of *C. aurea leaves extract and* atorvastatin *drug,* there was an increment in BMI rats of fed HFD alone though not as preceding numerically value of normal control during this study period (Table: 6).

Table: 6. BMI of rats on the 8th week during treatment with HMECA

BMI (g/cm^2) on 8th week	Normal Control	HFD.	Atorvastatin (3.5mg/kg)	HFD+200mg extract	HFD+300 mg extract	HFD+400 mg extract
	0.65	0.77	0.67	0.73	0.70	0.68

4.5.4. Effect of *C.aurea* leaves extract on serum lipid profile of rats fed high fat diet

From the results of study, keeping the animal on HFD significantly increased ($P<0.05$) the Serum total cholesterol levels of rats fed a high fat diet (92.0 ± 13.0 mg/dl) as compared to rats fed on normal diet (58.35 ± 6.15mg/dl). Serum LDL level was increased significantly ($p < 0.05$) in rats fed high fat diet (28.53±12.2 mg/dl) when compared with rats fed normal diet (7.7± 2.40 mg/dl) and serum triglyceride level increase significantly ($p < 0.05$) in rats fed a high fat diet (171.83 ±4.25 mg/dl) when compared with rats fed a normal diet (61.00 ± 2.82 mg/dl) and serum HDL level decreased significantly ($p < 0.05$) in rats fed a high fat diet (11.66 ±1.23 mg/dl) when compared with rats fed a normal diet (33.50± 2.12 mg/dl).

When HFD was co-administered with extracts the elevated levels of TC, TG and LDL-C have shown considerable decline. *Calpurnia aurea* leaf extracts treatment on 200 mg/kg in all cases treated did not cause any significant effect ($p > 0.05$) on serum total Cholesterol, triglyceride, LDL-cholesterol and circulating HDL-cholesterol level of rats fed a high fat diet when compared with rats fed high fat diet without treatment of *C.aurea* leaf extract.

But with the increase of the extract dose from 200mg to 300 mg when it was observed that serum total cholesterol, LDL-C were lowered and HDL-C was increased in rats, though not statistical significant and serum triglyceride level significantly reduced from 171.83 ± 4.25mg/dl

to 92.03 ± 4.54 mg/dl .*C.aurea* leaf extract treatment at 400mg cause significant decrease ($p < 0.05$) on serum total Cholesterol level of rats fed high fat diet (60.33± 8.60 mg/dl) when compared with non-treated rats fed a high fat diet only (92.00± 13.0 mg/dl). There were significant effect ($p < 0.05$) of *C. aurea* leaf extract treatment on 400 mg in serum triglyceride level of rats fed a high fat diet (84.73±19.4 mg/dl) when compared with non-treated rats fed a high fat diet only (171.83 ±4.25 mg/d). There were significant effect ($p < 0.05$) of *C .aurea* leaf extract on 400mg/kg treatment on serum HDL levels of rats fed high fat diet (29.66± 1.52 mg/dl) observed, when compared with non-treated rats fed a high fat diet only (11.66 ±1.23 mg/dl).

In addition *C.aurea* leaf extract treatment at 400mg/kg with rats fed high fat diet were significantly decreased ($p < 0.05$) Serum LDL levels of rats fed a high fat diet (9.70± 5.77mg/dl),when compared with rats fed high fat diet alone (28.53±12.2 mg/dl). On the other hand the Serum total cholesterol levels of rats fed a high fat diet with atorvastatin treated for 8 weeks (57.00 ± 3.60 mg/dl) significantly decreased ($p < 0.05$) when compared with rats fed a high fat diet alone (92.00± 13.0 mg/dl). Rats fed a high fat diet with atorvastatin treated for 60 days (71.00 ± 9.98) did cause significant effect ($p < 0.05$) on serum triglyceride levels when compared with rats fed a high fat diet only (171.83±4.25 mg/dl).

Serum HDL level increased significantly ($P < 0.05$) in rats fed high fat diet with atorvastatin treatment (32.73 ± 11.91mg/dl) when compared with rats fed a high fat diet only (11.66 ± 1.23 mg/dl). Atorvastatin treatment on rats fed high fat diet also caused Significant lowering ($p < 0.05$) of serum LDL level (9.433 ± 1.76 mg/dl) when compared with rats fed a high fat diet only (28.53±12.2 mg/dl). Therefore the result of the studies revealed *C.aurea* leaves extract at a dose of 400mg/kg has almost similar activity as that of atorvastatin 3.5 mg/kg during treatment period and antilipidemic activity effect *C.aurea* leaves extract has been increase as dose extract were elevated when compared to standard control (atorvastatin) (figure :8).

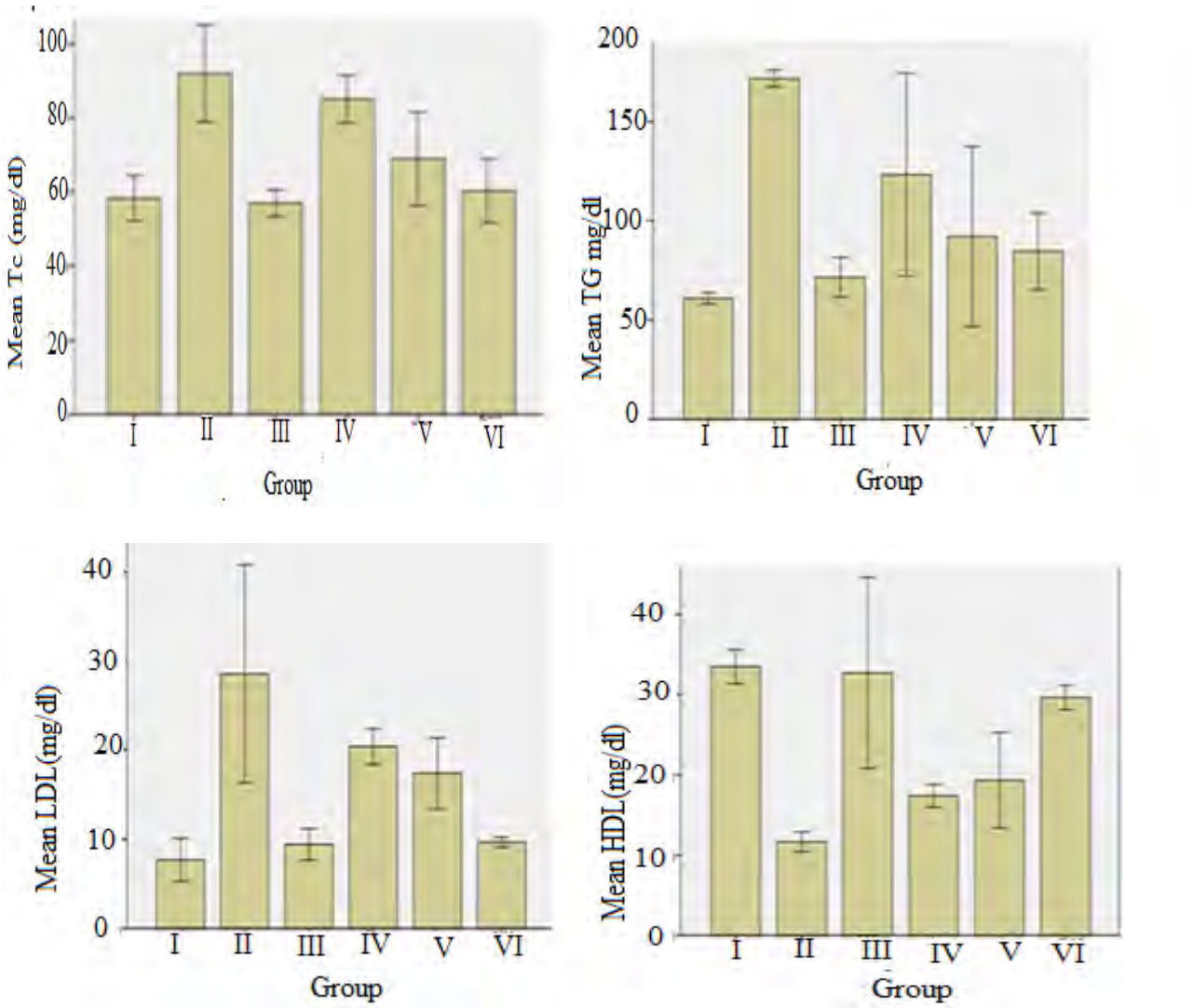

Figure: 8. Effect of different dose of methanolic extract of *calpurnia aurea* on serum lipid profile of albino wistar rats fed a a high fat diet. All treatments were for 60 days. The extract was given orally at (200,300,400) mg /kg /day.

4.6. Effect of HMECA treatment on histopathological changes of the albino rats liver

Light microscopic examination of liver histology of wistar albino rats fed a high fat diet Showed normal liver histology with no lipid deposition in the hepatocytes of the liver and no fatty liver was seen in the 8 weeks feeding period.

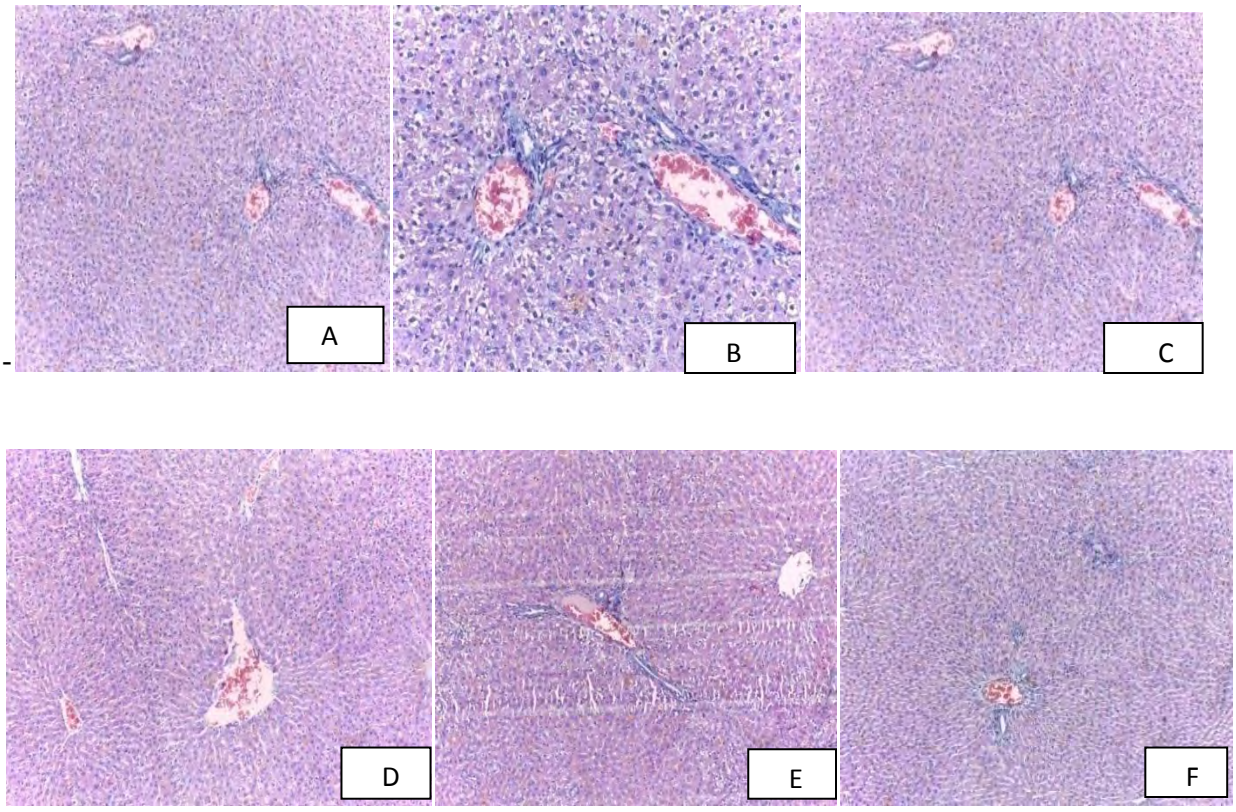

Fig. 9: Normal liver histology with no lipid deposition in the hepatocytes of the liver *tissue*. (Hematoxylin and eosin, 100×). (A) Normal control, (B) high fat diet control, (C) high fat diet + Atorvastatin (3.5mg/kg b.w), (D) high fat diet + MECA (200mg/kg b.w), (E) high fat diet + MEGG (300 mg/kg b.w) and (F) high fat diet + MEGG (400 mg/kg b.w) fed for 8 weeks.

5. Discussion

5.1. Association of increased fat intake and Hyperlipidemia

Hyperlipidemia has been documented as one of the major causative agent for atherosclerosis, which is resulting in coronary heart diseases (CHD).Consuming excessive portions of foods rich in calories, particularly dietary fats and carbohydrates have been identified as major contributing factors in the growing epidemic of hyperlipidemia and its associated cardiovascular diseases. Energy from dietary fat has a larger effect on body-weight gain than has energy from non-fat sources, because fat has more calories per gram than other macronutrients (Saris, 2006).

The high cholesterol level in tissue and serum may be due to increased uptake of exogenous cholesterol and subsequent deposition and decreased cholesterol catabolism as evidenced by a reduction in bile acid production and turnover of bile acids. The metabolism of free and ester cholesterol are impaired in liver tissue and the rate of turnover was specifically decreased in all tissues of hyperlipidemic rats. Lipid deposition is major clinical complication of hyperlipidemia (Venu *et al*., 2010). In order to explain the mechanism of action for the improvement in serum lipid profile, the drug atorvastatin was used in the present experiments, which is a lipid lowering agent by competitively inhibiting the enzyme HMG-CoA reductase in liver. It increases the expression of LDL receptors in the liver. These LDL receptors increases uptake and subsequent removal of LDL and then restore cholesterol homeostasis (Ray and Rege, 2000).

5.2. Effects of *Calpurnia aurea* leaves extract on High fat diet fed Albino rats

The results of the present study showed the dried and the coarse powder of *Calpurnia aurea* leaves was subjected to maceration technique in 80% methanol and the percent yield was 15.9 % (w/w).The preliminary Phytochemical analysis of methanolic leaves extracts of *C.aurea* revealed the presence of bioactive compounds such as alkaloid, saponin, flavanoid, and phenolic compound. They were found in our metanolic extract might be responsible for its medicinal properties like hypolipidemic effect of the plant

5.2.1. Effects of *Calpurnia aurea* leaves extract on food intake

The feed consumption result and direct observation of test animals using activity cages shows an increase in daily feed consumption and appetite initially (1-4week), in all over the groups while

this appetite was lost abruptly, then starting from the fifth week onwards, those rats fed HFD with atrovastatin revealed significant change or reduction in appetite as compared with HFD fed animals.

On the eight week treatment, there was very strong significance in the food intake reduction at high dose (400mg/kg) co administered with HFD fed rats as compared with HFD fed alone. Which were almost similar with standard drug (3.5mg/kg) supplement with HFD treatment during the final period of the experiment (Senecha *et al*., 2012).

The reduction in the food intake in the HFD fed group treated with *C.aurea leaves* extract may be due to its ability to suppress the animals' appetite indicating that, the action of bioactive components like saponins, and phenolic compound. Thus the identified class of components in single or in combination with other constituents present in the plant extract might be responsible for the antilipidemic in the treatment groups in dose dependent manner. This was similar to the results reported by (Chidrawar, 2011).

5.2.2. Effects of *Calpurnia aurea* leaves extract on the body weight

There was no significant difference in weight between non-treated rats fed a high fat diet and rats fed a high fat diet treated with extract 200mg/kg, 300mg/kg and 400mg/kg until the sixth week, though on subsequent treatment, On the seventh week in rats fed a high fat diet with 400mg/kg extract in drinking water revealed a significantly reduction on body weight of rats in their body weights when compared with non-treated rats fed a high fat diet alone. On the last eighth week in rats fed a high fat diet treated with 400mg extract in drinking water showed a significant reduction in their final body weights when compared with rats fed a high fat diet alone

On the last eighth week, in rats fed a high fat diet treated with lower dose of 300mg extract showed a significant change in their final body weights when compared with rats fed a high fat diet alone which had been seen in the preceding value of the weeks. Therefore the study revealed as there were similarity in the weight reduction pattern of animals treated at 400mg/kg with HFD diet, and those on standard drug treatment at 3.5mg/ kg on the last week of treatment.

The data showed that the HFD fed with extract treated animals did not gain weight as much as HFD fed alone, while the standard drug treated group with HFD only. Thus the result of the studies indicates hydromethanolic extract of *C.aurea* leaves extract which was co administered with HFD was effective in reduction of weight. This was due to the presence of bioactive constituent in the plant which responding in a dose dependent manner during the reaction (Anyanwu et al., 2013).

5.2.3. Effects of *Calpurnia aurea* leaves extract on body mass index

The BMI of the HFD fed control remained increased markedely as compared to the normal control. This is correlated with the work done by (Novelli *et al.* 2007), which showed that high caloric diet increased significantly the BMI of rats fed high fat diet as compared to the normal control .Although there was a decrease in the BMI of the rats treated with *C.aurea leaves extract* at higher dose and by atorovastatin which was used as positive control during this study.

5.2.4. Effects of *Calpurnia aurea* leaves extract on serum lipid profile

High fat diet rich food resulted in a significant increase in serum total cholesterol and triglyceride in plasma which is accompanied by increased serum LDL-C level, with decreased circulating HDL-C levels in hyperlipidemic control as compared to normal group (Shattat, 2014). Rats treated with atorvastatin (standard control) co administered with high fat diet showed marked reduction in all serum lipoproteins but it increase in circulating HDL level as compared with HFD fed untreated group (Ray and Rege, 2000). Treatment with HMECA at a dose of 200mg/kg showed marked reduction in TC, TG, and LDL-C levels with increase in HDL-C level as compared to serum lipid profile in HFD fed control, though not significant alteration, while at high dose of 400mg/ml extract there was a significant reduction in TC, TG, and LDL-C levels followed with significant rise in HDL cholesterol levels in the experimental animals. Even at 300mg/kg of the extract, causes of significant reduction in serum triglyceride level though not on TC, and LDL-C, these might be related with plants phytochemical constituents which are responsible for lowering the lipid profile, which were mostly acting on dose dependent manner.

Chávez-Santoscoy (*et al*., 2013) in their work reported that some of the chemical constituents of plants, such as flavonoids and saponins lower cholesterol absorption by the inhibition of cholesterol micellar solubility. The plants saponins fraction isolated from leaf extracts have an *in vivo* antiobesity activity which led to significant decrease in weight in visceral organs and lipid profile such as triglycerides, total cholesterol, low-density lipoproteins and very low-density lipoproteins (Marrelli *et al.,* 2016). Furthermore, as reported earlier (Son *et al*., 2010) similar results were confirmed on that plants chemical constituents like Phenolics compounds that have hypolipidemic effect, and lowers the risk of high fat diet-induced obesity and reduces serum cholesterol. Therefore, plants, flavonoids, saponins and Phenolics and other bioactive compounds found in our hydromethanolic extract of *C aurea* could be instrumental to its hypolipidemic effect in a dose dependent manner *(*Yanping *et al*., 2005; Patel *et al*., 2009; Ghasemzadeh , 2011).

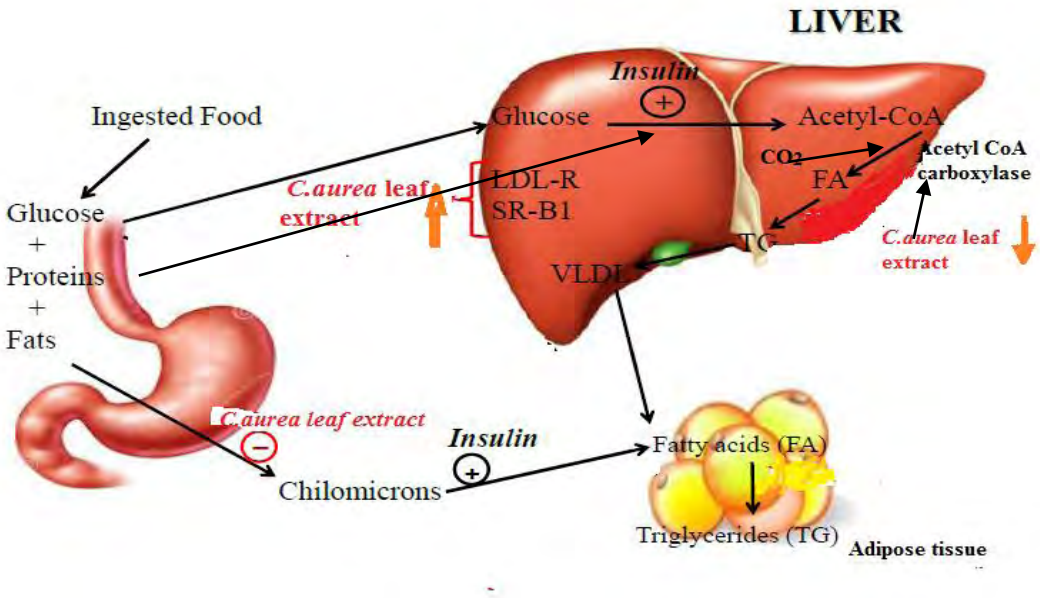

Figure 10: Hypothetical effect of *C.aurea* leaf extract reduces plasma lipid and hepatic triglyceride concentrations and increase cholesterol uptake in the liver via up-regulation of Low-Density Lipoprotein (LDL)-receptor and Scavenger receptor class B member 1 (SR-B1),down regulate Acetyl COA Carboxylase which is the regulated step during fatty acid synthesis and influenced cholesterol-regulating enzymes activities, 3-hydroxy-3-methylglutaryl-CoA reductase.

5.2.5. Effects of *Calpurnia aurea* leaves extract on DPPH free radical

Hyperlipidemia also relates to an increased oxidative stress causing significant production of oxygen free radicals, which may lead to oxidative modifications in low density lipoproteins, which plays a significant function in the initiation and progression of atherosclerosis and associated cardiovascular diseases (Mishra *et al.,* 2011).Natural antioxidants are present in herbs and flowers are responsible for preventing the deleterious consequences of oxidative stress.The1,1-diphenyl-2-picrylhydrazyl (DPPH) is free radical with a maximum absorption at 517 nm, that can readily undergo reduction by an antioxidant (Usmani, 2013). The DPPH scavenging capacity of the MECA plant may be related to the phenolics, Flavonoids and tannins present which play a central role for MECA to become one of the strongest antioxidant.

In-vitro antioxidant activity of HMECA was done by using thin layer chromatography technique qualitatively and by using spectrophotometer quantitatively, using DPPH as free radical in vitro in both cases. The reaction of HMECA at different dose against DPPH free radical using TLC plate were result in the formation of pale yellowish circles with a diameter proportional to dosing of plant leaves extract. The larger the diameter on TLC plate inhibited the higher the concentration of the extract. The development of this pale yellowish color during reaction shown were a reduction of DPPH by antioxidant that were, original deep violet color of DPPH, had been changed to DPPH-H (pale yellow) which is consistent, not only with the postulate of Muthusamy and his colleagues (Muthusamy *et al.,* 2009) but also with the work of Ebrahimzadeh and his colleagues. Therefore these results might be due to the transfer of electrons from the extract to DPPH during the reaction (Ebrahimzadeh *et al*., 2009).

The result of DPPH assay is also expressed in IC_{50} value. it express the amount of extract needed to decrease free radical absorbance by 50%. The lower IC_{50} value represents the higher antioxidant activity of the tested sample. In the present investigation, we have evaluated the free radical scavenger activity of HMECA during the experiment was 230µg/ml and IC_{50} value ascorbic acid , which standard antioxidant , as positive control was 160 µg/ml. The results were in line with previous reports made (Ebrahimzadeh *et al.,* 2009; Muthusamy *et al.,* 2009).

5.2.6. Effects of *Calpurnia aurea* leaves extract on the liver histopathology

Light microscopic examination of liver histopathology of albino wistar rats fed a high fat diet showed normal liver histology with no lipid deposition in the hepatocytes of the liver and no fatty liver was seen in the weeks of feeding period .This might be no histopathological data were available for direct comparison of the whole liver.

6. Conclusion

The results of this study revealed that the hydromethanolic extracts of of *Calpurnia aurea* leaves decreased food intake, body weight, and body mass index and altered the serum lipid profiles in albino wistar rats. This was interpreted to indicate that the plant bioactive molecule were involved in decreasing appetite, serum TC, TG, LDL-C and increasing serum HDL-C level. Besides, the extract *has* good antioxidant property and could be due to the presence of flavonoids, saponins and phenolics compounds against 1,1-diphenyl-2-picrylhydrazyl/DPPH free radicals. The DPPH free radicals when reacts with different dose of hydromethanolic extract of *Calapurnia aurea* leaves, a visible violet color of DPPH was changed to pale yellow during the study .This might be due to the acceptance of an electron donated from the extract by DPPH free radicals. The hydromethanolic extracts of *Calapurnia aurea* showed prominent IC_{50} value of 230µg/ml which was compared with ascorbic acid, 160µg/ml which is a well known antioxidant and its absorbance was measured at 517nm. Therefore, results of this study show that hydromethanolic extracts *Calpurnia aurea* leaves might be as a good candidate for lowering hyperlipidemia and an easily accessible source of natural antioxidants against free radical that was produced by different mechanism of action.

7. Limitations of the study

- Routes of administration of extract, atorovastatin and drinking water may also affect the significance of the results.
- The study was also unable to include the effect of *C.auera* extract on histopathological changes on the muscle of the experimental animals due to resource limitation.
- Small numbers of rats were used in this study due to limited resources, so that the study was somewhat restricted to thirty three rats. .
- No histopathological data of the whole liver were available for direct comparison

8. Recommendations

- The present study shows the traditional herbal plants like *Calpunia* aurea leaves extract have antilipidemic properties on albino wistar rats and antioxidant activities on *in vitro* DPPH radicals. However further investigation on:-
- Comprehensive biochemical and pharmacological investigations of active components of the *Calpurnia aurea* plant, in particular, those which are responsible for improving lipid profiles of rats should be evaluated .
- Individual components of the plant with *in vivo* antioxidant activities and their action should be investigated.
- *Calpurnia aurea plant* effect on hyperlipidemia ,using higher doses of extract.
- Studies are needed on lard-feeding model of hyperlipedemic in wistar albino rats using higher numbers of rats should be carried out for longer periods of time.
- Indigenous people of the study area should be involved in conservation and management plans of plant resources

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