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EVALUATION OF THE EFFECT OF METHANOL CRUDE EXTRACT OF *JUGLANS regia* LEAF ON LIVER ENZYMES AND LIPID PROFILES OF SWISS ALBINO MICE FED HIGH FAT DIET

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A RESEARCH THESIS SUBMITTED TO JIMMA UNIVERSITY, INSTITUTE OF HEALTH, FACULTY OF MEDICAL SCIENCE, DEPARTMENT OF BIOMEDICAL SCIENCES, BIOCHEMISTRY COURSE UNIT FOR PARTIAL FULFILLMENT OF MASTER'S DEGREE (MSc) IN MEDICAL BIOCHEMISTRY

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Evaluation of the Effect of Methanol Crude Extract of *Juglans regia* leaf on Liver Enzymes and Lipid profiles of Swiss Albino Mice Fed High Fat Diet

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A research thesis submitted to Jimma University, Institute of Health, Faculty of Medical science, Department of Biomedical Sciences, Biochemistry course unit for Partial Fulfillment of Master's Degree (MSc) in Medical Biochemistry

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Declaration

This is to certify that the thesis prepared by Meskelu Seyoum Moti, entitled: "Evaluation of the effect of methanol crude extract of *Juglans regia* leaf on liver enzymes and lipid profiles of Swiss albino Mice fed high fat diet" and submitted in partial fulfillment of the requirements for the Degree of Masters of Science (Medical Biochemistry) complies with the regulations of Jimma University and meets the accepted standards with respect to originality and quality.

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Abstract

Background: High fat diets feeding an important risk factor for obesity which accelerate over production of reactive oxygen species in hepatocytes cells and debilitating cellular antioxidant capacity that aggravate metabolic disturbances such as hyperlipidemia and liver diseases through increased of liver enzymes and lipid profiles. *Juglans regia* is a plant used by community to treat hyperlipidemia, high blood pressure and liver diseases, and its ingredients have antioxidant role through scavenge of accumulated fatty in hepatocytes. Thus, the current study intended to evaluate the effect of *Juglans regia* extract on liver enzymes and lipid profiles of mice fed high fat diet.

Methods: Study was conducted at Jimma University Tropical and Infectious Disease Research Center (JUTIDRC) and thirty male Swiss albino mice were randomly divided into six groups. Group I (normal control) were given distilled water, Group II were given HFD only, Group III were received atorvastatin, G- IV,V and VI of mice were received HFD and 100, 200 and 300mg/kg of *Juglans regia* crude leaf extract, respectively for thirty days. All mice were fasted overnight and sacrificed by cervical dislocation and 2-2.5ml of blood was drawn to evaluate lipid profiles and liver function tests, and liver histopathology was investigated. Data was entered into Epi-data 3.1 & exported to SPSS 25 software for analysis by using one way ANOVA.

Results: Serum level of ALP, AST and TG of mice fed high fat diet (G-II) were significantly elevated ($P<0.01$) when compared to normal control group (G-I).Group of mice treated with atorvastatin (G-III) and *Juglans regia* crude leaf extract at high dose (300mg/kg) significantly decreased ($P<0.05$) elevated AST, TG, TC and ALP when compared to group of mice fed high fat diet only (G-II). Furthermore, the liver section of a group of mice fed HFD (G-II) suffered moderate cell necrosis, vacuolar degeneration and mild-mixed inflammation when compared to normal control group, despite such abnormalities being absent in G-III and G-VI.

Conclusion and Recommendation: The finding showed that dose dependent of *Juglans regia* crude leaf extract as exhibit noticeable effect on liver enzymes and lipid profiles levels on mice fed high fat diet, which rationalize claimed use of *Juglans regia* in treatment of liver diseases. Molecular mechanisms by which medication acts should be contemplated.

Key words: High fat diet, *Juglans regia,* liver enzymes, albino mice, liver histopathology, lipid profiles

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Abbreviations and Acronyms

AED: Animal Equivalent dose

ALP: Alkaline phosphatase

ALT: Alanine aminotransferase

AST: Aspartate aminotransferase

BMI: Body mass index

B.W: Body weight

CHD: Coronary heart disease

CVD: Cardiovascular disease

CYPA1: Cytochrome P450 1A

EPA: Eicosapentaenoic acid

FAS: Fatty acid synthase

FFA: Free fatty acids

H0: Null hypothesis

HA: Alternative hypothesis

HDL: High- density lipoprotein

H&E: Heamatoxylin and Eosin

HFD: High fat diet

LCAT: lecithin cholesterol acyltransferase

LDL: Low- density lipoprotein

LFT: Liver Function test

MAG: Monoacyl glycerols

MUFA: Monounsaturated Fatty Acids

NAFLD: Non-Alcoholic fatty liver disease

OECD: Organizational Economic co-operation and development

P-AMPK: 5' AMP-activated protein kinase

PPARα: Peroxisome proliferator-activated receptor α

PUFA: Polyunsaturated fatty acids

ROS: Reactive Oxygen Species

Rpm: Revolution per Minute

SFA: Saturated fatty acids

SPSS: Statistical package for social sciences

SST: Serum separator tube

TBiL: Total bilirubin

TC: Total cholesterol

TFA: Trans fatty acids

TG: Triglyceride

WHO: World Health Organization

1. Introduction

1.1 Background

Dietary fat consumption, either in saturated or unsaturated forms, has long been studied in relation to human health, and an excessive intake of saturated fats induces obesity, which become a global epidemic and an important public health problem in many countries (1).

High intake of HFD has a direct impact on serum lipid profiles and fatty acid composition (2), by increasing amount of low density lipoprotein and decreasing high density lipoprotein in the bloodstream, both of which raise risk of cardiovascular diseases (3).

High fat diet consumption also associated with accumulation of triglycerides in adipose tissue and liver which were accelerate overproduction of ROS and debilitating cellular antioxidant activity, consequent for development of obesity and non-alcoholic hepatic steatosis by elevation of liver enzymes and lipid profiles (4).

The buildup of obesity, insulin resistance, hypertension, arteriosclerosis and dyslipidemia is referred to as metabolic syndrome (5). These disorders impact roughly 20-40% of the population in developed countries, and their frequency is predicted to rise in the future decades (6).

Highly intake of HFD can cause hyperphagia in humans (7), decreased lipolytic activity in fat tissue, hypothalamus neuron apoptosis (7), mitochondrial metabolism impairment (8), insulin resistance, and obesity (5).

Obesity is a serious metabolic disorder, characterized by abnormal accumulation or excessive fat accumulation caused by an imbalance between energy intake and expenditure (9).

Obesity is becoming an increasingly problematic issue in modern society's associated with Sedentary life styles, recognized a risk factor for the development of hyperlipidemia ,NALFD , Insulin resistance ,atherosclerosis and liver diseases (10).

Hyperlipidemia is caused by increased oxidative stress through causing oxidative modifications in low-density lipoproteins, which play role in the initiation of atherosclerosis and related cardiovascular diseases (11).

Non-alcoholic liver fatty acid disease (NALFD) refers to a group of more severe clinical pathological disorder that range from fatty liver to steato-hepatitis with or without cirrhosis of the liver (12). Furthermore, excessive deposition of fatty in adipocytes cause insulin resistance which lead to fatty accumulation in liver cells (4), which increases the rate of mitochondrial beta

oxidation of fatty acids and ketogenesis, promotes lipid peroxidation and increases the accumulation of ROS in hepatocytes cells (13).

The liver is an organ responsible for detoxification of hepatotoxic substances such as viruses, fungal products, bacterial metabolites, purified sugars, saturated fats, environmental pollutants, and chemotherapeutic agents that can cause various diseases in the organs (14).

The *Juglans* genus (Juglandaceae) comprises several species, and *Juglans regia* is its best-known member, which is widely distributed throughout the world, constituting an important species of deciduous trees found primarily in temperate areas and cultivated commercially throughout Southern Europe , Eastern Asia, United States and East Africa parts like Zambia , Mozambique Nigeria, Uganda ,Sudan and Ethiopia, Western Oromia (15).

Juglans regia is known by several names with walnut in English, Ferenj lawoz in Amharic and Ocholoni in Afan Oromo. Traditionally, the plant part is cultivated mainly as nuts ,which are cooked and consumed as a snack for treatment of constipation, high blood pressure, heart disease, liver diseases, high blood cholesterol, erectile dysfunction, as anti-hemorrhoid, astringent (16).

Pharmacological studies have shown methanol extracts of *Juglans regia* leaf are being studied as anthelminthic and antioxidant, confirming the use of traditional herbal medicine for treatment of microbial diseases (17), and the bark of the stem is used as laxative as well as chewed to reduce toothache (18)**.**

1.2 Statement of the Problem

According to Global health observatory data base, between 2016 and 2021,39% of world's population was overweight and 13.1% were obese (19). The situation is worsening in Latin America and Caribbean, with of 58% populations, 360 million overweight and 23% obese with a total of 140 million (20). Of these 15-25% obesity is because of increased consumption of high diet rich in saturated fatty acids (21).

Today, more than 41 million people die each year in the worldwide ,and more than 1.7 million people die every year in the United States due to high fat diet induced obesity associated diseases such as cardiovascular diseases, diabetes mellitus, metabolic syndrome and cancer (22).

In China, the situation is very serious and a number of patients reach 160 million with annually related high fat diet induced obesity associated disorders like diabetes mellitus, stroke, cerebral infarction and liver disease (23).

There were also study conducted in Saud Arabia that show that 35% of population die every year because of obesity and associated diseases like hyperlipidemia, and liver injury ,which caused by genetic factors (20%),snack foods (25%), high intake of saturated fat diet (31%) ,environmental factors (19%) , and others (5%) (24).

Hepatotoxicity (5-7%) is the common adverse effect reported with the use of statin drugs through increased level of aminotransferases (25). So, there is a need supplementing herbs as traditional medicine as alternatives of modern medicine.

However, such type of problems were increased in developing countries including Ethiopia, with different risk factors like adoption of western life style, decreased physical exercise and high intake diet rich in fat (26).

A variety of crude extracts and isolated compounds from plants, can induce body weight reduction and proven high fat diet induced obesity associated complication (27).

Data also revealed that most of the parts of plant as it possess antidiabetic, antihyperlipidemic, for cardiac stimulation (28),antidiarrheal and hepatoprotective (29).

Therefore, this study was designed to demonstrate the effects of *Juglans regia* leaf extract on liver enzymes and lipid profiles of mice nourished with high fat diet.

1.3 Significance of the Study

Traditionally, in different parts of Ethiopia *Juglans regia* plant parts has been used by a society for treatment of liver diseases, and hyperlipidemia so far than synthetic drugs due to local availability, minimal side effects and low cost.

However, such studies and reports are lacking in our country on protective effect of *Juglans regia* leaf extract on liver diseases and hyperlipidemia in animal's model. Therefore, this study is expected to enumerate the effect of *Juglans regia* leaf extract on liver enzymes and lipid profiles of Swiss albino mice fed high fat diet.

Additionally, this study may have a great contribution for alleviations of certain adverse effect use of statin drugs in treatment of hyperlipidemia. So, to mitigate these problems it is ought to search for alternative medicine/herbal drugs for treatment of hyperlipidemia and liver diseases.

Furthermore, the study will provide baseline data for future researchers and serve as premise for further investigation on this plant for better utilization of its claimed therapeutic values. Additionally, this study also helps to create awareness about the protective effect of *Juglans regia* in society and to scale-up the community consuming habit of *Juglans regia* particularly for liver diseases and hyperlipidemia.

2. Literature Review

High fat diets could lead to changes in adipose tissue deposition (adiposity), metabolic alteration and hepatocellular damages (30). These overall alterations lead individuals to obesity throughout the world by debilitating antioxidant effect of diet rich in saturated fatty acids (31).

Due to the adverse effects of synthetic compounds that have emerged in recent years, the clinical importance and potential use of herbal remedies/allopathic medicine as antioxidants and antimicrobial effects were increased throughout the world (32).

Among allopathic medicine *Juglans regia* is the one that comprising many compounds including omega-3-fatty acids, Phytosterols, sitosterols and antioxidants that were helped to reduce cancer risks and alter gene expression in breast cancer patients (33).

A Study done in Turkey on *Juglans regia* leaf extract as helps to reduce harmful effects of nitrite induced toxicity and prevent hepatotoxicity in rat liver (34) and *Juglans regia* fruit supplement with a diet able to reduce visceral fat, regulate lipid profiles like TG, TC, LDL and HDL in high fat diet fed induced Obesity in Wistar rats (35) while study done on *Juglans regia* kernel extract as a modulator against the initiation phase of NAFLD throughout modifying hepatic and systemic lipid homeostasis and decrease cholesterol levels in HFDs induced obesity in Zucker rats (36).

There is also a finding on effects *Juglans regia* stem bark extract as antimicrobial against oral micro flora throughout inhibiting the growth of different gram-positive bacteria responsible for dental plaques (37) and oral hygiene problems related due to the presence of phenolic compounds, terpenoids, alkaloids, flavonoids, and steroids (38).

2.1 Mechanisms of high fat diet induced obesity related dyslipidemia

After the ingestion of a meal containing fat, storage form of lipids, TG are lipolyzed in the intestinal lumen into FFA and 2-monoacylglycerols (MAG) and are taken up via the enterocytes by way of passive diffusion and specific Transporters like CD36 (39). Cholesterol is haunted by the enterocytes via the precise cholesterol transporter Nie man-Pick C1 like 1 protein (NPC1L1) (40).

Once within the enterocyte, cholesterol is transformed into cholesterol-esters, whereas FFA and MAG are assembled into TG again. Finally, cholesterol-esters and TG are packed alongside

phospholipids and apo-lipoprotein (apo) B48 to form chylomicrons (41). After assembly, the chylomicrons are secreted into the lymphatic system and finally enter the circulation via the lymph vessel (24).

The liver synthesizes TG-rich lipoproteins called very low lipoproteins, which increase postprandial when food derived TG and FFA reach the liver (42). The assembly of VLDL is nearly just like the synthesis of chylomicrons, but apo B100 is that the structural protein of VLDL and its remnants, and low density lipoproteins (43).

The human liver lacks the editing complex necessary to change apo B100 molecule into the smaller apoB48, by post-transcriptional modification of 1 base resulting in a premature stop codon (44).

The postprandial rise in insulin is one among the foremost important regulatory mechanisms for fuel storage and leads to the effective inhibition of hormone sensitive lipase, which is the key enzyme for hydrolysis of intracellular lipids (44).

Despite the uptake of FFA by adipocytes and myocytes, a proportion of FFA remains within the plasma compartment where the FFA are bound by albumin and transported to the liver (45). When delivery of FFA for energy expenditure is insufficient like within the fasting state, FFA are often mobilized by fat for oxidation in energy demanding tissues like cardio-myocytes which is a crucial regulator of FFA mobilization from fat (46).

2.2 Obesity induced changes in lipoprotein metabolism

Elevated fasting and postprandial TG, as well as a prevalence of small dense LDL and low HDL-C, are the hallmarks of obesity-related dyslipidemia (47). Hypertriglyceridemia, which causes a delay in the clearance of TG-rich lipoproteins and the development of tiny dense LDL, may be the primary cause of other lipid disorders (48).

Obesity impairs TG-rich lipoprotein lipolysis due to lower LPL mRNA expression levels in adipose tissue, lower LPL activity in skeletal muscle, and lipolysis competition between VLDL and chylomicrons (49).

Increased postprandial lipemia causes an increase in FFA levels, which causes LPL to detach from its endothelium surface. LPL may remain linked to VLDL and IDL, causing TG depletion to continue (50).

CETP's exchange of TG from these remnants for cholesterol-esters from HDL, together with hepatic lipase's concerted action, results in the production of tiny dense LDL (51).
The cholesterol-ester content of LDL reduces in the presence of hypertriglyceridemia, whereas the TG content of LDL increases due to CETP activity (52).

However, hepatic lipase hydrolyzes the increased TG content in LDL, resulting in the production of tiny, dense LDL particles (53). Increased TG concentrations cause the production of small dense LDL in obesity, which is independent of total body fat mass (54). Small dense LDL is degraded slowly, with five-day residence periods, which enhance atherogenity (55).

2.3 Medicinal Plants

The Global and National market for medicinal herbs has been growing rapidly and significant economic gains are being realized with global sales of herbal products which totaled an estimated US dollar 60 million in 2000 (56).

Phenolic compounds are secondary metabolites found in different parts of plants and have a pivotal role against stress induced by environmental, pathogen and injuries worldwide (57).
These phenolic compounds have a great role for a human beings in recent years by reducing the risk of cardiovascular disease and degenerative disease through scavenge free radicals and possess metal chelating properties (58).

Juglans regia have several species widely distributed throughout the world and comprises significant forms of deciduous trees and widely cultivated in Asia, South America, USA, South Europe and Africa (59).
Juglans regia are an excellent source of various compounds that express antioxidant and antimicrobial properties as well as antihistaminic, anti-inflammatory, antiulcer, antidiabetic and hepatoprotective properties (60).
There are studies conducted on health benefits of *Juglans regia*, which ranks of the highest content of antioxidant followed by Pistachios, and Hazelnuts (61).

Juglans regia is also considered as a good source of vitamin E and fatty acids like linoleic, linolenic acids, palmitic acids and stearic acids, helps to reduce the risk of cardiovascular disease by decreasing the concentration of LDL and enhancing the level of HDL (62).

The Study conducted by Kris-Etherton found that ellagic acids and flavonoids in *Juglans regia* have potential in modulating serum cholesterol and have cardio protective effects due to flavonoids (63).

2.4 Dietary fatty acid and lipid metabolism

There were comparisional studies done on male rats fed with a semisynthetic diet that contains fish oil(omega-3), sunflower oil(omega-6), Oliva oil(omega-9) ,or coconut oil (saturated medium with omega -3) and among those fed polyunsaturated fatty acids rich diets, PUFAs change to omega-3 that reduce LDL, TC, and selectively that increase biliary cholesterol secretion (64).

Another study conducted in Iran on Swiss albino mice fed with diet rich in omega-3 PUFAs (DHA and EPA) helps to reduce Triglycerides, phospholipids and cholesterol when compared to those fed with saturated fatty acids due to polyunsaturated fatty acids are rich with antioxidants than saturated fatty acids (65).

There was experimental Study conducted by Montoya, on the effects of different types of diets containing saturated diet (palm oil), MUFA (Oliva oil), PUFA (sunflower oil) supplementation with fish oil shows that MUFA and PUFA diet rich regulate better lipid profiles and liver enzymes than saturated rich diet (66).

A Study conducted in America on both female and male Wistar rats with moderate hypercholesterolemia on lipid profile, lipoprotein and oxidative stress, diet rich in PUFAs as decreases total cholesterol and LDL levels than those fed with the average American diet and Oliva oil -rich diets (67).

There was also a study done on HFD feeding in Swiss albino mice as it exacerbate metabolic syndrome and NAFLD, liver as an organ for fat metabolism, and lipid droplets become accumulated when there is excess body fat and causing fatty liver and liver damage (68).

However, administration of *Juglans regia* meal reduced concentration of elevated ALT and AST in serum due to Swiss albino mice fed HFDs, alleviating liver fat accumulation and hepatic tissue damage (69).

As CYPA1 and HMGR are rate-limiting enzymes for bile acid and plasma cholesterol biosynthesis respectively, they have a pivotal role in fat metabolism (70).

However, there is an increased expression of fatty acids and HMGR as well as a reduction in CYP7A1 and LCAT expression in the liver of rats fed with HFD (71). So, the consumption of

Juglans regia leaf achieves hypolipidemic effect by inhibiting the synthesis of cholesterol and fatty acids (72).

2.5 Dietary fatty acid and hyperlipidemia

Excessive consumption of diet rich in saturated fatty acids can induce dyslipidemia which is a risk factor for the development of cardiovascular diseases and its pervasiveness has drastically expanded through worldwide because of current ways of life which really increased utilization of high fat diet eating regimens (73).

A study done in Pakistan on Sprague Dawley rats shows that the consumption of high fat diet can induce hyperlipidemia and feeding of *Juglans regia* helps to improve lipid profile and lower body mass index in one to three because of they are rich in MUFAs and PUFAs (74).

Another study was conducted in USA on the effects of *Juglans regia* kernels extract on serum lipid profile of STZ induced diabetic mellitus on Wistar rats shows that feeding *Juglans regia* diet have the ability to lower serum total cholesterol than those fed with control diets (75) and study done in Quetta on effects of *Juglans regia* fruit consumption in HFDs induced obesity in males Sprague Dawley rats helps to reduce several cardiovascular disorders throughout decrease total cholesterol concentration in blood (76).

2.6 Protective effects of dietary *Juglans regia* on hepatic triglyceride and adipose tissue inflammation in mice fed HFD

Dietary *Juglans regia* also have more protective effects on HFD induced fatty liver and NAFLD in mice fed with regular rodent chow/HFD (45% of energy derived) than those without supplied 21.5% *Juglans regia* energy derived (77).

However, walnut supplementation for mice fed HFDs reduce amounts of hepatic triglycerides, altered levels of proteins involved in lipid homeostasis, like AMP- activated protein kinase, FAS, and peroxisome proliferator activated receptor protein (78).

The Signaling molecule, PPAR-α which is essential for modulating lipid transport and metabolism via increase fatty acid oxidation, protects the liver from the development and advancement of NAFLD in different mice fed with HFD (79).

So, supplementation of *Juglans regia* extract prevents HFD induced adipose tissue inflammation in mice through inhibition of activated P-AMPK and up-regulation of FAS, leads to restoration of their levels in normal range (80).

Many of line evidence also elucidated linkage between liver and adipose tissue role in causing intrahepatic TG accumulation associated with obesity (81).There are pro-inflammatory molecules like chemokines and cytokines that cause adipose tissue inflammation, accelerate hepatic fat accumulation in obesity. Indeed, these overall blocks development of inflammation in adipose tissue can prevent HFD induced hepatic steatosis (82).

2.7 Conceptual Framework

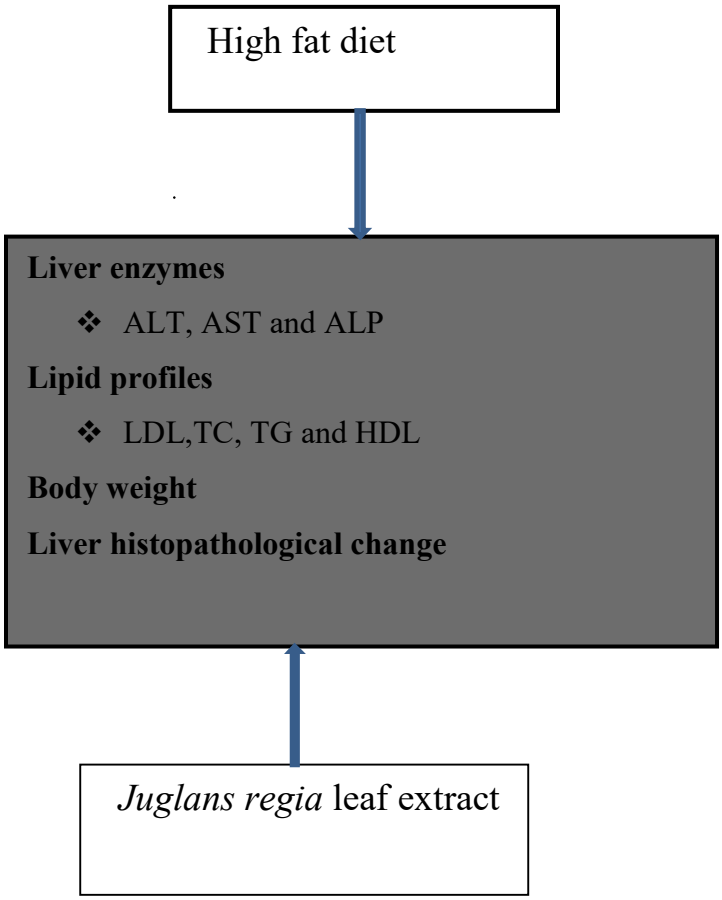

Figure 1: Conceptual framework adopted from (83)(84)(85).

3. Objectives

3.1 General Objective

To evaluate the effect of methanol crude extract of *Juglans regia* leaf on liver enzymes and lipid profiles of Swiss albino mice fed high fat diet at Jimma University Tropical and Infectious Diseases Research Center, Jimma zone, Sekoru district, Southwest Ethiopia, 2021

3.2 Specific Objectives

1. To assess effect of methanol crude extract of *Juglans regia* leaf on lipid profiles (TC, TG, LDL and HDL) of Swiss albino mice fed high fat diet
2. To evaluate effect of methanol crude extract of *Juglans regia* leaf on liver enzymes (ALT, AST and ALP) of Swiss albino mice fed high fat diet
3. To investigate histopathological changes in liver of Swiss albino mice fed high fat diet

3.3 Hypothesis

H0: Methanol crude extract of *Juglans regia* leaf has no effect on liver enzymes and lipid profiles of Swiss albino mice fed high fat diet

HA: Methanol crude extract of *Juglans regia* leaf has effect on liver enzymes and lipid profiles of Swiss albino mice fed high fat diet

4. Methods and Materials

4.1 Study Area and Period

The study was carried out at Jimma University Tropical and Infectious Disease Research Center(JUTIDRC), Jimma zone, Sekoru district 233km South west of the capital, Addis Ababa and 102 km East of Jimma town, South west Ethiopia from December 03, 2021-January 05, 2022.

4.2 Study Design

A Random posttest only control group experimental trial design was employed.

4.3 Reagents and Chemicals

Acetone, petroleum ether, Ethanol, Methanol, Normal saline, distilled water, lipid profiles and liver function test reagents,10 % formalin, Paraffin wax, xylene solution, ketamine, H$ E stain, atorvastatin were purchased from TherDose pharm private limited. All reagents and chemicals of analytical grade were used in this experiment.

4.4 Equipments

Mortar and Pestle, Test tube, Dropper, Flasks (2L,5L), Buchner funnel, Rotary evaporator(Buchi RotaVapor,Switzerland),Lyophilizer(Alpha12LDPlus,JohnMorrisscientific,PVt),Beaker(10ml,100ml,800ml), Blunt needle, Stainless Steel and Plastic Cage, Centrifuge(Gemmy industrial corp,Taiwan), Micropipettes (20µm, 20-200µm, 1000µm), Organ tubes, Electrical balance (A&D company, Limited Tokyo Japan), Refrigerator (Beko),Tissue embedder (Tissue-Tek), Mold(tissue cap), Wax dispenser (Electro-thermal Model: MH8523, China), ribbon Microtome (Leica Model: TP 1020,Germany), Slides, Light microscopy (Olympus CX21FS1, Philippines), Oven (Gallen Kamp), Desiccator, Fully automated Chemistry analyzer (ABX Pentra 400, China),SEM LipidoCare Analyzer (SEM Biosensor, INC., Korea), Tissue cassettes, and automated built-in digital photo camera (Camera KRUSS optronic Germany 3.0 MP USB 2.0) were used in the study.

4.5 Sample Size Determination

An appropriate sample size determination is important since too small size misses the real effect in an experiment while a sample size larger than necessary will lead to wasting resources and ethical issues on sacrificed animals (86).

Sample size for these experimental studies can be calculated based on Federer calculation formula as follows:

Federer calculation given by (t-1) (n-1) ≥ 15, whereas t was the number of the groups, n was the experimental animal per group; and drop-out size (do) was estimated 10%, the minimal size of sample was determined as:

$(6-1)(n-1) \geq 15$

$5(n-1) \geq 15$

$n \geq 15+5/5$

$n \geq 4$

$ndo = n/(1-do)^2$

$ndo = 4/(1-do)^2 = 4/(1-0.1)^2 = 4/0.81 = 4.9 \approx 5$ where ndo is minimal sample size (87). This research was using 30 experimental animals whereas there are five individuals in each six group of mice. The number of mice as the experimental animals was 30.

4.6 Plant Material Collection and Authentication

The fresh leaves of *Juglans regia* were collected from Oromia region farming area, Jimma Arjo district area of East Wollega which is 351 km away from Addis Ababa and was authenticated by a botanist and a voucher specimen MS001 was given and deposited at the National Herbarium, Addis Ababa University for further reference.

4.7 Preparation of Plant Extract

After collection, the leaves of *Juglans regia* was washed thoroughly with tap water to remove dust and dirty, and brought it to Jimma University, Health institute, Department of Biomedical sciences, Medical biochemistry postgraduate laboratory where it was air dried under shade area at room temperature and afterwards ground into fine powder using clean mortar and pestle.

The Powder was strained and put away in a glass holder. Then, a coarse powder of *Juglans regia* leaves was macerated in 99% absolute methanol (v/v) for three days with mechanical shaking three times each a day (88).

Then, the extract was filtered through Whatman filter paper 1.0 and the filtrate was evaporated to be dried by rotatory evaporator. Then, the filtrate was taken into thermostatic oven at 40°C to remove the rest of methanol.

From this point onward, the last concentrate which is free of methanol was taken into a profound cooler to have strong consistency and dried by freeze dryer and weighed. Finally,34.98g of extract was packed in airtight glass bottles by proper labeling and kept in the refrigerator until experiment was performed (89).

4.8 Preliminary phytochemical screening

Preliminary phytochemical analysis of *Juglans regia* leaves was performed by following standard methods endorsed beforehand of Trease and Evans and sofawara (90).

4.8.1 Testing for phenolic compounds (ferric chloride test)

3ml fraction of methanolic extract of *Juglans regia* was mixed with four drops of ferric chlorides and blue-black color was detected that shows the presence of phenolic compounds.

4.8.2 Testing for tannins

15ml of distilled water was added into the test tube, 2g of powdered *Juglans regia* was boiled for 3 minutes and cooled, then filtered. Then, 3 drops of ferric chlorides was added to the filtered sample and no brownish green color were observed that shows absence of tannin compounds.

4.8.3 Testing for alkaloids

2ml of methanolic extract of *Juglans regia* was treated with Mayer's test (1.525g mercuric chloride+2g of potassium iodide) and mixed well and shaked gently. Finally yellow colored precipitate was detected that shows the presence of alkaloid molecules.

4.8.4 Testing for cardiac glycosides

5ml of methanolic extract of *Juglans regia* was treated with 5ml of distilled water and shaked well and formation of foam shows the presence of glycoside.

4.8.5 Testing for flavonoids

2ml of methanolic extract of *Juglans regia* was treated with 3 drops of sodium hydroxide and initially an acute yellow color was formed and 1 drops of sulfuric acid was added, and makes colorless that shows presence of flavonoids molecules.

4.8.6 Testing for steroids

2ml of methanolic extract of *Juglans regia* was treated with 2ml of chloroform and filtered, then 6 drops of sulfuric acid was added to the filtered sample and shaken gently that allows it to stand carefully and yellow color was detected that shows the occurrence of steroid molecules.

4.8.7 Testing for Saponins

5ml of distilled water was mixed with 2ml of methanolic extract of *Juglans regia* in test tube and mixed vigorously by addition of 1 drop of sulfuric acid and foam was formed immediately that showed positive for saponin molecules.

4.8.8 Testing for Terpenoids

2ml of chloroform was added to 5ml methanolic extract of *Juglans regia* and evaporated on water bath and then boiled 3ml of diluted sulfuric acid and cooled. No Grey color was observed that shows the absence of terpenoids molecules.

4.8.9 Testing for Anthraquinones

5ml of benzene was treated with 3g methanolic extract of *Juglans regia* and mixed well in conical flask, and soaked for 10 minutes, and then filtered. Nextly, 5ml of ammonia solution (10%) was added to the filtrate and shaked vigorously for 30 seconds. Finally, violet color was detected that shows the plant was positive for Anthraqiunones compounds.

4.9 Acute oral toxicity test

Acute oral toxicity was conducted according to previous study administered with a single oral dose of *Juglans regia* leaf powder via oral gavage at a dose rate of 2000mg/kg body weight per a day (91), according to the Organization for Economic Cooperation and Development (OECD) guideline No. 425 (92).

Five female mice with 6-8 weeks old were selected and kept in dosing to allow for acclimatization to the laboratory conditions prior to dosing. Single female Swiss albino mice fasted for four hours on the first day of the test then; 2000 mg/kg of the extract was given by oral route using oral gavage and the mice were observed for the manifestation of behavioral and physical changes and special attention was given during the first four hours.

Depending on the results from the first mice, the next 4 female's animals were employed and fasted for about four hours and then a single dose of 2000 mg/kg of the extract was given orally and followed firmly in the same manner. The observation was sustained daily for a total of fourteen days (93).

Then the mice didn't show any sign of toxicity like lacrimation, hair erection, convulsion, coma and death during the first four hours as well as during fourteen days of observation. This finding indicates that LD50 of *Juglans regia* >2gm/kg.

This category includes that substance characteristic to comparable low acute toxicity and sub-chronic risk with oral LD50 between 2000 and 5000mg/kg/day.

Depending on this, 100mg/kg/day was taken as a single dose, 200 and 300mg/kg/day were taken as double and triple dose respectively (94).

Finally, individual weights of animals were determined before and after administering testing substance and weight changes calculated and recorded. At the end of the test survival animals were weighed and humanely killed (95).

4.10 Experimental Animals

A total of thirty healthy male Swiss albino mice aged 6-8 weeks old and weighing 25-35grams were purchased from Jimma University Tropical and Infectious Disease Research Center (JUTIDRC), Sekoru, Jimma, Ethiopia.

The experimental mice were situated in a plastic cage with a stainless steel cover and were kept in animal laboratory at room temperature with 12 hr light and dark cycles. The cages were kept clean during experiment.

Before initiation of experiment, all mice were left for acclimatization for one week at Jimma University Tropical and Infectious Disease Research Center on laboratory conditions and experimental animals were randomly assigned into six groups consisting of five mice per a group. Female mice were excluded from the study.

4.11 High Fat Diet (HFD) Preparation

Standard mouse pellets were obtained from Kality Agriculture and Reproduction Center, Addis Ababa, Ethiopia. Many experiments have used mice fed with commercial lard (animal fat) as an obesity model (96).

However, since Commercial lard is not available in Ethiopia, the HFD used in this experiment was prepared from locally available Palm oil. To prepare the HFD, palm oil (3L) was purchased from Sekoru, Jimma, Ethiopia. Then, palm oil was repeatedly heat five times on stainless steel stove; each lasting of twenty minutes.

At the end of heating session oil was allowed to cool for five hours. After cooled for five hours test diets were formulated by mixing of 15%(w/w) palm oil properly with eighty five (85%) of mice regular pellets. Then, the pellet were reformed and dried on black plastic bag containers (Pestal) overnight and given to mice at morning (97).

The standard pellet powder consists of 20% fat, 60% carbohydrate and 20% protein (98). Therefore, with addition of 15% (w/w) of thermo oxidized palm oil to the pellets this contains 20% of fat, a produced food mixture containing 27% fat.

Therefore, the term" high fat diet" in these study refers to a diet containing 39% fat by weight (27% from added thermo oxidized palm oil and 12% of standard pellet), since 20% standard pellet is equal to 12%.

The dose of daily HFD given to experimental animals was given by calculation formula:

$$D = BW \times 10 /100 \times K$$

Where D is the dose of HFD given to each mouse per a day (g), BW is the body weight of each mouse (g), 10/100 is the daily food consumption of mice relative to their body weight, and K is the proportion of HFD given to each mouse in a specific group in grams (99).

4.12 Extrapolation of atorvastatin dose to experimental animals

Regardless of the purpose of administration, choosing safe and effective drug in the initial dose in animals and humans is a concern. The human doses of atorvastatin drug were extrapolated to animals: Human equivalent dose mg/kg = Animal's dose in (mg/kg) × (Animal km ÷ Human km), where Km is a correction factor reflecting the relationship between body weight and body surface area (100).

AED mg / kg = Human dose (mg / kg) x (Animal Km / Human km)

= 20/60/3/37

= 0.33/0.081

= 4.07mg/kg per a day

4.13 Experimental and treatment protocol

A total of thirty male Swiss albino mice were randomly assigned into six groups with five animals per a cage. Each mouse in the given groups was identified by giving a number on their tails by permanent marker. The mice were grouped and treated as follows:

1) Group I (Normal control group): represent mice received regular pellets and 1ml of distilled water Po daily for 30 days.

2) Group II (Negative control group): represent mice received HFD and 1ml of distilled water Po daily for 30 days.

3) Group III (Positive control group): represent mice received HFD and atorvastatin 20mg/kg Po daily for 30 days.

4) Group IV (Experimental group): represent mice received HFD and 100mg/kg of *Juglans regia* crude leaf extract for 30 days.

5) Group V (Experimental group): represent mice received HFD and 200mg/kg of *Juglans regia* crude leaf extract for 30 days.

6) Group VI (Experimental group): represent mice received HFD and 300mg/kg of *Juglans regia* crude leaf extract for 30 days (101).

4.14 Measurement of body weight

Body weight of the experimental mice was measured using triple beam balance at the beginning of the experiment, in the middle of the weeks, immediately before being euthanized and recorded using the corresponding code given to each mouse in the groups to see bodyweight change in all groups of experimental animals. For the statistical analysis, the initial and final body weights were taken into account and expressed as mean ±SEM.

4.15 The food intake measurement

The daily food intake of the mice was measured in the morning using a weighing balance. Food intake was the difference in weight between food put into cage and the remaining at the end of 24 hours (g/day/cage). Then, for statistical analysis the mean average of food intake in weekly per a gram was taken, analyzed using repeated measure of ANOVA and expressed as mean ±SEM.

4.16 Blood collection and serum preparation from mice

At the end of the experimental study, mice were fasted overnight and anesthetized with 100 mg/kg ketamine/12.5 mg/kg xylazine injection. Thereafter, mice were euthanized by cervical dislocation after cardiac puncture for blood collection using 5-cc syringe.

Two to two and a half milliliter (2-2.5 ml) of blood was collected with a plain tube and placed in serum separator test gel, left for 30 minutes at room temperature to clot and centrifuged at 3000rpm for 10 minutes, and serum was isolated using a micropipette(1000µl) and stored at -20^0c until analysis of lipid profiles (HDL,LDL,TG and TC) were measured with the aid of auto analyzer machine(Human star 300SR,Germany) and LFTs (ALT,AST,ALP) were evaluated by using a fully automated chemistry analyzer (ABX Pentra 400, China).

4.16.1 Total cholesterol

Total cholesterol was measured enzymatically in serum in a series of coupled reactions that hydrolyze cholesteryl esters and oxidize the 3-OH group of cholesterol. Cholesterol esters are hydrolyzed to free cholesterol by cholesterol ester hydrolase. The free cholesterol produced was oxidized by cholesterol oxidase to cholest-4-en-3-one with the simultaneous production of

hydrogen peroxide, which oxidatively couples with 4-aminoantipyrine and phenol in the presence of peroxidase to yield Quinoneimine dye with maximum absorption between 500-550 nm.

Cholesterol ester + H2O ⟶ cholesterol+ FFA

Cholesterol + O2 ⟶ 4-Cholestinone + H2O2

2H2O2 + 4-aminoantipyrine + phenol ⟶ Quioneimine dye + 4H2O

Figure 2: Scheme of total cholesterol quantification reaction principles

4.16.2 Determination of Triglycerides

The method was based on the enzymatic hydrolysis of triglycerides to glycerol and frees fatty acids by lipoprotein lipase (LPL). Glycerol was converted to glycerol 3-phosphate and adenosine-5-phosphate (ADP) by glycerol kinase and ATP.

Glycerol-3- phosphate was oxidized by glycerol phosphate oxidase to form dihydroxyacetone phosphate and H2O2. In the presence of peroxidase and H2O2, 4-aminoantipyrine couples with phenol to form a colored product (quinoneimine) that can be measured spectrophotometrically at a wavelength of 500nm.

Triglyceride + 3H2O →Glycerol + 3FFA

Glycerol + ATP→ G-3-P + ADP

G-3-P + O_2→ DHAP + H2O2

2H2O_2 + 4-aminoantipyrine + phenol →Quininoid dye + 4H2O

Figure 3: determination of triglycerides

4.16.3 Determination of High density lipoprotein

The basic principle for determination of high density lipoprotein cholesterol was as follows. The apoB containing lipoproteins in the specimen react with antibodies to apoB that renders them nonreactive with the enzymatic cholesterol reagent under conditions of the assay. The enzymes used were also pegylated, and this allows them to react only with HDL-c and not with antibody bound LDL-c, VLDL-and chylomicrons.

The apoB containing 39 lipoproteins was thus effectively excluded from the assay and only HDL-c was detected under the assay conditions. The HDL-c test was a two reagent homogenous system for the selective measurement of serum or plasma HDL-c in the presence of other lipoprotein particles. The assay was comprised of two distinct phases.

In phase one, it was likely that in the presence of slightly alkaline buffer and magnesium sulfate and dextran sulfate selectively form water soluble complexes with LDL-c, LDL-c and chylomicrons, which were resistant to polyethylene glycol (PEG) modified enzymes.

In phase two the cholesterol concentration of HDL-c cholesterol was determined enzymatically by 22 cholesterol esterase and cholesterol oxidase coupled with PEG to the amino groups (approx.40%).

(1) ApoB containing lipoproteins + α-cyclodextrin + Mg+2 + dextran H2SO4) → soluble non-reactive complexes with apoB-containing lipoproteins

(2) HDL-cholesteryl esters → HDL-unesterified cholesterol + fatty acid

(3) Unesterified chol + O2 → cholestenone + H2O2

(4) H2O2 + 5-aminophenazone + N-ethyl-N-(3-methylphenyl)-N'succinyl ethylenediamine + H2O + H+ peroxidase→ quinoneimine dye + H2O

Absorbance is measured at 600 nm.

Figure 4: determination of high density lipoprotein

4.16.4 Determination of Low Density Lipoprotein

Circulating LDL-c is calculated from measured values of total cholesterol, triglycerides and HDL-c according to the Friedewald equation: LDL-c = TC [HDL-c + TG/5)].

Where [TG]/5 are an estimate of VLDL-c, all values are expressed in mg/dl. The equation is derived from another equation, [Total Cholesterol] = [VLDL-c] + [LDL-c] + [HDL-c

4.17 Liver Histopathological Studies

In order to see the effects of mice fed HFD and counter effects of *Juglans regia* crude leaf extract fed mice, histopathological examination of the liver was performed. Small pieces of liver tissue were taken from each mouse carefully and fixed with 10% of buffered neutral formalin saline.

Then, the tissue was taken to Jimma University Pathology department tissue grossing and processing room. Then, tissue was dehydrated using methanol for 2 hours and its sections was treated with xylene solution to remove methanol and replaced with paraffin wax and stained with Hematoxylin and eosin, and was examined by light microscope (olympusX2FS1, Philippines) at 40xmagnifications.

After examination, photos were taken by a microscope camera (camera KRUSS Optronic, Germany, 3.0 MP, USB 2.0), and the pictures were read and interpreted by a senior Pathologist.

4.18 Variables

4.18.1 Dependent variables

- Lipid profiles
- Liver enzymes
- Histopathological change

4.18.2 Independent variables

- High fat diet
- *Juglans regia* crude leaf extract

4.19 Operational Definitions

Hyperlipidemia: It is abnormally elevated of any of or all lipid and lipoproteins in the blood. It is described as a defect in lipoprotein metabolism; for example, increased total cholesterol, triglycerides, LDL, and decreased HDL (102).

Juglans regia: herbal medicine with a complex of macronutrients and micronutrients that have beneficial effects on many aspects of health (103).

Lipid profiles**:** a panel that is used to measure the amount of lipids and fatty substance in the blood. They include total cholesterol, triglycerides, LDL, and decreased HDL.

Liver enzymes: The most current indicators that are elevated due to liver diseases, injury, high consumption of diet rich in fat, etc. They include: AST, ALT and ALP (104).

Normal control group: the group of mice which were administered of only distilled water.

Negative control group: the group of mice which were received only HFD only.

Positive control group: the group of mice which were received atorvastatin and HFD only.

Experimental groups: groups of mice which were received HFD and different doses *Juglans regia* crude extract.

4.20 Data quality control and management

Before, during and after the analysis precautions were considered and the equipments used during the experiment were calibrated. All manipulations and procedures of biochemical tests were conducted by trained and experienced laboratory technicians under close supervision of investigators by following SOP guidelines.

Liver function tests (AST, ALT, ALP) and Lipid profiles (HDL, LDL, TC, TG) were analyzed by using the ABX Pentra 400 clinical chemistry auto analyzer (HORIBA ABX SAS, China) as

per the manufacturer's instructions. The liver histopathology investigation was performed by senior Pathologists.

4.21 Data Entry and Analysis

The quantitative data were collected, properly coded and entered through Epi-data version 3.1, processed; edited and analyzed using SPSS version 25 of statistical software after it was checked and cleaned.

A result were presented by tables and expressed as mean ± standard error of mean. The mean difference among groups was evaluated by using Paired t-test and one way analysis of variance (ANOVA). Post hoc Tukey test for multiple comparisons was conducted for determining level of significance difference between each treatment group, and ($P<0.05$) considered as statistically significant.

A microscopic evaluation was qualitatively analyzed through preparing microscopic slides for each group, Photos were taken by a microscope camera and pictures were read and interpreted by a Pathologist.

4.22 Ethical Consideration

Before conducting experiment, Ethical clearance was obtained from Jimma University Institutional Review Board (IRB) by approval letter with Ref.No.IHRPGD/7/2021 issued on 9/11/2021 and a supportive letter was written for Jimma University Tropical and Infectious Disease Research Center (JUTIDRC).

Then, any concerned administrative offices were communicated with a formal letter. An animal used in this study was kept from any unnecessary painful and terrifying situations (OECD, 2008). To make pain and suffering minimal during any surgical intervention (blood collection), the animal was anesthetized using ketamine/xylazine injection and the procedure was carried out by well-trained persons.

All animal handling, care and activities performed during the experiment was carried out as declaration of national and international guidelines of experimental animals and code of ethics of experimental animals.

4.23 Disseminations of Results

The results of the study were compiled in the form of a thesis and presented to the Department of Biomedical sciences. The result of the study is also communicated to all concerned institutions

including Department of Biomedical sciences, Ethiopian Public Health Institution (EPHI) and to advisors and will be published in peer reviewed journals and delivered in scientific conferences.

5. Results

5.1 Percentage yield of methanol crude leaf extract of *Juglans regia*

The amount of crude extract obtained from coarse powder of 250g of *Juglans regia* was 34.98g. Therefore, the percentage yield this extraction of 99% absolute methanol (10/90v/v) was given as:

Percent yield=actual yield/theoretical yield × 100

= (34.98g/250g) × 100 = 13.99% (w/w)

5.2 Phytochemical result present in methanol leaf extract of *Juglans regia*

The result of phytochemical screening of methanol extract of *Juglans regia* leaf showed that the presence of bioactive components like, phenolic compounds, alkaloids, flavonoids, terpenoids, heart glycosides, saponin, tannins, anthraquinone and steroids (Table:1).

Table 1: The result of phytochemical screening of methanolic extract of *Juglans regia* leaf

Phytochemical constituent	Status
Phenolic	+
Alkaloids	+
Flavonoids	+
Terpenoids	-
Heart glycosides	+
Saponin	+
Tannins	+
Anthraquinone	-
Steroids	+

+ stands for the presence of phytochemicals

- stands for the absence of phytochemicals

5.3 Effect of methanol leaf extract of *Juglans regia* on food intake

Food intake amount was determined based on by taking mean average of food intake weekly a (gram/cage) and analyzed by using repeated measure of ANOVA.

As described in (Table 2) below the effect of HFD and *Juglans regia* leaf extract on food intake, the food intake of HFD fed control (Group II) mice didn't show significant difference up to 3rd week despite significantly increased in 4th week as compared to normal group. The food intake significantly reduced in Group III mice (HFD + atorvastatin) from the 2nd week to 4th week at ($P<0.05$).

Interestingly, the food intake of mice fed on HFD supplemented with *Juglans regia* leaves extract at 100 mg/kg and 200mg/kg dose did not bring any significant alteration up to 3rd week when compared with group of mice fed HFD (G-II).

Further analysis indicate that highest dose of *Juglans regia* leaves extract (300 mg/kg) significantly decreased food intake (P=0.035) when compared to G-II from 1st week up to 4th week.

Table 2: Effect of different doses of *Juglans regia* leaf extract on food intake

Groups	1st week	2nd week	3rd week	4th week
Normal control	49.4±33.62	52.15±37.4	58.85±51.12	61.98±11.30
HFD control	67.08±6.26	75.29±17.03	76.06±18.12	78.13±12.21[a]
Atorvastatin +HFD	70.88±8.15	66.83±8.15[b]	63.12±6.35[ab]	61.49±9.01[b]
HFD+100mg/kg	55.40±13.92	76.12±15.37	67.01±0.49	76.39±15.76[ab]
HFD+200mg/kg	73.12±8.16	76.39±1.35	74.15±14.67	67.50±0.21[ab]
HFD+300mg/kg	77.67±6.75	78.42±6.25	73.52 ±7.52	71.61 ±8.07[b]

All values are expressed as mean ±SEM (n=5).*Values are statistically significant at P<0.05 using one-way ANOVA followed by post hoc Tukey test by comparing with group II. The superscript" a" and "b" shows that statistically significant (P < 0.05) when compared to normal control group and HFD groups respectively within the same column. The superscript" ab" shows that statistically significant (P< 0.01) when compared to HFD groups within the same column.

5.4 Effect of methanol extract of *Juglans regia* leaf on body weight of mice

Before starting of treatment with HFD and extract, there was no significance difference in mean body weight values in all groups of animals. However, at the end of experiment the mean body weight values of group of mice received HFD only (G-II) was increased in number from 3rd week to 4th week insignificantly from 29.05±0.91 to 30.5±0.56 at (P=0.835) when compared to normal control group.

Interestingly, the mean body weight of group of mice treated with atorvastatin, low and moderate dose of *Juglans regia* crude leaf extract groups insignificantly decreased (P= 0.33;0.095 and 0.178) respectively from 1st week to 4th week compared to negative control group (G-II).

There was a significant decrement on mean body weight in group of mice received high dose of *Juglans regia* crude leaf extract from 2nd week to 4th week when compared to Group of mice received HFD only (G-II) at (P=0.001) (Table 3).

Table 3: Average body weights measurement in gram during the 4 weeks of albino mice fed high fat diet during treatment with different doses of *Juglans regia*

Groups	1st week	2nd week	3rd week	4th week
Normal control	28.4±0.62	27.2±0.42	28.56±0.12	29.15±0.30
HFD control	27.90±0.70	28.65±0.63	29.05±0.91	30.5±0.56
Atorvastatin +HFD	30.2±0.72	28.16±0.95[a]	26.4±1.30	24.67±1.48
HFD+100mg/kg	29.15±0.59	28.13±0.77	27.87±1.49	26.83±2.07
HFD+200mg/kg	27.56±1.41	28.98±0.75	27.14±1.42	26.67±1.83
HFD+300mg/kg	30.01±1.86	27.33±1.74[a]	26.90 ±1.39[a]	24.23 ±1.95[a]

Values are mean±SEM (n=5). Values are statistically significant at * P<0.05 using one-way ANOVA followed by post hoc Tukey test. Superscript "a"P<0.05 compared to HFD fed control group.

A paired t-test was used to compare the initial and final body weight change of the mice shown in (Table 4) there was a body weight change with in different groups of animals during performing the experiment. A final body weight of group I and II were significantly increased when compared to initial body weight of group I and II at (P<0.05).

Atorvastatin treatment group (group III) brings numerical difference of final body weight decrement as compared to group (group II) insignificantly (P>0.05). Oral supplementation of *Juglans regia* leaf extract 200mg and 300mg b/w significantly (P<0.05) reduced the increased final body weight as compared with initial body weight HFD group (group II). However, the final bodyweight of group IV was slightly decreased insignificantly (P>0.05) (25.38+3.16) as compared to initial body weight (26.08+4.2).

Table 4: Effect of methanolic extract of *Juglans regia* leaf on body weight of mice

Groups	Initial body weight	Final body weight	Mean difference ±SD	P –value
Normal control	28.88±0.85	30.68±3.96	-1.82±4.29[a]	0.002
HFD control	27.86±3.05	37.83±11.93	-8.82±8.88[a]	0.003
Atorvastatin +HFD	27.99±1.09	26.51±3.32	1.48±0.53	0.402
HFD+100mg/kg extract	26.08±4.2	25.38±3.16	0.69±2.13	0.098
HFD+200mg/kg extract	24.17± 1.84	23.94 ±2.88	1.48±0.53[a]	0.010
HFD+300mg/kg extract	22.76±3.14	21.25 ±3.65	-0.48±1.80[a]	0.006

The results were expressed as mean ± SD. "a" implies statistically a significant (P < 0.05)

5.5 Effect of *Juglans regia* leaf extract on liver enzymes of albino mice fed high fat diet

5.5.1 Serum Alanine Transaminase

In this study serum ALT level of HFD control group (85.60±10.78) significantly increased when compared to control group (38.80±3.49) at (P<0.01). Serum ALT level atorvastatin treatment groups (positive groups) also shows that significant decrement (47.42±3.22) when compared to HFD control group (85.60±10.78) at (P<0.05).

Group-IV, V and group-VI mice show a significant difference from group-II mice. This shows that 100mg/kg, 200mg/kg and 300mg/kg *Juglans regia* extract significant decrement in serum level of ALT (P<0.01).

5.5.2 Aspartate aminotransferase

As shown in (Table 5) below, Serum AST level of group II (116.60±18.85) was significantly increased as compared to group I (22.60±7.36) at (P<0.05). Serum AST level of atorvastatin treatment groups (positive groups) also shows that significant decrement (97.60±8.26) when compared to HFD control group (116.60±18.85) at (P<0.01).

The *Juglans* regia extract treated groups; HFD+100mg/kg extract,HFD+200mg/kg extract and HFD+300mg/kg extract mice show a significant decrement in serum AST level when compared to HFD control group at (P<0.01).

5.5.3 Serum Alkaline phosphatase

Serum ALP level of group II (294.01±25.96) was significantly increased when compared to group I (71.80±15.23) at (P<0.05) alike serum ALP level of atorvastatin received group (197.40±73.04),HFD+200mg/kg extract (153.4±221.62),and HFD+300mg/kg extract (144.60±28.84) were significantly decreased when compared to group II at (P<0.01).

Table 5: Effect of different doses of *Juglans regia* leaf extract on liver enzyme parameters of Swiss albino mice fed high fat diet

Variables (IU/L)	Groups					
	Normal control	HFD control	atorvastatin +HFD	HFD+100mg/kg extract	HFD+200mg/kg extract	HFD+300mg/kg extract
ALT	38.80±3.49	85.60±10.78^{ab}	47.42±3.22^{b}	48.80±11.81^{c}	44.01±3.53	42.60±13.29^{c}
AST	22.60±7.36	116.60±18.85^{b}	97.60±8.26^{d}	54.60±14.04	34.40±17.37^{d}	24.60±3.51^{d}
ALP	71.80±15.23	294.01±25.96^{b}	197.40±73.04	169.60 ±108.61	153.4±221.62^{d}	144.60±28.84

The values were expressed as mean ± SEM. The sample size is 5 for each group. ∗indicates significant differences among all groups at (P < 0.05) as tested by one-way ANOVA. The superscript"ab" and "c" shows that statistically significant (P < 0.01) when compared to normal

control group and HFD groups respectively. Superscript “b” shows that statistically significant (P< 0.05) and superscript “d” showed that statistically significant (P<0.01) when compared to group of mice fed HFD only.

5.6 Effect of *Juglans regia* leaf extract on serum lipid profiles of albino mice which were fed high fat diet

5.6.1 Serum Total cholesterol

As indicated in (Table 6) below the serum total cholesterol in a group of mice treated with HFD control (65.01±2.91) significantly raised (P=0.011) as compared to normal control group mice (34.8±23.81)despite atorvastatin treated group was show numerical decrement without statistically significant (P=0.374) as compared to HFD control group(G-II).

Interestingly, group of mice treated with *Juglans regia* extract at low dose (G-IV) and moderate dose (G-V) shows numerical decrement of TC when compared to group of mice treated with HFD control only (G-II) despite group of mice treated with high dose of *Juglans regia* extract statistically significant (P=0.002) decreased serum TC when compared to group of mice treated with HFD only (G-II).

5.6.2 Serum Triglycerides (TG)

As indicated in (Table 6) below, HFD treated group and HFD plus atorvastatin treatment group (G-III) and the lowest dose (100 mg/kg) of *Juglans regia* leaf extract treated group (Group-IV) showed a significant increase (P<0.05) in the serum level of TG as compared to normal control group and group (G-II) respectively.

But, group-V and VI mice which were treated with 200mg/kg, 300mg/kg and HFD showed a decrement in the serum level of TG as compared to group-II insignificantly (P=0.524,P=0.206) respectively.

5.6.3 Serum Low Density Lipoproteins (LDL)

The serum level of LDL in group-II (92.6±6.42) increased significantly (P<0.05) as compared to normal control group-I (59.01±10.54). However there is a decrease in the serum level LDL of *Juglans regia* leaf extract treated group (III (49.01±11.93) and IV (44.01±13.41) at (P<0.05) as compared to group II mice (HFD control group).

Interestingly there is a numerical decrement of LDL of group V and VI as compared to HFD control (group II) without statistically significance.

5.6.4 Serum High density lipoprotein

As indicated in (Table 6) below the mean value of HFD control group (G-II) of mice decrement of HDL level statistically insignificantly (P>0.05) as compared to normal control group (G-I) while the mean value of HDL-C was significantly increased in low (P=0.005), moderate (P=0.023) and high (P=0.021) dose of *Juglans regia* leaf extract and atorvastatin (P=0.004) treated as compared to HFD control group (G-II).

Table 6: Effect of *Juglans regia* leaf extract on lipid profiles of albino mice fed high fat diet

Variables (mg/dl)	Groups					
	I	II	III	IV	V	VI
TC	34.8±23.81	65.01±2.91[a]	37.6±11.91	33.2 ±8.21	30.23±111.56	29.2±10.42[b]
TG	32.8 ±25.07	60.2 ±6.83[a]	43.02±13.27[b]	42.6±9.47[b]	40.03 ±21.21	35.2±14.75
LDL	59.2±7.56	92.6±7.82[a]	53.6±6.42[b]	34.6±2.88[b]	29.01±9.46	27.20±2.17
HDL	43.01 ±10.54	37.8±5.06	46.01±11.93[c]	43.01±13.41[c]	45.80 ±14.85[d]	47.60 ±6.76[d]

The values were expressed as mean ± SEM. the sample size is 5 for each group. I: normal control group; II: negative control group; III: positive control group; IV: received 100 mg/kg/day *Juglans regia* leaf extract; V: received 200 mg/kg/day of *Juglans regia* leaf extract; and VI: received 300 mg/kg/day of *Juglans regia* leaf extract, respectively.

The superscript"a" and "b" shows that statistically significant (P< 0.05) when compared to normal control group and HFD groups respectively. The superscript"c" and "d" shows that statistically significant (P < 0.01) and (P<0.05) when compared to HFD only groups.

5.7 Effect of *Juglans regia* leaf extract on histopathological changes of albino mice liver

The results of histopathological examination of the livers of mice fed high fat diet for 4 weeks were shown below (Figure 5).

The result showed that there were different extents of cell necrosis in the hepatic lobules in mice fed high fat diet. In the normal control group (G-I) there were no lipid droplets distributed in the hepatic lobules, with intact central vein, no vacuolar degeneration and no necrosis, which shows that the integrity of hepatic lobular structure was maintained.

The HFD control group (G-II), however, suffered from moderate cell necrosis of hepatic lobule, moderate vacuolar degeneration (20%) and mild-mixed inflammatory cell infiltration around hepatic lobule and portal vein.

Interestingly, atorvastatin treatment group (G-III) there was a significant regeneration of liver architecture when compared to group of mice fed high fat diet even though the appearances of minor cell infiltration. However, group IV, V and VI there were the alleviation of hepatic necrosis to less extent and a significant improvement observed in pathological changes of liver tissues group of mice received moderate (200mg/kg) and high dose (300mg/kg) when compared to (G-II) (Figure 5).

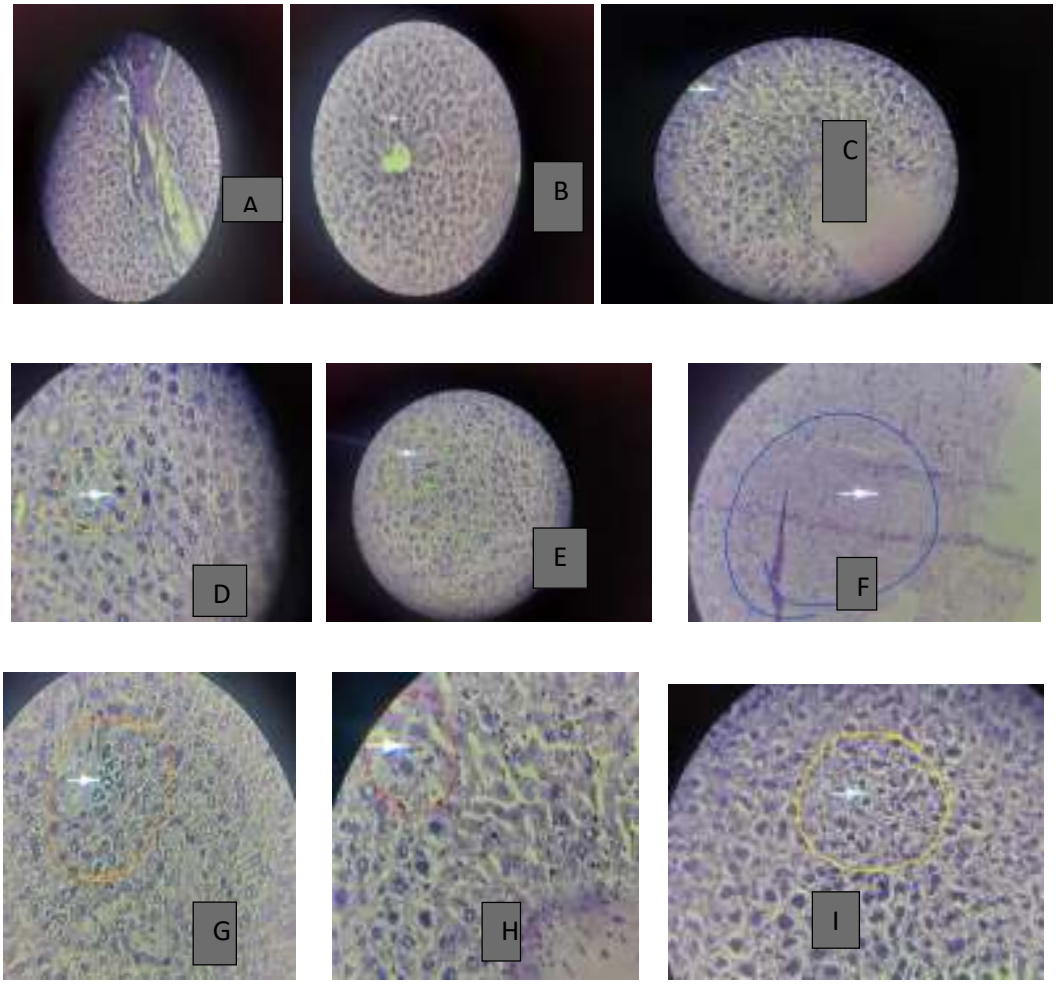

Figure 5: Photomicrographs (Hematoxylin and Eosin (HE)) showing the cytoarchitecture in the liver of mice treated with HFD and *Juglans regia* leaf extract.

(A–C) Control group, showing the characteristic architecture, with an intact hepatocytes, vacuolar degeneration and necrosis respectively (indicated by white arrow).

(D-F) HFD group where, purple, green and blue encircled arrow indicate damaged hepatocytes, moderate vacuolar degeneration and necrosis respectively.(G) showing atorvastatin received group, where orange encircled arrow indicate minor cell inflammation.

(H) Showing that group received 200mg/kg of extract, where encircled red arrow indicate damaged minor (<5%) hepatocytes cells with moderate hepatic necrosis.

(I) showing that group received 300mg/kg of extract yellow encircled arrow indicate that significant improvement of pathological change of liver with minor cell inflammation (<5%).

6. Discussion

In the present study, the crude leaf extract of *Juglans regia* was used to treat liver diseases that was exacerbated due to high fat diet in male Swiss albino mice fed high fat diet for 30 days.

The effect of *Juglans regia* on daily food intake, bodyweight, liver enzymes, liver histopathological change, and lipid profiles were investigated in Swiss albino mice fed high fat diet. In the present examination, high fat diet indicates a critical increment body weight, serum AST, ALT, ALP, serum TC,TG,LDL-C and a significant decrease of HDL-c in Swiss albino mice fed high fat diet group when compared to normal control group ($P<0.05$).

In this study, the daily food intake of HFD group (G-II) was higher than normal control group (G-I) from 1st week to 4th week while the food consumption of *Juglans regia* crude leaf extract at low, moderate and high dose treated groups of mice was significantly reduced than HFD group (G-II). The presence of dietary fibers molecules in *Juglans regia* leaf extract has appetite decreasing effect through slowing gastric emptying and increasing early satiety (102).

In present study, there were increased body weight found in group of mice fed HFD (G-II) when compared to normal control group (G-I) of mice because of accumulation of diet rich saturated palm oil adipose tissues (105).

However, a group of mice treated with standard drug (atorvastatin) and low (100mg/kg), moderate (200mg/kg), 300 (mg/kg) high dose of *Juglans regia* leaf extract, there was a significant weight reduction, which proves anti-obesity effects. These is due to hypophagic properties flavonoids bioactive compounds present in *Juglans regia* crude leaf extract (36).

High intake of diet rich in saturated fat leads to damage in the liver, causing metabolic function loss of the liver through increased of liver enzymes in the blood (106). In our study, we observed that a group of mice fed high fat diet (G-II) as it provoked an increase of AST, ALT and ALP level as compared to normal control group.

However, oral supplementations of methanol crude extract of *Juglans regia* leaf at low, moderate and high dose were able to reverse increased amount of liver enzymes. Our present results, was in harmony with study done on effects of green tea extract on mice fed HFD for 10 weeks (107). This is due to that phenolic compounds found in *Juglans regia* leaf extract have ability to attenuate hepatic steatosis in mice.

Treatment of methanol extract of *Juglans regia* leaf restored serum levels of liver enzymes near to normal level in group of mice fed high fat diet (G-II). Group of mice treated with *Juglans regia* leaf extract at moderate dose (200mg/kg) and higher dose (300mg/kg) showed significant reduction ($P<0.01$) of ALP, AST&ALP as compared to (G-II) fed high fat diet.

Additionally, low (100mg/kg) dose of *Juglans regia* leaf extract lowered the level less extent to normal ($P<0.01$) as compared to group of mice fed high fat diet (G-II). Group of mice received atorvastatin (G-III) was significantly reduced ($P<0.05$) liver enzymes (AST, ALT, ALP) as compared to group of mice fed with only high fat diet (G-II). This study was contradict with study done in Iran (88) and South Europe (108). This may be due to different extraction methods, duration of experiment, and use of different solvent used during extraction and different experimental animal's models.

In present study, the serum level of ALT and ALP of group of mice fed HFD (G-II) was significantly increased ($P<0.01$) as compared to normal control group. These increment was due to inability of the liver to metabolize accumulated fat in hepatic tissue (109).
This study was inconsistent with a study conducted in Turkey on effect of walnut oil consumption on nitrite induced hepatotoxicity on wistar rats (34). The exact cause of variation was unknown, but the possible explanation may be due to duration of treatment, geographical variation, induced dose and different experimental animals.

The groups of mice treated with moderate (200mg/kg), high (300mg/kg) and atorvastatin treatment group serum ALP and AST was significantly decreased ($P<0.05$) when compared to group of mice fed high fat diet (G-II). This study was agreed with study done on effects of *Juglans regia* stem bark extract on liver enzymes parameters of Sprague rat fed saturated Palmitic acid (34) and (60). This may be due to liver damage exacerbated by HFD may be restored with bioactive molecules with in methanolic extract of *Juglans regia* and standard drug used during experiment.

High fat diet feeding related with accumulation of liver lipids which cause damage of cellular heomostasis (110). In our present study showed that a group of mice treated with moderate (200mg/kg) and high dose (300mg/kg) of *Juglans regia* leaf extract were decreased level of TC and LDL when compared to group of mice fed HFD only. This study was consistent with study conducted on effects of chronic consumption of grape fruit juice on mice fed high fat diet for 6

weeks (111). This is due to that phenolic compounds present in *Juglans regia* leaf have capacity to inhibit the production of ROS and inhibit lipid peroxidation.

The current finding also showed that group of mice treated with *Juglans regia* leaf extract at 200mg/kg and 300mg/kg, their TC and LDL-c levels were significantly reduced when compared to group of mice fed with HFD (G-II) only despite group of mice received standard drug (atorvastatin) reduced their LDL-c insignificantly when compared to group (G-II). This finding is consistent to previous studies (63) and study done in Iran (4).

The possible reason may be due to antioxidant properties of plant extract bioactive components like steroids and flavonoids have ability to reduce cholesterol reabsorption in the intestine and promoting fecal cholesterol excretion, which in turn reduces the level of LDL-C in plasma, increasing the amount of cholesterol excreted from the body and decrease in hepatic cholesterol synthesis.

High fat diet (HFD) supplementation also elevate TG in group of mice received HFD (G-II) when compared to normal group (G-I) because of HFD have capable of reduce fatty acid oxidation, which in turn increase level of hepatic triacylglycerol (112).
But, level of TGs is insignificantly reduced with administration of atorvastatin and significantly reduced with treatment of high dose (300mg/kg) of *Juglans regia* leaf extract. Our finding is in line with a previous studies done in Pakistan (63) and Norway (113). This may be due the chemical constituents of plants, such as flavonoids and Saponins, lower cholesterol absorption by the inhibition of cholesterol micellar solubility.

There was also HDL-c result significant increment in group of mice treated with atorvastatin and treated with low, moderate and high dose of *Juglans regia* leaf extract with 100mg/kg, 200mg/kg and 300mg/kg respectively which is in harmony with result of study done on effects of *Juglans regia* fruit consumption on lipid profiles and liver histopathological changes on nitrite induced hepatotoxicity for 14 days (36) and study conducted in Pakistan (76).

The possible mechanisms by which *Juglans regia* plant extract enhances HDL-C serum level may be by regulating the hydrolysis of certain lipoproteins and their selective uptake, and metabolism by different tissues due to presence of tocopherols, phytosterols and polyphenols in leaf modifies the structure of the HDL-C lipoprotein by increasing paraoxonase-1 enzyme activity, which is responsible for the hydrolysis of lipid peroxides.

Moreover, a microscopic examination of the liver slide section was performed to evaluate the effect of HFD on both control and treatment groups. Group of mice in control group (G-I) revealed normal structure of liver without damage of central vein, no lipid droplets distributed in hepatocytes cell, no inflammation and vacuolar degeneration were detected (Figure 5).

High fat feeding were increased of lipid accumulated in hepatocytes cells through up regulation of PXR gene involved in fatty acid synthesis, uptake of these free fatty acid by liver from circulation has pivotal role for development of hepatic lipid droplets on hepatocytes cells (114).

In a group of mice fed HFD (G-II) there were moderate inflammation (25%), mild necrosis (15%), moderate apoptosis (20%), mild vacuolar degeneration (15%) around portal vein, lobular disarray (hexagonal) was observed (Figure 5).

In contrast, an obvious reduction in hepatocytes structured and significant regeneration of liver architecture was observed in groups of mice treated with moderate 200mg/kg and highest dose 300mg/kg of *Juglans regia* leaf extract (Figure 5).

This study was in a good agreement with study on effect of *Juglans regia* fruit supplementation on Wistar rat fed high fat diet for six weeks (115). This may be due to the anti-hyperlipidemic effect of glycosides, anti-oxidant effect of saponins, and ant-inflammatory effect of flavonoid in plant extract.

Additionally, group of mice treated with standard drug (atorvastatin) (G-III) there was a significant alteration of liver parenchymal cells with presence of mild cholestasis and sinusoidal dilation to lesser extent when compared to a group of mice fed HFD (G-II) (Figure 5.).

These study was in contradict with previous study reported on effect of *Juglans regia* leaf extract on CCL4 induced hepatotoxicity on Wistar rat for 10 weeks (116). This discrepancy may be due to different experimental animals used and duration of the treatment.

Limitations of the study

The exact content of fat in high fat diet was not determined.

Specific bioactive compounds that have effect on liver enzymes and lipid profiles were not identified.

The pharmacokinetics properties of *Juglans regia* leaf extract was not comparable with standard drug atorvastatin.

The specific organs of elevated enzymes were not identified.

7. Conclusions

The results of the study indicate that *Juglans regia* leaf extract decreased amount of food intake, body weight, altered liver enzymes and lipid profiles in Swiss albino mice fed high fat diet. This is due to phytochemicals present in leaves like flavonoids; phenolic and saponin were able to mop up free radicals generated by high fat diet feeding.

Weight reduction was also observed in a group of mice treated with *Juglans regia* leaf extract that helps to avoid abnormal accumulation of fatty acids in adipose tissue.

It was found that *Juglans regia leaf* extract has potential reducing effect on LDL, TC, TG and increasing HDL-c and effects of normalize AST, ALT, and ALP level of high fat diet induced histopathological changes in livers of mice. These indicate the protective effect of this plant via histopathological investigation.

The effect of *Juglans regia* leaf extract on lipid profiles at higher dose 300mg/kg is comparable with hypolipidemic effect of atorvastatin which is a standard drug for hyperlipidemia.

The present study also revealed that *Juglans regia* leaf extract have both hepatoprotective and antihyperlipidemic effects, and response is profoundly effective as dosage of *Juglans regia* leaf extract increased in Swiss albino mice fed high fat diet.

8. Recommendations

Our recommendations are the researcher;

To conduct further studies on active ingredients responsible for hepatoprotective and hypolipidemic properties of plant extract.

Further,investigation on molecular mechanisms by which these plant extract passes hepatoprotective and hypolipidemic effect as well as to conduct on different parts *Juglans regia* others than leaf.

Community should be preserve *Juglans regia* plant.

Community should be adopting habit of consuming of *Juglans regia* plant particularly for liver diseases.

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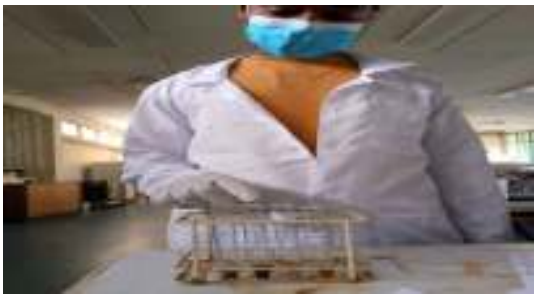
Annexs

Annex 1: Some of photomicrography taken during conducting preliminary phytochemical screening tests



- Phytochemical screening

✓ Basic steps from time of plant collection up to time of preparing solution for administering for experimental animals (with photography)

- Plant collection and coarse powder preparation(1-3)
- Soaking powder by using methanol(4)
- After 72 hrs filter and using rotary vapor to remove rest methanol(5-7)
- Preparing solution for adminstering experimental aniamls from crude extract(8-9)

- ✓ Basic steps from preparing solution for administering for experimental animals to serum and histopathology analysis (with photography).

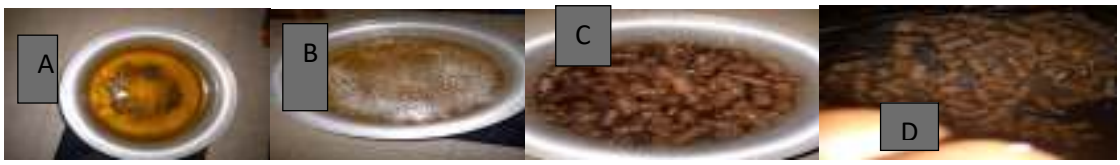
Annex 2.Bodyweight checklist for determining change of body weight during experimental methods

Table 7: checklist for determining change of body weight during experimental methods per a week

Group	no of mice	1st week	2nd week	3rd week	4th week	remark
Group-I	1. 2. 3. 4. 5.					

Group -III	1. 2. 3. 4. 5.				
Group –IV	1. 2. 3. 4. 5.				
Group -V	1. 2. 3. 4. 5.				
Group -VI	1. 2. 3. 4. 5.				

Annex 3: Photography of High fat diet (HFD) preparation Procedures



(A) Palm oil was liquefied by heating in a pan on a stove intermittently for five times with each lasting twenty minutes

(B) The liquefied and heated palm oil allows was allowed to cool for five hours

(C) And finally placed in powdered pellet while mixing properly and baking

(D) Reformed test diet were stored and dried on plastic bag container (Pestals) allows preventing further oxidation.

Annex 4: Principles and procedures for conducting Liver function tests

1.1 Alkaline phosphatase

Principle: The ALP acts upon the AMP buffered dodiumThymolphthalein monophosphate. The addition of an alkaline reagent stops enzyme activity and simultaneously develops a blue chromogen, which is measured photometrically.

Procedure**:**

1.0.5ml of Alkaline Phosphatase was dispensed and kept at 37oC for 3 minutes in labeled test tube.

2. At intervals, 0.5ml of standard, control, and sample was added and mixed gently.

3. De-ionized water will be used as blank and mixtures were incubated at 37oC for 10 minutes.

4.2.5ml of Alkaline Phosphatase color developer was added at intervals and mixed well.

5. The absorbance of the mixture was read at 590nm.

1.2 Alanine aminotransferase

Principle:

L-Alanine + α-ketoglutarate ⎯ (ALT) → Pyruvate + Glutamate

Pyruvate + 2, 4- DNPH-ine → Pyruvate + 2, 4-DNPH-one

Procedure:

1. Four test tubes labeled with blank, calibrator, control and sample was prepared.

2.0.5ml of ALT substrate was dispensed to each tube and placed in a 37oC heating bath for 3-5 minutes.

3. After five minutes 0.1ml of the corresponding sample was added to each labeled tube.

4. The solution was mixed and immediately returned to the heating bath for exactly 30 minutes.

5. After exactly 30 minutes, 0.5ml of ALT color reagent was added to each tube, heating bath for 10 minutes.

6. After exactly 10 minutes, 2.0ml of ALT color developer was added and heating bath for 5 minutes.

7. The absorbance was read at 505nm.

1.3 Aspartate aminotransferase

Principle: L-Aspartate + α-ketoglutarate → Oxalacetate + L-Glutamate

A diazonium was used which selectively reacts with the oxalacetate to produce a color complex that is measured photometrically.

Procedure:

1. Test tubes labeled as control, calibrator, and sample

2.0.5ml of AST was added to each tube and warmed in 37oC heating bath for 4 minutes.

3.0.1ml of samples was added into their respective tubes, mixed and returned to the heating bath for exactly 10 minutes.

4. After 10 minutes, in the same timed sequence, 0.5ml of AST color reagent was added, mixed and immediately returned to the heating bath for 10 minutes.

5. After 10 minutes, 2.0ml of 0.1N HCl was added and mixed.

6. The absorbance was read at 530nm.

Annex 5: Standard operating procedure (SOP) for serum preparation

Aim: Effective Separation of blood products

Purpose: To standardize separating procedures so that research samples was uniform in quality

1. Select test tube with no anticoagulant, serum separator tube (SST)

2. Draw enough amount of blood (1-2ml) from the animals

3. Allow to stand for 20-30min for clot formation at room temperature before spinning and separating. A delay in centrifugation may have a detrimental effect on the sample quality and may result inaccurate results. Avoid hemolysis

4. Centrifuge the sample to speed separation and affect a greater packing of cells. Clot and cells was separate from clean serum and settle to the bottom of the vessel.

The supernatant is the serum which can be now collected by dropper or pipette for testing purposes or stored (-20^{0}c) for subsequent analysis or use.

Annex 6: Preparations of working solutions

10% Neutral Buffered Formalin

40% formaldehyde	100 ml
Distilled water	900 ml
Sodium dihydrogen phosphate monohydrate	4 gm
Disodium hydrogen phosphate anhydrous	6.5 gm

Harris's Hematoxylin (H)

Hematoxylin crystals	2.5 gm
Absolute alcohol	25 ml
Potassium alum	50 gm
Distilled water	500 ml

Sodium iodates	0.5 gm
Glacial acetic acid	20 ml
1% Alcoholic Eosin (E)	
Eosin Y, water soluble (CI 45380)	1 gm
95% Ethanol	100 ml
Glacial acetic acid	0.5 ml
1% Acidic alcohol	
70% alcohol	500 ml
Hydrochloric acid, concentrated	5 ml
Bluing solution	
Sodium bicarbonate	2.5 gm
Distilled water	1000 ml

Annex 7: Tissue processing procedures

Fixation	
10% Neutral Buffered Formalin	24 hrs.
Washing	
Running tap water	several times
Dehydration	
70% Ethanol	2 hrs.
90% Ethanol	2 hrs.
Absolute alcohol I	11/2hrs
Absolute alcohol II	11/2hrs
Absolute alcohol III	11/2hrs
Absolute alcohol IV	overnight
Clearing	
Xylene I	11/2hrs
Xylene II	2 1/2hrs
Infiltration	
Paraffin wax I	2 1/2hrs
Paraffin wax II	overnight

Annex 8: Heamatoxylin & Eosin (H&E) Staining protocol

Deparaffinization	
Xylene I	5 min
Xylene II	5 min
Rehydration	
Absolute alcohol I	4 min
Absolute alcohol II	4 min
95% Ethanol	3 min
70% Ethanol	3 min
Rinse in distilled water	5 min
Stain in Hematoxylin	15 min
Rinse in running tap water	5 min
Decolorize in acid alcohol	1-3 sec
Rinse in running tap water	5 min
Immerse in Sodium bicarbonate solution	3-6 sec
Rinse in running tap water	5 min
Counterstain in Eosin	1 min
Dehydration	
70% Ethanol	2 min
95% Ethanol	2 min
Absolute alcohol II	3 min
Absolute alcohol I	3 min
Clearing	
Xylene II	4 min
Xylene I	4 min