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Evaluation of Ethanolic Seed Extract of Ziziphus Mauritiana on High-Fat Diet (HFD) Induced Fatty Liver in Wistar Rats

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Abstract: NAFLD is a chronic liver disease that affects a high proportion of the world's population which causing metabolic and hepatic damage. Ziziphus mauritiana is traditionally used as a dietary supplement for several diseases. This study was carried out to determine the beneficial effect of a standardized extract of Z.mauritiana on an animal model of nonalcoholic fatty liver disease (NAFLD). NAFLD was induced by a high-fat diet (HFD) in Wistar rats. Mainly, the terpenoids and flavonoids of this seed have antioxidant and anti-inflammatory activity, and it is identified by GC-MS. HFD rats will show an increase in the Serum lipid levels of total cholesterol (TC), triglyceride (TG), low-density lipoprotein (LDL),very low-density lipoprotein (VLDL), and a decrease in the high-density lipoprotein (HDL) levels and hepatic enzymes. Oxidative markers like SOD, CAT, GSH, and GPx are decreased in HFD rats. This study will significantly reduce the elevation of body weight, liver fat accumulation, TG, TC, LDL, VLDL, AI levels, and hepatic enzymes, and will increase antioxidant activities. In conclusion, ZMSE extract will significantly reduce the inflammation and a greater amount of deposition of fats in the liver, and its dietary supplementation will have a therapeutic effect on NAFLD.

Keywords: Non-alcoholic fatty liver disease (NAFLD), Superoxide Dismutase (SOD), Catalase (CAT), High-fat diet (HFD), Ziziphus mauritiana Seed Extract (ZMSE), High-density lipoprotein (HDL), Low-density lipoprotein (LDL),Very low-density lipoprotein (VLDL),Non-alcoholic steatohepatitis (NASH), Total Cholesterol (TC).

Introduction

One of the most common liver disorders in the world is non-alcoholic fatty liver disease (NAFLD)[1]. NAFLD is a range of liver diseases in which hepatic steatosis or a macro-vesicular accumulation of triglycerides in the hepatocyte grows in the absence of further causes (e.g., medications, excessive drinking of alcohol, or certain inherited conditions) [2]. The inflammatory subtype of NAFLD is called non-alcoholic steatohepatitis (NASH), which includes steatosis along with inflammation and evidence of hepatocyte damage (ballooning) and inflammation, either with or without fibrosis [2]. Diet-induced obesity is the most widespread inducer of NASH development because a severe form of accumulation of fat in the liver leads to the breakdown of lipid metabolism. Currently, the buildup of triglycerides of free fatty acids and hepatic cholesterol is the main cause. Accumulation of excess fat may be associated with insulin resistance, the incapacity to store energy

properly, and eventually the development of metabolic syndrome vulnerability [4]. Impaired mitochondrial oxidation of fatty acids is suggested to be the primary cause of obesity due to faulty cellular metabolism. Managing body weight and energy levels may also depend on subtle changes in thermogenesis linked to uncoupling proteins. Studying metabolic syndrome necessitates concentrating on being overweight, resistance to insulin, and energy metabolism [5]. Hepatic free cholesterol in particular is a significant lipotoxic substance that is essential for the advancement of NASH [3]. Liver inflammation is caused by cytokines such as TNF-α and IL-6. Both hepatic inflammation and lipid buildup brought on by a high-fat diet were reduced in a lack of any IL-6 or TNF [3]. Many antioxidant molecules, including vitamin C, terpenoids and terpenes, and flavonoids, are produced by plants, making them acceptable dietary supplements to guard against oxidative stress [6]. Among consumers, edible plants are the most often acknowledged alternative healthcare source. Although further investigation is still required to identify the biological activity of many ordinary plants, it has been demonstrated that the crude extracts and manufactured goods of several plants have antioxidant potential. Furthermore, the plants' varied parts exhibit different levels of biological activity. Because of this, Ziziphus mauritiana, a well-known medicinal plant in India, was selected for this investigation even though not much prior research had been done on its seeds. Ziziphus mauritiana, a fruit tree that is a member of the Rhamnaceae family, is also referred to as Indian jujube in English. Though present in Africa as well, its natural habitat is Asia, primarily India and China. Ziziphus mauritiana leaves are liver-protective against ethanol & CCl4-induced hepatotoxicity, and they have been associated with benefits for fever, asthma, and liver problems [7]. This study evaluated to determine how an ethanolic extract from Ziziphus mauritiana seeds affected Wistar rats' fatty livers caused by high-fat diets.

Materials and methods

Plant collection and authentication

Ziziphus mauritiana seeds were collected from Vaduvur, Tiruvarur, Tamilnadu, India in December 2023. (BSI/SRC/5/3/2023/Tech/968) They were authenticated by the Botanical Survey of India, Southern Regional Centre, TNAU campus, Coimbatore. 641 083. Seeds were collected, rinsed with distilled water, shadow-dried for a week, finely crushed, and stored in tightly sealed containers.

Ethanolic extraction of Ziziphus mauritiana seed[8]

A fine powder (200g) was obtained by grinding and sieving the dry seeds using a mesh screen. This was thoroughly extracted to yield the ethanol sections of the extract for 24 hours at a constant temperature in a Soxhlet device using 600 ml of 70% ethanol. The solvent was used throughout the extraction process. The extracts were kept at -20°C following the removal from the solvent at low pressure and <40°C. The yield for the extract's ethanol component was 41.5 w/w, respectively.

GC - MS Analysis

The Gas Chromatography-Mass Spectroscopy (GCMS) technique is used to determine the phytoconstituents found in an ethanolic extract of Ziziphus mauritiana seed (ZMSE). The GC-MS analysis was conducted out at Tamil Nadu Agricultural University (TNAU) in Coimbatore, Tamil Nadu, India. This extraction analysis was performed using Perkin Elmer's Clarus SQ 8C Gas Chromatography-Mass Spectrometer.

Determination of total flavonoid content[9]

Plant extracts were tested for flavonoid concentration using an aluminum chloride colorimetric technique. Following a room temperature incubation period, a UV-VIS spectrophotometer was used to detect the mixture's absorbance at 415 nm. Quercetin equivalent milligrams per gram of flavonoid content was used.

In-vitro anti-oxidant assay

DPPH free radical scavenging activity[10,11]

By evaluating an extract's capacity to scavenge or donate hydrogen to radicals, the study used Blois to assess an extract's antioxidant quality. Absorbance was measured using a blank after the extract and the stable DPPH radical were combined. Comparing the absorbance readings of the test and control samples allowed us to determine the percentage of inhibition.

Percentage inhibition (I%) = (Abs control- Abs sample /Abs control) X 100

ABTS free radical scavenging activity[12,13]

The ability of an extract to scavenge ABTS radicals was assessed using the Rice-Evans technique. After ABTS was dissolved in water, it reacted with potassium persulfate to create an ABTS radical cation (ABTS+). After diluting the ABTS solution with PBS and adding it to various chemical concentrations, the sample's % inhibition of ABTS+ was calculated.

Percentage inhibition (I %) = (Abs control- Abs sample /Abs control) X 100

In vitro Anti-inflammatory Assays

Inhibition of protein denaturation assay[14]

A test solution including BSA and Ziziphus mauritiana seed extract was utilized in the study's modified technique of BSA anti-denaturation assay. After cooling the mixture and incubating it for 20 minutes at 37°C, phosphate buffer saline was added. The absorbance of the sample was calculated at 416 nm, and its protein denaturation percentage inhibition was computed.

Percentage inhibition (I%) = (Abs of negative control - Abs sample /Abs of negative control) X 100

Animal models and experimental design

Protocols for the study using male 10-week-old Wistar-albino rats were authorized by the Institutional Animal Ethical Committee (IAEC) KMCRET/ReRc/MPharm/77/2023 for Animal Care. Temperature (20–25°C), light/dark cycle, relative humidity (55–10%), and standard pellet diet were all maintained for the rats under standard settings. The research was carried out in compliance with authorized criteria and adhered to CPCSEA recommendations. Five groups with varying diets and treatments had been developed for the rats. To aid with histopathology research, blood samples were obtained.

Estimation of Serum lipid and liver parameters

To analyze serum and lipid biomarkers, blood samples were collected into tubes and centrifuged at 10,000 rpm for 15 minutes. The serum was maintained at 80 °C, and measurements of TC, TG, HDL, LDL, VLDL, AI, SGOT, and ALP were performed to determine the following biomarkers.

Estimation of oxidative stress biomarkers

In liver tissues, levels of both non-enzymatic and enzymatic antioxidants, including SOD, CAT, GSH, and GPx, were assessed. Liver tissues are used to test LPO as well.

Histopathology Analysis

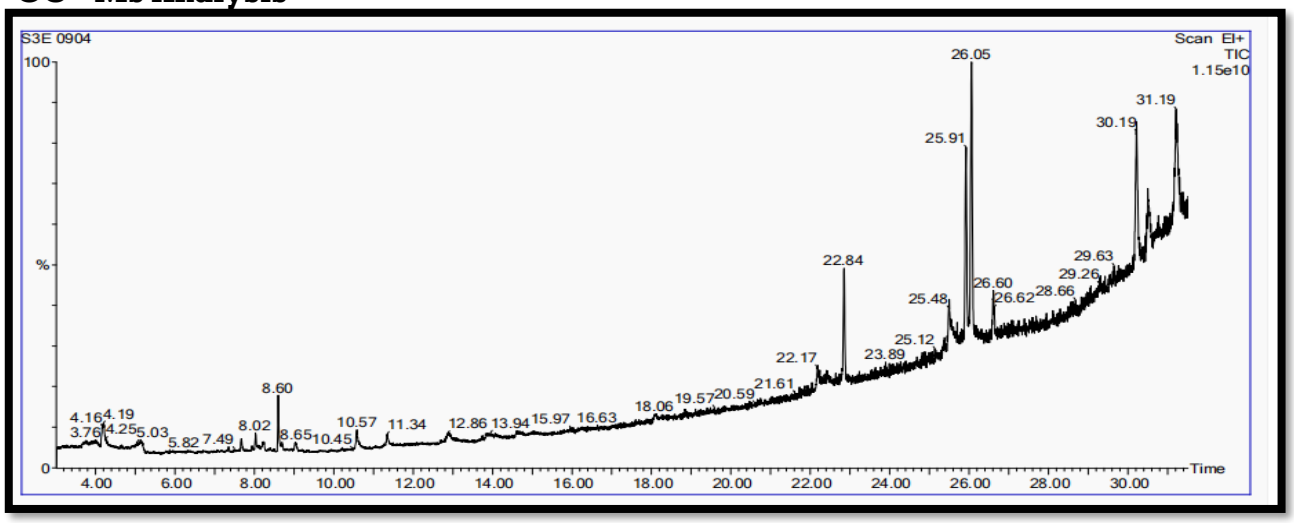
Paraffin-embedded fixed segments were stained with formalin 10% to detect hepatic steatosis, and hematoxylin and eosin staining were used to assess the histological examination.

Statistical Analysis

Data were expressed as Mean ± SD. Statistical analysis was carried out by one-way ANOVA, followed by post hoc analysis, Tukey's multiple comparison test, and in two-way ANOVA, followed by Bonferroni multiple comparison test using Prism 5.0, and considered statistically significant. Data were analyzed using Graph Pad Prism 5.0 (La Jolla, CA., USA).

Results

GC - Ms Analysis



S.No	RT	COMPOUNDS	PROPERTY
1	30.485	Dodecanoic acid, heptyl ester	Anti-inflammatory
2	29.289	7,3',4',5'-Tetramethoxyflavanone	Anti-oxidant
3	22.841	Hexadecanoic acid, ethyl ester	Anti-inflammatory
4	11.337	L-Glutamine	Anti-inflammatory
5	5.124	2,6-Dihydroxybenzoicacid, 3TMS derivative	Anti-oxidant

Fig 1

Table 1: Various compounds identified in Ziziphus mauritiana using GC-MS

Determination of Total Flavonoid Content

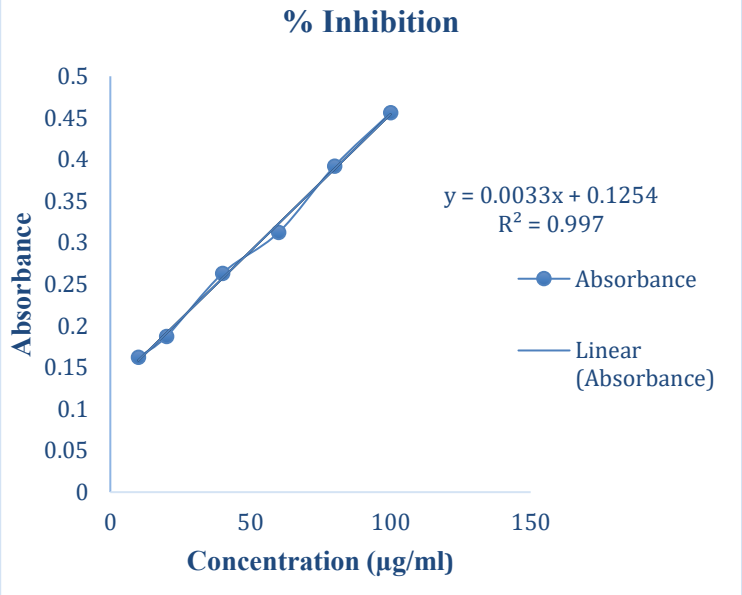


Fig 2

Figure 2 demonstrated that the total flavonoid content in the ethanolic extract of Ziziphus mauritiana seed was found to be 61.2µg/ml, equivalent to quercetin.

DPPH Free Radical Scavenging Activity

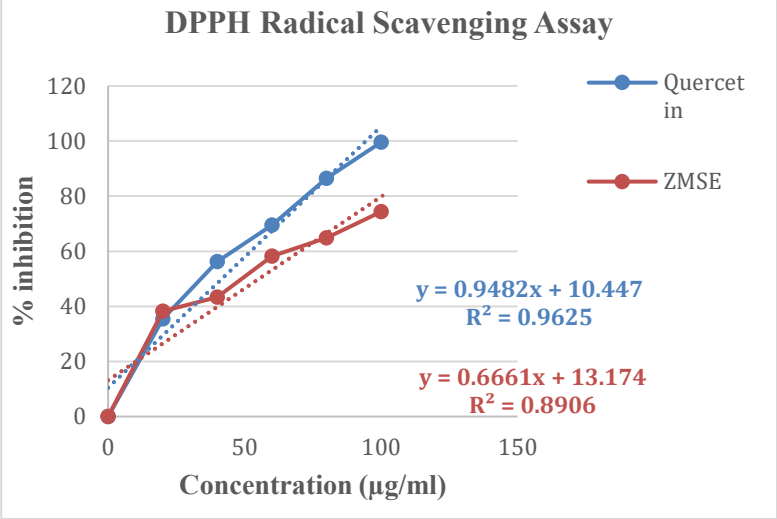


Fig 3

Figure 3 demonstrated that the DPPH Scavenging activity of ethanolic extract of Ziziphus mauritiana seed (ZMSE) (20-100µg/ml) and Quercetin as reference standard (20-100 µg/ml) are concentration dependent. The IC_{50} Values of ZMSE and Quercetin were found to be 70.29µg/ml and 52.26µg/ml, respectively.

ABTS Free Radical Scavenging Activity

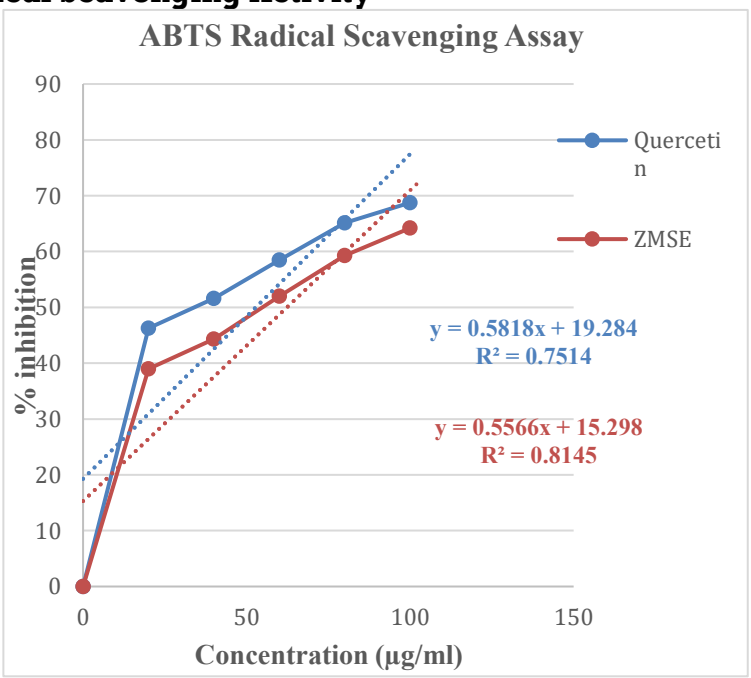


Fig 4

Figure 4 demonstrated that the ABTS scavenging activity of ethanolic extract of Ziziphus mauritiana seed (ZMSE) (20-100µg/ml) and as the reference standard (20-

100µg/ml) is concentration dependent. The IC_{50} Values of ZMSE and Quercetin were found to be 80.31µg/ml and 69.98µg/ml, respectively.

Inhibition of Protein Denaturation ASSAY

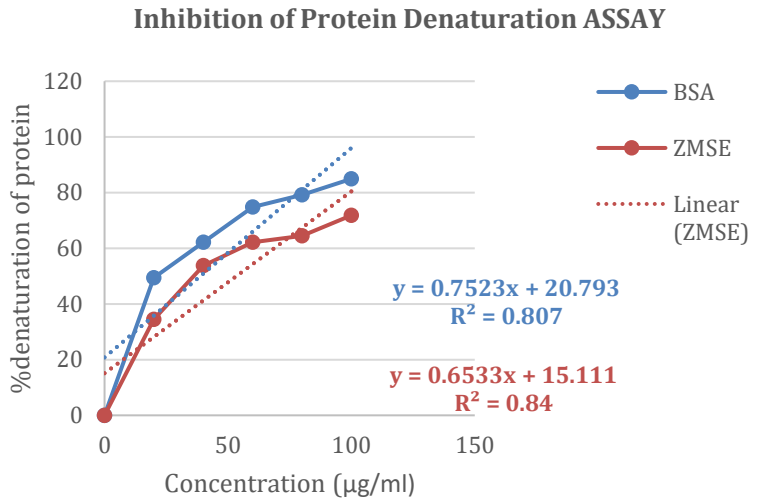


Fig 5

Figure 5 data demonstrated the concentration-dependent inhibition of heat-induced protein denaturation by Ziziphus mauritiana seed extract (ZMSE)and Diclofenac sodium. For both the ethanol extract and Diclofenac sodium, maximum inhibition was seen at 500 µg/mL.Additionally, compared to the methanol extract of Ziziphus mauritiana seed (IC_{50} = 303.0 µg/mL), diclofenac sodium (IC_{50} = 261.4 µg/mL) exhibited a stronger anti-denaturation capacity.

Effect on body weight and lipid profile

When compared to the group fed a standard diet, the animals fed an HFD gained weight far more quickly. Compared to rats given HFD with a vehicle, weight growth was decreased by all dosages of ZMSE and simvastatin. (Fig.6)

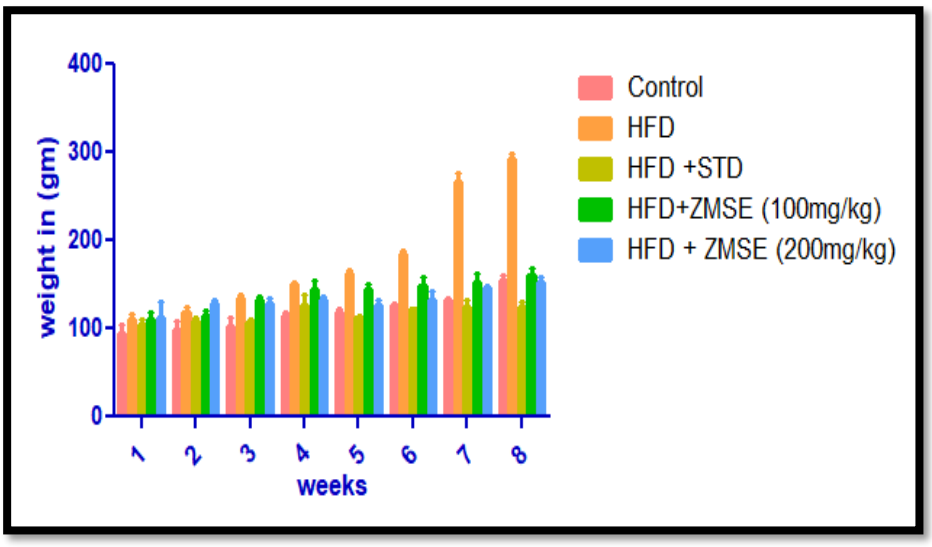


Fig 6

(Fig. 6-12) shows how the lipid profile varies throughout animal groupings. When compared to rats on an HFD diet, the blood levels of TG, TC, LDL, and VLDL were much lower in normal animals; on the other hand, HDL-C was significantly greater in normal rats. Simvastatin and various dosages of ZMSE were used to significantly lower the TG level in comparison to HFD animals treated with a vehicle ($p < 0.05$). Furthermore, as compared to the negative control, both doses of ZMSE therapy decreased the levels of LDL and TC. ZMSE at higher dosages showed statistically greater action in regulating lipid profile markers.

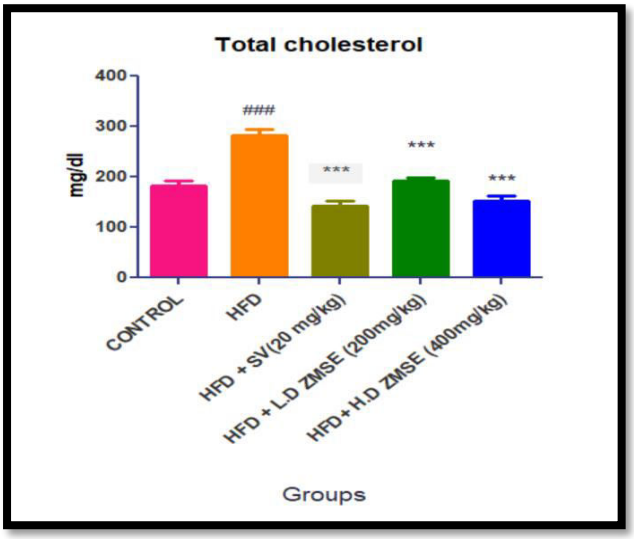


Fig 7

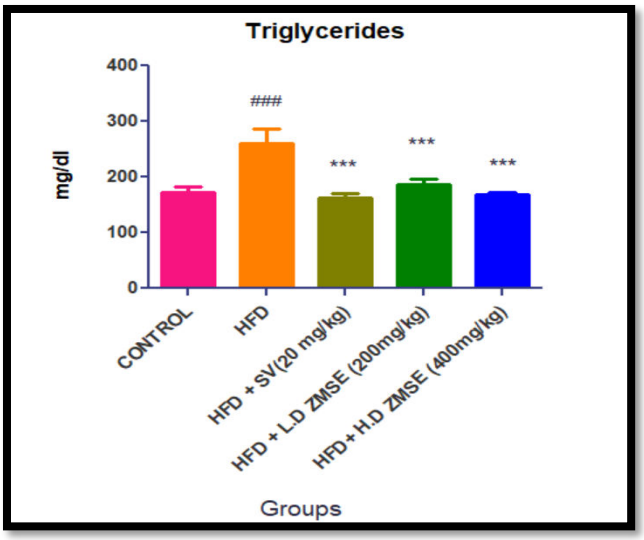


Fig 8

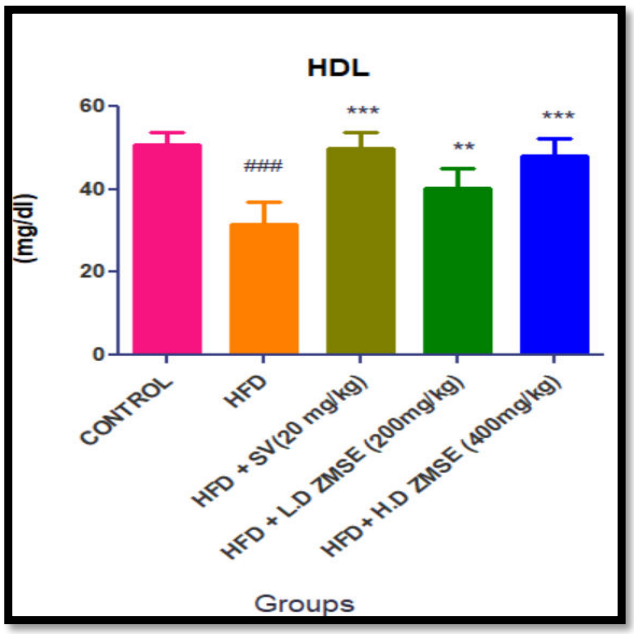


Fig 9

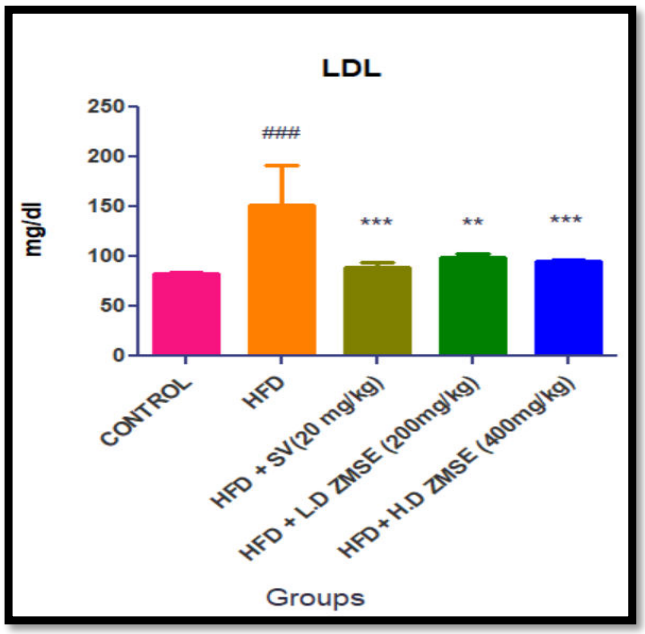


Fig 10

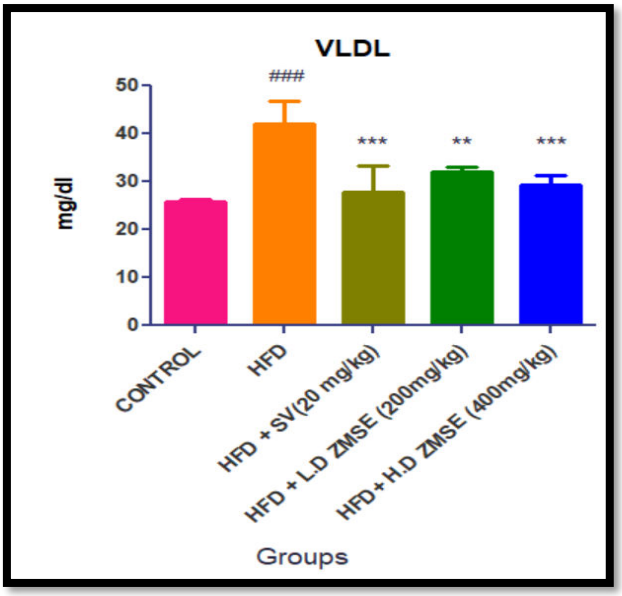


Fig 11

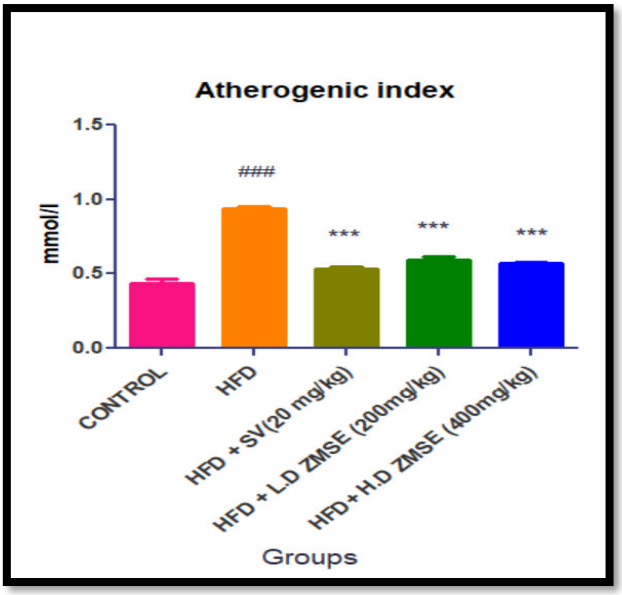


Fig 12

Data are expressed as the mean ± SEM. Statistical significance (p) was calculated by using one-way ANOVA followed by the Turkey multiple comparison test using GraphPad Prism 5.0. The level of significance shows ns- not significant, *p<0.05, **p<0.01, ***p< 0.001, ****P<0.0001 calculated by comparing the treated group with a diseased group. ###P < 0.001, ##P < 0.01, #P < 0.05 vs control.

Effect of Liver Parameters

The HFD group treated with vehicle showed significantly elevated serum levels of SGPT, ALP, and SGOT; however, these levels were decreased in a dose-dependent manner by simvastatin and two doses of ZMSE. These are shown in **(Figs 13,14,15).** The total protein level also decreased in the fed group and increased in the treated groups, as shown in **Fig. 16**. The total bilirubin was significantly decreased in the ZMSE-treated groups compared to the HFD-fed group, as shown in **Fig. 17**.

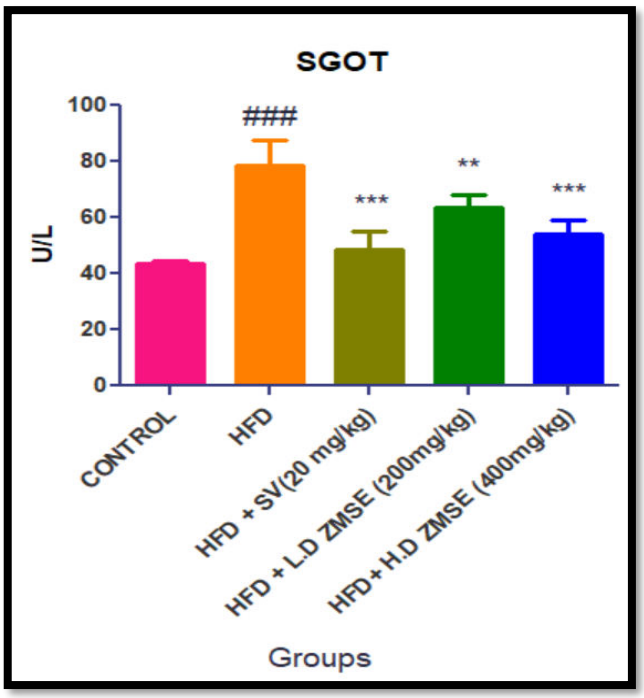


Fig 13

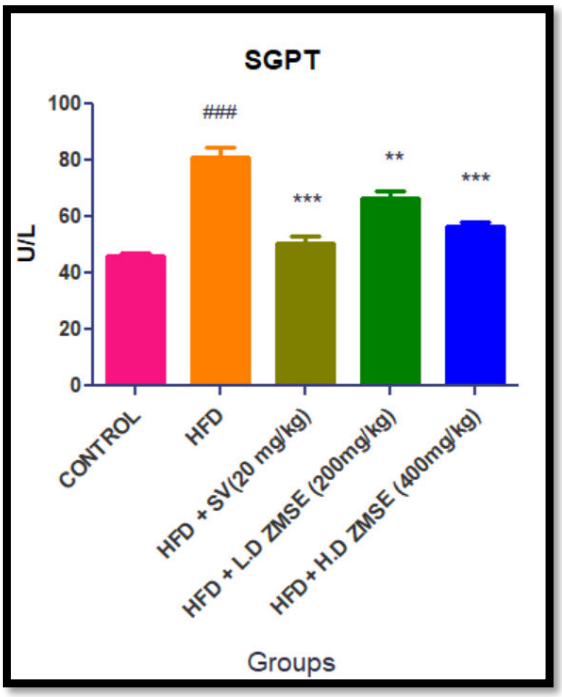


Fig 14

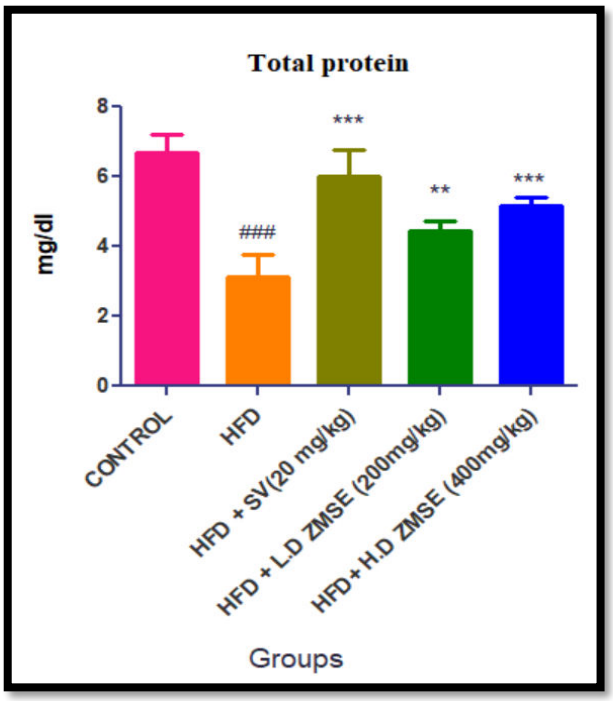


Fig 15

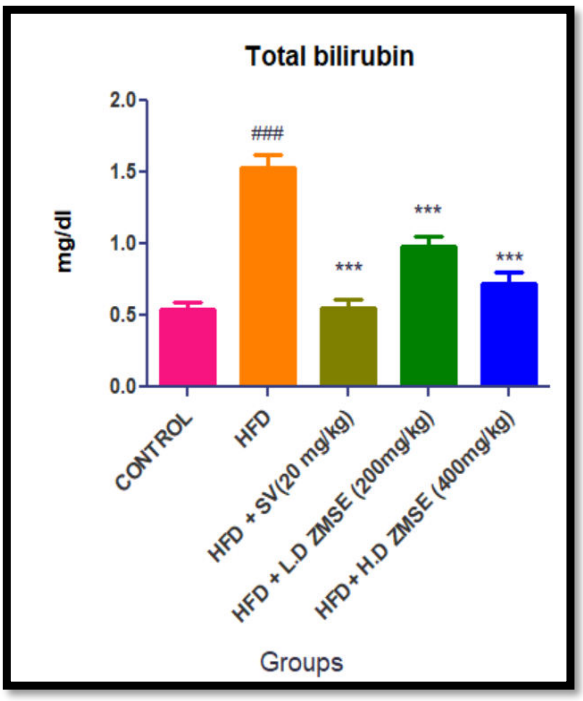


Fig 16

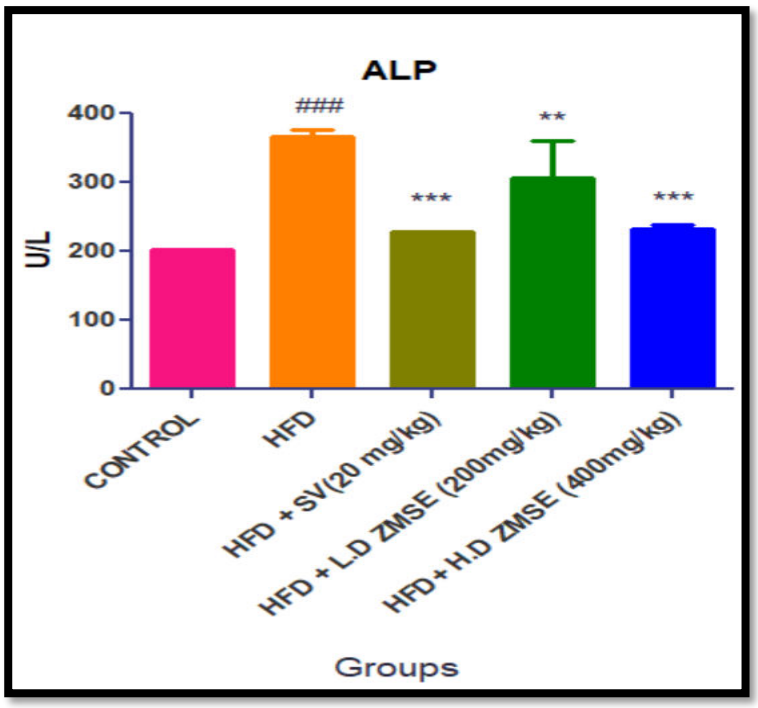


Fig 17

Data are expressed as the mean ± SEM. Statistical significance (p) was calculated by using one-way ANOVA followed by the Turkey multiple comparison test using GraphPad Prism 5.0. The level of significance shows ns- not significant, *p<0.05, **p<0.01, ***p< 0.001, ****P<0.0001 calculated by comparing the treated group with a diseased group. ###P < 0.001, ##P < 0.01, #P < 0.05 vs control.

Estimation of Oxidative Biomarker

HFD caused significant oxidative damage in mice with NAFLD treated with a vehicle. Different doses of ZMSE were used to modify oxidative stress-related parameters. Doses of ZMSE lowered increased levels of LPO **(Fig. 18)**. Treatment with 200 mg/kg of ZMSE had the greatest effect on SOD, CAT, GSH, and GPx, as demonstrated in **(Fig.19-22)**.

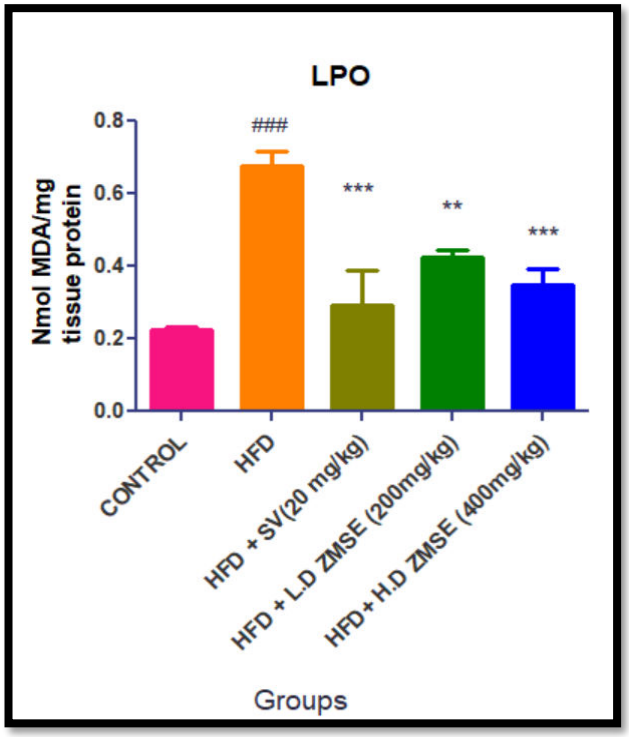


Fig 18

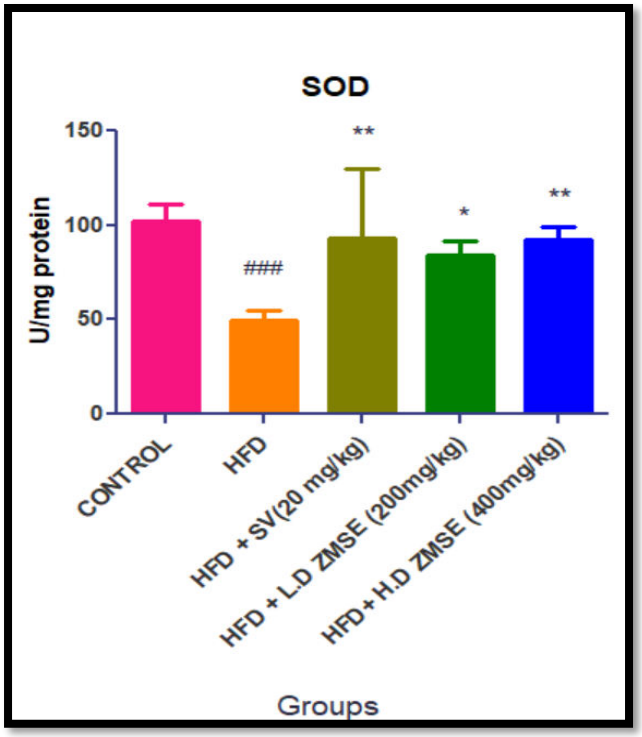


Fig 19

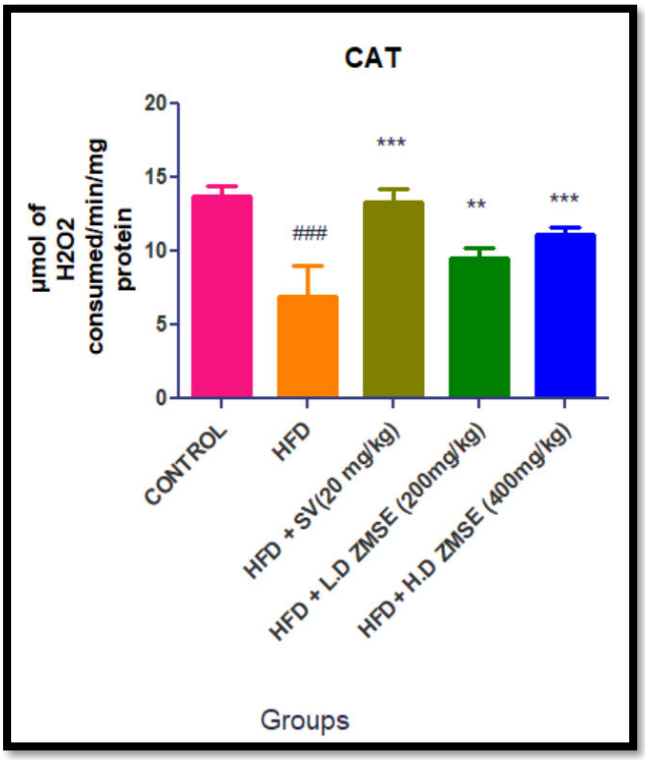


Fig 20

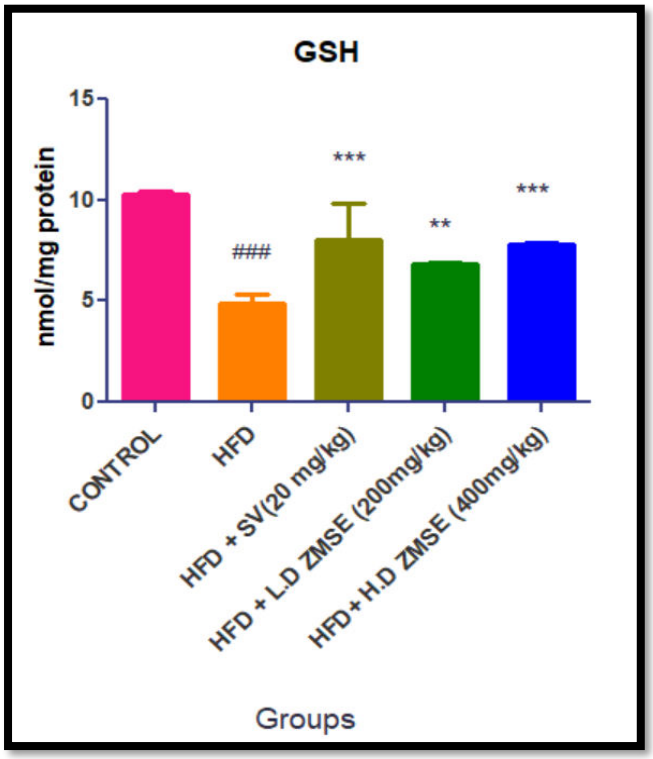


Fig 21

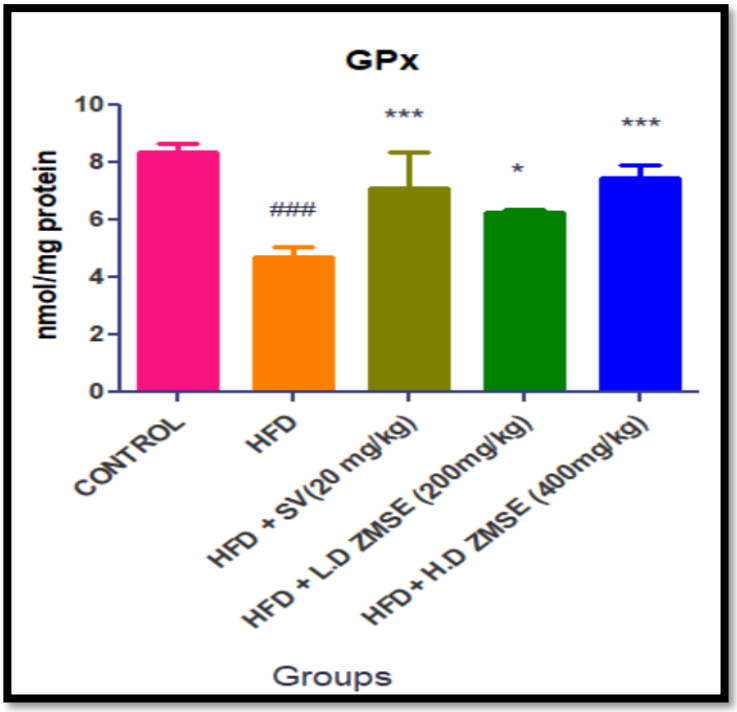


Fig 22

Data are expressed as the mean ± SEM. Statistical significance (p) was calculated by using one-way ANOVA followed by the Turkey multiple comparison test using GraphPad Prism 5.0. The level of significance shows ns- not significant, *p<0.05, **p<0.01, ***p< 0.001, ****P<0.0001calculated by comparing the treated group with a diseased group. ###P < 0.001, ##P < 0.01, #P < 0.05 vs control.

Histopathology analysis

Group 1: Control

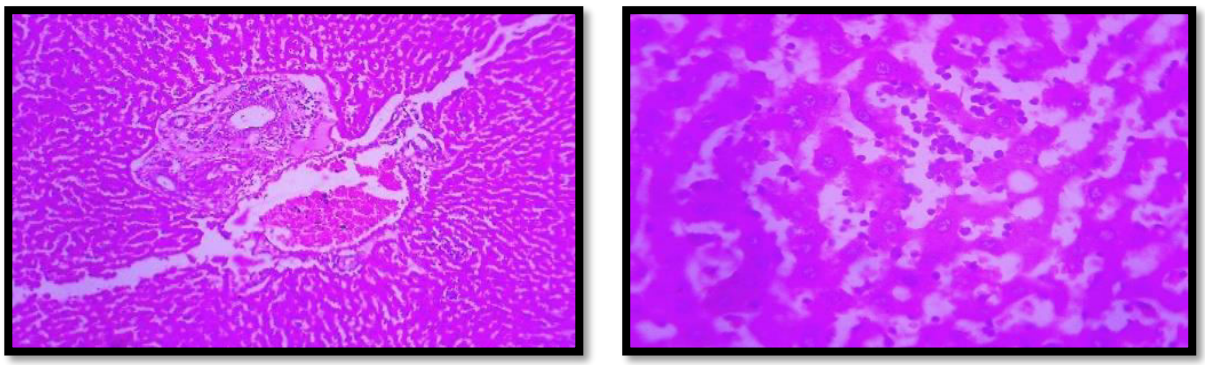


Fig 23 (a)

Fig. 23 (a) demonstrates that the control liver has normal lobular architecture. Individual hepatocytes have no substantial disease. The Portal triad demonstrates modest periportal inflammation. The central vein is congested. Sinusoids exhibit moderate dilation. There is no sign of a fatty liver.

Group 2: Hfd

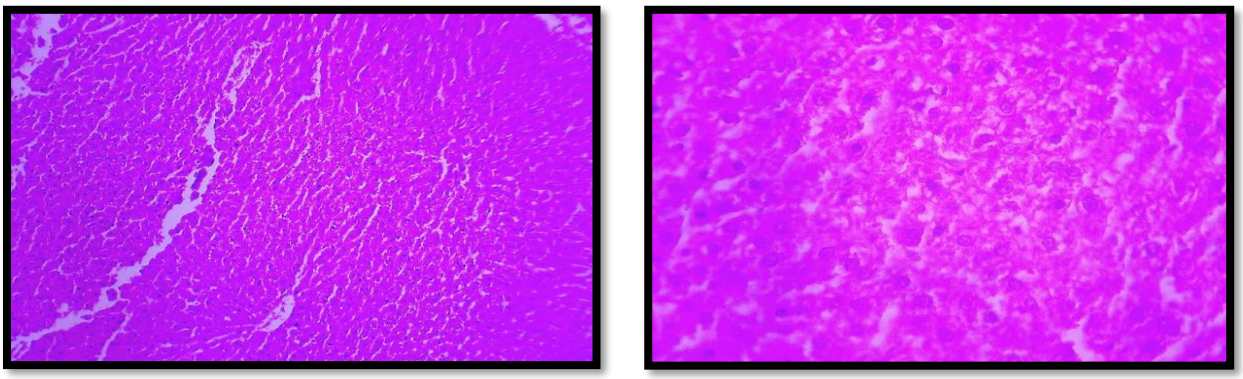


Fig 23 (b)

Figure 23 (b) demonstrated that the diseased group showed altered lobular architecture. Individual hepatocytes showed mild interface hepatitis. Sinusoidal showed dilatation, central vein dilatation. The portal tract showed mild hyperplasia.

Group 3: HFD + STD (Simvastatin) (20mg/kg)

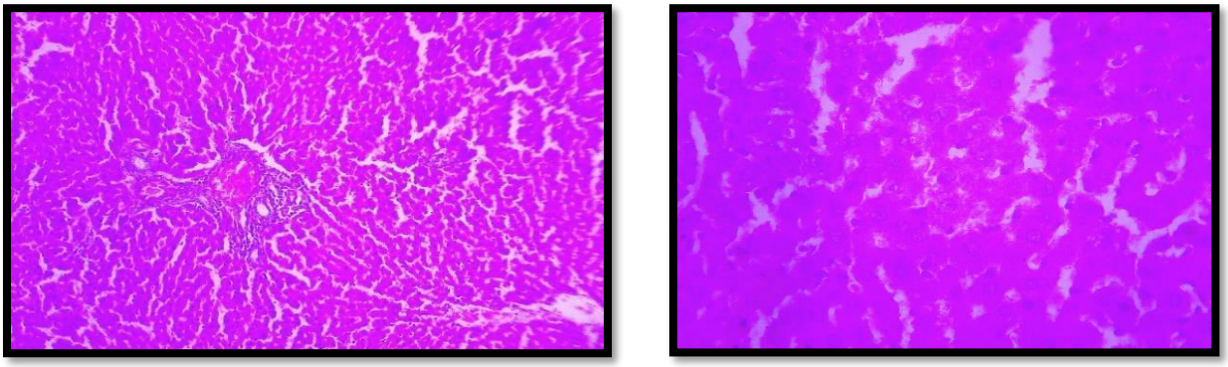


Fig 23 (c)

Figure 23 (c) The HFD + STD (SIMVASTATIN) (20mg/kg) treated liver exhibited normal-appearing liver architecture. Individual hepatocytes revealed mild interface hepatitis with localized necrosis. Dilation of the central veins and sinusoids. The portal tract morphology is normal.

Group 4 HFD +ZMSE (100mg/kg)

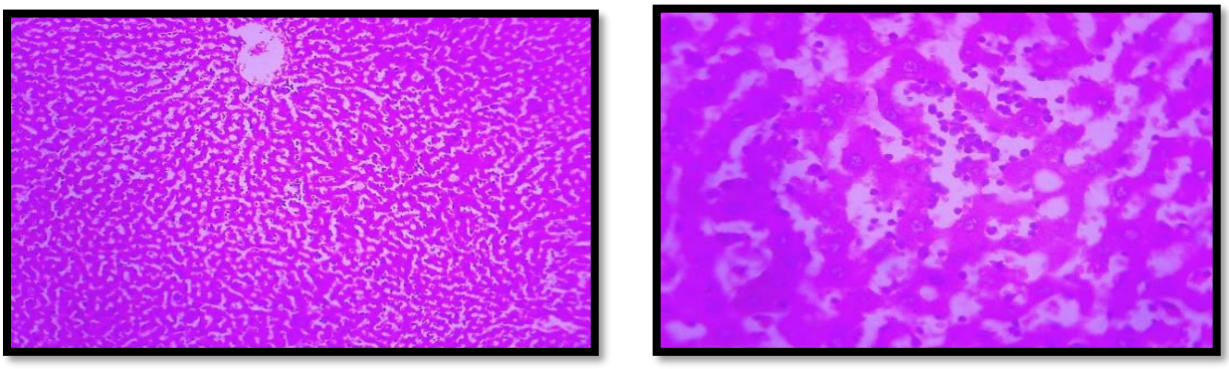


Fig 23 (d)

Figure 23 (d) demonstrated that theHFD + ZMSE (100mg/kg) treated liver showed altered normal architecture. Individual hepatocytes showed mild interface hepatitis. Portal tracts show periportal inflammation. Central vein and sinusoids showed no significant pathology.

GROUP 5 HFD +ZMSE (200mg/kg)

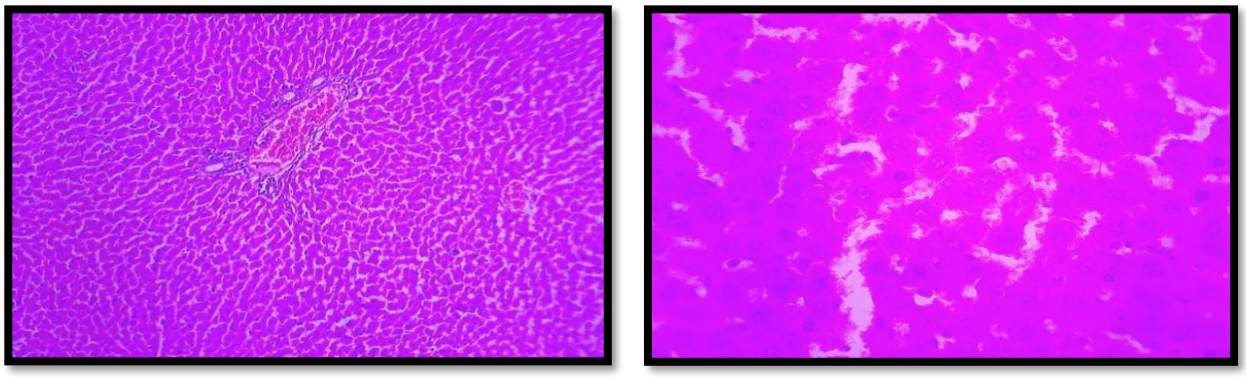


Fig 23 (e)

Figure 23 (e) demonstrated that theHFD + ZMSE (200mg/kg) treated liver showed altered normal architecture. Individual hepatocytes showed normal pathology. Portal tracts showed normal. Central vein and sinusoids showed no significant pathology.

Discussion:

NAFLD is strongly associated with the known metabolic syndrome, which also includes obesity, hypertriglyceridemia, diabetes, atherosclerosis, and hypertension. Eating meals rich in calories is one of the main risk factors. It has been shown, however, that the Mediterranean diet, the usage of certain herbal remedies, and the intake of unsaturated fats can reduce the incidence and severity of liver illnesses, such as nonalcoholic fatty liver disease (NAFLD) [15]. Since there aren't many effective conventional treatments for NAFLD and they all have various side effects, herbal therapies with lipid-lowering, antioxidant, and anti-inflammatory properties can help manage the condition [16]. **Ziziphus mauritiana** has been used as traditional medicine and also it has various pharmacological activities [17].In this study, the preliminary phytochemical screening of seed extracts indicates the presence of flavonoids, alkaloids, tannins, terpenoids, and glycosides may account for antioxidant and anti-inflammatory potential, and also confirmed by the GC-MS analysis.Reactive molecules known as free radicals are produced during a variety of physiological processes. These molecules can cause oxidative damage and are implicated in the pathogenesis of a wide range of diseases, such as dementia, cancer, and aging. Antioxidants are critical components of cellular defense mechanisms because they prevent the start or spread of highly reactive chain reactions that shield biological molecules from oxidative damage.Plant extracts may be evaluated for their antioxidant properties using a sensitive and quick test called DPPH radical scavenging activity [18]. This assay examined the

ability of the extracts to neutralize the stable free radical DPPH (2,2-diphenyl-1-picrylhydrazyl). It was assessed by measuring their percent inhibition and comparing it to the standard antioxidant, quercetin. In our study, ZMSE exhibited dose-dependent scavenging of DPPH with IC_{50} at 70.29 µg/ml. The antioxidant capacity of the ZMSE extract was also assessed using the ABTS+ radicals cation test. The utilization of a particular absorbance wavelength outside of the visible range (734 nm) and a quick response time are two benefits of this test. ZMSE extracts demonstrated concentration-dependent free radical scavenging of ABTS with IC_{50} 80.31 µg/ml. This finding suggests that ZMSE extracts exhibit a good ability to scavenge ABTS+ radicals, their overall antioxidant activity may be influenced by interactions with the assay components. Protein denaturation has been correlated with the formation of inflammatory disorders. preliminary screening for anti-inflammatory activity was performed with the protein denaturation method using bovine serum albumin protein. In this study, ZMSE produced an effective inhibitory profile towards protein denaturation with IC_{50} at 65.81 µg/ml.Administration of the HFD diet induces metabolic syndrome characterized by obesity, IR, and liver steatosis [19]. Numerous studies show that the buildup of fat in the liver makes it more vulnerable to various incidents, including oxidative stress and the inflammation that follows, which causes steatosis to advance to fibrosis, cirrhosis, and hepatitis [20]. In conclusion, the rat's body weight was significantly elevated in the diseased group.

Microvesicular steatosis and hepatocellular injury were linked to HFD treatment. Liver damage is suggested by the elevated activity of marker enzymes, including SGOT, SGPT, & ALP, Total protein, and Total bilirubin [21]. This study's findings showed that ZMSE treatment significantly reduced the rise of those enzymes to a level similar to that of the conventional medication Simvastatin. ZMSE protects liver cells from harm and maintains their normal state, which may help the functionally living cells survive. Hepatic levels of the whole lipid profile, including TC, TG, HDL, LDL, and VLDL, were significantly elevated[22]. One major risk factor in the onset of liver damage is fatty liver. In this study, lipid markers such as TC, TG, HDL, LDL, and VLDL were significantly decreased in the treated groups.One major risk linked to the pathogenesis of non-alcoholic fatty liver disease (NAFLD) is oxidative stress. Long-term oxidative stress has been shown to oxidize cytotoxic free fatty acids, which can lower the antioxidant levels in the liver and increase the expression of cytokines [23]. Free radicals are currently thought to be a key possibility for inflammation and damage to tissues in many chronic illnesses, including liver disorders [19]. Therefore, one of the present pharmacological therapies for NAFLD may be the consumption of antioxidant supplements, which might be helpful.Tissue damage is caused by the accumulation of free radicals that reduce the enzymatic anti-oxidants [24]. This study showed that ZMSE-treated groups significantly increased the levels of enzymatic antioxidants such as SOD, CAT, and GPx.Non-enzymatic anti-oxidants where GSH is the most abundant antioxidant in the liver, with higher concentrations than any other. The concentration of GSH in the liver indicates the liver's capacity for detoxification. This study showed that ZMSE-

treated groups significantly increased the levels of non-enzymatic antioxidants. The lipid peroxidation process is a form of free radical reaction that degenerates cell membranes and produces a majority of compounds that are known to possess mutagenic or carcinogenic qualities [25]. Increased production of reactive oxygen species (ROS) causes oxidative stress, which in turn causes lipid peroxidation to rise and antioxidant enzyme levels to fall (superoxide dismutase, catalase, etc.). In this study, ZMSE-treated groups significantly decreased the level of Lipid Peroxidation. Histopathology studies showed the progression of NAFLD in all groups. However, the untreated groups showed hepatitis and altered lobular architecture, thus it confirms the liver damage caused by the HFD. While the ZMSE-treated groups showed normal hepatocytes.

Conclusion

Plant compounds are found in pharmaceuticals, over-the-counter medications, and home cures all around the world. Thus, the current study's findings demonstrated that the chosen medicinal plants have strong anti-inflammatory and antioxidant properties in rats with fatty liver disease induced by a high-fat diet. It also showed the benefits of Ziziphus mauritiana as an alternative medication and dietary supplement for the treatment of non-alcoholic fatty liver disease (NAFLD). Ziziphus mauritiana was shown to significantly decrease elevated levels of liver enzymes, cholesterol, LDL, and serum TG. The main potential modes of action for this natural treatment are hepatoprotective activity and improvement of antioxidant status, which are mediated by the component's flavonoids and terpenoids. The present study concludes that the extracts of Ziziphus mauritiana seedpossess significant anti-oxidant, anti-hyperlipidemic, and anti-inflammatory activity.

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