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Downregulation of hepatic fat accumulation, inflammation and fibrosis by nerolidol in purpose built western-diet-induced multiple-hit pathogenesis of NASH animal model

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ABSTRACT

Western diet style (fast food), which includes fatty frozen junk food, lard, processed meats, whole-fat dairy foods, cream, mayonnaise, butter, snacks, and fructose, is a primary etiological determinant for developing nonalcoholic steatohepatitis (NASH) worldwide. Here the primary focus is to see the impact of naturally identified essential oil on disease mechanisms developed in an animal model using the same ingredients. Currently, symptomatic therapies are recommended for the management of NASH due to non-availability of specific treatments. Therefore, the present study was designed to evaluate the potential anti-NASH effect of nerolidol in a rat model fed with a purpose-built diet. The diet substantially induced insulin resistance, hepatic steatosis, dyslipidemia, and elevation of liver enzymes in the experimental animals. The levels of liver oxidative stress markers, nitrites (NO_2^-), serum pro-inflammatory cytokine (TNF-α) and hepatic collagen were increased in disease control rats. Nerolidol oral treatment in ascending dose order of 250 and 500 mg/kg substantially reduced the steatosis (macrovesicular and microvesicular), degeneration of hepatocytes, and inflammatory cells infiltration. The amounts of circulatory TNF-α and tissue collagen were also reduced at 500 mg/kg dose of nerolidol, expressing its anti-fibrotic effect. The current study described the multiple-hit pathophysiology of NASH as enhanced steatosis, pro-inflammatory markers, and oxidative stress in rats, which resulted in the development of vicious insulin resistance. Nerolidol treatment significantly reduced hepatic lipid accumulation and halted disease progression induced by a hypercaloric diet.

1. Introduction

Nonalcoholic fatty liver disease (NAFLD) is a leading cause of chronic hepatic disorders in both developed and developing economies (30% in industrialized and about 10% in underdeveloped) [1]. The prevalence has increased to up to 90% in obese and 55% in diabetic patients in Western nations during the previous two decades, affecting 20–30% of adults and 3–10% of children [2]. Similarly, in Pakistan, with an increase in the number of patients suffering from metabolic syndrome (MetS), the disease epidemic is growing day by day, although the actual figure is much higher than the recently reported hospital-based study which is 14% [3]. The disease initiates as a benign illness progresses to fatal nonalcoholic steatohepatitis (NASH) and liver fibrosis. It is estimated that 15–25% of early-stage NAFLD clinical cases lead to NASH, and 15–20% of NASH cases develop into cirrhosis [4,5]. Visceral obesity and NASH can cause systemic inflammation and extrahepatic illnesses,

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including chronic renal disease, cardiovascular diseases (CVD), malignancies and Type 2 diabetes mellitus (T2DM) [6].

The mechanisms of NAFLD progression are currently unclear [7]. According to the classic "double-hit" hypothesis, intrahepatic fat deposition or simple steatosis is believed to be the "first hit" and prelude for the development of hepatocyte damage [8]. In contrast, excessive mitochondrial fatty acids (FAs) oxidation is expected to be the "second hit" (NASH), producing reactive oxygen species (ROS), cellular oxidative stress (OS), inflammation, and hepato-apoptosis [9]. The "multiple parallel hits" concept has recently questioned this theory, where insulin resistance (IR) and dyslipidemia are the antecedents to NAFLD [10], and liver is vulnerable to hyperinsulinemia-induced oxidative damage, activation of profibrogenic and proinflammatory mediators, as well as hepatic stellate cell (HSCs) proliferation [11,12]. Secondly, the order of the "two hits" has been debated, as inflammation might precede steatosis, and metabolic changes seen in disease are thought to develop in parallel rather than sequentially [13]. On the other hand, the "distinct hit" theory presupposes that following the IR, independent and distinct pathways are triggered, resulting in either simple steatosis or NASH [4].

The chronic consumption of high-calorie food rich in saturated and trans-fats, and fructose has a major role in the etiology and involves initiating disease mechanisms [14–17]. People in both the West and East, particularly the younger generation, are increasingly following the Western diet style (fast food), which include fatty frozen junk food, lard, processed meats, whole-fat dairy foods, cream, mayonnaise, butter and snacks, and are deficient in cereals, whole grains, fruits, and vegetables [18]. Meanwhile, fructose corn sirup is regularly utilized in soft drinks, beverages, canned fruits, jellies, desserts, and morning breakfasts. Globally, its intake is projected to have risen by 16% per capita due to its correlation with high food consumption in modern society [19].

Despite severe challenges to health systems, NASH treatment is still lacking [7]. The pharmaceutical industry faces significant difficulty in developing an effective combination therapy against the disease with all the characteristics of MetS [20]. Scientists are seeking new multi-target therapeutic agents. Most pharmacological strategies involve antioxidants and insulin sensitizers, as both IR and OS are attractive targets and constitute a significant area of research [21]. Polyphenols, flavonoids, and terpenoids are the most researched phytochemicals [22,23]. Terpenoids are reported to have antioxidant, anti-hyperglycemic and anti-inflammatory effects [24,25] and have a potential anti-NASH impact. We previously established a successful high-fat diet (HFD)-induced NAFLD animal model in our lab. It was reported that safranal (a monoterpene) was effective against various attributes of steatosis and inflammation, evidenced both biochemically and histopathologically [26].

In light of the recent discovery of novel compounds that may be useful against this metabolic disorder, the study was designed to determine the anti-NASH efficacy of nerolidol in a rat model of HFD with fructose (western diet). It is categorized as acyclic-aliphatic sesquiterpene alcohol with the systemic name of 3,7,1-trimethyl-1,6,10-dodecatrien-3-ol. Nerolidol is found in essential oils of *Eriobotrya japonica*, *Amaranthus retroflexus*, and *Canarium schweinfurthii* [27]. It is declared a safe molecule by the Food and drug administration, and the LD50 value in rats is more than 5000 mg/kg body weight with no toxicity through the oral route [28]. It has been shown to possess antioxidant, anti-inflammatory, hepatoprotective, neuroprotective, gonadoprotective and cardioprotective [28–35] activities in various drug-induced animal models.

2. Materials and methods

2.1. Chemicals and drugs

Nerolidol (Shanghai Xian-Dinn Biotech Co., Ltd. CAS# 7212–44–4). Glutathione (GSH) and N-(1-naphthyl) ethylenediamine dihydrochloride (NED)' Chem Impex Intl. USA, chloramine-T from Uni-chem chemical reagents and Ellman's reagent was purchased from BDH Chemicals Ltd. England. Nicotinamide adenine dinucleotide phosphate (NADPH), Ehrlich's reagent and hydroxyproline (HXP) were purchased from AmBeed USA. Dithionitro-bis-benzoic acid (DTNB) from AnalaR BDH Germany, sulfanilamide from MP biomedicals Inc. France, and 1-chloro-2,4-dinitrobenzene (CDNB), thiobarbituric acid (TBA), pyrogallol and bovine serum albumin (BSA), was obtained from Sigma Aldrich Germany. The standard botanical mixture (LivLiv®) was obtained from Hinucon Pharma. Karachi Pakistan, combination of siliphos (silybin), *Cynara cardunculus* (artichoke), and *Curcuma longa* (turmeric) extracts.

2.2. Animal ethics and care

Six-weeks-old-male albino Sprague-Dawley (SD) rats weighing 150–200 g were used in the study. The animals were collected from animal house at College of Pharmacy, University of Sargodha, Pakistan. These were housed and handled in accordance with the guidelines approved for this research protocol by the "Animal Ethics Committee of the College of Pharmacy, University of Sargodha, Sargodha, Pakistan" with voucher number (SU/ORIC/2861/2021). A cycle of 12-hr light and dark, at the temperature of 25 °C (25°C ± 2) with a relative humidity of 40–60% and adequate ventilation were provided to the rats kept in wide plastic cages. The animals were provided free access to tap water and a diet of regular chow food pellets during the acclimatization phase.

2.3. Experimental protocol

Initially, the model was validated on rats fed a HFD with 20% fructose in their drinking water (HFHFr-diet) for eight weeks, commonly known as the NASH diet or western-style diet (WD). In some randomly sacrificed animals, anthropometric, histological, and blood data demonstrated the induction of disease for NAFLD. Thirty rats were separated into five groups (n = 6) for a total duration of twelve weeks in the proper treatment model (Fig. 1). All the groups of rats, except normal control group (NC), received HFHFr diet for eight weeks following four weeks of drugs treatment through oral gavage, in addition to the food pattern previously used for the normal, diseased, and treated groups. Doses of the tested drug' nerolidol were calculated using the rat's body weight, and initially estimated through preliminary pilot screening. The dose of standard botanical mixture (SBM) was calculated using the Human Equivalent Dose (HED) formula for rats. Before administration, nerolidol and the standard mixture were dissolved in the 2% Tween-80. The normal vehicle control (NC) and HFHFr (DC) groups were only received 10 mL/kg' 2% Tween 80 during the treatment period. Fresh food (150 g in each cage) was provided daily to the animals 30 min after drug treatment, and body weight and blood sugar levels of each rat were measured weekly.

2.4. Composition and energy of WD and normal diet (ND)

A similar composition of HFD and ND was utilized in the current study, prepared and validated in our previous model. The ND provided 4.5 kcal/g of energy, whereas 60% HFD gave 6.1 kcal/g of energy [26]. Additionally, in drinking glass bottles, 20 g fructose was dissolved in the 100 mL water and given to rats for 12 weeks along with the HFD. The additional energy in kilojoules (KJ) consumed by the rats was estimated by using their food (g), and drinking water (mL) intake (which would have been equivalent to fructose (g) consumption). Fructose has a value of 4 kcal/g energy, and an individual rat consumed 0.8 kcal or 3.4 KJ of energy in 1 mL water. The total energy intake of rats from WD was determined after measuring the energy obtained from drinking water, added to the energy obtained from the solid food of the ND or HFD, respectively.

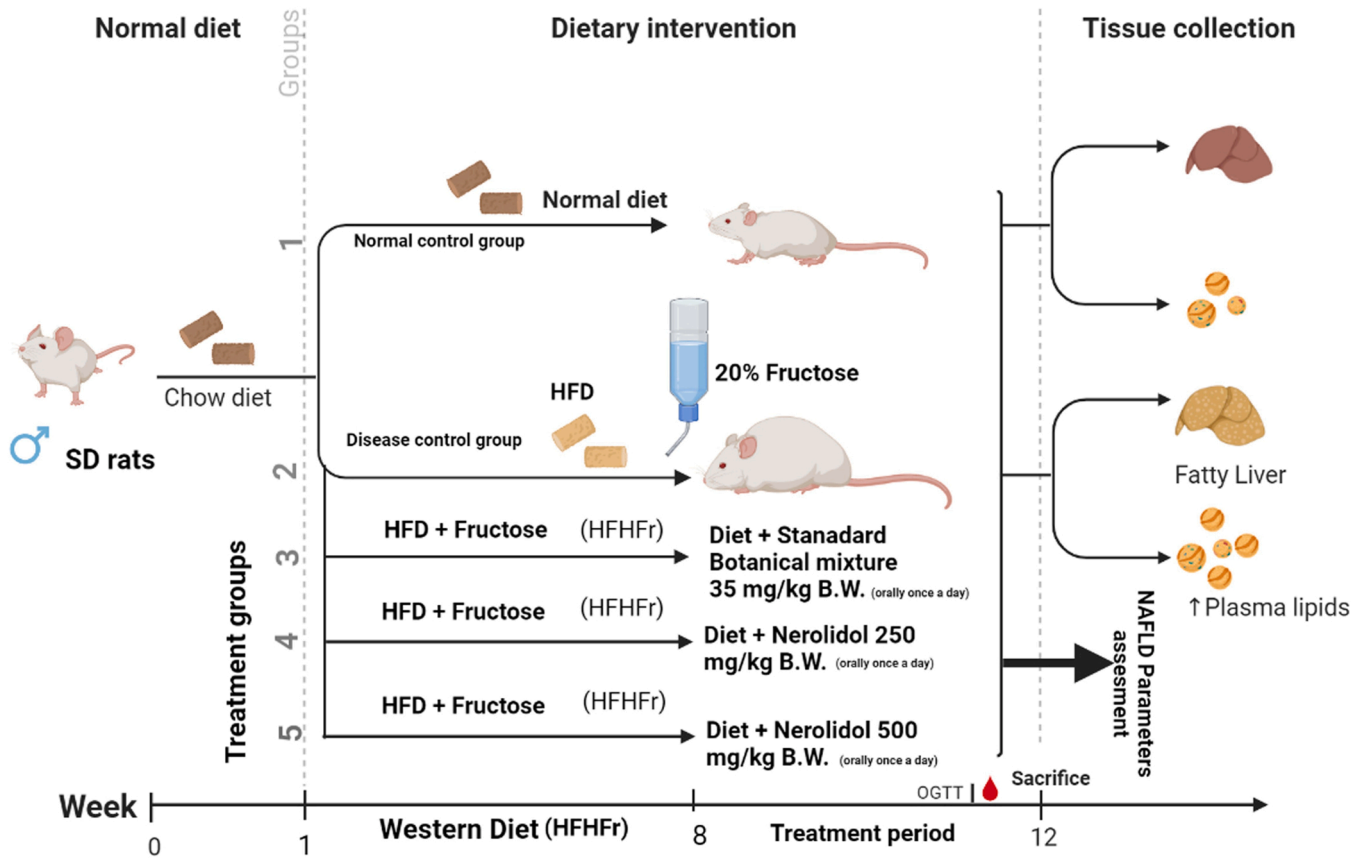

Fig. 1. Animal grouping and treatments.

2.5. Measurement of rat's glucose metabolism parameters

The effect of nerolidol on the quantity of consumed food, water and calorie intake by treated the animals was daily assessed by calculating the amount of food and water left for each batch. The response of the drugs on body mass index (BMI) was calculated after completion of study. Food efficiency was estimated from each rat's weight gain and total food intake. The oral glucose tolerance test (OGTT) was done prior to the final day of the investigation. Each group of rats was fasted overnight and then given 2 g/kg oral glucose load, with blood glucose levels (BGL) measured at 0, 30, 60, and 120 min. Total area under the curve of glucose (AUC) was computed by using trapezoidal rule.

2.6. Animal's sacrifice and organs indices

On the last day of study, rats were weighed, levels of fasting blood glucose (FBG) were noted and sacrificed using a ketamine/xylazine cocktail (90 mg/kg) in peritoneal area. The blood samples were obtained by cardiac puncture and equally divided into plasma and serum tubes. The samples were centrifuged (Spectrafuge 6C-Labnet int.) at 2500 rpm for the 15 min and kept at − 20 °C till analysis. Each animal's liver and the adipose tissues (epididymal, retroperitoneal, and perirenal fat pads) were collected and weighed. The adiposity and liver indices were computed as relative weights of organs in relation to the total body weight of individual rat. Liver was divided into two parts after being washed with physiological saline solution. It was stored at − 20 °C for the assays and in 10% neutral formalin for histological evaluation.

2.7. Preparation of hepatic supernatants and estimation of total protein contents

Rat's hepatic tissue sample (100 mg) was homogenized (Lambgic TH-mini-USA, L-C119- 0524) in 1 mL of 50 mM chilled phosphate buffer saline (PBS) (pH 7.4) and then centrifuged for 15 min at 10,000 rpm and 4 °C. The resulting mitochondrial supernatant (PMS) was separated from the 10% homogenate, and used for further analysis of liver proteins and antioxidants. Sample's total protein content (mg/mL) was estimated by the technique given by Lowry et al. [36]. It was modified for 96-well plates, using BSA as a standard in the range of 25–250 μg per well. The final absorbances of samples were measured at 750 nm on the microplate reader (Synergy, USA), and measured protein values were employed for the estimation of antioxidants.

2.8. Liver fat accumulation, and collagen deposition

The total hepatic lipids were measured employing Folch's [37] assay, with minor modifications. The tissue mixture was homogenized in the extracting solvent composed of chloroform and the methanol in a 2:1 ratio. Supernatant was centrifuged and separated into layers by washing with normal saline (NS) and dried at 70 °C. After adding PBS, the sample was taken to quantify triglycerides (TGs) and total cholesterol (TC) by using standard biochemical kits, and according to the manufacturer's protocol (BIOMED MDSS GMBH Sciffgr aben Germany) on a biosystems bioanalyzer (Biosystems BTS 350 Spain).

Assay of Reddy and associates [38] was used to determine the amount of hydroxyproline (HXP) in the liver. Initially, liver samples were hydrolyzed with NaOH and supernatant was oxidized with chloramine-T reagent. In the 96-well microplate, chromophore was formed following the pipetting of 1 M Ehrlich's reagent, measured for absorbance at 550 nm. The standard curve of HXP (20–2 μg/well) was used to estimate the concentrations of unknown sample. The amount of collagen was estimated by multiplying the concentration of HXP by 7.46 and reported as mg/100 mg of hepatic tissue.

2.9. Estimation of biochemical markers involved in energy production processes

The serum concentrations (U/L) of hepatic enzymes such as aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) were determined through standard enzymatic kits (LABKIT Chemilex S.A. Pol. Ind. Spain) in conjunction with an automated blood bio-analyzer. As a non-invasive measure, the AST and ALT ratio (De-Ritis ratio) was computed to evaluate the progressive phase of NASH. Serum uric acid was assayed from the kit purchase from Randox Laboratories UK. Similarly, plasma lipid profile consisting of TC, TGs, and the high-density lipoprotein cholesterol (HDL-C) were calculated using conventional biochemical kits. Friedewald's formula was used to calculate VLDL-C, and LDL-C concentrations.

2.10. ELISA assessment of circulatory insulin and cytokine

The plasma insulin and serum TNF-α levels were measured by using the ELISA kits and as per manufacturer's instructions (Zokeyo biotechnologies Co. Ltd. Wuhan, China). Insulin has a standard curve range of 1.5 μIU/mL - 48 μIU/mL with a sensitivity of 0.1 μIU/mL. Similarly, ELISA kit of TNF-α has a standard range of 10–320 pg/mL with the sensitivity of 1.0 pg/mL. The reaction was stopped by adding acidic solution, and absorbance was determined at 450 nm using multimode reader. HOMA-IR index was determined from the FBG (mM/L) and insulin (mIU/L) levels [39].

2.11. Determination of the hepatic oxidative stress markers

The hepatic advanced oxidation protein products (AOPP) level was calculated by spectrophotometric technique given by the Tiwari et al. [40]. Chloramine-T absorbance was measured at 340 nm and calibration curve was made for a concentration range of 0–100 nM/mL. Amount of AOPP in the unknown samples was calculated as equivalent of chloramine-T in nM/mL and nM/g tissue, respectively. Malondialdehyde (MDA) levels in hepatic tissues were measured by assaying thiobarbituric acid reactive species (TBARS) formed, using technique of Ohkawa et al. [41] with slight modification. Tetra-methoxy propane was used to generate MDA solution and through serial dilution, its standard concentrations ranging from 10 to 0.5 μM/mL were prepared. The optical density of supernatant was noted at 532 nm and MDA level was estimated by standard curve, and represented as μM/mg protein.

The amount of hepatic nitric oxide (NO) was measured as its metabolite nitrite (NO_2^-) by the procedure given by Green et al. [42]. Trichloroacetic acid (TCA) was initially used to deproteinize 5% tissue supernatant (sample: TCA, 1:9, v/v) and centrifuged. The supernatant was aliquoted for analysis using the Griess reagent. The azo compound produced a purple color, examined for absorbance at 520 nm. The sample concentration was determined by NO_2^- standard curve (100–1.56 μM) and reported as μM/mg protein.

2.12. Antioxidant enzymes

The concentration of reduced glutathione (GSH) in the liver was determined by using DTNB (Ellman's Reagent) through the assay described by Sedlak and Lindsay [43]. The standard curve of GSH was prepared by serial dilutions of concentrations ranging from 1 to 0.2 μM/mL. The absorbance of was calculated at 412 nm, and quantity of GSH in each sample was calculated from calibration curve and presented as μM/mg tissue protein. Activity of Glutathione-S-transferase (GST) was estimated using Habig's assay [44]. Initially, substrate master mix was prepared from reduced GSH and CDNB, and change in absorbance (ΔA) was observed for 5 min at 340 nm (at 1 min interval). Enzyme activity of GST was measured as μM/min/mg protein, where value of molar extinction coefficient (ε) for CDNB conjugate was 9.6 mM/cm.

The specific activity of hepatic glutathione peroxidase (GPx) was determined by the Paglia and Valentine [45] assay. The amount of enzyme oxidize 1 nM of NADPH for one minute was defined as one unit (U) of the GPx activity. The reaction was initiated with addition of hydrogen peroxide (H_2O_2) into sample wells, and ΔA was measured for 5 min at 340 nm. GPx enzymatic activity was represented as nM of NADPH oxidized/min/mg ($U.mg^{-1}$ tissue protein), where ε of NADPH was 0.00373 μM^{-1} per well.

Superoxide dismutase (SOD) assay was performed by the method described by Marklund and improved by Li et al. [46]. The rise in absorbance at 325 nm was measured for the 5 min (at 30 s intervals) against a blank Tris-HCl buffer. The findings were presented as U/mg protein, with one-unit of SOD equaling the amount of enzyme that suppresses pyrogallol autoxidation by 50%. Similarly, the enzyme activity of catalase (CAT) was calculated by the procedure given by Aebi et al. [47], modified for microplates. The hepatic PMS was mixed with ethanol to release active CAT enzyme, and after adding hydrogen peroxide (H_2O_2), the decline in absorbance was observed at 240 nm (for 3 min) on the kinetic mode of multimode reader. The amount of enzyme required to decompose 1 M H_2O_2 in the one milligram of protein within one minute time period is specified as a unit of CAT. Specific enzyme activity of was presented as U/mg hepatic protein in the samples, where the value of ε for H_2O_2 was 43.6 LM^{-1} cm^{-1}.

2.13. Liver tissue section examination

Liver tissues were fixed in the 10% neutral buffered formalin, followed by embedding in paraffin, and then cut into 6-μm sections for microscopic examination. After staining of the hematoxylin and eosin, hepatic architecture was observed for steatosis, necrosis, and inflammatory cell infiltration. Histological sections were photographed at the resolution of (100×, 300× and 400×) in the camera-attached light microscope (Olympus CX31, UK).

2.14. Statistical analysis

The data was reported as Mean ± S.E.M. To examine the significance of variation in body weight, glucose levels, and food utilization during the treatments, a two-way analysis of variance (ANOVA) with Bonferroni multiple comparisons test was used. A one-way ANOVA following the Dunnett's test was used to determine the statistical significance in lipid accumulation, oxidative stress, liver enzymes, collagen, cytokine, and plasma lipid profiles using Graph Pad Prism 8.4. The findings in treatment groups were compared to HFHFr (DC) group of rats to determine the percentage protections for all parameters. A probability level of $P < 0.05$ was selected as statistical significance.

3. Results

3.1. Food intake and adiposity index

Table 1 and Fig. 2 summarize the results of change in diet intake and rat's body weight throughout the study period. Rats fed with HFHFr diet (WD) gained significantly ($p < 0.001$) more weight in contrast to NC group and had a higher adiposity index. Nerolidol at both dose levels markedly ($p < 0.05$) decreased the rat's BMI and adiposity index. There was no statistically significant difference in daily food and water intake in the groups, but fructose-fed rats consumed much more water than the ND-fed rats. Although treatment groups with HFD plus fructose diet, consumed somewhat less food and had a slightly lower average calorie intake in a rat (Kj/day/rat), this difference was non-significant when compared to DC group for total food and calorie intake. Overall, a substantial ($p < 0.001$) increase observed in the daily calories intake by each diseased rat, and nerolidol treatment has shown no effect on percentage food efficiency.

Table 1
Effect of Nerolidol on adiposity, blood liver function enzymes, uric acid, fasting blood glucose and insulin concentrations in rat.

	NC	HFHFr	SBM	NER 250	NER 500
Food efficiency (%)	10.2 ± 0.91[a]	22.98 ± 2.98	19.48 ± 3.32	20.59 ± 1.85	19.16 ± 3.98
BMI (g/cm^2)	0.94 ± 0.06[b]	1.22 ± 0.05	0.94 ± 0.03[b]	1.15 ± 0.04	1.01 ± 0.03[c]
Adiposity Index	1.52 ± 0.09[a]	5.32 ± 0.28	4.16 ± 0.08[b]	4.95 ± 0.23	4.51 ± 0.22[c]
AST (U/L)	35.17 ± 1.47[a]	121.22 ± 5.49	85.61 ± 2.79[a]	95.17 ± 5.01[a]	89.67 ± 3.23[a]
ALT (U/L)	26.83 ± 1.94[a]	125.67 ± 2.69	68.17 ± 2.36[a]	83.17 ± 3.41[a]	71.83 ± 2.96[a]
ALP (U/L)	49.53 ± 4.26[a]	150.52 ± 12.88	119.30 ± 7.82[c]	138.22 ± 6.56	121.81 ± 4.77[c]
AST/ALT	1.37 ± 0.08[a]	0.97 ± 0.15	1.25 ± 0.02[a]	1.14 ± 0.04[b]	1.24 ± 0.06[a]
Uric Acid (mg/dl)	2.77 ± 0.14[a]	4.85 ± 0.23	3.98 ± 0.13[c]	4.58 ± 0.21	4.10 ± 0.16
Fasting Insulin (mIU/L)	11.07 ± 0.59[a]	24.45 ± 0.99	17.02 ± 0.89[a]	20.6 ± 0.79[a]	18.22 ± 0.88[a]
FBG (mM/L)	4.23 ± 0.12[a]	7.15 ± 0.19	6.12 ± 0.13[b]	6.85 ± 0.21	6.41 ± 0.17[c]

Results are presented as Mean ± SEM (n = 6). Where ap< 0.001, bp< 0.01 and cp< 0.05: statistically significant in contrast to disease control (DC) group by one-way ANOVA with multiple comparison of Dunnett's test.
NC, Normal diet group; HFHFr, High fat diet+ 20% fructose given group for 12-weeks (DC); and SBM, standard botanical mixture treated (positive control); NER 250, Nerolidol 250 mg/kg; NER 500, nerolidol 500 mg/kg dose administered groups for the last 4-weeks along with HFHFr diet.
Abbreviations: ALT, alanine transaminase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; BMI, body mass index.

3.2. Effect of nerolidol on glucose metabolism

Fig. 3 (A-B) shows the OGTT findings for the DC and obese treated rats. WD notably reduced rat's glucose tolerance, as evidenced by increased BGL at all the time periods. The maximum elevation in BGL was noted in the NC group after 1 h of glucose ingestion and decreased about baseline at 120 min, but in the HFHFr-induced group of rats, a rise in BGL was noticed after 60 min and persisted high for the coming 60 min. The BGL at and after the 60 min was observed considerably lower ($p < 0.05$) in the rats administered nerolidol (500 mg/kg/day). Nerolidol-treated rats showed an increasing protection trend (22.2%) for OGTT values when the AUC was used as a quantitative measure.

Similarly, WD considerably ($p < 0.001$) raised up the fasting blood glucose (FBG) levels in rats (69.73%), measured on the last day (Table 1). The results showed that its level was markedly ($p < 0.05$) reduced by nerolidol (500 mg/kg) treated group. Moreover, the plasma insulin was reported remarkably ($p < 0.001$) high (1.2-fold) in HFD plus fructose-treated rats. With the protection of 15.41% and 21.40% respectively, nerolidol (250 and 500 mg/kg) treatment groups significantly ($p < 0.001$) reduced the hyperinsulinemia in contrast to the DC group. As shown in Fig. 3C, calculated value of the HOMA-IR index was markedly ($p < 0.001$) decreased (29.8%) in rats administered with nerolidol in ascending order of its doses, revealing that it alleviated the systemic insulin resistance.

3.3. Impact of nerolidol on diet-induced liver injury and hyperuricemia

The serum concentrations of ALP, AST, ALT, and uric acid were remarkably ($p < 0.001$) raised in rats with the HFD plus 20% fructose diet, showing that NASH diet caused the liver injury. As expressed, in Table 1, the treated drug' nerolidol markedly ($p < 0.001$) reduced serum AST and ALT levels in dose-dependent fashion with percentage inhibition of 30.65% and 44.53%, respectively. The value of the AST/ALT

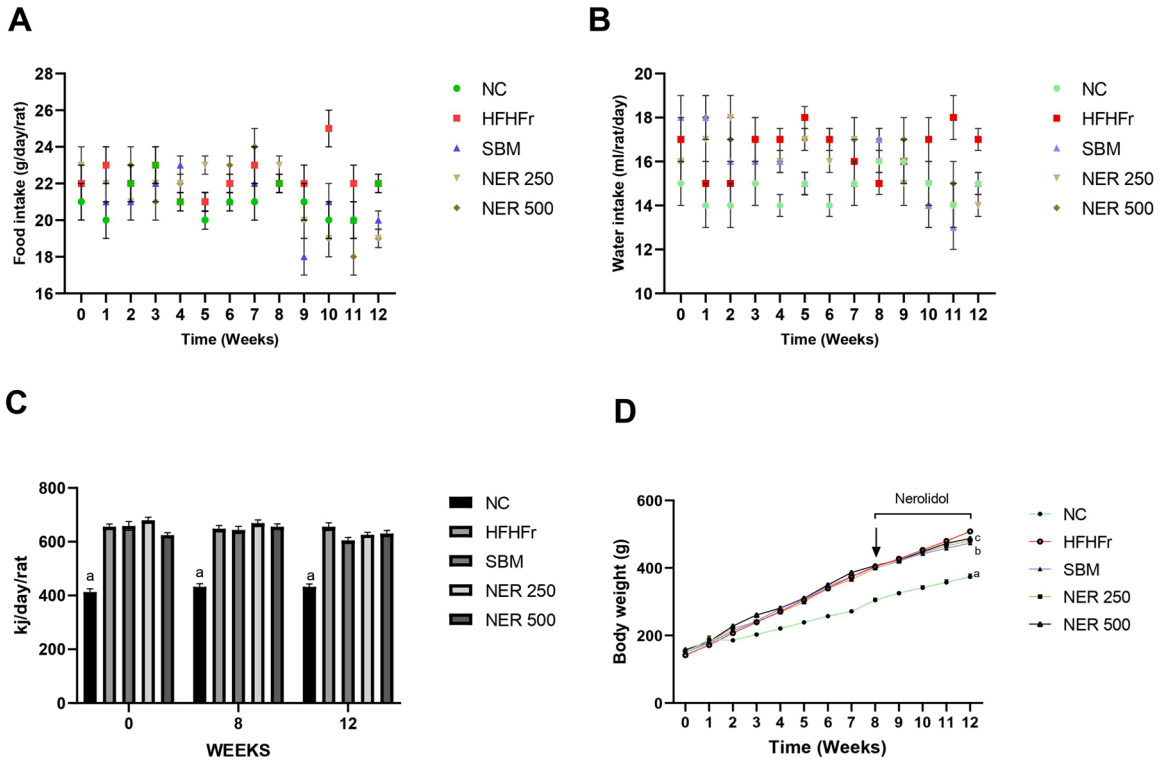

Fig. 2. Nerolidol effect on (A) food (B) water (C) energy intake and (D) weight gain of rats. Results are presented as mean ± SEM (n = 6). Where [a]$p < 0.001$, [b]$p < 0.01$ and [c]$p < 0.05$: significantly different versus disease control group of rats by applying two-way (A, B & D) and one-way ANOVA (C) with multiple comparisons of Bonferroni and Dunnett's tests. NC, Normal control group; HFHFr, High fat diet+ 20% fructose given group (DC); SBM, standard botanical mixture treated (positive control); NER 250, Nerolidol 250 mg/kg; NER 500, nerolidol 500 mg/kg treated group.

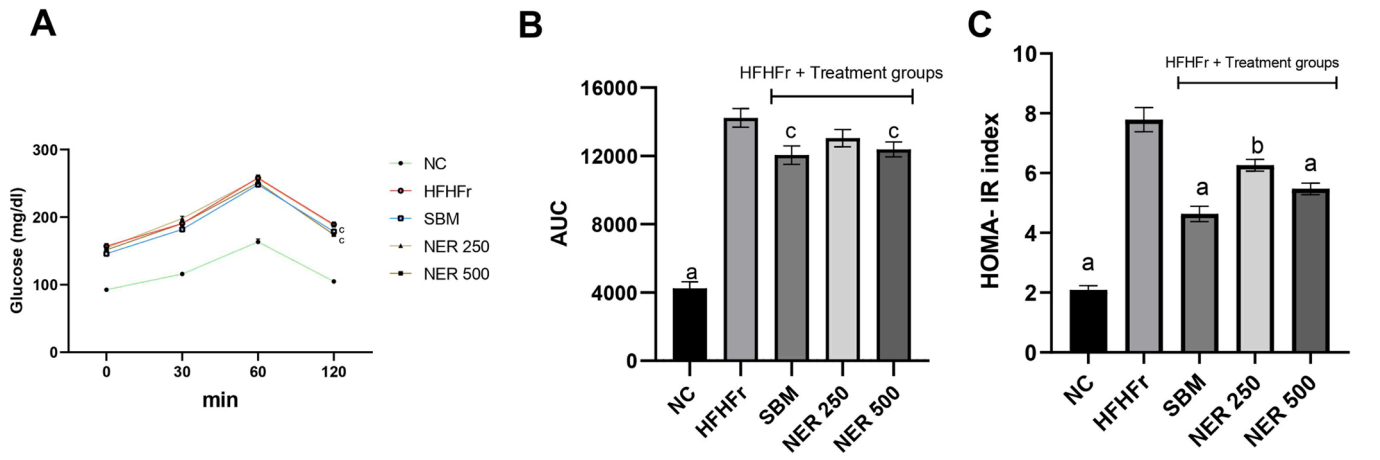

Fig. 3. Effect of nerolidol on glucose metabolism. OGTT (A), AUC (B) and HOMA-IR index (C) in Western Diet-induced NAFLD model. Results are presented as mean ± SEM (n = 6). Where $^{a}p < 0.001$, $^{b}p < 0.01$ and $^{c}p < 0.05$: significantly different versus disease control group of rats by applying two-way and one-way ANOVA with multiple comparisons of Bonferroni and Dunnett's tests. NC, Normal control group; HFHFr, High fat diet+ 20% fructose given group (DC); SBM, standard botanical mixture (positive control group); NER 250, Nerolidol 250 mg/kg; NER 500, nerolidol 500 mg/kg treated group.

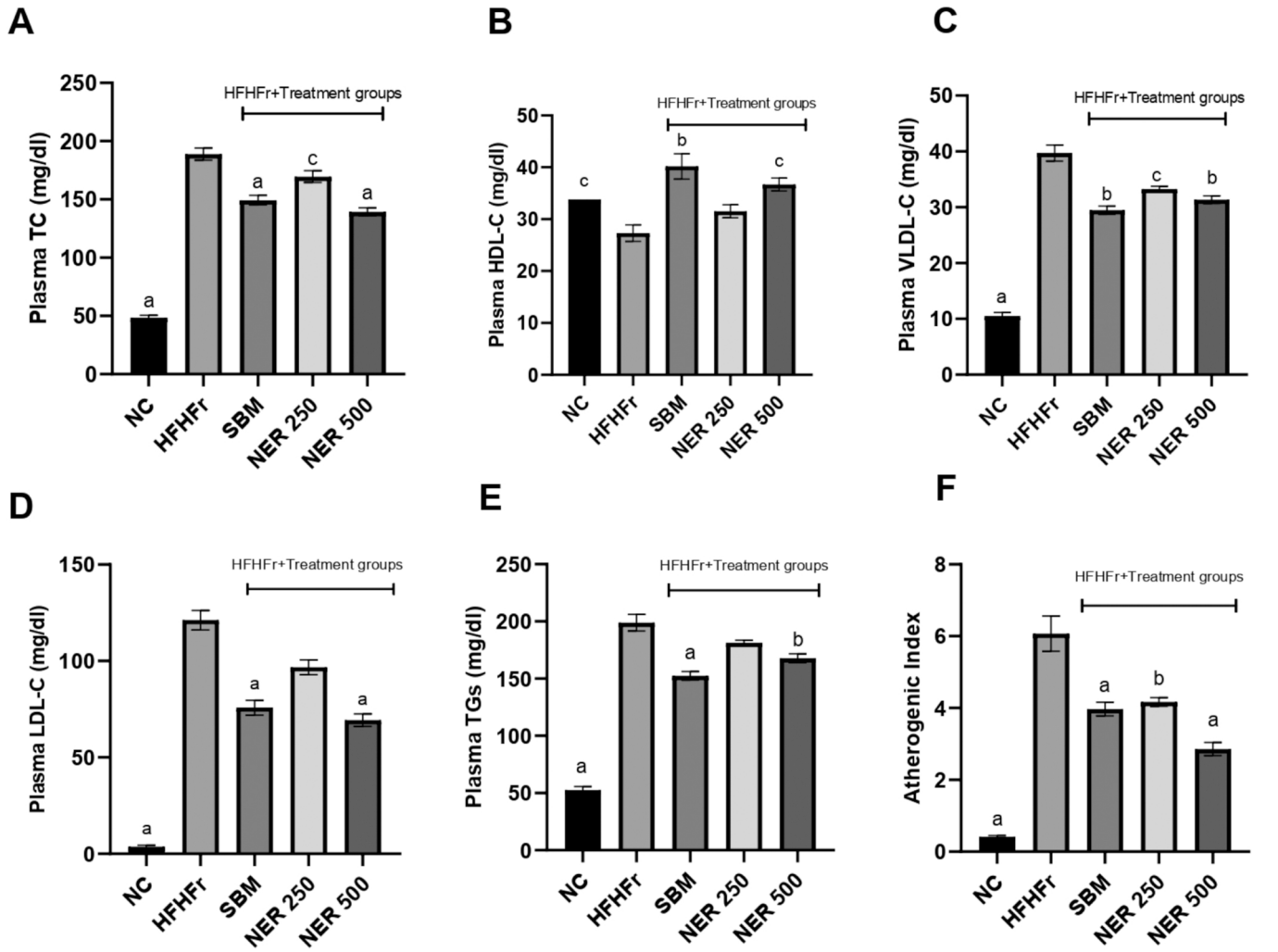

Fig. 4. Effect of Nerolidol on plasma lipids profile of the rats in HFD plus fructose diet induced NAFLD model. Results are presented as mean ± SEM (n = 6). Where $^{a}p < 0.001$, $^{b}p < 0.01$ and $^{c}p < 0.05$: significantly different versus disease control group by applying one-way ANOVA with Dunnett's tests. NC, Normal diet group; HFHFr, High fat diet+ 20% fructose given group; SBM, standard botanical mixture (positive control); NER 250, Nerolidol 250 mg/kg; NER 500, nerolidol 500 mg/kg dose group. TC, total cholesterol; TGs, triglycerides; VLDL-C, very low-density lipoprotein-cholesterol; HDL-C, high density lipoprotein-cholesterol, LDL-C, low density lipoprotein-cholesterol.

ratio was substantially ($p < 0.001$) decreased in DC group indicates that the disease was progressing and where ALT was increasing more rapidly than AST. The group treated by nerolidol (500 mg/kg) showed a hepatoprotective effect by significantly ($p < 0.001$) increasing the AST/ALT ratio (30.50%). Furthermore, at a higher dose, it has shown an increasing trend of protection (16.43%) in lowering serum uric acid.

3.4. Nerolidol effects on lipid metabolism

Fig. 4 (A-F) shows the results where the DC group fed with HFD and fructose, plasma TC, LDL-C, VLDL-C, and TGs levels were substantially ($p < 0.001$) elevated. Nerolidol (500 mg/kg) treated group remarkably ($p < 0.001$) reduced the levels of TC and related lipoproteins. The amount of LDL-C was considerably ($p < 0.001$) decreased with inhibition of 26.45%. The levels of HDL-C were markedly lowered ($p < 0.05$) in the HFHFr fed rats, and its concentration was substantially (30.40%, $p < 0.05$) raised after treatment with nerolidol (500 mg/kg). Furthermore, the atherogenicity index was also notably ($p < 0.001$) lowered by nerolidol in a dose-dependent way with 56.44% protection.

Similarly, a substantial ($p < 0.001$) rise in the liver weight (liver index), hepatic TGs, and TC accumulation was observed with WD, ultimately causing steatosis. Fig. 5 (A-D) express the results of individual fat content, where in the ascending dose order of drug, percentage inhibition against hepatic TC content was more significant than TGs (44.24% and 22.15%, respectively). Nerolidol at a higher dose level markedly ($p < 0.001$) reduced the hepatic total fat buildup (33.13%) and liver index (20.90%.), and the effect was more significant than SBM treated group.

3.5. Nerolidol ameliorated hepatic AOPP, MDA, and nitrites (NO_2^-) levels

After 12 weeks on the WD, hepatic MDA, AOPP, and NO_2^- levels were observed significantly ($p < 0.001$) high in the HFHFr group **(**Table 2**).** Nerolidol at both doses has been demonstrated to have a substantial effect in reducing oxidative stress markers. Compared to the DC group, nerolidol (500 mg/kg) treated rats remarkably ($p < 0.001$) reduced liver MDA levels by 51.72%. Similarly, AOPP levels were considerably ($p < 0.001$) diminished at this dose with 49.70% inhibition, greater than SBM treated rats. The concentration of liver NO_2^- was raised 4-fold in the DC rats, while nerolidol treated (250 and 500 mg/kg) rats remarkably ($p < 0.001$) lowered these levels dose-dependently with inhibition of 47.47% and 61.72%, respectively.

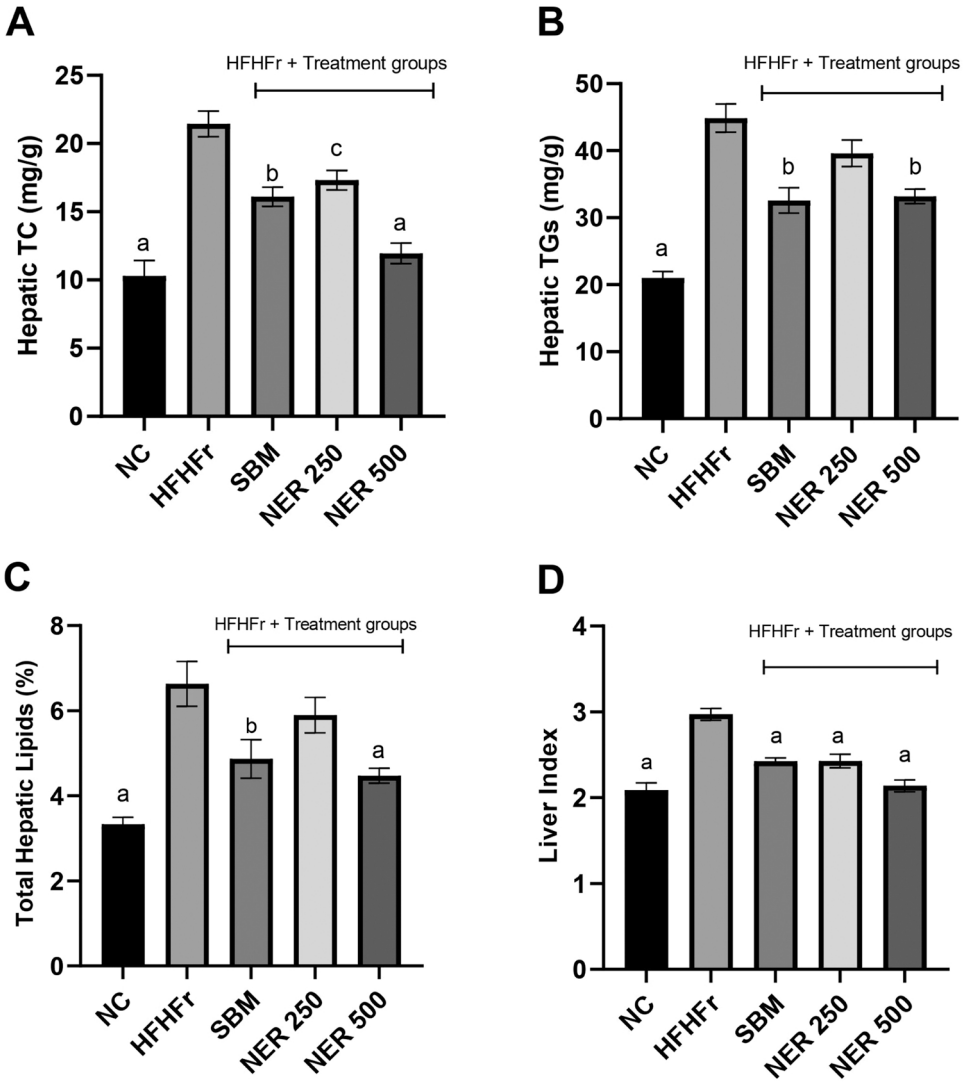

Fig. 5. Effect of Nerolidol on Hepatic lipids accumulation and liver index in NAFLD rats. Results are presented as mean ± SEM (n = 6) and statistically significant in contrast to disease control group by applying one-way ANOVA with Dunnett's test. Where $^{a}p < 0.001$, $^{b}p < 0.01$ and $^{c}p < 0.05$: NC, Normal control; HFHFr, High fat diet+ 20% fructose given group; SBM, standard botanical mixture treated (positive control); NER 250, Nerolidol 250 mg/kg; NER 500, nerolidol 500 mg/kg group.

Table 2
Effect of Nerolidol treatment on hepatic oxidative stress markers and antioxidants enzymes.

	NC	HFHFr	SBM	NER 250	NER 500
AOPP (nM/g tissue)	471.3 ± 68.18[a]	1250.5 ± 101.3	805.4 ± 57.92[b]	954.4 ± 72.98[c]	770.8 ± 64.56[a]
MDA (μM/mg protein)	0.49 ± 0.06[a]	2.37 ± 0.34	1.02 ± 0.17[a]	1.34 ± 0.12[b]	1.15 ± 0.17[a]
NO_2^- (μM/mg protein)	2.87 ± 0.31[a]	15.1 ± 1.05	5.09 ± 0.23[a]	7.86 ± 0.95[a]	4.95 ± 0.27[a]
GSH (μM/mg protein)	0.25 ± 0.02[a]	0.11 ± 0.01	0.23 ± 0.01[a]	0.17 ± 0.02[c]	0.19 ± 0.03[b]
GST (μM/min/mg protein)	0.13 ± 0.01[a]	0.05 ± 0.01	0.11 ± 0.02[a]	0.06 ± 0.01	0.10 ± 0.01[a]
GPx (nM of NADPH oxidized/min/mg protein)	35.70 ± 5.24[b]	15.01 ± 3.29	34.05 ± 2.86[b]	30.45 ± 3.18[c]	38.16 ± 3.40[a]
SOD (U/mg protein)	2.53 ± 0.32[a]	0.58 ± 0.23	1.88 ± 0.25[b]	1.28 ± 0.13	1.73 ± 0.18[b]
CAT (U/mg protein/min)	7.21 ± 0.97[a]	1.78 ± 0.25	5.25 ± 0.74[b]	3.11 ± 0.42	5.88 ± 0.44[a]

Results are presented as Mean ± SEM (n = 6). Where ap< 0.001, bp< 0.01 and cp< 0.05: statistically significant in contrast to disease control (DC) group by one-way ANOVA with multiple comparison of Dunnett's test.
NC, Normal diet group; HFHFr, High fat diet+ 20% fructose given group for 12-weeks (DC); and SBM, standard botanical mixture treated (positive control); NER 250, Nerolidol 250 mg/kg; NER 500, nerolidol 500 mg/kg dose administered groups for the last 4-weeks along with HFHFr diet.
Abbreviations: AOPP, advance oxidation protein product; MDA, malondialdehyde; GSH, reduced glutathione; NO_2^-, nitric oxide (as nitrite); GST, glutathione-S-transferase; GPx, glutathione peroxidase; SOD, superoxide dismutase; CAT, catalase.

3.6. Nerolidol restored hepatic antioxidant enzymes and glutathione system

Due to chronic intake of HFD and fructose diet, hepatic levels of GSH, GST and GPx were significantly depleted ($p < 0.001$) in DC group, as shown in (Table 2). Nerolidol has shown a dose-dependent efficacy on the hepatic glutathione system. At the dose of 500 mg/kg, the treated group markedly ($p < 0.01$) restored (70.12%) GSH levels in the liver. Similarly, it had a substantial ($p < 0.001$) effect on GPx levels at this dose level, increasing it by 1.9-fold. The hepatic content of GST was also remarkably ($p < 0.001$) increased by 74.15% at ascending order of treatment. Similarly, the depleted levels of hepatic SOD and CAT due to WD were also markedly ($p < 0.001$) restored by nerolidol with a dose-dependent effect. The treated group with 500 mg/kg dose has notably raised the SOD (1.8-fold, $p < 0.01$), and the CAT (1.9-fold, $p < 0.001$) levels in the liver extract.

3.7. Nerolidol reduced serum cytokine and hepatic fibrosis

The amount of serum TNF-α was raised ($p < 0.001$, 116.1%) in NASH diet-fed animals as compared to NC group. The results are shown in Fig. 6A, that its quantity was remarkably ($p < 0.001$) and dose-dependently ameliorated by both nerolidol treated groups with the protection of 30.89%. The reported anti-inflammatory effect of the phytochemical was more significant than the SBM treated group. Furthermore, as a diagnostic marker to measure hepatic fibrosis, the liver deposition of collagen was remarkably ($p < 0.001$) augmented in DC rats (Fig. 6B). The biochemical levels were significantly ($p < 0.01$) decreased in nerolidol treated groups at 500 mg/kg dose with 53.46% inhibition.

3.8. Effect of nerolidol on liver histology

As shown in photomicrographs (Fig. 7a-c) the WD given to rats for 12 weeks led to severe necrosis, fat droplets deposition and inflammation in liver sections. Nerolidol treatment in ascending dose order substantially reduced the steatosis (macrovesicular and microvesicular), degeneration of hepatocytes, and inflammatory cells infiltration.

4. Discussion

NASH is a multifactorial illness that falls under the umbrella of the metabolic syndrome and is primarily characterized by insulin resistance as a major contributor to its pathophysiological manifestation [20]. In humans, the prolonged consumption of a western-style diet (WD) high in HFD, cholesterol, and fructose is a major risk factor for causing systemic and hepatic features of disease [7]. Thus, the present study has often explored the possibility of promptly inducing disease-related clinical characteristics in a rat model utilizing an HFD (60% of energy from a fat source) in solid food and 20% fructose in drinking water. As a result, a successful framework for NASH was established for screening and evaluating drugs.

Drug discovery for NASH and associated complications is the need of the time [21]. Natural compounds are ubiquitous in the world's flora and possess antioxidant, insulin-sensitizing, anti-inflammatory, and hypolipidemic properties and are becoming more popular and targeted for the effective management of NASH [22]. It has been established that the sesquiterpene class exhibits hepatoprotective effects [48]; hence present work evaluated nerolidol against a rat model of NASH. This is the first study; in which effects of nerolidol was examined on steatosis caused by chronic intake of a purpose-built food, and its effect on

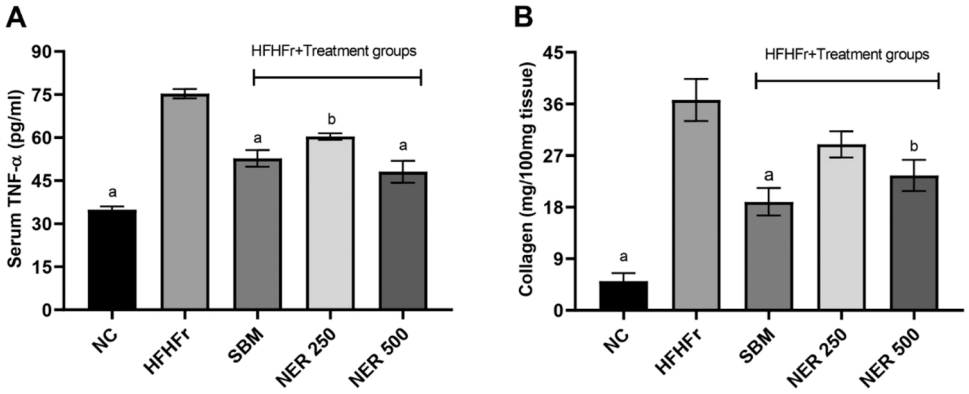

Fig. 6. Nerolidol effect on (A) TNF-α, and (B) Collagen levels. Results are presented as mean ± SEM (n = 6). Where [a]$p < 0.001$ and [b]$p < 0.01$: significantly different versus disease control by applying one-way ANOVA with Dunnett's tests. NC, Normal control; HFHFr, High fat diet+ 20% fructose given group; SBM, standard botanical mixture (positive control); NER 250, Nerolidol 250 mg/kg dose; NER 500, nerolidol 500 mg/kg/day group.

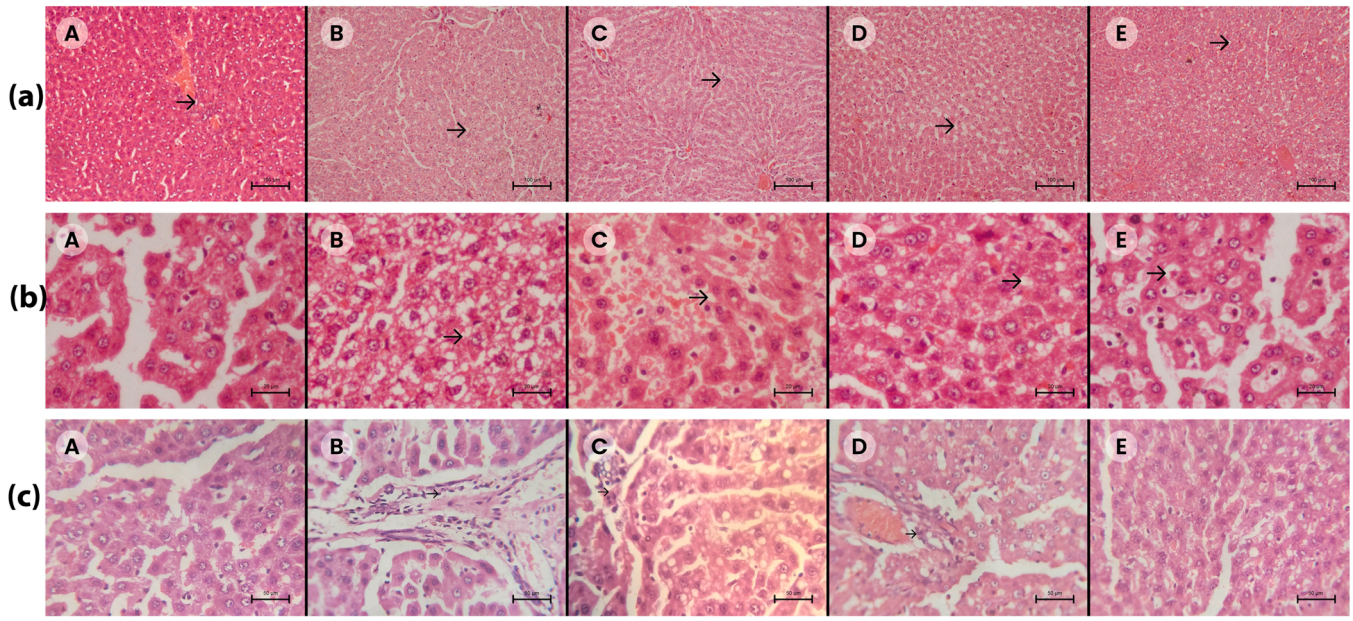

Fig. 7. Effect of Nerolidol on the histopathology of Western-diet fed rat's liver stained with hematoxylin-eosin. Photomicrographs of representative sections of each group are shown macrovesicular and microvesicular steatosis [magnification, 100× (a) and 400× (b)] and lobular inflammation around central vein (c) 300x, indicate by black arrows. (A) Liver of normal control group; (B) Disease control; C-E are treated groups fed with HFHFr diet, where (C) Rat liver treated with standard botanical mixture; (D-E) Liver section of rat treated with nerolidol 250 and 500 mg/kg/day.

subsequent pathology of NASH and fibrosis.

In clinical settings, in addition to increasing body weight, HFD and a fructose diet can cause hyperglycemia and hyperinsulinemia in obese people, predisposing them to diabetes [15,17]. Similarly, in the current study, over 12 weeks, WD was significantly increased fasting blood glucose and insulin levels and impaired OGTT of rats. It was due to selective-hepatic insulin resistance caused by increased gluconeogenesis and decreased utilization by peripheral tissues that ultimately led to systemic insulin resistance [11]. The HOMA-IR index is utilized to measure it, and nerolidol at a high dose level significantly lowered its value to normal, indicating that it has a more likely influence on insulin release or sensitization. Hashiesh et al. [49] reported that β-caryophyllene (another sesquiterpene) showed an anti-NAFLD effect through ameliorating insulin resistance.

A high-calorie diet enriched in saturated fatty acids (SFAs) and fructose causes steatosis and hepatomegaly, enlarging the liver's size and weight [4,16]. Overall, 60% of free fatty acids (FFAs) are generated from the lipolysis of adipose tissue and 15% from HFD, resulting in increased intrahepatic lipid accumulation [8]. The phases of steatosis entailed increasing uptake of FFAs and de novo-lipogenesis (DNL), and decreasing FAs mitochondrial β-oxidation, and VLDL-C release from hepatocytes [1]. Fructose serves as both substrate and an inducer of hepatic DNL and inhibits TGs clearance. Moreover, its breakdown in the liver causes increased VLDL secretion and decreased FAs oxidation [14]. The insulin pathway regulates hepatic lipid metabolism, and the "multihit" theory postulates that hepatic lipid deposition results in decreased insulin signaling [13]. On the other hand, insulin resistance triggers the secretion of FFAs from adipose tissues and activates the proteins involved in DNL, which adds to steatosis and its progression. Additionally, it inhibits apolipoprotein B (ApoB) synthesis and decreases the hepatic release of VLDL-C particles [20].

In the current study, nerolidol treated groups have shown a substantial and dose-dependent effect on lowering liver index and hepatic fat contents, suggesting that it may target any of the processes involved in steatosis. Previously, total sesquiterpenes from Loquat (*Eriobotrya japonica*) leaf alleviated HFD-induced NAFLD and expressed a similar effect [48]. Nerolidol at the dose of 500 mg/kg was found to be more efficacious at normalizing hepatic total cholesterol levels, possibly by inhibiting proteins involved in its metabolism like hydroxy-3-methylglutaryl-coenzymeA reductase (HMGCR), sterol regulatory element-binding protein-2 (SERBP2), and low-density lipoprotein receptors (LDLR) [50]. The data corroborates the previously reported effect of safranal, demonstrating that terpenoids may have a more significant potential effect on cholesterol production [26]. Furthermore, some other phytochemicals such as oxymatrine, plumbagin, and phytosterols esters reported similar anti-steatosis effects in various animal studies involving HFD and fructose [39,51,52].

HFD with fructose may cause blood lipids abnormalities due to insulin resistance, resulting in lipotoxicity and the steatosis in a vicious cycle [53,54]. Secondly, dyslipidemia is the common clinical symptom in NAFLD patients and may relate to cardiovascular diseases owing to the atherosclerotic effect of hepatic steatosis [6]. Initially, disease lowers both the qualitative and quantitative clearance of various lipoproteins. The excessive formation and release of large TG-enriched VLDL molecules by the liver contribute to the kinetic derangement of the lipid profile [5]. The conversion rate to small and dense atherogenic low-density lipoprotein (LDL-C) particles increases, which are more susceptible to oxidation and macrophage uptake. Furthermore, NAFLD patients also have fewer plasma HDL-C particles, which possesses an anti-atherogenic impact [50].

Rats developed hypertriglyceridemia and hypercholesterolemia in the current study after 12 weeks of WD. The effect of nerolidol on blood lipid profile normalization was comparable to that of measured hepatic dyslipidemia. At the dose of 500 mg/kg, remarkable antihyperlipidemic activity was reported, most notably on total cholesterol and related lipoproteins such as HDL-C and LDL-C. Moreover, its treatment significantly reduced the atherogenic index, showing its cardioprotective impact. Previously, Asaikumar et al. [35] revealed that oral treatment with nerolidol restored dyslipidemia in isoproterenol-induced myocardial infarction in rats. Clinical investigations have found that increased blood aminotransferases (ALT and AST) in the absence of other hepatic disorders may support the diagnosis in up to 50% of patients with simple liver steatosis and 80% of patients with NASH [2]. Their levels were substantially higher in NASH diet-treated rats in the current

investigation, indicating liver injury. The reduction in these liver enzymes by nerolidol treatment correlates with its hepatoprotective activity. Additionally, the drug substantially increased the De-Ritis ratio, a critical diagnostic marker for disease progression, suggesting a slowdown in disease development.

Chronic overconsumption of HFD and fructose has long been recognized as a crucial etiological factor in the development of oxidative stress and inflammation [9,17,52]. Oxidative stress may be measured by determining the alteration in physiological balance between pro-oxidant species and antioxidant enzymes [55]. Fructose catabolism lowers ATP levels in the liver, causing hyperuricemia and produces ROS [14]. Some of the most critical oxidative damage products include peroxidation of biological lipids and proteins, such as MDA and AOPP [56]. The MDA has a longer half-life as compared to ROS and may permeate readily into the extracellular space, intensifying the effect of oxidative stress [41]. AOPPs are cross-linked protein products of dityrosine and a novel oxidative marker formed by the action of chloraminated per-oxidants. Their amounts are found high in blood and liver of patients with NASH [40]. Similarly, in the context of reactive nitrogen species and nitrative stress, increased NO formation by the inducible nitric oxide synthase (iNOS) is also linked to the illness [55].

Likewise, in NASH, cholesterol and SFAs accumulation in mitochondria and endoplasmic reticulum deplete glutathione (GSH) system and SOD levels, cause their dysfunction, and sensitize to inflammatory cytokines [57]. Moreover, peroxisomal β-oxidation increases fivefold to detoxify ROS, reducing catalase enzyme [4]. The cellular redox imbalance may impair the insulin signaling pathway and alter key enzymes involved in lipid metabolism, innate immune signaling, and liver apoptotic pathways [11]. Furthermore, hepatic mitochondrial dysfunction may precede hepatic steatosis via insulin resistance [10]. On the other side, insulin resistance and accompanying hyperglycemia are, themselves, significant contributors to ROS generation by upregulating the lipid peroxidation in microsomes and downregulating mitochondrial β-oxidation, as well as depleting endogenous antioxidants, and inducing inflammation [58]. So, this is a vicious cycle, oxidative stress produces steatosis, and steatosis causes oxidative stress. These scenarios collectively contribute to NAFLD development and progression, further exacerbating the NASH stage [56].

In the current study, prolonged intake of WD showed an increase level of hepatic oxidative stress moieties, such as NO, MDA, and AOPP. The findings are in-line with some earlier research showing tissue endogenous antioxidant defense system may be depleted in HFD and fructose-fed animals and lead to insulin resistance and NASH [59,60]. Nerolidol treatment significantly and dose-dependently reduced their levels and showed antioxidant effect. In a previous study by Javed et al. [34], nerolidol has remarkably diminished the MDA levels in the rotenone-induced neuroinflammation model of animals. Similarly, Iqubal et al. [30] reported that nerolidol oral dosing to rats reduced NO levels in the cyclophosphamide (CPx)-induced cardiotoxicity model by inhibiting inducible nitric oxide synthase (iNOS).

Furthermore, the levels of hepatic antioxidants such as the GSH system (reduced GSH, GST, and GPx), and CAT and SOD were remarkably restored by nerolidol treated rats. These antioxidants scavenge ROS to water (H2O) and dismutase the superoxide radicals, thus alleviating the second hit [55]. Nerolidol (500 mg/kg) has a more noticeable effect on hepatic CAT, GST and GPx than reduced GSH factor. In previous work, Celik et al. [33] found that nerolidol restored antioxidants in thioacetamide-induced gonadotoxicity model of Swiss albino mice. The hypothesized mechanism is stimulation of the nuclear factor erythroid 2–related factor 2 (Nrf-2) cellular signaling pathway, as found upregulated in the Cpx-induced neuroinflammation model [32].

The adipocyte dysfunction is related with increased systemic levels of specific cytokines, such as tumor necrosis factorα (TNFα) [8]. Furthermore, oxidative stress indicators like MDA and NO, and metabolic signals like FFAs may trigger intracellular inflammatory processes in the hepatocytes and Kupffer cells in the HFD and fructose-induced NASH [7,16]. These include activating the nuclear factor-κB (NF-κB), mitogen-activated protein kinase (MAPK), and c-Jun N-terminal kinases (JNK) related downstream signaling cascades [58]. TNFα affects lipid and glucose metabolism enzymes and may disrupt insulin signaling by hindering insulin receptor tyrosine kinase activity and increasing protein kinase C (PKCε) activation and serine phosphorylation of IRS-1 [12]. Our data showed serum TNFα levels increased all through the 12-week NASH diet, and nerolidol therapy with increasing doses decreased its concentration. The previous research reported that oral treatment of nerolidol downregulated the MAPK, NF-kB, and c-JNK signaling proteins in doxorubicin-induced cardiotoxicity models in rats [28,29]. Moreover, multiple studies have demonstrated that terpenoids have anti-inflammatory properties in NASH models [61].

Cytokines, lipid peroxidation aldehydes, and ROS play critical roles in further progression of NASH by triggering necrosis, and apoptosis cascades in the parenchymal and extra-parenchymal liver [57]. The HSCs in its activating form synthesize collagen, which is accumulated in the extracellular matrix of the liver, allowing the pathology to proceed from steatohepatitis to fibrosis and cirrhosis [4]. Hydroxyproline is an amino acid that is a key constituent of collagen and is formed by the irreversible post-translational hydroxylation of proline by prolyl hydroxylase [38]. Its measurement has been used to diagnose various diseases associated with collagen degradation. Chronic HFD and fructose diet intake in humans and animals promote the development of hepatic fibrosis by increasing collagen levels [18]. The current study endorsed the notion of hepatic injury by finding elevated collagen (HXP) levels in the rats livers given WD. Nerolidol at the dose of 500 mg/kg reported an anti-fibrotic impact by reducing HXP amount in liver, so have potential to inhibit disease progression. Previous preclinical studies have shown other natural compounds, such as swertiamarin, pterostilbene, and resveratrol, to ameliorate oxidative stress and NASH induced by a high fructose-high fat diet [59,62].

Furthermore, nerolidol treatment has been shown to substantially affect histopathological indicators such as reducing steatosis and lobular inflammation, correlated with biochemical findings of antioxidants, cytokine, and hepatic lipotoxicity. The current study found that slowing down of pathogenicity and anti-steatotic effect of the drug was more significant than standard botanical mixture. In another study, zerumbone, a naturally occurring cyclic sesquiterpene of *Zingiber zerumbet*, attenuated NAFLD in hamsters fed on HFD [63]. Moreover, results of hepatic enzymes and histopathology are in-line with earlier research where nerolidol has shown potent anti-apoptotic and anti-inflammatory effects described by enhanced expression of Bcl-2 and decreased expression of Bax and caspase-3 in Cpx-induced hepatic injury [31].

5. Conclusion

In the current study, multiple-hit pathogenesis of NASH was described by increased steatosis, inflammation and oxidonitrative stress led to development of vicious insulin resistance in rats. Nerolidol treatment significantly reduced hepatic lipid accumulation, and halted disease progression induced by a hypercaloric diet. Furthermore, drug treatment diminished oxidative stress markers, fibrosis, and cytokine while significantly increased hepatic antioxidants. The ensuing hepatic insulin resistance was alleviated, as evidenced by a reduction in blood glucose, and insulin levels, and dyslipidemia, established the co-relevance among study parameters. Histological testing revealed that improved insulin sensitivity might enhance liver function and indicated promising effects of the drug against NASH caused by HFD and fructose.

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Ethics approval statement

The research work on animals were conducted after animal ethics approval from university ethics committee with voucher number (SU/ORIC/2861/2021).

CRediT authorship contribution statement

Usman Sabir: Conceptualization, Methodology, **Hafiz Muhammad Irfan**: Supervision, Writing – original draft preparation. **Alamgeer**: Visualization, Investigation. **Aman Ullah**: Data curation: **Yusuf S Althobaiti**: Validation. **Fahad S Alshehri**: Writing – reviewing and editing, **Zahid Rasul Niazi:** Software.

Declaration of Competing Interest

We all authors declare no conflict of interest.

Data availability

The data that support the findings of this study are available on request from the corresponding author.

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Conflict of interest statement

The authors report no declarations of competing interest.

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