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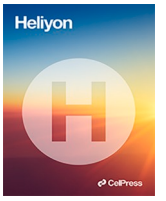

Research article

Milk thistle protects against non-alcoholic fatty liver disease induced by dietary thermally oxidized tallow

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ABSTRACT

Non-alcoholic fatty liver disease (NAFLD) is a chronic condition caused by several factors including thermally oxidized tallow. Various strategies have been considered to ameliorate NAFLD. However, the role of milk thistle (MT) in ameliorating NAFLD caused by thermally oxidized tallow has not been reported. The purpose of this study was to evaluate the ability of milk thistle to protect rabbits from the toxicity of oxidized tallow (OT). The rabbits were given OT and an extract of MT. The composition of MT was analyzed using HPLC-DAD, and tallow samples were studied using GC-MS. The study also examined liver histology, antioxidant levels, liver-related inflammatory markers, and serum lipid profile. The results showed that the major components of the MT extract were silybin B, formononetin-glucuronic acid, proanthocyanidin B1, silychristin B, silydianin, and isosilybin A. The group given OT showed elevated lipid profiles, lower antioxidant status, higher levels of hepatic inflammatory markers, and lower levels of anti-inflammatory markers. This group also had higher fat storage in the liver compared to the control or treatment groups. However, when MT was supplemented, the pro-inflammatory cytokines (IL-1, IL-4, IL-6, and TNF-α) and antioxidant status (CAT, SOD, GSH-Px, GSH, and TBARS) of the liver returned to normal. This suggests that MT extract is an excellent source of hepatoprotective compounds. It protects the liver by increasing antioxidant enzymes, decreasing pro-inflammatory cytokines, and increasing anti-inflammatory markers.

1. Introduction

The modern world's leading cause of chronic liver disorders is the rapidly rising prevalence of non-alcoholic fatty liver disease (NAFLD). In general, NAFLD is linked to metabolic syndrome, a higher risk of type 2 diabetes, and controllable signs of insulin resistance including dysglycemia and dyslipidemia [1]. NAFLD is thought to be the primary cause of altered liver enzymes, which are linked to an increased risk of diabetes along with coronary artery disease [2]. The assessed prevalence of NAFLD worldwide ranges from 3 to 45 %. Wide ranges in prevalence are dependent on the study population as well as the diagnostic methods (liver biopsy, imaging, liver enzyme, etc.) employed [3].

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Globally, the prevalence of metabolic syndrome as well as the fatty liver is rising, and this is now the main social issue [4]. Numerous population studies have documented that the incidence of fatty liver illnesses rises with age and that these individuals are also more vulnerable to morbidity and death [5]. People who have fatty liver disease are more likely to develop type 2 diabetes, heart disorders, cirrhosis, and several types of cancer [6]. Six characteristics of fatty liver that increase the risk of cardiovascular disease (CVD) have been identified. These include prothrombotic and pro-inflammatory states, elevated blood pressure, atherogenic dyslipidemia, insulin resistance with glucose intolerance, and abdominal obesity. An individual who satisfies three or more of these criteria must receive a clinical diagnosis of metabolic syndrome or fatty liver [7].

Epidemiological studies show that the prevalence is 15 % in Asian countries and 20–30 % in Western countries [8]. Long-term exposure of liver cells to high concentrations of free fatty acids (FFA) in the form of triglycerides can also cause the liver to become chronically inflammatory [9]. Oxidized lipids are one of the common causes of NAFLD [10]. Thermally oxidized dietary lipids have been shown to cause fatty accumulation in the liver of the experimental animals [11,12]. Thus, it is warranted to look for remedies for NAFLD.

Herbs and herbal remedies have been utilized for a long time to treat liver diseases, such as NAFLD [13]. The herbaceous plant *Silybum marianum* L. commonly known as milk thistle (MT), which belongs to the Compositae family, is native to Southern Europe as well as North Africa [14]. It has been documented that as early as the 4th century BC, Westerners utilized *S. marianum* to cure gallbladder and liver ailments. Additionally, it was discovered that in patients with drug-induced liver injury, nonalcoholic fatty liver disease, viral hepatitis, and alcoholic liver disease, *S. marianum* demonstrates anti-inflammatory, immunomodulating, antifibrotic, antioxidant, as well as regenerating qualities of the liver [15]. Isosilychristin, isosilybin, silybin, silychristin, silydianin, and toxifolin are the primary components of silymarin, a combination of lipophilic and flavonoid lignans that is isolated directly from the dried seeds of the *S. marianum* [16]. According to reports, silymarin has pharmacological effects on immunological modulation, neuroprotection, antioxidant, reducing blood lipids, avoiding diabetes, and anti-inflammatory. It also inhibits certain cancers [17]. Moreover, silymarin has a range of pharmacological actions that can guard the membranes of the liver cells, stop the deterioration of the liver cells, encourage liver cleansing, and aid in liver detoxification [18]. Recently, silymarin was found to protect against renal injury induced by a high-fat diet, through the normalization of lipid metabolism [19]. Silymarin has been found to ameliorate the oleic acid-induced lipid accumulation in HepG2 cells [20]. Silymarin was hepatoprotective against the viral-induced infection in mice, and thus effective in Mayaro fever [21]. However, there is a lack of information on the effects of milk thistle on the thermally oxidized lipids induced NAFLD, which thus warranted the present work. Milk thistle rich in silymarin was found to protect against dietary oxidized lipids-induced toxicity in rabbits.

2. Materials and methods

The methanol, eugenol, quercetin, catechin, and acetic acid were supplied by Sigma (USA). Glutathione peroxidase (GSH-Px), alanine aminotransferase (ALT), low-density lipoproteins (LDL-C), high-density lipoproteins (HDL-C), total cholesterol (TC), and triglycerides (TG) were among the commercial kits that were utilized. All the chemicals as well as the reagents are of the highest purity and analytical grade.

2.1. Sample preparation

The tallow had been thermally oxidized at 160 °C for ten continuous hours on a hot plate. After the thermal oxidation, the samples were stored at −20 °C in a refrigerator [22]. The sample of milk thistle powder was collected from Lower Dir, Pakistan, and identified using standard protocols as reported recently [23]. Samples of fresh fruits were taken from the plant and washed. After being shade-dried, the samples were finely powdered. Then 10 mL of a methanol-water (90:10) mixture was used to dissolve 1 g of the powder sample, which was then shaken for 10 h. After filtering, the mixture was centrifuged for 10 min at 5000 rpm. After filtering, the samples were added to the HPLC vials. For animal studies, 50 g of MT fruit powder was mixed with 500 mL of 90 % methanol water for 48 h while being constantly shaken in a shaking incubator. Methanol was evaporated and the sample was lyophilized to obtain dry extract for animal feeding.

2.2. Characterization of samples

The oxidized tallow was examined using the GC-MS (GC-MS-5977B, Agilent Technology, USA) with Agilent DB-1 column and helium as a carrier gas (24 mL/min). The experimental setup included a 36-min runtime, an oven temperature range of 70–270 °C, and 1 μL of injection volume. The quantification of compounds was based on the percent peak area [11].

A reverse-phase Agilent 1260 Infinity system was used to identify compounds in milk thistle. Phenolic compounds were separated using a C18 high-resolution column (Agilent Technologies, Germany) which was regulated at a temperature of 25 °C, and had a specification of 4.6 × 100 mm. A binary solvent system, consisting of solvents A (methanol-acetic acid-water, 10: 2: 88, v/v/v), and B (methanol-acetic acid-water, 90: 2: 8, v/v/v). The injection volume was 50 μL, and the flow rate was 1 mL/min. The initial procedure as described in our laboratory [24] served as the foundation of the elution process. Absorption spectra were recorded between 200 and 700 nm using a DAD detector. Silybin A and B, Silychristin, and cyanidin-3-glucoside standard solutions were made in methanol for calibration curves.

2.3. Animal protocols

The Helsinki criteria, which outline the appropriate care and use of animals in experiments, have been approved by the ethics committee and the Graduate Studies Committee of the Department of Biotechnology. Subsequently, the work was approved by the Advanced Studies and Research Board of the University of Malakand (No. UOM/Admin/2023/712, item-04) in its 83rd meeting.

The rabbits (Himalayan breed), all in good health, were purchased from the local vendor. These animals had been kept in a climate-controlled environment with a 12-h day and 12-h night cycle, and a temperature of 24 °C. They were provided with frequent access to food and water in their cages and were given a period of seven days to acclimate before any experimental feeding took place. Rabbits were divided into seven groups at random. Group 1 was control, group 2 was fed with 2 g/kg un-oxidized tallow (labeled as NT), group 3 was fed with 2 g/kg thermally oxidized tallow (labeled as OT), group 4 was fed with MT 200 mg (labeled as MT), group 5, 6 and 7 were fed with 2 g/kg thermally oxidized tallow and 100, 200 and 300 mg of MT, which were labeled as OT + MT1, OT + MT2, and OT + MT3, respectively. Previous studies showed that 2 g/kg of thermally oxidized tallow produced significant fatty accumulation in the liver of rabbits [25]. All the groups with the replicate (n = 5) animals were fed orally for 14 days. The blood samples were collected after 7 and 14 days of treatments.

After completing the feeding cycle, the animals were sacrificed. Blood samples were collected and placed in gel tubes, which were then centrifuged at 5000 rpm for approximately 10 min to obtain serum. Afterward, the serum underwent further biochemical analysis and/or stored at −20 °C. The liver was extracted and preserved in a 10 % formalin solution.

2.4. Hematology

Blood was extracted from the rabbit's jugular veins for hematological examination and placed in EDTA tubes. An automated digital machine to provide hematology or blood profile was used to measure the concentrations of white blood cells, blood platelets, hemoglobin (Hb) levels, red blood cells (RBC), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and hematocrit (HCT%) [11].

2.5. Biochemical analyses

The serum lipid profile, which includes total cholesterol, triglycerides (TG), high-density lipoprotein (HDL) cholesterol, and low-density lipoprotein (LDL) cholesterol were examined using a biochemistry analyzer, Mindray, Model BA 88A (China).

2.6. Liver histological examination

The livers of the rabbits from each group were removed in order to collect liver samples, which were then preserved in 10 % (v/v) paraformaldehyde/PBS solution. The preserved samples were embedded in paraffin blocks and sliced into 5-μm slices using a microtome (Leica, Type RM 2235, Germany). These sections were stained with hematoxylin and eosin dye. Histological observations were conducted using a Zeiss Axioscope optical microscope with a magnification of 200× [26].

2.7. Catalase assay

The quantitative enzyme immunoassay method was applied (Cusabio Technology LLC, USA) for catalase analysis at PHC Diagnostics, Lahore, Pakistan. Precoated CAT antibodies were introduced into microplate wells containing standards as well as specimens (100 μL each). Next, the wells were treated with avidin-conjugated horseradish peroxidase (100 μL) and a biotin-conjugated antibody specific for CAT. The mixtures were incubated for 1 h and then were combined with 90 μL of substrate and further incubated for 30 min. Following the addition of 50 μL of stop solution mixture, the color developed at 450 nm was measured, and the CAT values were calculated and expressed in picogram per milliliter.

2.8. Superoxide dismutase assay

Superoxide dismutase (SOD) was analyzed using a quantitative enzyme immunoassay technique at PHC Diagnostics, in Lahore, Pakistan. Microplates, previously coated with a specific anti-SOD antibody, were filled with 100 μL of samples and standards. After cleaning, a 100 μL biotin-conjugated antibody treatment and another 100 μL avidin-conjugated horseradish peroxidase treatment was administered. A 90 μL substrate solution was then added to each well, and they were incubated for approximately 30 min before adding a stop solution. The color strength was evaluated at 450 nm. SOD was then calculated using the curve, and the findings were expressed in pg/mL.

2.9. Glutathione peroxidase assay

Glutathione peroxidase (GSH-Px) analysis was conducted using an enzyme immunoassay methodology (Cusabio Technology LLC, USA) at PHC Diagnostics, in Lahore, Pakistan. Microplates coated with an antibody specific to GSH-Px were filled with 100 μL of standards and samples in each case. The next step was to apply 100 μL of the biotin-conjugated antibody, wash the area, and then apply 100 μL of the avidin-conjugated horseradish peroxidase. A 90 μL substrate solution was added to each well and allowed to incubate for

about 30 min before a stop solution was added. The SOD was calculated using the curve from the color's intensity measured at 450 nm and expressed as pg/mL.

2.10. Total glutathione contents

The total glutathione content (reduced) of the liver was extracted using 100 mg of pulverized liver samples, 2 mL of pH 7.5 buffer (made of sodium phosphate dibasic heptahydrate and sodium phosphate monobasic monohydrate), and 8 mL of 3 % phosphoric acid. After shaking for 30 min, 0.5 μL of the sample was combined with 1500 μL of buffer solution. Then, 0.03 mL of dithio-nitrobenzene was added, and the mixture was incubated at 37 °C for 2 min. The instrument used for measurement was a Shimadzu UV–visible spectrophotometer (Japan). Absorbance was measured at 412 nm relative to a reference blank. The resulting amount was expressed as mmol/g [27].

2.11. Glutathione-S-Transferase assay

The activity of glutathione S-transferase (GST) in the liver was also examined. A very specific hydrolyzing enzyme called glutathione transferase is used to detoxify glutathione conjugates with various stressors or carcinogens. A 1-chloro-2,4-dinitrobenzene (CDNB) reagent at a concentration of 1 mmol/L was used to test the activity of GST. The extract (100 μL) made in ethanol and buffer was combined with 700 μL of potassium phosphate buffer (pH 7.5, made of sodium phosphate dibasic heptahydrate and sodium phosphate monobasic monohydrate), 100 μL of GSH, and 100 μL of CDNB [25]. The enzyme activity was expressed as unit/g of liver.

2.12. Lipid peroxidation

Hepatic lipid peroxidation was measured as thiobarbituric acid reactive substances (TBARS) as per the established spectrophotometric protocol in our laboratory (Zeb and Ullah, 2016).

2.13. Measurement of hepatic inflammatory markers

2.13.1. Total RNA isolation and cDNA synthesis

Hepatic inflammatory markers were studied at PHC Diagnostics, in Lahore, Pakistan. The Magen Biotechnology HiPure Total RNA Kit (Catalogue No. IVD4121) was used to extract total RNA from tissue samples. Thermo Fisher's cDNA kit (HRP013 100T) from ZOKEYO, China was used to reverse transcribe mRNA samples into cDNA. The SYBR Select Master Mix was then used with cDNA as a template and the matching duplicate primers (see supplementary file, Table S1) to perform real-time expression of each of the targeted genes (IL-1, IL-4, IL-6, TNF-α, PPAR-α, and PPAR-γ). The samples' pertinent Ct values were assessed about controls and control samples in terms of housekeeping genes.

2.13.2. Real-time qPCR

The RT-qPCR technique was performed using a 2× SYBR qPCR mixture (ZOKEYO, China) with a total reaction volume of 15 μL. The mixture consisted of cDNA (1 μL), SYBR Green mix (10 μL), and primers (0.5 μM each). Housekeeping genes (GAPDH) were used as a reference to measure the samples' pertinent Ct values. The amplification conditions consisted of 95 °C for 30 s and 40 cycles of 95 °C for 5s and 60 °C for 20s [28].

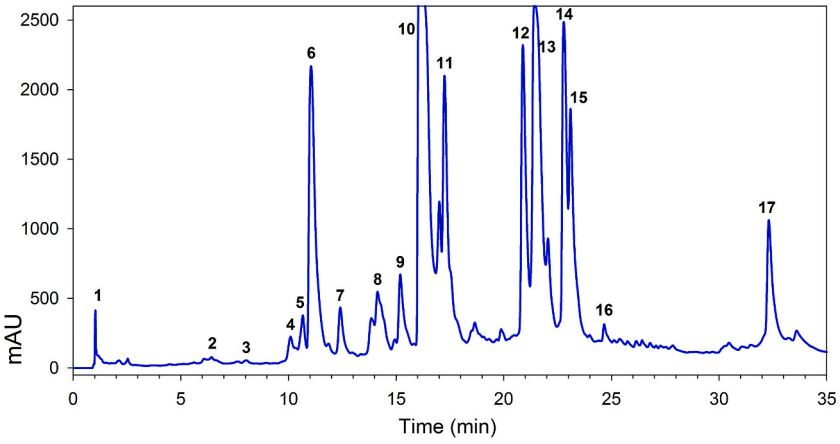

Fig. 1. HPLC-DAD chromatogram at 320 nm of the phenolic compounds of milk thistle. Each peak number represents an individual compound with characteristics shown in Table 1.

2.14. Statistical analysis

GraphPad Prism (version 10.2.2; Graph Pad Software, USA) was used for the analysis. To compare the results, a one-way ANOVA was conducted, with Dunn's multiple comparison tests. For gene expression analysis, an unpaired *t*-test was employed. The significance of the differences between the groups was determined at different p-values.

3. Results

3.1. Composition of extract and tallow

Fig. 1 displays a chromatogram of the composition of milk thistle using HPLC-DAD. As indicated in Table 1, nineteen compounds were identified and quantified (μg/g). The first component to elute was coumaroyl hexoside, which had a λ_{max} of 263 nm and an amount of 103.7 μg/g. The second compound was hydroxylated silibinin, which had a λ_{max} of 284 nm and the second-lowest concentration of 62.08 μg/g. This was followed by caffeic acid hexoside, which had the lowest amount at 56.45 μg/g. Daidzein-sulfate was the fourth compound to elute, with a concentration of 264.5 μg/g. Peak 5 had a concentration of 304.8 μg/g and was identified as silybin A. Proanthocyanidin B1 exhibited peak 6 at a concentration of 2480.0 μg/g. Taxifolin and isosilychristin, with quantities of 430.6 and 775.4 μg/g, respectively, constituted peaks 7 and 8. Peak 9 represented silychristin A, which had a concentration of 722.1 μg/g. Peak 10 showed the presence of silybin B, which had the highest quantity at 5233.1 μg/g. Silychristin B and silydianin, with concentrations of 804.9 and 2037.0 μg/g, were present in Peaks 11 and 12. Formononetin-glucuronic acid exhibited peak 13 with the second-highest concentration of 3921.9 μg/g and a λ_{max} of 300, 280 nm. With a concentration of 2017.6 μg/g and a λ_{max} of 359, 299, and 262 nm, myricetin hexoside constituted peak 14. Isosilybin A and B, with concentrations of 1951.8 and 132.3 μg/g, respectively, were the subjects of peaks 15 and 16. Petunidin-3-glucoside was the final compound, with a concentration of 1337.1 μg/g and a distinctive λ_{max} of 523, 276 nm. According to these findings, the top six compounds in decreasing order of quantity were above 1900 μg/g and were silybin B (23 %), formononetin-glucuronic acid (17 %), proanthocyanidin B1 (11 %), myricetin hexoside (9 %), silydianin (9 %), and isosilybin A (9 %). The entire amount of the extract was made up of 78 % of these six components.

The GC-MS profile of the unoxidized tallow showed fourteen compounds (Supplementary file, Table S2). These were ethyl cyclohexane (2.15 %), ethylbenzene (1.19 %), p-xylene (5.25 %), o-xylene (1.34 %), nonane (1.39 %), mesitylene (0.613 %), decane (4.507 %), undecane (0.739 %), dodecane (0.468 %), tridecane (0.418 %), n-hexadecanoic acid (0.723 %), oleic acid (1.16 %), bis(2-ethylhexyl) phthalate (80.7 %), and cholesterol (1.542 %). Thermally oxidized tallow contains ethyl cyclohexane (2.36 %), ethylbenzene (1.31 %), p-xylene (5.71 %), o-xylene (1.5 %), nonane (1.54 %), decane (1.22 %), undecane (0.499 %), dodecane (0.468 %), 2,3-dihydro-4-methoxy-6,7-methylenedioxyfuro (2,3-*b*)quinoline (1.38 %), and bis(2-ethylhexyl) phthalate (83.8 %).

3.2. Effects on serum lipid profile

The serum cholesterol concentrations of the untreated group were 107.0 and 119.7 mg/dL after 7 and 14 days of treatments, respectively. The MT group had serum total cholesterol concentrations of 71.66 and 77.00 mg/dL, after 7 and 14 days of treatments, respectively. These values were similar to the serum cholesterol concentrations of the control group. In the OT and NT groups, blood cholesterol levels significantly increased ($p < 0.05$) when compared to both 7- and 14-day levels and control groups. The OT + MT1 group had higher total cholesterol levels than the control group. The blood cholesterol levels of the OT + MT3 were notably lower ($p < 0.05$) than those of the OT + MT2 group. These levels were 84.33 and 85.33 mg/dL, after 7- and 14-day levels, respectively (Table 2).

Table 1
Characteristics, and composition of phenolic compounds in milk thistle.

Peak	Rt (min)	Identity	λ_{max} (nm)	Quantity (μg/g)[a]
1	1	Coumaroyl hexoside	263	103.7 ± 0.7
2	6.1	Hydroxylated silibinin	284	62.08 ± 1.1
3	8.1	Caffeic acid hexoside	328, 287sh	56.45 ± 1.1
4	10	Daidzein-sulfate	308	264.5 ± 3.3
5	10.7	Silybin A	288	304.8 ± 3.2
6	11.1	Proanthocyanidin B1	306, 280	2480 ± 5.7
7	12.4	Taxifolin	290	430.6 ± 2.7
8	14.1	Isosilychristin	290	775.4 ± 3.7
9	15.1	Silychristin A	288	722.1 ± 4.3
10	16.2	Silybin B	289	5233.1 ± 9.3
11	17.2	Silychristin B	288	804.9 ± 1.9
12	20.9	Silydianin	288	2037 ± 2.1
13	21.4	Formononetin-glucuronic acid	300, 280	3921.9 ± 4.5
14	22.8	Myricetin hexoside	359, 299, 262	2017.6 ± 1.3
15	23.1	Isosilybin A	288	1951.8 ± 8.3
16	24.6	Isosilybin B	288	132.3 ± 3.1
17	32.3	Petunidin-3-glucoside	523, 276	1337.1 ± 6.8

[a] Values are means with a standard deviation of the triplicate independent measurements.

The total triacylglycerols (TGs) of control animals were 34.33 and 26.33 mg/dL, for 7- and 14-day treatments, respectively. The only MT-treated group's TGs, which were 38.66 and 25.97 mg/dL, respectively, corresponding to 7 and 14 days of dosing, did not differ significantly ($p < 0.05$) from the control group. Comparing OT and NT groups to the untreated group, the TGs rose significantly ($p < 0.05$). The OT + MT1 group showed an increase in comparison to the control, whereas the OT + MT2 group showed a highly significant ($p < 0.05$) decrease in TG over the OT group.

The serum HDL-cholesterol concentrations in the control group were 52.66 and 55.33 mg/dL, corresponding to 7 and 14-day treatments, respectively. The MT treatment alone showed a small elevation in serum HDL-cholesterol in comparison to the control group; however, the groups given OT + MTl, OT + MT2, and OT + MT3 showed significant increases. In the OT group, there was no discernible decline in serum HDL cholesterol.

The blood LDL-cholesterol values of the control group were 21.00 and 43.66 mg/dL, corresponding to 7 and 14-day treatments, respectively. The blood LDL-cholesterol levels did not significantly differ ($p < 0.05$) between the group that received only MT and the control group. A significant ($p < 0.05$) rise in the OT group in comparison to the control group was observed. After 7 and 14 days of treatments, the OT + MT1 group's serum LDL-cholesterol levels were significantly ($p < 0.05$) lower than those of the control group. The OT + MT2 and OT + MT3 groups also exhibited a significant ($p < 0.05$) reduction in LDL-cholesterol. The reduction was more pronounced at 14 days of treatments.

3.3. Effects on hematology

The hemoglobin levels in the control group were 15.13 and 15.50 g/dL, respectively, corresponding to 7 and 14-day treatments. The MT group did not show a significant ($p < 0.05$) change from the control group; however, the OT group did show a significant ($p < 0.05$) decline. In contrast to the OT group, the OT + MT2 and OT + MT3 groups showed an increase in hemoglobin levels as shown in Table 3.

The red blood cells (RBC) of the control groups had 5.30 and 5.66 ($\times 10^6$ cells/μL) values after 7 and 14 days, respectively. There was no significant ($p < 0.05$) difference in the MT and control values. There was a significant ($p < 0.05$) increase in the RBC in comparison to the 7- and 14-day doses when comparing the OT + MT1, OT + MT2, and OT + MT3 groups to the OT group.

Following 7 and 14 days of treatments, the while blood cells (WBC) of the control group was 5.46 and 6.70 ($\times 10^6$ cells/μL). There was no significant difference in the WBC of the control and MT groups. The WBC levels in the OT and NT groups were found to be considerably ($p < 0.05$) higher than those in the control group. However, the rise in the OT + MT1 group was not statistically significant in comparison to the groups that were only given milk thistle (MT) treatment and the control group. There was no discernible difference between the OT + MT2 and OT + MT3ml groups and the control group.

The platelet count within the control group was 39.35 and 38.83 ($\times 10^3$ cells/μL), corresponding to treatments for seven and fourteen days, respectively. The elevation in blood platelets in the OT and NT during the 7 and 14-day treatments was statistically significant ($p < 0.05$) compared to the control. The OT + MT1, OT + MT2, and OT + MT3 groups illustrated a substantial ($p < 0.05$) drop in comparison to the OT group.

With mean corpuscular hemoglobin (MCH) values of 26.53 and 29.56 pg/cell, respectively, corresponding to 7 and 14-day investigations, the untreated group's MCH was quite close to that of the MT group. There was no significant difference OT + MT1, OT + MT2, and OT + MT3 groups, and a significant decrease when comparing the OT and NT groups to the control group. The mean corpuscular volume (MCV) and hematocrit values were found to be comparable.

3.4. Effects on liver histology

The liver sections of the rabbits on a regular diet showed typical morphology with acceptable microvascular contents of fat (Fig. 2A), in contrast to the rabbits fed the oxidized tallow and unoxidized tallow groups, whose liver sections primarily had

Table 2
Effects of MT extract on serum lipid profile at 7 and 14 days of treatments.

Group	Total cholesterol (mg/dL)		Triglycerides (mg/dL)		HDL-cholesterol (mg/dL)		LDL-cholesterol (mg/dL)	
	Treatment Days		Treatment Days		Treatment Days		Treatment Days	
	7	14	7	14	7	14	7	14
Control	107.0 ± 2.8a	119.7 ± 2.0a	34.3 ± 2.8a	26.3 ± 3.7a	55.3 ± 2.0a	52.7 ± 2.0a	43.6 ± 3.2a	21.3 ± 3.6a
NT	180.7 ± 2.0b	190.4 ± 1.5b	102.3 ± 2.9b	107.7 ± 6.1b	54.3 ± 1.0a	45.3 ± 0.3b	106.0 ± 2.3b	116.3 ± 4.9b
OT	216.7 ± 1.4c	226.7 ± 1.8c	129.1 ± 3.9c	149.5 ± 7.2c	26.7 ± 4.0b	22.0 ± 3.7c	136.0 ± 5.5c	137.6 ± 7.7c
MT	71.7 ± 2.3d	77.0 ± 1.5d	38.4 ± 2.2d	25.9 ± 3.9d	65.6 ± 3.0c	67.4 ± 1.0d	17.6 ± 1.8d	21.3 ± 5.0d
OT + MT1	94.3 ± 5.0e	101.3 ± 2.5e	99.4 ± 7.1e	84.7 ± 5.1e	71.0 ± 4.0d	74.4 ± 2.0e	36.8 ± 0.08e	29.7 ± 5.0e
OT + MT2	87.3 ± 2.6e	95.3 ± 3.9e	74.5 ± 3.5f	65.5 ± 2.7f	76.5 ± 1.1d	81.4 ± 1.3f	31.8 ± 1.2f	25.4 ± 1.0e
OT + MT3	84.3 ± 3.2e	85.3 ± 5.0e	52.7 ± 6.8g	41.5 ± 1.4g	87.5 ± 2.5e	89.2 ± 3.3g	28.3 ± 2.8g	21.3 ± 3.6e

*Values are means with a standard deviation of the triplicate independent measurements. Abbreviations: HDL, high density lipoproteins; LDL, low density lipoproteins; NT, none oxidized tallow; OT, oxidized tallow; MT, milk thistle; OT + MT1, oxidized tallow 2 g/kg with milk thistle 100 mg; OT + MT2, oxidized tallow 2 g/kg with milk thistle 200 mg; and OT + MT3, oxidized tallow 2 g/kg with milk thistle 300 mg. Different letters (a-g) in the column represent significant at $p < 0.05$, Dunnet test.

Table 3
Effects of MT on the hematological parameters of rabbits.

Group	Hb (g/dL)		RBC,s (cells/μL) × 10^6		WBC,s (cells/μL) × 10^3		Platelets (cells/μL) × 10^3		MCH (pg/cell)		HCT (%)		MCV (fL/cell)	
	Treatment Days		Treatment Days		Treatment Days		Treatment Days		Treatment Days		Treatment Days		Treatment Days	
	7	14	7	14	7	14	7	14	7	14	7	14	7	14
Control	15.1 ± 1.2a	15.5 ± 3.4a	5.3a	5.66a	5.46a	6.70a	39.3a	38.8a	26.5 ± 3.7a	29.5 ± 3.0a	40.4 ± 4.3a	44.5 ± 4.8a	67.0 ± 2.3a	67.9 ± 2.2a
NT	10.5 ± 1.3b	9.43 ± 1.7b	3.8b	3.85b	14.1b	14.3b	51.5b	58.0b	17.9 ± 2.7b	16.1 ± 1.2b	23.3 ± 3.7b	22.1 ± 1.1b	52.9 ± 5.3b	52.0 ± 4.2b
OT	6.36 ± 0.1c	5.66 ± 0.8c	2.87c	2.53c	16.6c	17.4c	76.8c	85.4c	13.6 ± 1.6c	12.3 ± 2.1c	17.7 ± 4.4c	16.5 ± 0.3c	45.5 ± 3.2c	43.0 ± 7.8c
MT	15.9 ± 2.4a	15.7 ± 1.3a	5.45a	5.33a	4.15d	4.30d	40.5a	39.0a	26.1 ± 2.5a	26.4 ± 2.4a	23.7 ± 1.0b	27.4 ± 2.0d	73.6 ± 2.7d	71.5 ± 3.7a
OT + MT1	14.4 ± 1.8a	14.6 ± 3.5a	4.46d	4.52a	6.30e	8.26e	46.8d	47.7d	20.5 ± 3.4b	24.5 ± 4.1a	33.6 ± 6.6d	39.5 ± 0.09e	60.3 ± 4.4a	68.6 ± 3.7a
OT + MT2	15.9 ± 1.3a	15.8 ± 2.3a	5.63a	5.68a	5.30a	7.26a	42.7a	43.7e	21.3 ± 2.3b	26.5 ± 2.3a	36.0 ± 4.8d	43.5 ± 5.96a	62.7 ± 2.4a	71.5 ± 7.6a
OT + MT3	15.8 ± 3.3a	18.1 ± 2.0a	7.39e	7.49b	4.70a	6.26a	38.4a	39.4a	23.5 ± 3.5b	28.7 ± 3.2a	40.6 ± 2.6a	47.6 ± 3.3a	64.5 ± 3.5a	72.5 ± 3.7a

*Values are means with a standard deviation of the triplicate independent measurements. Abbreviations: Hb, hemoglobin; RBC, red blood cells; WBC, white blood cells; MCH, mean corpuscular hemoglobin; HCT, hematocrit; MCV, mean corpuscular volume; NT, none oxidized tallow; OT, oxidized tallow; MT, milk thistle; OT + MT1, oxidized tallow 2 g/kg with milk thistle 100 mg; OT + MT2, oxidized tallow 2 g/kg with milk thistle 200 mg; and OT + MT3, oxidized tallow 2 g/kg with milk thistle 300 mg. Different letters (a-e) in the column represent significant at $p < 0.05$, Dunnet test.

macrovascular fat contents (Fig. 2B–C). Both the oxidized and unoxidized groups exhibited necrotic features. The MT group had a similar histology to that of the control group as shown in Fig. 2D. The treatment MT in combination with OT resulted in recovery of lipid accumulation and cellular alteration in the liver of rabbits as shown in Fig. 2E and F. Thus, the MT supplementation was effective in recovering the structure of the liver.

3.5. *Effects on liver antioxidant status*

Fig. 3 showed that CAT significantly decreased in the livers of the NT and OT groups as compared to the control and enhanced significantly in the MT group. The activity of CAT in the liver tissue enhanced significantly in dose dose-dependent manner in OT + MT1, OT + MT2, and OT + MT3 groups reaching the highest CAT concentrations (132.26 pg/mL). Likewise, the livers of the OT group had significantly lower SOD levels than the control group ($p < 0.001$). When compared to the OT and control groups, the MT groups had lower SOD, and treatment groups had higher SOD and reached significantly higher in the OT + MT3 group. Glutathione peroxidase (GSH-Px) was significantly reduced in NT and OT as compared to control. The MT has the highest GSH-Px both alone or when given in combination with OT. The GSH contents were reduced significantly in the NT and OT groups as compared to the control and enhanced with supplementation of MT either alone or in combination with OT. There was no significant difference in the GSH contents of the

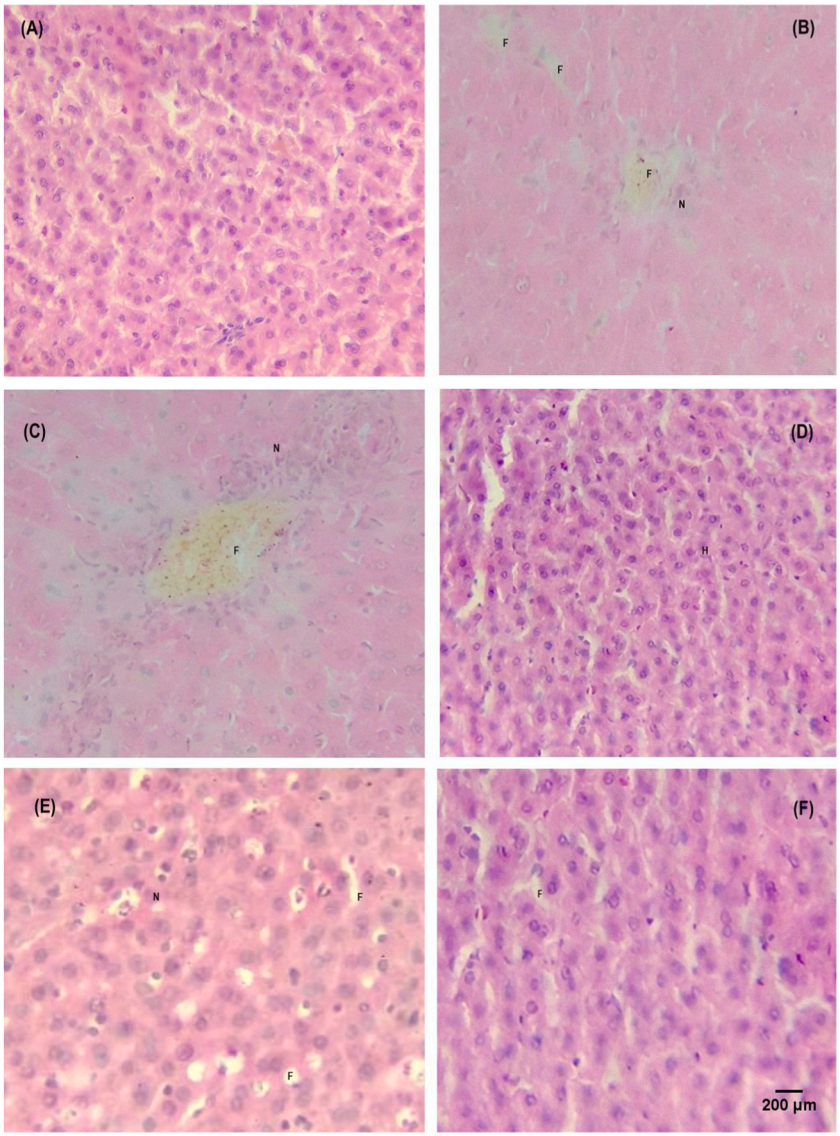

Fig. 2. Effects of MT extract on rabbit's liver histopathology. (A) control, (B) NT, (C) OT, (D) MT, (E) OT + MT1, and (F) OT + MT3. Abbreviations: hepatocyte (H), fats (F), necrosis (N), NT, none oxidized tallow; OT, oxidized tallow; MT, milk thistle; OT + MT1, oxidized tallow (2 g/kg) with MT (100 mg); OT + MT2, oxidized tallow (2 g/kg) with MT (200 mg); and OT + MT3, oxidized tallow (2 g/kg) with MT (300 mg).

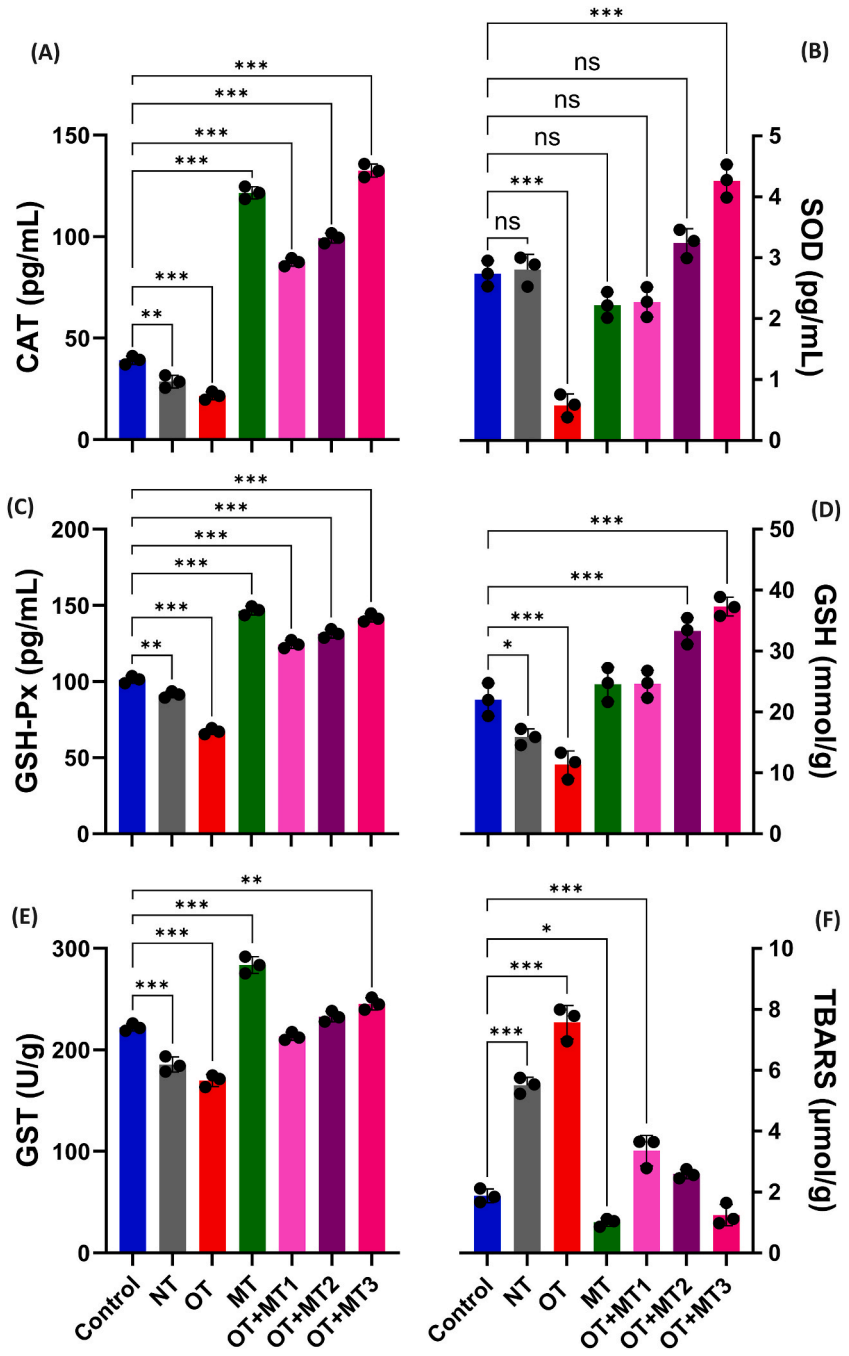

Fig. 3. Effect of MT extract on the liver antioxidant status of rabbits. (A) Catalase, (B) superoxide dismutase, (C) glutathione peroxidase, (D) total reduced glutathione, (E) glutathione-S-transferase, (F) thiobarbituric acid reactive substances. Data are displayed as the mean with a standard deviation of replicates (n = 3). Non-significant are not shown, *** <0.001 versus control in each treatment using Dunnett's multiple comparison tests.

control, MT, and OT + MT1 groups. The GST contents significantly declined in the NT and OT groups as compared to the control. Supplementation of MT alone significantly enhanced GST contents, while the increase was also significant in OT + MT groups. There was no significant difference between the control, OT + MT1, and OT + MT2 groups showing beneficial effects of MT.

3.6. Effects on liver inflammation

The expression of pro-inflammatory cytokines TNF-α as well as IL-1, IL-4, and IL-6 in liver tissues were determined. With reference

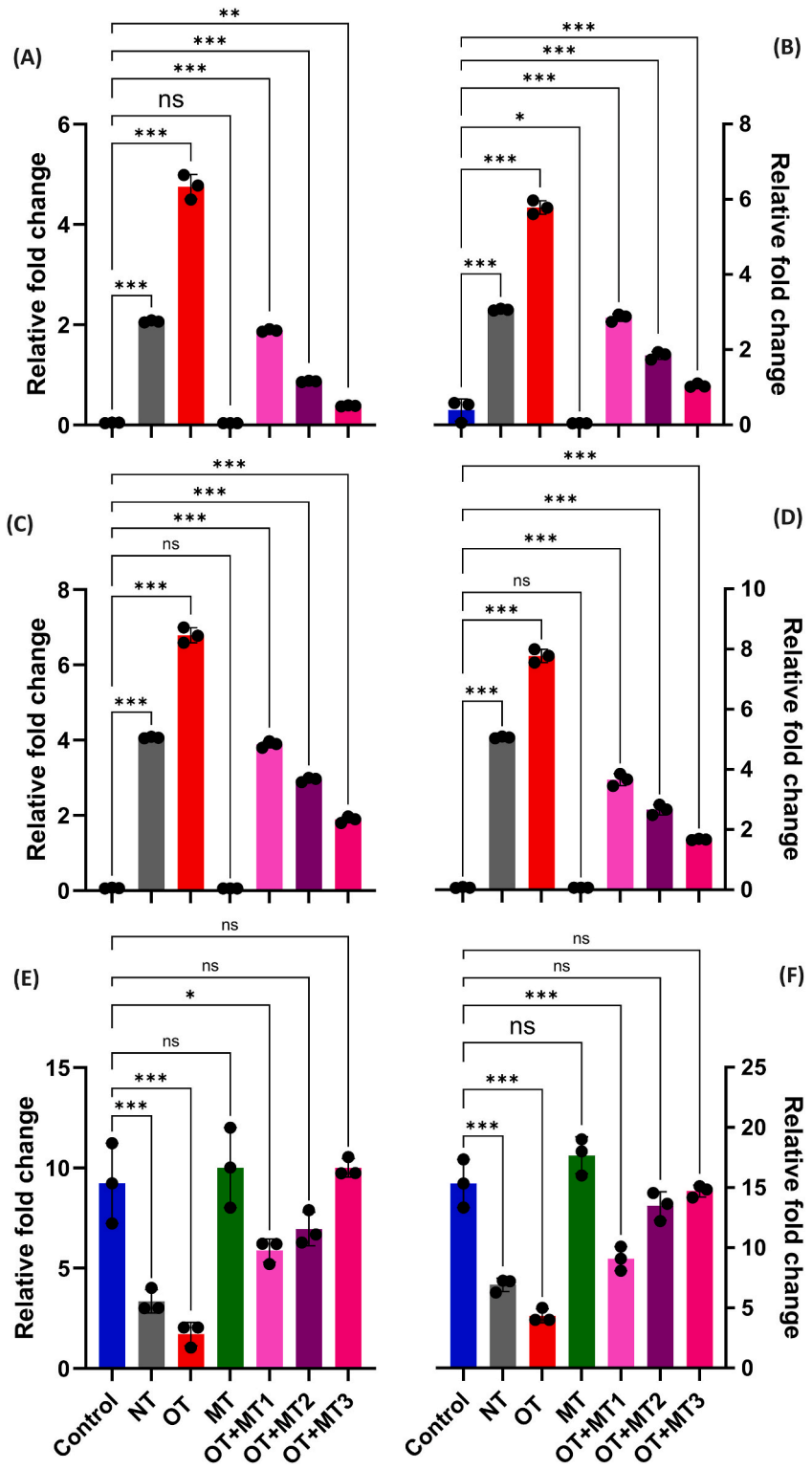

Fig. 4. Effect of MT extract on relative fold change in expression of pro- and anti-inflammatory markers in the liver of rabbits. (A) IL-1, (B) IL-4, (C) IL-6, (D) TNF-α, (E) PPAR-α and (F) PPAR-γ. Data displayed as mean with a standard deviation of replicates (n = 3), ns = nonsignificant, *0.033, **0.002, *** < 0.001 versus control in each treatment using Dunnett's multiple comparison tests. Abbreviations: NT, none oxidized tallow; OT, oxidized tallow; MT, milk thistle; OT + MT1, oxidized tallow (2 g/kg) with MT (100 mg); OT + MT2, oxidized tallow (2 g/kg) with MT (200 mg); and OT + MT3, oxidized tallow (2 g/kg) with MT (300 mg).

to the housekeeping gene (GADPH) and control gene, the relative fold change was used to determine the expression of these genes in the treated groups. The results demonstrated that, in contrast to the OT + MT1, OT + MT2, and OT + MT3 rabbit groups, these gene levels were upregulated in the OT-fed rabbits group, followed by NT. The levels of IL-1, IL-4, and IL-6 in the OT-fed rabbits were considerably higher ($p > 0.05$) than in the control group (Fig. 4). The expressions of IL-1 (Fig. 4A), IL-4 (Fig. 4B), and IL-6 (Fig. 4C) were significantly down-regulated in the OT + MT1, OT + MT2, and OT + MT3 treated groups in dose-dependent manner. Similarly, when compared to the OT group, the expression of TNF-α (Fig. 4D), was significantly up-regulated in the OT group and down-regulated in treated groups.

In contrast, the peroxisome proliferator-activated receptor-alpha (PPAR-α) expression significantly decreased in the livers of the NT and OT group as compared to the control group (Fig. 4E). The MT treatment alone or in combination with OT significantly increased the expression back to normal levels. There were no significant differences in PPAR-α fold change values among the control, MT, OT + MT1, and OT + MT2 groups, suggesting the beneficial role of MT. Similarly, PPAR-γ was downregulated in NT and OT groups and up-regulated in MT and OT + MT1, OT + MT2, and OT + MT3 treated groups (Fig. 4F). These findings suggested that MT treatment protected the liver from inflammation brought on by normal or oxidized tallow.

4. Discussion

The most prevalent liver disease globally is NAFLD. Research has shown that consuming beef tallow, vegetable ghee, and animal ghee can cause NAFLD in animals [29,30]. When tallow is heated, it becomes oxidized and forms more toxic compounds, which significantly contribute to the development of NAFLD [31]. The process of preparing tallow from the animal tissues and resulting thermal oxidation also lead to the formation of various aromatic compounds that can impact the flavor and quality of the food products [32]. Tallow has been found to contain hydrocarbons like decane, dodecane, tridecane, and undecane, as well as aldehydes, ketones, alcohols, nitrogen, or sulfur compounds, esters, acids, ethers, and phenols [33]. Some of these compounds have been identified in our research, suggesting that tallow is a good source of them. The induction of NAFLD in animal models through the consumption of tallow has been the focus of ongoing research. The primary approach to treating NAFLD is lifestyle modification, with numerous studies evaluating the effectiveness of weight loss, specific diets, and regular physical activity. However, there is divided opinion on the uses of pharmacological treatments [34]. Nevertheless, it is considered the most effective way to mitigate NAFLD. Different natural remedies, including milk thistle, are therefore used.

The lipophilic extract of MT derived from plant fruit has an active component called silymarin. Silymarin is made up of isomer flavonolignans, including silibinin, isosilibin, silidianin, and silichristin. In this study, 17 compounds were identified in the extract of MT. Silybin B, formononetin-glucuronic acid, myricetin hexoside, silydianin, and isosilybin A were the major compounds. Standardized MT has been shown to contain silibinin, silichristin, and silidianin as an active ingredient [35]. Romanian and German commercial samples of MT have been reported to contain myricetin, silybin A, and B [36]. Chinese samples of MT seeds have been found to contain taxifolin, silychristin, silydianin, isosilybin A and B, and silybin A as major compounds [37]. Therefore, the whole fruit extract of MT contains a group of compounds known as silymarin.

We have demonstrated that supplementing with MT extract significantly reduces body fat in rabbits fed with OT, while also altering serum lipid profiles, including TG, HDL, and LDL. The administration of MT decreases plasma levels of cholesterol and LDL-cholesterol in OT-fed rabbits, but it does not lower plasma cholesterol levels in normal rabbits. However, it does reduce phospholipids, particularly those carried in LDL, as previously reported [38]. The main mechanism behind the lipid-lowering effect may presence of important phenolic compounds in MT, which help to lower lipids. In rats with diet-induced hypercholesterolemia, silymarin from MT has been shown to have an anticholesterolemic effect, as evidenced by increased levels of HDL cholesterol and a decrease in total and biliary cholesterol [39]. Additionally, another study has revealed that MT may be beneficial for individuals at risk of diabetes and high cholesterol due to its antioxidant properties [40]. In conclusion, MT plays a hepatoprotective and hypolipidemic role in experimental animals.

In the present study, cellular antioxidant enzymes such as CAT, SOD, GSH-Px, TBARS, and GST as well as antioxidant GSH were found to be increased by the supplementation of MT extract. Previous research has shown that the flavonoids present in MT have strong antioxidant properties [41]. These strong antioxidant effects were demonstrated by increases in superoxide anions and lipid oxygen radicals induced by lipid peroxidation [42]. Studies have also shown that MT's *in-vivo* antioxidant activity increased glutathione levels, which is an important antioxidant that helps detoxify hormones, medications, and toxins. MT has the potential to act as both an antioxidant and a cholesterol-lowering agent, as it has been shown to boost superoxide dismutase in cell cultures [43]. It has been observed that oxidative imbalance, DNA damage, and enzyme impairment all contribute to the decline in SOD and CAT activities. Previous reports have indicated that oxidative stress develops in the tissues of rabbits administered OT. This chronic oxidative stress leads to lipid peroxidation and damage to liver tissue [44]. In conclusion, supplementation of MT extract can help mitigate the oxidative stress caused by a high-fat diet such as tallow and thermally oxidized tallow.

Our results indicated that the reduction in Hb and RBC levels was induced by feeding oxidized tallow; however, supplementing with MT promotes recovery. Compared to the control and MT groups, the WBC as well as platelet counts of the OT-treated group were considerably higher. This shows that supplementing with oxidized tallows severely stresses these blood markers. The current results are consistent with the work of Al-Quraishy et al. [45], which shows that lipid oxidation causes a decrease in hemoglobin concentration, while our WBCs findings are in agreement with Cam et al. [46], who suggested that the severity concerning the infection in the liver may be the cause of the drop in hemoglobin and rise in leucocyte counts. These results suggest that MT can withstand oxidative stress caused by oxidized lipids while maintaining normal hematological parameters.

Owing to its abundant supply of fat, the liver is the organ most susceptible to lipotoxicity. This is because it absorbs massive

amounts of fat straight from the portal vein in addition to lipoproteins and circulating lipids [47]. Ingestion of oxidized tallow for two weeks caused considerable changes in the hepatic structure. The alterations in the liver included the presence of fat macro- and microvesicles, fatty contents, and cellular and nuclear disintegration that suggested a process of cell death in the presence of hepatic steatosis. As previously noted, MT has anti-inflammatory, anti-metastatic, and chemo- and radioprotective properties [48]. It also controls the imbalance between cell survival and death by interfering with the production of cell cycle regulators and proteins implicated in apoptosis. Additionally, anti-apoptotic and anti-inflammatory properties of MT have been used to treat rats' steatohepatitis (fatty liver) [49]. The current investigation showed that taking MT fruit extract effectively corrected the liver abnormalities as revealed by histopathological examinations of the liver. The extract from MT lessened the severity concerning non-alcoholic fatty liver.

In the current study, rabbits fed with OT showed significantly higher levels of expression of inflammatory cytokines (IL-1, IL-4, IL-6, and TNF-α). However, the supplementation of MT reduced these markers. These findings are consistent with those of Jiang et al. [50], who found that AKT1 protein expression decreased in the model group compared to the control group, but levels of IL-6, MAPK1, Caspase 3, p53, and VEGFA were higher. In contrast, the AKT1 protein was up-regulated in the low-dose silymarin group and down-regulated in the high-dose silymarin group, along with IL-6, MAPK1, Caspase 3, p53, and VEGFA proteins. These results suggest that silymarin may protect against NAFLD. The downregulation of inflammatory markers is caused by phenolic compounds and silymarin. The anti-inflammatory and anticarcinogenic effects of silymarin may be due to its suppression of transcription factor NF-κB, which regulates the expression of many genes associated with inflammation, cytoprotection, and carcinogenesis. However, the precise molecular mechanisms underlying these effects are unknown [51]. According to a report, silymarin may potentially work by altering the activity of molecules that regulate the biological cycle and mitogen-activated protein kinase [52].

Nuclear receptor peroxisome proliferator-activated receptor A (PPARα) is a therapeutic target for several metabolic diseases and demonstrates strong anti-inflammatory properties [53]. PPARα and PPARγ regulate several key enzymes in lipid metabolism. In this study, both PPARα and PPARγ were significantly downregulated in the NT and OT groups. However, MT alone improved the levels of these markers. In the OT + MT groups, the levels of these markers significantly increased in a dose-dependent manner suggesting a protective role. On the other hand, Orolin et al. [54] showed that silymarin was hypolipidemic in high cholesterol-fed animals but this effect was not mediated by PPARα. Our work, however, agrees with the study of Zhu et al. [55], who showed that HFD caused a decline in liver PPARα levels, which were enhanced by the oils from MT. Our findings are also in agreement with the study of Mohamed Kamel et al. [56], who showed that MT upregulated PPARγ expression and was protective in the liver. Therefore, a high-fat diet consisting of tallow or lard causes NAFLD, which can be ameliorated by MT extract activation of PPARα and PPARγ in the liver.

5. Conclusions

Milk thistle (MT) possesses potent antioxidant properties. This study demonstrates how MT can protect against the negative consequences induced by tallow. Consuming normal or oxidized tallow raises blood levels of triglycerides (TG), LDL cholesterol, and total cholesterol. In addition to liver degenerative effects, oxidized tallow also results in decreased RBC, Hb, and hematocrit levels as well as increased WBC and platelet counts. However, when tallow consumption was followed by MT supplementation, serum lipid profile, and hematological indices were normalized. Furthermore, the expression of pro-inflammatory markers like TNF-α, IL-1, IL-4, and IL-6 was significantly reduced with the consumption of MT. Therefore, it can be concluded that MT plays a unique role in repairing liver damage caused by both unoxidized and oxidized tallow. MT helps shield the liver from oxidative stress by activating antioxidant enzymes and suppressing pro-inflammatory markers, while also upregulating peroxisome proliferator-activated receptors.

Data availability

The data is included in article and supplementary file.

CRediT authorship contribution statement

Neelab: Jamil, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation. **Alam Zeb:** Writing – review & editing, Supervision, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Muhammad Jamil:** Investigation, Formal analysis.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e31445.

References

[1] A.J. McCullough, Pathophysiology of nonalcoholic steatohepatitis, J. Clin. Gastroenterol. 40 (2006) S17–S29.
[2] R. Loomba, L.S. Friedman, G.I. Shulman, Mechanisms and disease consequences of nonalcoholic fatty liver disease, Cell 184 (2021) 2537–2564.
[3] Z.M. Younossi, P. Golabi, J.M. Paik, A. Henry, C. Van Dongen, L. Henry, The global epidemiology of nonalcoholic fatty liver disease (NAFLD) and nonalcoholic steatohepatitis (NASH): a systematic review, Hepatology 77 (2023) 1335–1347.
[4] S. Le Garf, V. Nègre, R. Anty, P. Gual, Metabolic fatty liver disease in children: a growing public health problem, Biomedicines 9 (2021) 1915.
[5] B. Hildrum, A. Mykletun, T. Hole, K. Midthjell, A.A. Dahl, Age-specific prevalence of the metabolic syndrome defined by the international diabetes federation and the national cholesterol education program: the Norwegian HUNT 2 study, BMC Publ. Health 7 (2007) 220.
[6] C. Lorenzo, K. Williams, K.J. Hunt, S.M. Haffner, The national cholesterol education program–adult treatment panel III, international diabetes federation, and world health organization definitions of the metabolic syndrome as predictors of incident cardiovascular disease and diabetes, Diabetes Care 30 (2007) 8–13.
[7] A.T. Kraja, I.B. Borecki, K. North, W. Tang, R.H. Myers, P.N. Hopkins, D. Arnett, J. Corbett, A. Adelman, M.A. Province, Longitudinal and age trends of metabolic syndrome and its risk factors: the Family Heart Study, Nutr. Metabol. 3 (2006) 41.
[8] Z. Younossi, F. Tacke, M. Arrese, B. Chander Sharma, I. Mostafa, E. Bugianesi, V. Wai-Sun Wong, Y. Yilmaz, J. George, J. Fan, Global perspectives on nonalcoholic fatty liver disease and nonalcoholic steatohepatitis, Hepatology 69 (2019) 2672–2682.
[9] K. Pafili, M. Roden, Nonalcoholic fatty liver disease (NAFLD) from pathogenesis to treatment concepts in humans, Mol. Metabol. 50 (2021) 101122.
[10] C. Hoebinger, D. Rajcic, T. Hendrikx, Oxidized lipids: common immunogenic drivers of non-alcoholic fatty liver disease and atherosclerosis, Front. Cardiovasc. Med. 8 (2022) 824481.
[11] A. Zeb, S. Ullah, Sea buckthorn seed oil protects against the oxidative stress produced by thermally oxidized lipids, Food Chem. 186 (2015) 6–12.
[12] A. Zeb, S.U. Rahman, Protective effects of dietary glycine and glutamic acid toward the toxic effects of oxidized mustard oil in rabbits, Food Funct. 8 (2017) 429–436.
[13] R.K. Dhiman, Y.K. Chawla, Herbal medicines for liver diseases, Dig. Dis. Sci. 50 (2005) 1807–1812.
[14] I. Marmouzi, A. Bouyahya, S.M. Ezzat, M. El Jemli, M. Kharbach, The food plant *Silybum marianum* (L.) Gaertn.: phytochemistry, ethnopharmacology and clinical evidence, J. Ethnopharmacol. 265 (2021) 113303.
[15] L. Abenavoli, A.A. Izzo, N. Milić, C. Cicala, A. Santini, R. Capasso, Milk thistle (*Silybum marianum*): a concise overview on its chemistry, pharmacological, and nutraceutical uses in liver diseases, Phytother Res. 32 (2018) 2202–2213.
[16] A.A. Elateeq, Y. Sun, W. Nxumalo, A.M. Gabr, Biotechnological production of silymarin in *Silybum marianum* L.: a review, Biocatal. Agric. Biotechnol. 29 (2020) 101775.
[17] S. Drouet, E.A. Leclerc, L. Garros, D. Tungmunnithum, A. Kabra, B.H. Abbasi, É. Lainé, C. Hano, A green ultrasound-assisted extraction optimization of the natural antioxidant and anti-aging flavonolignans from milk thistle *Silybum marianum* (L.) gaertn. fruits for cosmetic applications, Antioxidants 8 (2019) 304.
[18] A. Gillessen, H.H.-J. Schmidt, Silymarin as supportive treatment in liver diseases: a narrative review, Adv. Ther. 37 (2020) 1279–1301.
[19] B. Feng, R. Meng, B. Huang, Y. Bi, S.M. Shen, D.L. Zhu, Silymarin protects against renal injury through normalization of lipid metabolism and mitochondrial biogenesis in high fat-fed mice, Free Radical Biol. Med. 110 (2017) 240–249.
[20] Z. Malekpour-Dehkordi, M. Nourbakhsh, M. Shahidi, N. Sarraf, R. Sharifi, Silymarin diminishes oleic acid-induced lipid accumulation in HepG2 cells by modulating the expression of endoplasmic reticulum stress markers, J. Herb. Med. 33 (2022) 100565.
[21] A.C. Ferraz, L.T. Almeida, C.C. da Silva Caetano, M.B. da Silva Menegatto, R.L. Souza Lima, J.P.N. de Senna, J.M. de Oliveira Cardoso, L.O. Perucci, A. Talvani, W. Geraldo de Lima, B. de Mello Silva, A. Barbosa Reis, J.C. de Magalhães, C. Lopes de Brito Magalhães, Hepatoprotective, antioxidant, anti-inflammatory, and antiviral activities of silymarin against mayaro virus infection, Antivir. Res. 194 (2021) 105168.
[22] A. Zeb, W. ur Rahman, Effects of oxidized tallow on the rabbit serum lipids and antioxidant activity of the in-vitro lipids, Toxicol. Res. 28 (2012) 151–157.
[23] N. Khan, R. Ullah, K. Ali, D.A. Jones, M.E.H. Khan, Invasive milk thistle (*Silybum marianum* (L.) Gaertn.) causes habitat homogenization and affects the spatial distribution of vegetation in the semi-arid regions of northern Pakistan, Agriculture 12 (2022) 687.
[24] A. Zeb, A reversed phase HPLC-DAD method for the determination of phenolic compounds in plant leaves, Anal. Methods 7 (2015) 7753–7757.
[25] A. Zeb, A. Akbar, Ellagic acid suppresses the oxidative stress induced by dietary-oxidized tallow, Oxid. Med. Cell. Longev. 2018 (2018) 7408370.
[26] M. Shoaib, A. Saleem, A. Zeb, M.I. Khan, M.F. Akhtar, Chemical characterization and ameliorating effect of *Centratherum anthelminticum* extract against polycystic ovary syndrome in wistar rats, Internet J. Endocrinol. 2023 (2023) 4978562.
[27] D. Naz, A. Zeb, N. Nazir, R. Ullah, A.U. Rahman, A. Muhammad, Hepatoprotective and nephroprotective effects of *Sedum adenotrichum* in paracetamol-induced hepatotoxicity in rabbits, 3 Biotech 13 (2023) 217.
[28] P. Darabi, H. Khazali, M. Mehrabani Natanzi, Therapeutic potentials of the natural plant flavonoid apigenin in polycystic ovary syndrome in rat model: via modulation of pro-inflammatory cytokines and antioxidant activity, Gynecol. Endocrinol. 36 (2020) 582–587.
[29] M. Rahmadi, A.D. Nurhan, E.D. Pratiwi, D.A. Prameswari, S.M. Panggono, K. Nisak, J. Khotib, The effect of various high-fat diet on liver histology in the development of NAFLD models in mice, J. Basic Clin. Physiol. Pharmacol. 32 (2021) 547–553.
[30] M.N. Al-Hayder, R.S. Al-Mayyahi, A.S. Abdul-Razak, Effects of sunflower oils and beef tallow on serum parameters and liver histopathology in experimental rats, Obes. Med. 18 (2020) 100232.
[31] L.P. Barreto, R.Y. de Oliveira Silva, B.B. de Almeida, P.P. Ovídio, A.A. Jordao, Metabolic Effects of coconut oil on fatty liver and oxidative stress induced by a high-fat diet in rats, Gene Expr. 22 (2023) 10–18.
[32] S. Song, F. Zheng, X. Tian, T. Feng, L. Yao, M. Sun, L. Shi, Evolution analysis of free fatty acids and aroma-active compounds during tallow oxidation, Molecules 27 (2022) 352.
[33] J. Wang, L. Chen, Y. Liu, T.M. Olajide, Y. Jiang, W. Cao, Identification of key aroma-active compounds in beef tallow varieties using flash GC electronic nose and GC × GC-TOF/MS, Eur. Food Res. Technol. 248 (2022) 1733–1747.
[34] L. Abenavoli, N. Milic, Dietary intervention in non-alcoholic fatty liver disease, J. Acad. Nutr. Diet. 113 (2013) 211.
[35] L. Abenavoli, R. Capasso, N. Milic, F. Capasso, Milk thistle in liver diseases: past, present, future, Phytother Res. 24 (2010) 1423–1432.
[36] A.C. Raclariu-Manolica, Q. Mauvisseau, R. Paranaiba, H.J. De Boer, C. Socaciu, Authentication of milk thistle commercial products using UHPLC-QTOF-ESI plus MS metabolomics and DNA metabarcoding, BMC Complement. Med. Ther. 23 (2023) 257.
[37] S. Mukhtar, X.X. Zeng, W. Khalid, A. Moreno, J.M. Lorenzo, In vitro antioxidant capacity of purified bioactive compounds in milk thistle seed (*Silybum marianum*) along with phenolic profile, Food Anal. Methods 16 (2023) 651–663.
[38] N. Škottová, V. Krečman, Silymarin as a potential hypocholesterolaemic drug, Physiol. Res. 47 (1998) 1–7.
[39] V. Krečman, N. Škottová, D. Walterova, J. Ulrichová, V. Šimánek, Silymarin inhibits the development of diet-induced hypercholesterolemia in rats, Planta Med. 64 (1998) 138–142.
[40] N. Suksomboon, N. Poolsup, S. Boonkaew, C.C. Suthisisang, Meta-analysis of the effect of herbal supplement on glycemic control in type 2 diabetes, J. Ethnopharmacol. 137 (2011) 1328–1333.

[41] S.I. Ramadan, M. Shalaby, N. Afifi, H. El-Banna, Hepatoprotective and antioxidant effects of *Silybum marianum* plant in rats, Agro. Veter, Med. Sci. 5 (2011) 541–547.
[42] E. Shaker, H. Mahmoud, S. Mnaa, Silymarin, the antioxidant component and *Silybum marianum* extracts prevent liver damage, Food Chem. Toxicol. 48 (2010) 803–806.
[43] G. Müzes, G. Deak, I. Lang, K. Nekam, P. Gergely, J. Feher, Effect of the bioflavonoid silymarin on the in vitro activity and expression of superoxide dismutase (SOD) enzyme, Acta Physiol. Hung. 78 (1991) 3–9.
[44] R. Zhu, Y. Wang, L. Zhang, Q. Guo, Oxidative stress and liver disease, Hepatol, Res. 42 (2012) 741–749.
[45] S. Al-Quraishy, M.S. Metwaly, M.A. Dkhil, A.-A.S. Abdel-Baki, F. Wunderlich, Liver response of rabbits to Eimeria coecicola infections, Parasit, Res. 110 (2012) 901–911.
[46] Y. Çam, A. Atasever, G. Eraslan, M. Kibar, Ö. Atalay, L. Beyaz, A. İnci, B.C. Liman, Eimeria stiedae: experimental infection in rabbits and the effect of treatment with toltrazuril and ivermectin, Exp. Parasitol. 119 (2008) 164–172.
[47] S.A. Vielma, R.L. Klein, C.A. Levingston, M.R. Young, Adipocytes as immune regulatory cells, Int. Immunopharm. 16 (2013) 224–231.
[48] K. Ramasamy, R. Agarwal, Multitargeted therapy of cancer by silymarin, Cancer Lett. 269 (2008) 352–362.
[49] S. Aghazadeh, R. Amini, R. Yazdanparast, S.H. Ghaffari, Anti-apoptotic and anti-inflammatory effects of *Silybum marianum* in treatment of experimental steatohepatitis, Exp. Toxicol. Pathol. 63 (2011) 569–574.
[50] G. Jiang, C. Sun, X. Wang, J. Mei, C. Li, H. Zhan, Y. Liao, Y. Zhu, J. Mao, Hepatoprotective mechanism of *Silybum marianum* on nonalcoholic fatty liver disease based on network pharmacology and experimental verification, Bioengineered 13 (2022) 5216–5235.
[51] C. Saliou, B. Rihn, J. Cillard, T. Okamoto, L. Packer, Selective inhibition of NF-κB activation by the flavonoid hepatoprotector silymarin in HepG2: evidence for different activating pathways, FEBS Lett. 440 (1998) 8–12.
[52] X. Zi, R. Agarwal, Modulation of mitogen-activated protein kinase activation and cell cycle regulators by the potent skin cancer preventive agent silymarin, Biochem. Biophys. Res. Commun. 263 (1999) 528–536.
[53] N. Bougarne, B. Weyers, S.J. Desmet, J. Deckers, D.W. Ray, B. Staels, K. De Bosscher, Molecular actions of PPARα in lipid metabolism and inflammation, Endocr. Rev. 39 (2018) 760–802.
[54] J. Orolin, R. Vecera, D. Jung, U.A. Meyer, N. Skottova, P. Anzenbacher, Hypolipidemic effects of silymarin are not mediated by the peroxisome proliferator-activated receptor alpha, Xenobiotica 37 (2007) 725–735.
[55] S.Y. Zhu, N. Jiang, J. Yang, J. Tu, Y. Zhou, X. Xiao, Y. Dong, *Silybum marianum* oil attenuates hepatic steatosis and oxidative stress in high fat diet-fed mice, Biomed. Pharmacother. 100 (2018) 191–197.
[56] G.A. Mohamed Kamel, E. Harahsheh, S. Hussein, Mechanisms underlying the hepatoprotective effect of silymarin on fluoxetine-induced liver injury in rats: the implication of peroxisome proliferator–activated receptor-gamma (PPAR-γ), Comp. Clin. Pathol. 31 (2022) 689–698.