

Evaluating the Hepatoprotective and Anti-Inflammatory Efficacy of Herbal Extracts for NAFLD Prevention

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Abstract: Natural products may offer a safer approach for mitigating the increasing prevalence of Non-Alcoholic Fatty Liver Disease (NAFLD). This study aimed to evaluate the hepatoprotective and anti-inflammatory effects of a water-based extract of Moringa Oleifera leaf, Milk Thistle, and Cassia (MO-MT-CA) against High-Fat, High-Sugar Diet (HFSD)-induced fatty liver. The research focuses on assessing lipid profiles and inflammation as key markers of its potential effect. The experimental animals were allocated randomly into 3 groups: 1st healthy group was fed a standard diet, 2nd group was fed a HFSD for 12 weeks, and 3rd group was given HFSD or MO-MT-CA for 4 weeks. Lipid profiles, hepatic function, and inflammatory biomarkers were measured in each group. HFSD-fed rats showed elevated serum triglycerides, cholesterol, and low-density lipoproteins LDL levels. Hepatic function tests were significantly higher than in healthy rats. HFSD also increased inflammatory markers, such as Tumor Necrosis Factor (TNF- α) and interleukin 6 (IL-6), as well as lipid metabolism hormones (leptin and resistin). The study introduces a potentially novel herbal blend, MO-MT-CA, and examines its beneficial impact on lipid metabolism, liver function, and inflammation, which may contribute to new therapeutic approaches for NAFLD.

Keywords: Hepatosteatosis, Herbs, Lipid Profile, Anti-Inflammatory, Hormone of Adiposity

Introduction

NAFLD is epidemiologically associated with Type 2 Diabetes Mellitus (T2DM), indicating a reciprocal cause-and-effect relationship and a vicious cycle of pathology. NAFLD is widespread in individuals with type 2 diabetes and is primarily associated with uncontrolled diabetes. Liver diseases are among the most prevalent health issues globally and typically result from chemicals, drugs, alcohol, or dietary disturbances (Yao and Liu, 2022).

NAFLD is characterized by fat overload in hepatocytes. It is strongly associated with cardiovascular risk factors related to atherosclerosis. Elevated lipid intake results in hyperlipidemia, followed by an increase in β -oxidation of fatty acids and the generation of Reactive Oxygen Species (ROS), triggering the activation of the Nuclear Factor Kappa B [NF- κ B] pathway, thereby

increasing the release of proinflammatory cytokines in various tissues (Lian *et al.*, 2020; Nani *et al.*, 2021).

Herbal treatments for NAFLD are widely available, have few side effects, and have proven therapeutic mechanisms and impact. Safe, affordable, and widely available options for treating NAFLD include the use of medicinal plants, which are rich in bioactive compounds known to support health. These plants offer cytoprotective effects, antioxidant, and anti-inflammatory properties. They also provide additional benefits such as reducing obesity, lowering lipid levels, and/or antidiabetic actions (Xu *et al.*, 2020). While many studies use plant extracts made with ethanol or methanol, using water-based extracts may indicate a focus on safety and suitability for human use.

Recent studies have revealed that the adipose tissue–liver axis and gut–liver axis are associated with NAFLD. Interestingly, herbal formulas have been reported to target both the adipose tissue–liver and gut–liver axes and to treat



inflammatory problems in NAFLD. Chinese herbal medicines may play key roles in controlling intestinal permeability, lowering inflammatory reactions, protecting hepatocytes, improving fat metabolism, and modifying nuclear receptors (Yang *et al.*, 2021). Therefore, our goal was to evaluate the impact of a mixture of MO-MT-CA, as a promising therapeutic approach or prevention of steatosis, as well as for the development of therapeutic strategies. We hypothesized that MO-MT-CA would prevent fatty liver disease by regulating lipid profiles, inflammatory mediators, adipose hormones, and steatosis through the action of its active compounds. Moreover, we explored the treatment mechanism and investigated the biochemical relationships among lipid profiles, inflammatory mediators, adipose hormones, and steatosis. The changes observed due to the treatment provide insight into its mechanism and further analysis in a rat model of fatty liver triggered by a high-fat diet.

Materials and Methods

Plant Materials

Moringa Oleifera Leaf, Milk Thistle, and *Cassia Acutifolia* (MO-MT-CA) plants were purchased from a local marketplace in Dammam Province, KSA. Medicinal samples were stored in the Chemistry Department, Faculty of Science, Imam Abdulrahman Bin Faisal University, Kingdom of KSA.

Preparation of Plant Extracts

The plant leaves were rinsed with running tap water to eliminate dust and left to air-dry in the dark, and ground into powder using an electric mortar. The resulting powder was then extracted with hot water. Approximately 500 g of powdered leaf material was immersed in a sufficient amount of water for 24 hours, with continuous mixing and shaking at ambient temperature. The hot water extraction was conducted at approximately 80–100°C to enhance the solubility and release of bioactive compounds such as alkaloids, phenolics, flavonoids, and polysaccharides. The application of heat helps break down plant cell walls, improving the extraction efficiency of compounds that are less soluble at lower temperatures. This approach was complemented by an ambient temperature extraction (~25°C) to preserve thermolabile constituents.

The extract was subsequently strained through a muslin cloth. The filtrate underwent concentration by evaporation into a semi-solid mass, applying a rotary evaporator to recover the extract. Viscid brown and green plant leaf extracts were obtained and preserved at -20°C for later use (Ahmed *et al.*, 2021). A mixture of Moringa oleifera leaf, Milk Thistle, and Cassia extracts [1:1:1], coded as (MO-MT-CA), was used for the animal experiments.

Animal Diet

Two kinds of rat diets were administered: Standard rat rations and HFSD (PE Enterprise, Riyadh, KSA). The composition of the standard rat ration included fat (5%), carbohydrates (65%), crude protein (20%), vitamins, minerals (calcium carbonate, dicalcium phosphate, magnesium oxide, and sodium chloride) (5%), and dietary fiber (5%). The energy content of this food was 2813 kcal/kg. The HFSD consisted of lipids (20%) (saturated fatty acids, 200 g/kg), carbohydrates (55%), crude protein (20%), and vitamins and minerals (5%). The energy content of this ration was 5100 kcal/kg.

Experimental Animals

Male albino rats (n = 60), each weighing 80–90 g and one month old, were used for this investigation. Rats were obtained from the laboratories of the Institute for Research and Medical Consultation [IRMC], Imam Abdulrahman Bin Faisal University, Dammam, Saudi Arabia. The rats were acclimatized for 1 week after arrival at the laboratory. The rats were housed in plastic cages at 24±3°C with 50% humidity, under a 12-hour light/dark cycle. Food and water were available *ad libitum*.

Sample Size Justification: A total of 60 rats were used, with 20 rats per group. This sample size was selected based on commonly accepted standards in the literature and is considered sufficient to achieve adequate statistical power for detecting medium to large effect sizes in preclinical animal studies. Specifically, previous research indicates that a group size of 15–20 animals typically provide ≥80% power to detect biologically meaningful differences at a significance level of $\alpha = 0.05$ using standard statistical tests such as ANOVA or t-tests. Additionally, 20 blood samples per group were collected, with each sample corresponding to a single animal, ensuring reliable biochemical analysis. This design balances scientific rigor with ethical considerations by minimizing animal use while maintaining statistical validity.

Chemicals, Kits, Investigation, and Analysis

Lipid profiles, including the levels of Triacylglycerol (TG), Total Cholesterol (TC), Low-Density Lipoprotein (LDL), High-Density Lipoprotein (HDL), and bilirubin, were assessed in blood samples calorimetrically using scientific kits from Human Gesellschaft für Biochemica und Diagnostica mbH (Germany). Hepatic function was assessed through the quantification of alanine aminotransferase (ALT), albumin, and bilirubin levels.

TG levels were quantified enzymatically by lipase hydrolysis. The resulting glycerol was oxidized using hydrogen peroxide (H₂O₂) and glycerol oxidase, producing a quinoneimine dye, which was measured at an absorbance of 520 nm. Cholesterol levels were determined through the hydrolysis of esters to produce cholesterol, which was then oxidized.

The equipment used for biochemical analysis includes: Spectrophotometer (Colorimeter), model, Human HumaLyzer series (HumaLyzer 3000, 4000), Wavelength range 340–700 nm. Diagnostic Reagent Kits (Human GmbH), contain specific enzymes, substrates, and color reagents for each analyte. Incubator or Water Bath to maintain optimal conditions. Centrifuge, used to separate serum or plasma from blood samples before analysis. Speed: 3000–4000 rpm for 10–15 minutes. Data Handling Equipment, Computer, and Printer for result analysis, calibration curves, and documentation. Micropipettes and Tips, Essential for accurate sample and reagent dispensing, their volume ranges from 10 to 1000 μ L.

The Hematoxylin and Eosin (H&E) staining technique was applied for microscopic examination to evaluate liver cell morphology, lipid droplet incidence, and the detection of inflammatory cells. The lipid profile, liver function indicators, and amount of leptin, resistin, and cytokines (TNF- α and IL-6) were determined using kits.

HPLC Analysis of the Extract

High-Performance Liquid Chromatography (HPLC) was employed to identify the flavonoid and phenolic substances in the extracts, including gallic acid, quercetin, and caffeic acid.

The analysis used an Eclipse XDB-C18 column (150 $\times$ 4.6 μ m; 5 μ m) with a C18 protection column (Phenomenex, Torrance, CA). The mobile phase consisted of acetonitrile (solvent A) and an aqueous solution of 2% acetic acid (solvent B), in a volume/volume ratio of 60:40 (ACN: 2% acetic acid in water). This ratio is commonly used because it provides a balanced polarity, enabling effective retention and separation of phenolic acids and flavonoids. The flow rate was preserved for a total time span of 60 minutes. An injection volume of 50 μ L was used, and the peaks were determined by correlating the retention times and UV–vis spectra to known standards. The samples were monitored at 280 and 320 nm and filtered using an Acrodisc 0.45 μ m syringe filter (Gelman's Laboratory, MI) before injection.

Work Plan and Grouping of the Animals

The research continued for 16 weeks and included 2 stages: 1) Inducing fatty liver (1-12 weeks) and 2) treatment (12-16 weeks). A total of sixty male albino rats were randomly assigned to three groups, with twenty rats in each group. The first group, the healthy group, was given a standard rat diet for the entire 16-week study period. Forty rats received a diet consisting of HFSD. Between weeks 12 and 16, the 40 rats in this group were subdivided into two smaller groups for further interventions. The second group, the positive reference group (n = 20), continued on the HFSD. In contrast, the remaining third group was administered the MO-MT-CA

mixture daily by oral administration at a dose equivalent to 5 ml/kg for 4 weeks.

Blood and Tissue Sampling

Blood was retrieved from the medial canthus of the rat's eye via a microhematocrit vessel. Blood samples were placed in dry centrifuge tubes, allowed to clot at 25 $\pm$ 2°C, and then centrifuged at 3000 rpm for 20 minutes to separate the serum. The non-hemolyzed clear serum was removed with a syringe and preserved at -80°C for subsequent analysis. For histopathology, the rats were euthanized under anesthesia, and the hepatic tissue was fixed in 10% formalin.

Histopathological Examination of the Liver

Histopathological inspection was carried out using H&E staining, and the liver was examined under a microscope. NAFLD grades were assessed at low [4 $\times$ and 10 $\times$] and high [40 $\times$ and 100 $\times$] magnifications (Liang *et al.*, 2014). The grade and severity were categorized based on the presence of lipid globules, macro- and microvesicular hepatocellular steatosis, and lobular and cellular inflammation (Al-Muzafar and Amin, 2017). Hepatosteatorosis was classified as normal [0, $\leq$ 5%], mild [1, 6–30%], moderate [2, 31–60%], or severe [3, 0 $\geq$ 60%].

Statistical Analysis

For each dataset, the mean and Standard Error of the Mean (SEM) were computed. Prior to conducting ANOVA, assumptions of normality and homogeneity of variances were evaluated using the Shapiro-Wilk and Levene's tests, respectively. Upon confirmation of these assumptions, group comparisons were conducted using one-way analysis of variance (ANOVA) to assess overall differences among groups. When a significant ANOVA result was observed, pairwise comparisons were performed using the Newman-Keuls post hoc test to identify specific group differences. This post hoc method was chosen for its ability to compare all possible pairs of means while controlling for Type I error. All statistical analyses were carried out using GraphPad Prism software (version 8.0). A p-value of less than 0.05 was considered statistically significant.

Results and Discussion

HPLC Analysis of Several Phenolic Compounds

The flavonoid and phenolic compounds, such as quercetin, apigenin, luteolin, gallic acid, fraxetin, caffeic acid, catechin, epicatechin, ferulic acid, ellagic acid, cinnamic acid, and myricetin, in the leaf and stem extracts of MO-MT-CA were analyzed by HPLC and are presented in Table (1).

Effects of MO-MT-CA Extract on Body Weight and Lipid Profiles in HFSD-Induced Steatosis

Figure (1) represents the impact of a regular diet or HFSD on body weight gain in rats. The key implication illustrated is that rats on the HFSD show significantly greater weight gain compared to those on a normal diet. This suggests that the combination of high fat and high sucrose promotes excess energy intake and storage, leading to adipose tissue accumulation. In particular, the diet appears to favor the development of visceral fat, a type of fat associated with metabolic disorders. Furthermore, the increase in body weight correlates with fat deposition in the liver, indicating the early stages of Non-Alcoholic Fatty Liver Disease (NAFLD). This result supports the conclusion that an HFSD promotes unhealthy weight gain and organ fat accumulation, with implications for metabolic disease development.

HFSDs show significantly greater weight gain compared to those on a normal diet.

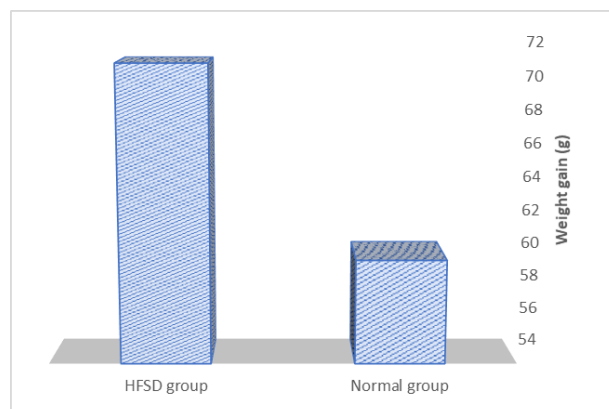


Fig. 1: Effect of normal diet or HFSD on rat body weight gain

Table 1: Some Phenolic and flavonoids compounds in the MO-MT-CA extract

RT	m/z	Compound Name	Molecular Formula	Molecular Weight
7.6	247	Fraxetin	C ₁₀ H ₈ O ₅	208.04
5.99	120	hydroxycoumarin	C ₉ H ₆ O ₃	162.14
7.45	84	2,3-Butanedithiol	C ₄ H ₁₀ S ₂	122.02
-	-	Methyl propyl disulfide		
-	-	2-(1-Mercaptoethyl) furan	C ₆ H ₈ OS	128.2
-	-	1,3-Dihydroxyanthraquinone	C ₁₄ H ₈ O ₄	240
	240.09	Phenolic acids	C ₁₂ H ₁₆ O ₅	240.25 g/mol
12.1	170.12	Gallic acid	C ₁₀ H ₁₂ O ₅	170.12 g/mol
41.52	131.0 100	Cinnamic acid	C ₉ H ₈ O ₂	148.16 g/mol
8.97	181	Caffeic acid	C ₉ H ₈ O ₄	180.16 g/mol
Flavonoids				
5.723	117.03	Apigenin	C ₁₅ H ₁₀ O ₅	270.24 g/mol
3.670	286.2	Luteolin	C ₁₅ H ₁₀ O ₆	286.24 g/mol
7.9	302.2	Quercetin	C ₁₅ H ₁₀ O ₇	302.23 g/mol

Table 2: Effect MO-MT-CA extract on lipid profiles in HFSD fed rats

	Normal	HFSD	HFSD+MO-MT-CA
TG (mg/dl)	111.03±9.01	229.33±10.9*a	181.94±6.89b
TC (mg/dl)	78.08±4.35	110.6±2.19**a	87.16±3.79b
LDL mg/dl	35.7±2.33	64.5±6.59***a	42.7±5.52bc
HDL (mg/dl)	88.99±8.082	37.79±4.52***a	68.97±6.02 b

These values represent means and standard errors, with different superscript letters indicating significant differences at $p < 0.05$

Table 3: Effect of MO-MT-CA extract on liver function in HFSD fed rats

	Normal	HFSD	HFSD+MO-MT-CA
ALT	30.8±1.94	49.3±4.39***a	37.1±3.14b
Albumin (g/dl)	3.13±0.19	3.00±0.20	3.05±0.21
T. proteins (g/dl)	8.41±0.69	8.41±0.56	7.88±0.57
T. Bilirubin	1.42±0.16	2.36±0.23**a	1.74±0.15 a
D. Bilirubin	0.79±0.09	2.08±0.1***a	1.55±0.06c
LDH	621.49±24.21	825.31±56.110**	799.72±33.67
Glucose (mg/dl)	114.64±2.93	189.44±17.25***a	148.43±11.92a

These values represent means and standard errors, with different superscript letters indicating significant differences at $P < 0.05$

Blood TAG, TC, and LDL concentrations were significantly higher ($p \leq 0.05$). In contrast, HDL levels were reduced in the HFSD group relative to the normal group (Table 2). Additionally, compared to the HFSD group, the MO-MT-CA group exhibited notably lower serum TAG, TC, and LDL levels, while showing higher HDL levels (Table 2).

This study investigates the hepatoprotective and anti-inflammatory properties of the MO-MT-CA mixture, assessing its impact on lipid profiles, liver function, metabolic hormones, and inflammatory biomarkers. These effects can be attributed to the phenolic and quercetin catech compounds present in the mixture (Stohs and Hartman, 2015; Devaraj *et al.*, 2011; Asgari-Kafrani *et al.*, 2020; Bellassoued *et al.*, 2021; Pareek *et al.*, 2023). The findings are discussed as follows.

Our results demonstrated that HFSD-induced steatosis led to dyslipidemia, including hypertriglyceridemia, elevated LDL levels, and reduced HDL levels (Table 2). This was accompanied by hepatic damage, with increased lipid droplet accumulation and varying degrees of hepatosteatosis, including micro- and macrovascular steatosis and significant periportal inflammation (Lian *et al.*, 2020). These changes suggest a significant link between lipid profile abnormalities and fatty liver (Kathak *et al.*, 2022). The consumption of sucrose-enriched fatty acids likely contributed to adipose tissue accumulation, particularly in visceral fat, leading to weight gain and fat deposition in the liver.

Compared to the HFSD group, the MO-MT-CA extract treatment led to a decrease in the steatosis grade, along with reductions in lipid profile levels and liver function markers (Table 3), indicating the potential anti-steatosis effects of the extract (Pais & D'Amato, 2014).

Moringa oleifera in our mixture is known to be relatively safe for oral administration and may protect against liver damage, as demonstrated in rats given an overdose of APAP (Sharifudin *et al.*, 2013; Younis *et al.*, 2022). The obesity prevention and anti-diabetic effects of *Moringa*'s isothiocyanates are primarily due to their ability to inhibit hepatic gluconeogenesis and promote hepatocyte regeneration through antioxidant properties (Waterman *et al.*, 2015).

The hypolipidemic activity of MO-MT-CA is likely attributed to its polyphenolic compounds. Flavonoids, such as quercetin, stabilize HDL-C and prevent its breakdown. *Moringa oleifera*, rich in polyunsaturated fatty acids, may facilitate LDL-C catabolism, leading to lower serum LDL-C levels. Additionally, *Cassia acutifolia* contains anthraquinone glycosides and flavonoids that help mobilize fat droplets from fatty liver tissues, improving lipid profiles. Milk thistle further supports hepatic cell regeneration and enhances metabolism (Zhang *et al.*, 2023), while *Moringa oleifera* helps maintain normal blood glucose levels (Sebii *et al.*, 2024).

Existing literature on *Cassia* largely highlights its laxative and anti-inflammatory effects, with limited evidence regarding its role in NAFLD, especially in combination with other herbs. Although *Cassia acutifolia* is not traditionally recognized as a primary agent for NAFLD treatment, it has anti-inflammatory and antioxidant properties, gut-cleansing, and reduction in gut-derived endotoxins. may lower lipopolysaccharide (LPS) absorption, reducing gut-liver axis-related endotoxemia, which contributes to hepatic inflammation and supports liver health, therefore complements the actions of *Moringa* and milk thistle in a multi-herbal approach. our study's investigation into this novel combination could help define a previously unexplored synergistic role for *Cassia* in NAFLD prevention.

Effect of MO-MT-CA Extract on Hepatic Function Biomarkers in HFSD-Fed Rats

The HFSD group exhibited a significant increase in the activities of ALT as a liver enzyme, LDH, total bilirubin, and direct bilirubin compared with the normal group. In contrast, MO-MT-CA administration for 4 weeks decreased ALT activity, total and direct bilirubin levels ($p \leq 0.05$) in comparison with the HFSD group (Table 3).

In the HFSD group, we observed significant increases in blood bilirubin, ALT, and LDH levels. These elevations were associated with fat accumulation and enhanced lipogenesis in hepatocytes (Jeon *et al.*, 2023), leading to liver damage, increased conjugated bilirubin levels, and the release of intracellular transaminases into the bloodstream. Additionally, Reactive Oxygen Species (ROS) generated through lipid peroxidation contributed to liver dysfunction (Zhang *et al.*, 2023).

Treatment with MO-MT-CA extract significantly reduced these biomarkers compared to the HFSD group (Table 3). *Moringa oleifera* extract, a key component of the formulation, improved liver function and lowered liver enzyme levels (Kim *et al.*, 2022). Kim *et al.* (2022) examined *Moringa oleifera* alone, emphasizing its ability to reduce hepatic lipid accumulation and oxidative stress, but did not explore its interaction with other phytotherapeutic agents. Quercetin, known for its antioxidant and anti-inflammatory properties, further reduced the expression of fibrotic factors such as Transforming Growth factor (TGF) and decreased hepatic enzymatic activity (Li *et al.*, 2024a). Other bioactive compounds, such as silymarin, may also contribute to the hepatoprotective effects of *Moringa*, enhancing its therapeutic potential.

Effect of MO-MT-CA Herbal Mixture Extract on Lipid Metabolic Hormones in HFSD-Fed Rats

HFSD increased leptin and resistin levels, but MO-MT-CA normalized them significantly (Table 4).

Table 4: Effect of MO-MT-CA extract on hormones and inflammatory markers in HFSD fed rats

	Normal	HFSD	HFSD+MO-MT-CA
Leptin (pg/ml)	1525.2 ±166.41 a	2525.29±226.36 b	1898.13±146.65 a
Resistin (ng/ml)	2.75±0.55	5.71±0.32 a	4.33±0.78
IL-6 (pg/ml)	0.095±0.01	0.270±0.02a	1.70±0.38b
TNFα (pg/ml)	0.0089±0.004	0.06±0.02*a	0.029±0.015ab

These values represent means and standard errors, with different superscript letters indicating significant differences at $P < 0.05$

This study not only evaluates traditional lipid profile markers (triglycerides, cholesterol, LDL) but also examines the role of lipid metabolism hormones, such as leptin and resistin, which are less commonly studied in relation to NAFLD and natural product interventions.

The HFSD group exhibited increased leptin levels, a hormone essential for lipid metabolism and energy homeostasis, which also contributes to progressive weight gain (Fig. 1). Chronic HFSD exposure likely elevated leptin secretion. Leptin exerts pleiotropic effects on multiple metabolic pathways and is closely linked to liver function, a key regulator of systemic metabolism (Martínez-Uña *et al.*, 2020). While leptin plays a crucial role in maintaining liver health by promoting triglyceride utilization, leptin resistance, commonly observed in obesity and NAFLD, impairs this function and contributes to hyperlipidemia (D'Souza *et al.*, 2017; Jiménez-Cortegana *et al.*, 2021; Qamar *et al.*, 2024). Moreover, a reduction in the soluble leptin receptor and elevated leptin levels has been reported in human biopsy-confirmed NAFLD. Interestingly, Canbakan *et al.* (2008) observed increased leptin concentrations in NAFLD patients and suggested a potential protective role of leptin in advanced hepatic impairment. Further investigation into the molecular mechanisms, signaling pathways, and metabolic regulation of leptin could pave the way for more effective NAFLD treatment strategies. Leptin treatment is effective for congenital leptin deficiency but remains controversial for other patients. Ongoing research is needed to develop safer leptin analogues and sensitizers. Despite the need for more long-term studies, leptin shows promise as a biomarker for diagnosing and monitoring NAFLD when combined with metabolic, lipid, and glucose analyses (Jiménez-Cortegana *et al.*, 2021).

Resistin, an adipocyte-specific hormone, is closely connected with obesity, diabetes, insulin resistance, and hepatosteatosis (Gupta *et al.*, 2023). In this study, HFSD feeding led to elevated levels of both resistin and leptin, accompanied by hypertriglyceridemia, lipid droplet accumulation in hepatic cells, and the progression of hepatosteatosis.

The current findings indicate that obesity and insulin resistance contribute to hepatosteatosis (NAFLD) and hepatic cirrhosis through lipid infiltration and adipocytokine-induced inflammation. As an endocrine organ, adipose tissue synthesizes adipocytokines such as

adiponectin, leptin, resistin, interleukins, and TNF- α , which regulate appetite, inflammation, metabolism, and immunity. However, in obesity, insulin resistance, and NAFLD, these regulatory processes become dysregulated (Kirichenko *et al.*, 2022; Clemente-Suárez *et al.*, 2023).

However, treatment with MO-MT-CA effectively normalized these hormonal imbalances (Table 4), enhancing lipolysis in hepatocytes and potentially mitigating NAFLD progression. These findings underscore the critical role of hormonal balance and enzymatic regulation in lipid metabolism, encompassing both lipogenesis and lipolysis, in the development of fatty liver disease. The hepatoprotective effects of MO-MT-CA may be attributed to its ability to modulate lipid profiles, enzymatic activities, and hormonal regulation, highlighting its therapeutic potential for HFSD-induced hepatosteatosis.

Effect of MO-MT-CA Herbal Mixture Extract on Inflammatory Marker Levels

TNF- α and IL-6 levels increased in the HFSD group, and MO-MT-CA ameliorated this effect (Table 4). Figure (2) shows a schematic representation of the mechanism of action of MO-MT-CA in hepatosteatosis. While individual components, such as milk thistle, are well known for their hepatoprotective properties, this study specifically investigates the anti-inflammatory effects of the MO-MT-CA blend by assessing key biomarkers, including TNF- α and IL-6, in a high-fat diet-induced fatty liver model.

HFSD feeding led to a significant increase in proinflammatory cytokines TNF- α and IL-6 in the liver, indicating an inflammatory response that accelerates fatty liver progression (Table 4). This effect was likely mediated by hepatic activation of NF- κ B and the production of inflammatory cytokines from adipose tissue (Nani *et al.*, 2021; Echeverría *et al.*, 2019; Muzurović *et al.*, 2021).

Adipose tissue in obesity is recognized to express elevated levels of IL-6 and TNF- α (Al Madhoun *et al.*, 2023). The mechanism of HFSD-induced hepatic inflammation involves hepatic macrophage infiltration mediated by IL-6 and TNF- α , proinflammatory cytokines produced by adipose tissue. These cytokines play a crucial role in the progression of NAFLD and fibrosis development (Gong *et al.*, 2023).

Table 5: Hepatosteatosis score in the differently treated groups of rats

Hepatosteatosis score (%)	Normal (%)	HFSD (%)	HFSD+MO-MT-CA (%)
0 (≤ 5)	80	0	55
1 (6:30)	15	60	30
2 (31:60)	5	35	10
3 (≥ 60)	0	5	5

Hepatosteatosis, Normal, HFSD, HFSD+ MO-MT-CA score (%), indicates the percentage area affected in the inspected hepatic slides.

Another mechanism involves the catabolism of fatty acids in mitochondrial hepatocytes through β -oxidation, where increased fatty acid transport induces lipotoxicity by generating excess ROS, leading to mitochondrial damage, accumulation of injured mitochondria, and hepatocellular injury (Mooli and Ramakrishnan, 2022). However, treatment with MO-MT-CA significantly reduced TNF- α and IL-6 levels, suggesting that the bioactive compounds in the extract exert anti-inflammatory effects that protect the liver from further damage. This reduction may be attributed to the synergistic action of these compounds, which not only regulate lipid metabolism but also modulate inflammatory pathways. These findings highlight the potential of MO-MT-CA as a promising therapeutic intervention for managing NAFLD and associated metabolic disorders.

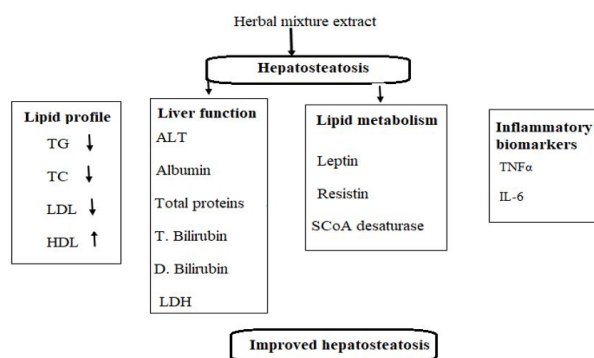


Fig. 2: Schematic representation of the mechanism of MO-MT-CA on hepatosteatosis

Effect of the Herbal Mixture Extract on Steatosis Grade

HFSD led to an increase in the fatty liver grade. The steatosis grade was 0 for the normal group in 80% of rats and 1 in 15% of rats, whereas in the HFSD group, it was 1 in 60% of rats and 2 in 35% of rats (Table 5). This indicates that the steatosis grade was elevated in the HFSD group compared to the normal group. In the HFSD +MO-MT-CA group, steatosis grades were 0 in 55% and 1 in 30% of rats. These results show that MO-MT-CA supplementation significantly reduced the steatosis grade to levels similar to those in the normal group.

Chemical Constituents and Biological Activity of MO-MT-CA Extract

The MO-MT-CA extract contains several bioactive compounds, including flavonoids (quercetin and kaempferol), polyphenols (e.g., caffeic acid), silymarin, and sennosides, all of which contribute to its therapeutic potential. These compounds exhibit significant antioxidant activity by scavenging free radicals, combating oxidative stress, and preserving hepatic cells from injury (Li *et al.*, 2024b; Chanu *et al.*, 2023).

Quercetin, a key flavonoid, is particularly noteworthy for its antioxidant defense mechanisms and inflammation, which are major contributors to chronic diseases as cancer, cardiovascular disease, diabetes, and hepatic steatosis. It may improve steatosis, reduce weight, and reduce visceral and hepatic fat by increasing fatty acid oxidation. Moreover, fat is shifted from the abdomen to other areas or converted into nonfatty tissue (Yang *et al.*, 2020; Chen *et al.*, 2021). Silymarin, a primary active component of the extract, plays a crucial role in hepatoprotection by acting as a potent antioxidant and promoting liver tissue regeneration. Additionally, milk thistle, rich in silymarin, has been widely recognized for its benefits in managing liver diseases, including chronic hepatitis and cirrhosis (Li *et al.*, 2024b), who, focused on the antioxidant and anti-inflammatory properties of milk thistle (particularly silymarin) in liver diseases but considered it only as a monotherapy, without investigating its synergistic potential with other botanicals like Moringa or Cassia.

Sennosides, derived from *Senna*, contribute to the extract's laxative effects by stimulating bowel movements, indirectly supporting liver health through the reduction of systemic inflammation. While Senna may not directly treat fatty liver disease, its antioxidant and anti-inflammatory properties provide mild, supportive effects for liver function (Nayan *et al.*, 2021).

Our study presents several novel contributions not addressed in prior research. These include a new methodology for extraction, identification, characterization, dosing, and experimental application. The dataset used was particularly effective for modeling NAFLD scores. The mechanisms of action—such as anti-inflammatory activity, hormonal regulation, and other biological effects—associated with the MO-MT-CA extract are distinct from those reported in earlier studies. While the hepatoprotective effects of individual

components such as *Moringa oleifera*, milk thistle, and *Cassia* are documented, this study uniquely investigates their synergistic combination and its application in NAFLD prevention. Key innovations include the first evaluation of the MO-MT-CA combination in a preventive NAFLD model, emphasizing synergistic rather than individual effects. Comprehensive biochemical assessments offering deeper insight into lipid metabolism, oxidative stress, and inflammatory pathways. Dose optimization and safety profiling of the combination—areas often overlooked in single-herb studies. A holistic phytotherapeutic approach aligned with integrative medicine trends, potentially offering a safer and more accessible alternative to synthetic pharmacological treatments.

Conclusion

HFSD-induced hepatosteatosis was characterized by increased body weight, dyslipidemia, triglyceride accumulation in the liver, disrupted metabolic markers (LDH, glucose, liver function tests), and hormonal imbalances (leptin and resistin), along with elevated inflammatory biomarkers (TNF- α and IL-6). The combination of these specific natural ingredients appears to be a novel approach. Our data highlight the potential of medicinal plants, such as MO-MT-CA, for preventing and treating hepatosteatosis and related complications, which is a growing concern in modern healthcare. MO-MT-CA administration improved NAFLD markers, lipid profiles, liver enzyme levels, and inflammatory biomarkers, suggesting its therapeutic potential. The combination of antioxidants, anti-inflammatory agents, and regulators of lipid metabolism in these mixtures could offer a multifaceted approach to liver health. However, further clinical trials and evidence-based research are needed to verify its safety and efficacy for long-term use.

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Authors Contributions

Kamal Adel and Hessah Mohammed Al-Muzafar contributed equally to this work. They jointly conceived, designed, and planned the study. Both authors conducted the experimental work, performed biochemical and

statistical analyses, and interpreted the results. Additionally, they collaborated on drafting, writing, and revising the manuscript. Both authors have read and approved the final version of the manuscript.

Ethics

All animal procedures and experiments were conducted in accordance with the standard guidelines of the Institutional Animal Care and Use Committee (IACUC). This study was conducted in compliance with local ethical guidelines and was approved by the Institutional Review Board of Imam Abdulrahman University, IRB, No. 2104-09-066.

Conflict of Interest

The authors declare no conflicts of interest.

Data Statement

Data will be available on request and provided in the manuscript.

References

- Ahmed, O. M., Abd El-Twab, S. M., Al-Muzafar, H. M., Amin, K. A., Abdel Aziz, S. M., & Abdel-Gabbar, M. (2021). *Musa paradisiaca* L. leaf and fruit peel hydroethanolic extracts improved the lipid profile, glycemic index and oxidative stress in nicotinamide/streptozotocin-induced diabetic rats. *Veterinary Medicine and Science*, 7(2), 500–511. <https://doi.org/10.1002/vms3.389>
- Al Madhoun, A., Kochumon, S., Haddad, D., Thomas, R., Nizam, R., Miranda, L., Sindhu, S., Bitar, M. S., Ahmad, R., & Al-Mulla, F. (2023). *Adipose tissue caveolin-1 upregulation in obesity involves TNF- α /NF- κ B mediated signaling*. *Cells*, 12(7), 1019. <https://doi.org/10.3390/cells12071019>
- Al-Muzafar, H. M., & Amin, K. A. (2017). *Probiotic mixture improves fatty liver disease by virtue of its action on lipid profiles, leptin, and inflammatory biomarkers*. *BMC Complementary and Alternative Medicine*, 17(1), 1–9. <https://doi.org/10.1186/s12906-016-1540-z>
- Asgari-Kafrani, A., Fazilati, M., & Nazem, H. (2020). *Hepatoprotective and antioxidant activity of aerial parts of Moringa oleifera in prevention of non-alcoholic fatty liver disease in Wistar rats*. *South African Journal of Botany*, 129, 82–90. <https://doi.org/10.1016/j.sajb.2019.01.014>
- Bellassoued, K., Hamed, H., Ghrab, F., Kallel, R., Van Pelt, J., Makni Ayadi, F., & Elfeki, A. (2021). *Antioxidant and hepatopreventive effects of Cassia angustifolia extract against carbon tetrachloride-induced hepatotoxicity in rats*. *Archives of*

- Physiology and Biochemistry, 127(6), 486–496.
<https://doi.org/10.1080/13813455.2019.1650778>
- Canbakan, B., Tahan, V., Balci, H., Hatemi, I., Erer, B., Ozbay, G., Sut, N., Hacibekiroglu, M., Imeryuz, N., & Senturk, H. (2008). *Leptin in nonalcoholic fatty liver disease*. Annals of Hepatology, 7(3), 249–254.
[https://doi.org/10.1016/S1665-2681\(19\)31856-3](https://doi.org/10.1016/S1665-2681(19)31856-3)
- Chanu, N. R., Gogoi, P., Barbhuiya, P. A., Dutta, P. P., Pathak, M. P., & Sen, S. (2023). *Natural flavonoids as potential therapeutics in the management of diabetic wound: A review*. Current Topics in Medicinal Chemistry, 23(8), 690–710.
<https://doi.org/10.2174/1568026623666230419102140>
- Chen, L., Liu, J., Mei, G., Chen, H., Peng, S., Zhao, Y., Yao, P., & Tang, Y. (2021). *Quercetin and non-alcoholic fatty liver disease: A review based on experimental data and bioinformatic analysis*. Food and Chemical Toxicology, 154, 112314.
<https://doi.org/10.1016/j.fct.2021.112314>
- Clemente-Suárez, V. J., Redondo-Flórez, L., Beltrán-Velasco, A. I., Martín-Rodríguez, A., Martínez-Guardado, I., Navarro-Jiménez, E., Laborde-Cárdenas, C. C., & Tornero-Aguilera, J. F. (2023). *The role of adipokines in health and disease*. Biomedicines, 11(5), 1290.
<https://doi.org/10.3390/biomedicines11051290>
- D'souza, A. M., Neumann, U. H., Glavas, M. M., & Kieffer, T. J. (2017). *The glucoregulatory actions of leptin*. Molecular Metabolism, 6(9), 1052–1065.
<https://doi.org/10.1016/j.molmet.2017.04.011>
- Devaraj, V. (2011). *Simultaneous determination of quercetin, rutin and kaempferol in the leaf extracts of Moringa oleifera Lam. and Raphanus sativus Linn. by liquid chromatography–tandem mass spectrometry*. Journal of Chinese Integrative Medicine, 9(9), 1022–1030. <https://doi.org/10.3736/jcim20110914>
- Echeverría, F., Valenzuela, R., Espinosa, A., Bustamante, A., Álvarez, D., González-Mañán, D., Ortiz, M., Soto-Alarcón, S. A., & Videla, L. A. (2019). *Reduction of high-fat diet-induced liver proinflammatory state by eicosapentaenoic acid plus hydroxytyrosol supplementation: Involvement of resolvins RvE1/2 and RvD1/2*. The Journal of Nutritional Biochemistry, 63, 35–43.
<https://doi.org/10.1016/j.jnutbio.2018.09.012>
- Gong, J., Tu, W., Liu, J., & Tian, D. (2023). *Hepatocytes: A key role in liver inflammation*. Frontiers in Immunology, 13, 1083780.
<https://doi.org/10.3389/fimmu.2022.1083780>
- Gupta, A., Gupta, P., Singh, A. K., & Gupta, V. (2023). *Association of adipokines with insulin resistance and metabolic syndrome including obesity and diabetes*. GHM Open, 3(1), 7–19.
<https://doi.org/10.35772/ghmo.2023.01004>
- Jeon, Y. G., Kim, Y. Y., Lee, G., & Kim, J. B. (2023). *Physiological and pathological roles of lipogenesis*. Nature Metabolism, 5(5), 735–759.
<https://doi.org/10.1038/s42255-023-00786-y>
- Jiménez-Cortegana, C., García-Galey, A., Tami, M., del Pino, P., Carmona, I., López, S., Alba, G., & Sánchez-Margalet, V. (2021). *Role of leptin in non-alcoholic fatty liver disease*. Biomedicines, 9(7), 762.
<https://doi.org/10.3390/biomedicines9070762>
- Kathak, R. R., Sumon, A. H., Molla, N. H., Hasan, M., Miah, R., Tuba, H. R., Habib, A., & Ali, N. (2022). *The association between elevated lipid profile and liver enzymes: A study on Bangladeshi adults*. Scientific Reports, 12(1), 1–10.
<https://doi.org/10.1038/s41598-022-05766-y>
- Kim, C. G., Chang, S. N., Park, S. M., Hwang, B. S., Kang, S.-A., Kim, K. S., & Park, J. G. (2022). *Moringa oleifera mitigates ethanol-induced oxidative stress, fatty degeneration and hepatic steatosis by promoting Nrf2 in mice*. Phytomedicine, 100, 154037.
<https://doi.org/10.1016/j.phymed.2022.154037>
- Kirichenko, T. V., Markina, Y. V., Bogatyreva, A. I., Tolstik, T. V., Varaeva, Y. R., & Starodubova, A. V. (2022). *The role of adipokines in inflammatory mechanisms of obesity*. International Journal of Molecular Sciences, 23(23), 14982.
<https://doi.org/10.3390/ijms232314982>
- Li, L., Lei, X., Chen, L., Ma, Y., Luo, J., Liu, X., Xu, X., Zhou, G., & Feng, X. (2024a). *Protective mechanism of quercetin compounds against acrylamide-induced hepatotoxicity*. Food Science and Human Wellness, 13(1), 225–240.
<https://doi.org/10.26599/fshw.2022.9250019>
- Li, S., Duan, F., Li, S., & Lu, B. (2024b). *Administration of silymarin in NAFLD/NASH: A systematic review and meta-analysis*. Annals of Hepatology, 29(2), 101174. <https://doi.org/10.1016/j.aohp.2023.101174>
- Lian, C.-Y., Zhai, Z.-Z., Li, Z.-F., & Wang, L. (2020). *High-fat diet-triggered non-alcoholic fatty liver disease: A review of proposed mechanisms*. Chemico-Biological Interactions, 330, 109199.
<https://doi.org/10.1016/j.cbi.2020.109199>
- Liang, W., Menke, A. L., Driessen, A., Koek, G. H., Lindeman, J. H., Stoop, R., Havekes, L. M., Kleemann, R., & van den Hoek, A. M. (2014). *Establishment of a general NAFLD scoring system for rodent models and comparison to human liver pathology*. PLoS ONE, 9(12), e115922.
<https://doi.org/10.1371/journal.pone.0115922>
- Martínez-Uña, M., López-Mancheño, Y., Diéguez, C., Fernández-Rojo, M. A., & Novelle, M. G. (2020). *Unraveling the role of leptin in liver function and its relationship with liver diseases*. International Journal of Molecular Sciences, 21(24), 9368.
<https://doi.org/10.3390/ijms21249368>

- Mooli, R. G. R., & Ramakrishnan, S. K. (2022). *Liver steatosis is a driving factor of inflammation*. Cellular and Molecular Gastroenterology and Hepatology, 13(4), 1267–1270. <https://doi.org/10.1016/j.jcmgh.2022.01.007>
- Muzurović, E., Mikhailidis, D. P., & Mantzoros, C. (2021). *Non-alcoholic fatty liver disease, insulin resistance, metabolic syndrome and their association with vascular risk*. Metabolism, 119, 154770. <https://doi.org/10.1016/j.metabol.2021.154770>
- Nani, A., Murtaza, B., Sayed Khan, A., Khan, N. A., & Hichami, A. (2021). *Antioxidant and anti-inflammatory potential of polyphenols contained in Mediterranean diet in obesity: Molecular mechanisms*. Molecules, 26(4), 985. <https://doi.org/10.3390/molecules26040985>
- Nayan, S. I., Chowdhury, F. I., Akter, N., Rahman, M. M., Selim, S., Saffoon, N., Khan, F., Subhan, N., Hossain, M., Ahmed, K. S., Hossain, H., Haque, M. A., & Alam, M. A. (2021). *Leaf powder supplementation of Senna alexandrina ameliorates oxidative stress, inflammation, and hepatic steatosis in high-fat diet-fed obese rats*. PLOS ONE, 16(4), e0250261. <https://doi.org/10.1371/journal.pone.0250261>
- Pais, P., & D'Amato, M. (2014). *In vivo efficacy study of milk thistle extract (ETHIS-094™) in STAM™ model of nonalcoholic steatohepatitis*. Drugs in R&D, 14(4), 291–299. <https://doi.org/10.1007/s40268-014-0068-2>
- Pareek, A., Pant, M., Gupta, M. M., Kashania, P., Ratan, Y., Jain, V., Pareek, A., & Chuturgoon, A. A. (2023). *Moringa oleifera: An updated comprehensive review of its pharmacological activities, ethnomedicinal, phytopharmaceutical formulation, clinical, phytochemical, and toxicological aspects*. International Journal of Molecular Sciences, 24(3), 2098. <https://doi.org/10.3390/ijms24032098>
- Qamar, M., Fatima, A., Tauseef, A., Yousufzai, M. I., Liaqat, I., & Naqvi, Q. (2024). *Comparative and predictive significance of serum leptin levels in non-alcoholic fatty liver disease*. Cureus. <https://doi.org/10.7759/cureus.57943>
- Sebii, H., Karra, S., Ghribi, A. M., Danthine, S., Blecker, C., Attia, H., & Besbes, S. (2024). *Moringa, milk thistle, and jujube seed cold-pressed oils: Characteristic profiles, thermal properties, and oxidative stability*. Foods, 13(9), 1402. <https://doi.org/10.3390/foods13091402>
- Sharifudin, S. A., Fakurazi, S., Hidayat, M. T., Hairuszah, I., Aris Mohd Moklas, M., & Arulselvan, P. (2013). *Therapeutic potential of Moringa oleifera extracts against acetaminophen-induced hepatotoxicity in rats*. Pharmaceutical Biology, 51(3), 279–288. <https://doi.org/10.3109/13880209.2012.720993>
- Stohs, S. J., & Hartman, M. J. (2015). *Review of the safety and efficacy of Moringa oleifera*. Phytotherapy Research, 29(6), 796–804. <https://doi.org/10.1002/ptr.5325>
- Waterman, C., Rojas-Silva, P., Tumer, T. B., Kuhn, P., Richard, A. J., Wicks, S., Stephens, J. M., Wang, Z., Mynatt, R., Cefalu, W., & Raskin, I. (2015). *Isothiocyanate-rich Moringa oleifera extract reduces weight gain, insulin resistance, and hepatic gluconeogenesis in mice*. Molecular Nutrition & Food Research, 59(6), 1013–1024. <https://doi.org/10.1002/mnfr.201400679>
- Xu, Y., Guo, W., Zhang, C., Chen, F., Tan, H. Y., Li, S., Wang, N., & Feng, Y. (2020). *Herbal medicine in the treatment of non-alcoholic fatty liver diseases: Efficacy, action mechanism, and clinical application*. Frontiers in Pharmacology, 11, 601. <https://doi.org/10.3389/fphar.2020.00601>
- Yang, D., Wang, T., Long, M., & Li, P. (2020). *Quercetin: Its main pharmacological activity and potential application in clinical medicine*. Oxidative Medicine and Cellular Longevity, 2020, 1–13. <https://doi.org/10.1155/2020/8825387>
- Yang, X.-F., Lu, M., You, L., Gen, H., Yuan, L., Tian, T., Li, C.-Y., Xu, K., Hou, J., & Lei, M. (2021). *Herbal therapy for ameliorating nonalcoholic fatty liver disease via rebuilding the intestinal microecology*. Chinese Medicine, 16(1), 1–13. <https://doi.org/10.1186/s13020-021-00470-x>
- Yao, P., & Liu, Y. (2022). *Terpenoids: Natural compounds for non-alcoholic fatty liver disease (NAFLD) therapy*. Molecules, 28(1), 272. <https://doi.org/10.3390/molecules28010272>
- Younis, N., Khan, M. I., Zahoor, T., & Faisal, M. N. (2022). *Phytochemical and antioxidant screening of Moringa oleifera for its utilization in the management of hepatic injury*. Frontiers in Nutrition, 9, 1078896. <https://doi.org/10.3389/fnut.2022.1078896>
- Zhang, L., Liu, S., Gu, Y., Li, S., Liu, M., & Zhao, W. (2023). *Comparative efficacy of Chinese patent medicines for non-alcoholic fatty liver disease: A network meta-analysis*. Frontiers in Pharmacology, 13, 1077180. <https://doi.org/10.3389/fphar.2022.1077180>