Journal of
Babol University of Medical Sciences
JBUMS
e-ISSN: 2251-7170 p-ISSN: 1561-4107

The Effect of Thymoquinone on Liver Function and Inflammatory Gene Expression in an Animal Model of Metabolic Dysfunction-Associated Fatty Liver Disease

F. Koohpeyma (MD)[1], M. R. Koohpeyma (MD)[2], M. Kaviani (MD)[3], N. Montazeri-Najafabady (PhD)[*1], S. Dastghaib (PhD)[1], M. Shams (MD)[1]

1.Endocrinology and Metabolism Research Center, Shiraz University of Medical Sciences, Shiraz, I.R.Iran.
2.Student Research Committee, Shiraz University of Medical Sciences, Shiraz, I.R.Iran.
3.Student Research Committee, Faculty of Nursing and Midwifery, Yasuj University of Medical Sciences, Yasuj, I.R.Iran.

***Corresponding Author: N. Montazeri-Najafabady (PhD)**
Address: Endocrinology and Metabolism Research Center, Shiraz University of Medical Sciences, Shiraz, I.R.Iran.
Tel: +98 (71) 32262745. **E-mail:** montazerin898@gmail.com

Article Type
Research Paper

Received:
Oct 15th 2024
Revised:
Dec 7th 2024
Accepted:
Feb 3rd 2025

ABSTRACT

Background and Objective: Metabolic dysfunction-associated fatty liver disease (MAFLD) is now widely recognized as the hepatic presentation of the metabolic syndrome. Owing to the well-established anti-inflammatory and antioxidant properties of thymoquinone, the current study was conducted to assess the effect of thymoquinone on serum liver enzyme activities, lipid profile parameters, as well as the transcriptional expression of key genes involved in inflammatory and antioxidant signaling pathways, using an animal model of MAFLD.

Methods: In this interventional-experimental animal study, 18 rats were randomly divided into 3 groups of 6: healthy control, experimental 1, and experimental 2. With the exception of the healthy control group, all animals were subjected to a high-fat diet over an eight-week period. Experimental group 2 received oral administration of thymoquinone at a dose of 10 mg/kg body weight for 35 days, while experimental group 1 received no therapeutic intervention. At the end of the experiment, tissue and serum samples were collected for colorimetric biochemical analyses (ALT, AST, cholesterol, triglyceride, LDL, HDL), RT-PCR measurement of NRF2, VCAM, ICAM, and NFkB gene expression, and histological examination.

Findings: The results of study demonstrated that thymoquinone treatment significantly decreased serum levels of ALT (94.5±10.85) and AST (202.16±32.12) compared to the MAFLD group ($p<0.001$). Thymoquinone also reduced cholesterol (257±50.37, $p<0.003$), triglycerides (90.16±12.73, $p<0.007$), and LDL (51.26±15.19, $p<0.001$), and increased HDL (48.83±6.5, $p<0.001$). Gene expression analysis revealed significant downregulation of VCAM (1.02±0.49, $p<0.001$), ICAM (0.89±0.16, $p<0.001$), and NFκB (1.44±0.44, $p<0.003$), and significant upregulation of NRF2 (0.99±0.22, $p<0.001$) following thymoquinone treatment compared to the MAFLD group. Histological assessments showed considerable improvement in tissue damage.

Conclusion: Based on the results of this study, thymoquinone can exert protective effects in MAFLD through the amelioration of lipid profile and hepatic enzyme levels.

Keywords: *Thymoquinone, Metabolic Dysfunction-Associated Fatty Liver Disease (MAFLD), Inflammatory Proteins, Cell Adhesion Molecules, Oxidative Stress, Gene Expression, Lipid Profile*.

Cite this article: Koohpeyma F, Koohpeyma MR, Kaviani M, Montazeri-Najafabady N, Dastghaib S, Shams M. The Effect of Thymoquinone on Liver Function and Inflammatory Gene Expression in an Animal Model of Metabolic Dysfunction-Associated Fatty Liver Disease. *Journal of Babol University of Medical Sciences*. 2026; 28: e23.

Introduction

The term 'metabolic dysfunction-associated fatty liver disease' (MAFLD) was proposed in 2020 to define fatty liver disease associated with systemic metabolic dysregulation. Since the introduction of this term, compelling evidence has emerged demonstrating the superiority of MAFLD over traditional terms such as non-alcoholic fatty liver disease (NAFLD) in several critical domains, including the prediction of liver-related and extrahepatic mortality, disease correlations, and identification of at-risk populations (1). MAFLD is now widely recognized as the hepatic manifestation of metabolic syndrome. Mirroring the ongoing diabetes and obesity pandemics, the global prevalence of MAFLD is steadily rising (2).

The clinical and pathological spectrum of MAFLD encompasses a continuum ranging from simple steatosis-defined as the accumulation of lipids within more than 5% of hepatocytes-to a considerably more aggressive inflammatory phenotype termed non-alcoholic steatohepatitis (NASH). NASH is histologically characterized by the concurrent presence of steatosis, evidence of hepatocellular injury manifesting as ballooning degeneration, and lobular inflammation (3).

As of the present time, there remains no approved pharmacological agent specifically indicated for the treatment of NASH. Therefore, current clinical management is predominantly centered on lifestyle modifications aimed at reducing body weight. Ongoing efforts in drug development are primarily directed toward the restoration of metabolic homeostasis and the suppression of pro-inflammatory and pro-fibrotic pathways (4). Over the last decade, more than twenty distinct molecular entities have been investigated as potential NASH therapies. The majority of these drugs were discontinued following unsuccessful clinical trials; some remain in early-phase development (phase 1 and 2), and only a few have advanced to phase 3 trials. To date, the most promising pharmaceutical approaches include antidiabetic drugs, FXR agonists, peroxisome proliferator-activated receptor (PPAR) agonists, and thyroid hormone receptor agonists (2).

Nigella sativa, a plant species belonging to the Ranunculaceae (buttercup) family, is commonly referred to as black seed or black cumin (5). Thymoquinone (chemically designated as 2-isopropyl-5-methylbenzo-1,4-quinone) represents the principal active pharmacological constituent of Nigella sativa and is recognized to possess a remarkably broad array of biological effects. An expanding volume of scientific literature has investigated the diverse therapeutic activities of thymoquinone, encompassing antioxidant, antitumor, antidiabetic, anti-inflammatory, and lipid-lowering properties (6). In recent years, the hepatoprotective potential of thymoquinone against various forms of hepatic injury has been experimentally validated. Specifically, this compound has been demonstrated to ameliorate hepatic steatosis, attenuate the progression of liver fibrosis, and function as an inhibitor of NAFLD initiation in rodent models of the disease (7). Furthermore, a substantial body of evidence indicates that thymoquinone has very low adverse effects and a low degree of toxicity (6).

A substantial body of research has been conducted to evaluate the therapeutic effects of thymoquinone across a spectrum of hepatic disorders. Asgharzadeh et al. demonstrated that thymoquinone administration effectively restores liver fibrosis induced by chronic inflammation, an effect likely mediated through its modulatory influence on oxidative stress status (7). Thymoquinone has consistently been shown to ameliorate liver injury across diverse experimental models, including cholestatic liver diseases (8), carbon tetrachloride (CCl4)-induced hepatotoxicity (9), and non-alcoholic steatohepatitis (NAFLD), via restoration of pro-oxidant antioxidant balance (PAB), modulation of inflammatory cascades, and enhancement of hepatic enzyme function (10). The compound exhibits considerable promise as a hepatoprotective agent, as evidenced by its capacity to effectively suppress thioacetamide-induced hepatic fibrosis in rat models through the inhibition of oxidative stress and inflammatory pathways, specifically via TGF-β/Smad-

dependent signaling (11). In a separate investigation, Nigella sativa seed oil was proposed as a potential dietary supplement for the prevention of fatty liver inflammation (12). Moreover, Yildiz et al. reported that treatment with Nigella sativa confers protection against hepatic ischemia-reperfusion injury in rats (13). Sayed-Ahmed et al. further noted that thymoquinone attenuates diethylnitrosamine-induced liver carcinogenesis through antioxidant signaling (14). Additional evidence indicates that thymoquinone mitigates non-alcoholic fatty liver disease in rodent models by suppressing oxidative stress, inflammatory mediators, and apoptotic cell death (15). Finally, thymoquinone has been shown to reverse experimental hypothyroidism-associated NAFLD (16).

Based on our comprehensive review of the existing literature, no experimental study has been conducted to date investigating the therapeutic effect of thymoquinone on MAFLD. Furthermore, recent evidence strongly supports the use of natural medicines-as opposed to synthetic drugs-for the treatment of various pathological conditions. The additional advantages offered by herbal medicines include their accessibility, straightforward administration, low cost, and a favorable safety profile with regard to adverse side effects. Taking into consideration the well-documented anti-inflammatory and antioxidant properties of thymoquinone across a wide range of diseases, including various liver disorders as demonstrated in previous studies, and given the absence of any prior research examining the specific effect of thymoquinone on MAFLD, the present study was designed and conducted with the following objectives. Specifically, this study aimed to evaluate the effect of thymoquinone on the lipid profile (including total cholesterol, triglycerides, low-density lipoprotein (LDL), and high-density lipoprotein (HDL)), on liver function enzymes (alanine aminotransferase (ALT) and aspartate aminotransferase (AST)), on the gene expression of key pathways involved in inflammation and antioxidant defense (namely NRF2, VCAM, ICAM, and NFkB), as well as on the histological architecture of liver tissue.

Methods

The present study is an interventional-experimental animal study conducted after approval from the Ethics Committee of Shiraz University of Medical Sciences, under the ethical approval code IR.SUMS.AEC.1402.066. All procedures were carried out in strict accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals. A total of 18 male rats of the C57BL/6 strain were used as experimental animals in this research. The rats had an average body weight of 25 grams and were approximately 12 weeks of age at the start of the study. These animals were selected using a completely randomized method and were kept under standard housing conditions for a period of two weeks prior to the experiment in order to allow for proper acclimatization. The sample size was determined utilizing the Resource Equation method, with an additional 20% allowance factored in to account for potential animal attrition during the study. The animal housing facility was maintained with an ambient temperature of approximately 20 to 22 degrees Celsius. A controlled photoperiod of 12 hours of light followed by 12 hours of darkness was provided. The relative humidity within the animal room was kept consistently between 40% and 55%. The rats were housed in cages made of polycarbonate material; each fitted with a mesh ceiling constructed of stainless steel. For the purposes of this study, the rats were randomly allocated into three distinct experimental groups, with six rats assigned to each group (n=6 per group). The grouping of the animals was carried out as detailed below:

Control group: In this group, no therapeutic intervention of any kind was performed on the animals throughout the study period. The animals in this group had access only to standard water and food.

Experimental group 1: This group of animals received a specially formulated high-fat diet for a duration of 8 weeks. The composition of this high-fat diet was as follows: 20% carbohydrates, 20% protein, and 60% fat.

Experimental group 2: This group similarly received the same high-fat diet for a period of 8 weeks. Following this dietary intervention, the animals then received thymoquinone (manufactured by Sigma Aldrich, USA, with catalog number 274666) at a dosage of 10 mg/kg. The thymoquinone was dissolved in 1 cc of normal saline solution and was administered to the animals via oral gavage on a daily basis for 35 consecutive days. Upon completion of the treatment period, all animals were anesthetized using a combination of ketamine at a dose of 80 mg/kg (supplied by Alfasan, Netherlands) and xylazine at a dose of 5 mg/kg (also supplied by Alfasan, Netherlands). Following anesthesia, blood samples were collected from each animal. These blood samples were placed into sterile centrifuge tubes and were left undisturbed at room temperature for a period of 20 minutes to allow for proper clot formation. Thereafter, the samples were subjected to centrifugation at a speed of 3000 revolutions per minute (rpm) for 15 minutes. The serum layer, which separated from the clotted blood during centrifugation, was carefully isolated and subsequently used for the measurement of various biochemical parameters. It is important to note that the euthanasia of the rats was carried out either by placing the animals inside a chamber filled with carbon dioxide (CO_2) or by following other relevant and approved guidelines for animal sacrifice.

Method of inducing the fatty liver model: In order to establish an experimental model of non-alcoholic fatty liver disease, a high-fat diet was administered to all rats included in the study, with the sole exception of the healthy control group animals. This dietary regimen was continued for a total of 8 weeks. The high-fat diet utilized for this purpose provides an energy content of 2.5 kilocalories per gram (kcal/g). In terms of its macronutrient composition, the diet contains 20% protein, 20% carbohydrates, and 60% fat, as previously described (17). Complete details regarding the precise composition of this high-fat diet are presented in Table 1.

Table 1. Details of the diet formula

Class Description	Ingredients	Grams
Protein	Casein, Lactic, 30 Mesh	200
Protein	Cystine, L	3
Carbohydrate	Lodex 10	125
Carbohydrate	Sucrose, Fine Granulated	72.80
Fiber	Solka Floc, FCC200	50
Fat	Lard	245
Fat	Soybean Oil, USP	25
Mineral	S10026B	50
Vitamin	Choline Bitartrate	2
Vitamin	V10001C	1
Dye	Dye, Blue FD&C #1, Alum. Lake 35-42%	0.05
	Total	773.85

Assessment of biochemical parameters: Measurement of biochemical parameters such as alanine aminotransferase, aspartate aminotransferase, cholesterol, triglycerides, LDL, and HDL concentrations was performed using the colorimetric method and a VIS spectrophotometer (model S2150, manufactured by UNICO, USA).

Molecular evaluations and expression level assessments of NRF2, VCAM, ICAM, and NFκB genes: For the purpose of conducting molecular investigations and quantifying the expression levels of NRF2, VCAM, ICAM, and NFκB genes using RT-PCR, tissue fragments obtained from the liver, aorta, and heart were harvested and immediately preserved in liquid nitrogen. Total RNA was extracted from liver samples collected from the experimental animals using TRIzol reagent (Invitrogen) and the procedure was carried out strictly in line with the manufacturer's guidelines. cDNA synthesis was performed using one microgram of RNA following the manufacturer's protocol (PrimeScript™ RT Reagent Kit, Takara).

The yield and quality of the extracted RNA were assessed spectrophotometrically using a NanoDrop 1000 instrument (NanoDrop Technologies, Montchanin, DE). Quantitative RT-PCR was performed on an Applied Biosystems StepOnePlus™ Real-Time PCR System using SYBR Premix Ex Taq™ II (Takara, Cat. #R820L) to determine mRNA expression levels.

Quantitative real-time PCR was performed on a StepOnePlus™ Real-Time PCR System (Thermo Fisher Scientific, USA). The primer sequences employed for each target gene are presented in Table 2. Relative expression levels between experimental groups were calculated from threshold cycle (Ct) values. Ct values of the target genes were normalized to the endogenous reference genes β-actin and GAPDH within each sample. Fold-change in relative gene expression was determined using the $2^{-\Delta\Delta Ct}$ method.

Table 2. List of oligonucleotide primer sequences employed for RT-PCR analysis

Gene	Sequences (5'→3')
NF-κB: Sense primer	TAGATGACCATGAGTCGCTTGC
NF-κB: Anti-sense primer	GCCAAACTTGCTCCATGTCC
NRF2: Sense primer	TGCCAAAGAAGGACACGACA
NRF2: Anti-sense primer	GCAGGCTATTGCTCATCACAG
ICAM-1: Sense primer	CAATTTCTCATGCCGCACAG
ICAM-1: Anti-sense primer	AGCTGGAAGATCGAAAGTCCG
VCAM-1: Sense primer	TGAACCCAAACAGAGGCAGAGT
VCAM-1: Anti-sense primer	GGTATCCCATCACTTGAGCAGG
GAPDH: Sense primer	AAAGAGATGCTGAACGGGCA
GAPDH: Anti-sense primer	ACAAGGGAAACTTGTCCACGA

Histological studies: Immediately after blood collection, the experimental animals were euthanized and subjected to complete necropsy. The target organs, including the liver, heart, and aorta, were carefully dissected and excised. The harvested tissues were thoroughly washed with normal saline to remove blood and debris, and were then immersed in 10% buffered formalin solution for fixation to preserve tissue architecture for subsequent pathological examination. Following routine tissue processing, paraffin-embedded sections were prepared and stained with hematoxylin and eosin (H&E). The stained histological slides were then examined under a light microscope for histopathological evaluation.

For the purpose of statistical analysis, all collected data were processed using SPSS software, version 23 (SPSS Inc., Chicago, Illinois, USA). Prior to selecting the appropriate statistical tests, the normality of the data distribution was assessed using the Kolmogorov–Smirnov test. Additionally, the assumption of homogeneity of variances was verified. As the data were found to follow a normal distribution and demonstrated equal variances across groups, parametric tests were deemed suitable for further analysis. To compare the mean values of the measured parameters in different experimental groups, a one-way analysis of variance (ANOVA) was performed. This was followed by Tukey's post hoc test to identify specific group differences. The results are expressed as mean±standard deviation (SD). A probability value (p-value) of

less than 0.05 was considered to indicate statistical significance. All graphical representations and figures were created using GraphPad Prism software, version 9.5.0.

Results

Effect of thymoquinone on hepatic enzyme parameters in the MAFLD model: The establishment of the MAFLD model was associated with a statistically significant increase in the serum levels of hepatic enzymes, specifically alanine aminotransferase (ALT) and aspartate aminotransferase (AST), when compared with the healthy control group ($p<0.001$). By contrast, therapeutic intervention with thymoquinone produced a marked and significant decrease in the levels of both ALT and AST in comparison with the untreated MAFLD group ($p<0.001$). Notably, the ALT and AST levels measured in the thymoquinone-treated group were also significantly lower than those observed in the control group, with the difference reaching statistical significance for ALT ($p=0.028$) and for AST ($p=0.045$). These findings are presented in Figure 1.

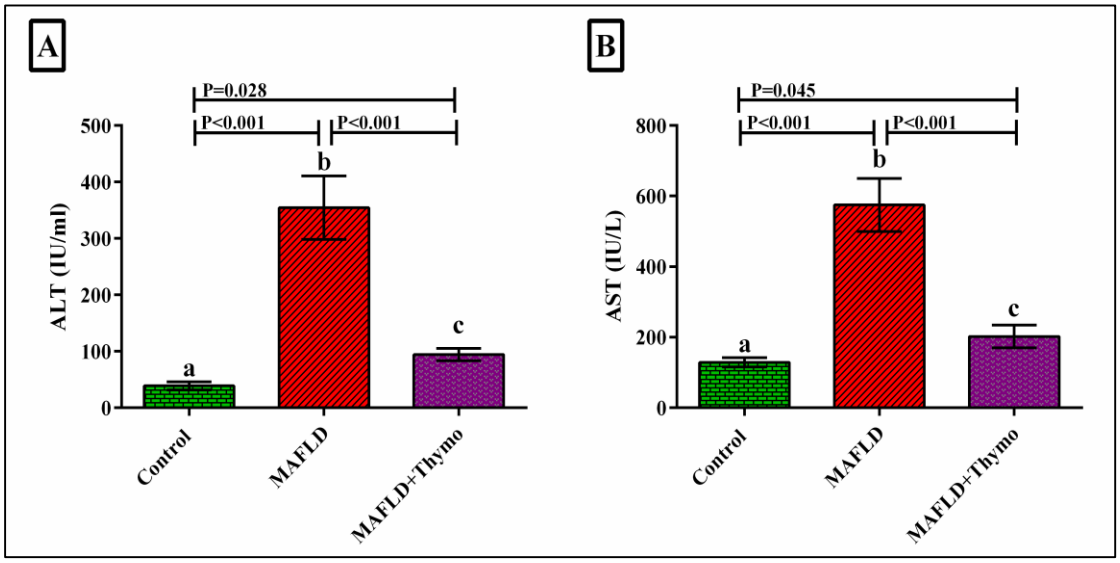

Figure 1. Comparison of the effect of thymoquinone on liver enzymes in the MAFLD model

The effect of thymoquinone on biochemical parameters in the MAFLD model: The modulatory effects of thymoquinone on key serum biochemical markers, specifically total cholesterol, triglycerides, high-density lipoprotein (HDL), and low-density lipoprotein (LDL), in the MAFLD model are presented in Table 3. The establishment of the MAFLD model led to a significant ($p<0.001$) decrease in serum HDL levels, accompanied by marked increases in total cholesterol ($p<0.001$), triglycerides ($p<0.001$), and LDL ($p<0.001$) when compared with the healthy control group. Therapeutic intervention with thymoquinone in MAFLD-affected rats resulted in beneficial modulations of the lipid profile. Specifically, thymoquinone treatment produced significant reductions in cholesterol ($p<0.003$), triglycerides ($p<0.007$), and LDL ($p<0.001$), while concomitantly increasing HDL levels ($p<0.001$). It should be noted, however, that despite these improvements, the levels of cholesterol ($p<0.001$), triglycerides ($p<0.008$), and LDL ($p<0.002$) in the thymoquinone-treated group remained significantly elevated compared with those observed in the healthy control group.

Table 3. Effect of thymoquinone on serum biochemical parameters in the MAFLD model

Groups	TG (mg/dl) Mean±SD	Cholesterol (mg/dl) Mean±SD	HDL (mg/dl) Mean±SD	LDL (mg/dl) Mean±SD
Control	66.17±13.24[a]	104.33±6.02[a]	50.50±3.78[a]	9.25±1.43[a]
MAFLD	114.33±8.66[b]	361.71±59.88[b]	34.00±1.26[b]	134.67±25.87[b]
MAFLD+Tymo	90.17±12.73[c]	257.00±50.37[c]	48.83±6.55[a]	51.27±15.20[c]

Values are expressed as mean±standard deviation (SD). The letters a, b, and c on the columns show which groups are statistically similar. If columns share the same letter, they are not significantly different. If they do not share a letter, the difference is significant (p<0.05).

The effect of thymoquinone on the expression levels of NRF2, VCAM, ICAM, and NFκB genes in the MAFLD model: The effect of thymoquinone administration on the mRNA expression levels of the key molecular markers NRF2, VCAM, ICAM, and NFκB in the MAFLD model is presented in Table 4. The induction of the MAFLD model was associated with a significant increase in the expression levels of the pro-inflammatory and adhesion-related genes VCAM, ICAM, and NFκB, accompanied by a significant decrease in the expression of the antioxidant-related gene NRF2, when compared with the healthy control group. The significance values were p<0.001 for VCAM, p<0.001 for ICAM, p=0.002 for NFκB, and p<0.001 for NRF2. Therapeutic intervention with thymoquinone resulted in a significant reversal of these molecular alterations, as evidenced by marked reductions in VCAM, ICAM, and NFκB expression and a marked increase in NRF2 expression relative to the untreated MAFLD group. The significance values for these changes were VCAM (p<0.001), ICAM (p<0.001), NFκB (p=0.003), and NRF2 (p<0.001). Despite the positive modulatory effects of thymoquinone on the expression of these genes, no statistically significant differences remained between the thymoquinone-treated group and the healthy control group.

Table 4. Effect of thymoquinone on the expression levels of NRF2, VCAM, ICAM, and NFκB genes in the MAFLD model

Groups	NRF2 Mean±SD	NF-κB Mean±SD	ICAM-1 Mean±SD	VCAM-1 Mean±SD
Control	0.98±0.027[a]	0.997±0.024[a]	1.06±0.046[a]	1.01±0.04[a]
MAFLD	0.30±0.23[b]	2.49±0.99	1.83±0.41[b]	2.61±0.78[b]
MAFLD+Tymo	0.99±0.22[a]	1.046±0.45[a]	0.89±0.18[a]	1.03±0.49[a]

Values are expressed as mean ± standard deviation (SD). The letters a, b, and c on the columns show which groups are statistically similar. If columns share the same letter, they are not significantly different. If they do not share a letter, the difference is significant (p<0.05).

Effects of thymoquinone on hepatic histopathological changes: Histological examination of liver sections from the high-fat diet group revealed the presence of numerous lipid-laden vacuoles and marked hepatocyte hypertrophy, which together confirmed the successful induction of non-alcoholic fatty liver disease. In contrast, liver sections from the thymoquinone-treated group exhibited substantial histopathological improvement. Specifically, the accumulation of fatty vacuoles within the hepatic tissue was significantly reduced in the treatment group, indicating a favorable therapeutic effect of thymoquinone on liver histology (Image 1).

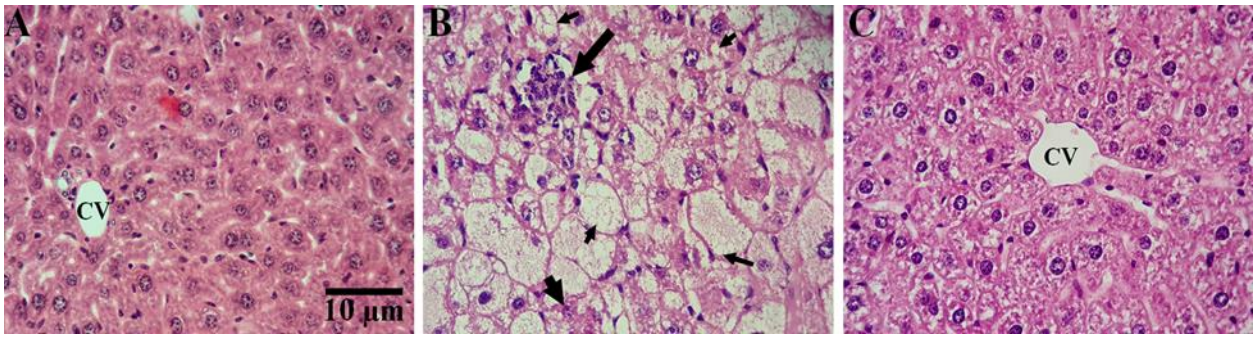

Image 1. Effects of thymoquinone on hepatic histopathological changes

Discussion

In the present study, following the successful induction of hepatic steatosis, the results revealed a statistically significant increase in serum levels of total cholesterol, triglycerides, and LDL, as well as a significant decrease in high-density lipoprotein cholesterol (HDL-C) levels in the MAFLD group when compared with the healthy control group. Subsequent investigations into the therapeutic effects of thymoquinone, administered at a dose of 10 mg/kg body weight over a period of 35 days, demonstrated that this bioactive compound exerted beneficial modulatory effects on the serum lipid profile in rats. Specifically, thymoquinone treatment resulted in a marked reduction in cholesterol, triglyceride, and LDL levels, alongside a significant elevation in HDL levels. These observations are in close accordance with the findings reported by Kolbus et al. (18). Furthermore, the results of our study are consistent with previously published reports that have highlighted the beneficial effects of thymoquinone on dyslipidemia and lipid metabolism disturbances (19). In an investigation conducted by Pei et al., it was similarly demonstrated that thymoquinone, via its lipid-lowering properties-specifically reducing cholesterol, triglycerides, and LDL-can exert protective effects on the cardiovascular system (20).

In the present study, ALT and AST levels were found to be significantly elevated in the MAFLD group relative to controls. Thymoquinone administration resulted in a significant reduction in both ALT and AST levels in the treated group compared to the MAFLD group. These observations are corroborated by Ahmad et al., who reported that thymoquinone at 80 mg/kg over 30 days was associated with beneficial alterations in hepatic enzyme activity, thereby explaining its potential antihyperglycemic properties (21). Consistent with this, several other investigations have documented the hepatoprotective and therapeutic effects of thymoquinone against various liver injury models (22, 23). Furthermore, another study concluded that thymoquinone, owing to its antioxidant capacity, reduces liver cancer induction by stabilizing cellular membranes, which consequently minimizes the leakage of liver enzymes (ALT, AST, ALP) into the circulation (24). In a similar study conducted in Iran, Jafari Nia et al. reported that 30-day treatment with thymoquinone at 5 and 10 mg doses significantly decreased triglyceride, total cholesterol, phospholipid, total bilirubin, glucose, insulin, VLDL, and LDL levels in an animal model of fatty liver (25).

In this study, molecular investigations were conducted to evaluate whether thymoquinone treatment could alter the concentrations of certain genes in a model of fatty liver disease. The results showed that thymoquinone significantly decreased the expression of the NF-κB gene, which had been markedly elevated as a result of fatty liver induction. This finding is consistent with the work of Salah et al., who observed that NF-κB gene expression declines progressively as thymoquinone concentrations increase (26). It is well established that thymoquinone exerts a direct inhibitory effect on NF-κB activation, and this mechanism, which has been documented in numerous previous reports, is likely responsible for the subsequent decrease

in pro-inflammatory mediators observed after thymoquinone administration (27). In addition, thymoquinone treatment also led to reduced expression of two other important genes: ICAM and VCAM. This observation is in line with prior studies, which have reported decreased VCAM-1 expression-both at the mRNA level and in terms of its functional activity-following thymoquinone exposure (28). Furthermore, evidence from another study indicates that pretreating cells with thymoquinone effectively suppresses the production of inflammatory cytokines such as interleukin-6 and interleukin-8, while also blocking the TNF-α-induced upregulation of adhesion molecules including ICAM-1 and VCAM-1 (29). VCAM-1 is a surface protein that plays a key role in the adhesion of monocytes to the vascular endothelium and their subsequent migration out of the bloodstream into tissues. Previous research has shown that pharmacological inhibition of VCAM-1 in NASH models leads to a reduction in VCAM-1 levels, decreased infiltration of macrophages into hepatic tissue, and a notable reduction in fibrosis (30).

In the present study, thymoquinone treatment was also found to increase the expression of the NRF2 gene within liver tissue. This observation is in agreement with the work of Elsherbiny et al., who have previously documented that thymoquinone is capable of raising NRF2 expression levels. NRF2 is a well-known transcriptional activator that plays an essential and multifaceted role in how cells respond to oxidative stress and exposure to heavy metals. It achieves this by driving the expression of a range of protective genes, including those encoding antioxidant enzymes and metallothioneins, which help neutralize reactive species and bind toxic metals (31). With respect to the underlying mechanism, thymoquinone appears to act by increasing NRF2 expression, and this upregulation subsequently leads to enhanced activity of important antioxidant molecules such as superoxide dismutase (SOD) and glutathione (GSH). Together, these changes help preserve the oxidation-reduction balance within the cell (32). It is proposed that the hepatoprotective effects of thymoquinone are mediated through inhibition of reactive oxygen species production and NF-κB signaling, modulation of various eicosanoid levels, and attenuation of fibrogenic events (33).

Histological analysis of liver tissue samples revealed that the healthy control group exhibited no signs of structural abnormality or pathological change. In contrast, the vehicle-treated group-which received the solvent without active treatment-developed severe hepatic steatosis. This steatosis presented predominantly as microvesicular fatty infiltration, with occasional macrovesicular changes, and was accompanied by noticeable swelling of the hepatocytes, indicative of cellular injury. When we examined the experimental groups in the current study, which were treated with thymoquinone at a dosage of 10 mg/kg body weight over an 8-week period, the histological findings showed that the extent of liver cell damage was markedly and significantly reduced compared to the vehicle group. These results are supported by a body of previous research, which has consistently demonstrated that thymoquinone exerts protective effects against liver injury across a range of experimental models. These include cholestatic liver disorders (8), hepatotoxicity induced by carbon tetrachloride (9), and non-alcoholic fatty liver disease. The underlying mechanisms proposed for this protection include the normalization of oxidant/antioxidant balance, the modulation of inflammatory responses, and the enhancement of liver enzyme activities, all of which contribute to improved hepatic function (10).

Several limitations of this study warrant acknowledgment, including the modest sample size, the omission of additional inflammatory and lipid parameters, the incomplete profiling of all genes implicated in fatty liver pathogenesis, and the lack of human clinical data. Accordingly, future human trials incorporating a broader range of biomarkers and larger cohorts are recommended to yield more definitive evidence regarding the therapeutic potential of thymoquinone in MAFLD.

The findings of the present study indicate that thymoquinone, at the tested dose and after a 35-day treatment period, significantly reduced serum lipid parameters as well as ALT and AST levels. Additionally, it downregulated the expression of oxidative stress- and inflammation-related genes, including NRF2, VCAM, ICAM, and NF-κB. These molecular changes were accompanied by marked improvements in hepatic histopathology, characterized by diminished fatty vacuole accumulation and attenuation of hepatocyte hypertrophy, collectively demonstrating the hepatoprotective efficacy of thymoquinone in an animal model of MAFLD.

The findings of this study provide clear and compelling evidence that thymoquinone has the potential to play a meaningful therapeutic role in the management of metabolic dysfunction-associated fatty liver disease. This protective effect appears to be mediated through several complementary mechanisms. First, thymoquinone reduces serum lipid profile levels, thereby addressing the dyslipidemia commonly associated with the condition. Second, it lowers the levels of hepatic functional enzymes such as ALT and AST, indicating reduced hepatocellular injury. Third, it mitigates oxidative stress and the inflammatory response triggered by the disease-specifically through the upregulation of the antioxidant-related gene NRF2 and the downregulation of the pro-inflammatory transcription factor NF-κB. Additionally, thymoquinone prevents the progression of histopathological damage to liver tissue. Considering these promising results, and given the well-established safety profile and minimal side effects of medicinal plants, there is considerable optimism that traditional medicine-particularly through the use of Nigella sativa (commonly known as black seed)-could pave the way for the development of new, effective, and well-tolerated pharmaceutical agents for the treatment of fatty liver disease. In this context, thymoquinone stands out as a particularly promising candidate for clinical evaluation across different subtypes of fatty liver disease. However, it must be emphasized that further research involving human participants is essential before these preclinical findings can be translated into clinical practice and definitive conclusions can be drawn.

Acknowledgment

The authors wish to acknowledge the Vice-Chancellor for Research of Shiraz University of Medical Sciences for providing financial support for this research.

References

1.Gofton C, Upendran Y, Zheng MH, George J. MAFLD: How is it different from NAFLD?. Clin Mol Hepatol. 2023;29(Suppl):S17-31.

2.Sangro P, de la Torre Aláez M, Sangro B, D'Avola D. Metabolic dysfunction-associated fatty liver disease (MAFLD): an update of the recent advances in pharmacological treatment. J Physiol Biochem. 2023;79(4):869-79.

3.Chalasani N, Younossi Z, Lavine JE, Diehl AM, Brunt EM, Cusi K, et al. The diagnosis and management of non-alcoholic fatty liver disease: practice Guideline by the American Association for the Study of Liver Diseases, American College of Gastroenterology, and the American Gastroenterological Association. Hepatology. 2012;55(6):2005-23.

4.Romero-Gómez M, Zelber-Sagi S, Trenell M. Treatment of NAFLD with diet, physical activity and exercise. J Hepatol. 2017;67(4):829-46.

5.Hannan MA, Rahman MA, Sohag AAM, Uddin MJ, Dash R, Sikder MH, et al. Black Cumin (*Nigella sativa* L.): A Comprehensive Review on Phytochemistry, Health Benefits, Molecular Pharmacology, and Safety. Nutrients. 2021;13(6):1784.

6.Darakhshan S, Bidmeshki Pour A, Hosseinzadeh Colagar A, Sisakhtnezhad S. Thymoquinone and its therapeutic potentials. Pharmacol Res. 2015;95-96:138-58.

7.Asgharzadeh F, Bargi R, Beheshti F, Hosseini M, Farzadnia M, Khazaei M. Thymoquinone restores liver fibrosis and improves oxidative stress status in a lipopolysaccharide-induced inflammation model in rats. Avicenna J Phytomed. 2017;7(6):502-10.

8.Oguz S, Kanter M, Erboga M, Erenoglu C. Protective effects of thymoquinone against cholestatic oxidative stress and hepatic damage after biliary obstruction in rats. J Mol Histol. 2012;43(2):151-9.

9.Hassanein KM, Al-Emam A, Radad K. Prophylactic effects of thymoquinone against carbon tetrachloride-induced hepatic damage in Sprague-Dawley rats. J Appl Pharm Sci. 2016;6(2):167-71.

10.Beheshti F, Hosseini M, Shafei MN, Soukhtanloo M, Ghasemi S, Vafaee F, et al. The effects of Nigella sativa extract on hypothyroidism-associated learning and memory impairment during neonatal and juvenile growth in rats. Nutr Neurosci. 2017;20(1):49-59.

11.Raghunandhakumar S, Ezhilarasan D, Shree Harini K. Thymoquinone protects thioacetamide-induced chronic liver injury by inhibiting TGF-β1/Smad3 axis in rats. J Biochem Mol Toxicol. 2024;38(4):e23694.

12.Al-Okbi SY, Mohamed DA, Hamed TE, Edris AE. Potential protective effect of Nigella sativa crude oils towards fatty liver in rats. Eur J Lipid Sci Technol. 2013;115(7):774-82.

13.Yildiz F, Coban S, Terzi A, Ates M, Aksoy N, Cakir H, et al. Nigella sativa relieves the deleterious effects of ischemia reperfusion injury on liver. World J Gastroenterol. 2008;14(33):5204-9.

14.Sayed-Ahmed MM, Aleisa AM, Al-Rejaie SS, Al-Yahya AA, Al-Shabanah OA, Hafez MM, et al. Thymoquinone attenuates diethylnitrosamine induction of hepatic carcinogenesis through antioxidant signaling. Oxid Med Cell Longev. 2010;3(4):254-61.

15.Awad AS, Abd Al Haleem EN, El-Bakly WM, Sherief MA. Thymoquinone alleviates nonalcoholic fatty liver disease in rats via suppression of oxidative stress, inflammation, apoptosis. Naunyn Schmiedebergs Arch Pharmacol. 2016;389(4):381-91.

16.Ayuob NN, Abdel-Hamid AAHM, Helal GMM, Mubarak WA. Thymoquinone reverses nonalcoholic fatty liver disease (NAFLD) associated with experimental hypothyroidism. Rom J Morphol Embryol. 2019;60(2):479-86.

17.Cho J, Lee I, Kim D, Koh Y, Kong J, Lee S, et al. Effect of aerobic exercise training on non-alcoholic fatty liver disease induced by a high fat diet in C57BL/6 mice. J Exerc Nutrition Biochem. 2014;18(4):339-46.

18.Kolbus D, Ramos OH, Berg KE, Persson J, Wigren M, Björkbacka H, et al. CD8+ T cell activation predominate early immune responses to hypercholesterolemia in Apoe^{-}(/)$^{-}$ mice. BMC Immunol. 2010;11:58.
19.Asgary S, Sahebkar A, Goli-Malekabadi N. Ameliorative effects of Nigella sativa on dyslipidemia. J Endocrinol Invest. 2015;38(10):1039-46.
20.Pei ZW, Guo Y, Zhu HL, Dong M, Zhang Q, Wang F. Thymoquinone Protects against Hyperlipidemia-Induced Cardiac Damage in Low-Density Lipoprotein Receptor-Deficient (LDL-R$^{-/-}$) Mice via Its Anti-inflammatory and Antipyroptotic Effects. Biomed Res Int. 2020;2020:4878704.
21.Ahmad S, Beg ZH. Elucidation of mechanisms of actions of thymoquinone-enriched methanolic and volatile oil extracts from Nigella sativa against cardiovascular risk parameters in experimental hyperlipidemia. Lipids Health Dis. 2013;12:86.
22.Rahmani AH, Almatroudi A, Babiker AY, Khan AA, Alsahli MA. Thymoquinone, an Active Constituent of Black Seed Attenuates CCl4 Induced Liver Injury in Mice via Modulation of Antioxidant Enzymes, PTEN, P53 and VEGF Protein. Open Access Maced J Med Sci. 2019;7(3):311-7.
23.Galaly SR, Ahmed OM, Mahmoud AM. Thymoquinone and curcumin prevent gentamicin-induced liver injury by attenuating oxidative stress, inflammation and apoptosis. J Physiol Pharmacol. 2014;65(6):823-32.
24.Yki-Järvinen H. Nutritional Modulation of Non-Alcoholic Fatty Liver Disease and Insulin Resistance. Nutrients. 2015;7(11):9127-38.
25.Jafari Nia L, Haeri Rouhani A, Yaghmaei P. Effect of thymoquinone on improvement of lipid profile and liver enzymes in fatty liver-induced NMRI mice. Iran J Med Aromat Plants. 2018;34(2):193-205.
26.Salah A, Sleem R, Abd-Elaziz A, Khalil H. Regulation of NF-κB Expression by Thymoquinone; A Role in Regulating Pro-Inflammatory Cytokines and Programmed Cell Death in Hepatic Cancer Cells. Asian Pac J Cancer Prev. 2023;24(11):3739-48.
27.Zhang L, Bai Y, Yang Y. Thymoquinone chemosensitizes colon cancer cells through inhibition of NF-κB. Oncol Lett. 2016;12(4):2840-5.
28.Abbasnezhad A, Niazmand S, Mahmoudabady M, Rezaee SA, Soukhtanloo M, Mosallanejad R, et al. Nigella sativa L. seed regulated eNOS, VCAM-1 and LOX-1 genes expression and improved vasoreactivity in aorta of diabetic rat. J Ethnopharmacol. 2019;228:142-7.
29.Umar S, Hedaya O, Singh AK, Ahmed S. Thymoquinone inhibits TNF-α-induced inflammation and cell adhesion in rheumatoid arthritis synovial fibroblasts by ASK1 regulation. Toxicol Appl Pharmacol. 2015;287(3):299-305.
30.Carr RM. VCAM-1: closing the gap between lipotoxicity and endothelial dysfunction in nonalcoholic steatohepatitis. J Clin Invest. 2021;131(6):e147556.
31.Elsherbiny NM, El-Sherbiny M. Thymoquinone attenuates Doxorubicin-induced nephrotoxicity in rats: Role of Nrf2 and NOX4. Chem Biol Interact. 2014;223:102-8.
32.Sabir S, Saleem U, Akash MSH, Qasim M, Chauhdary Z. Thymoquinone Induces Nrf2 Mediated Adaptive Homeostasis: Implication for Mercuric Chloride-Induced Nephrotoxicity. ACS Omega. 2022;7(8):7370-9.
33.Bai T, Lian LH, Wu YL, Wan Y, Nan JX. Thymoquinone attenuates liver fibrosis via PI3K and TLR4 signaling pathways in activated hepatic stellate cells. Int Immunopharmacol. 2013;15(2):275-81.