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Original Article

Comparative effects of thymol and vitamin E on nonalcoholic fatty liver disease in male Wistar rats

Efeitos comparativos de timol e de vitamina E em doença hepática gordurosa não alcoólica em ratos Wistar machos

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Abstract

Following the obesity epidemics, nonalcoholic fatty liver disease (NAFLD) has grown in prevalence and become a main cause of morbidity and death, intimately linked to cardiovascular disease, cancer, and cirrhosis. The key factor in the evolution of NAFLD is thought to be oxidative stress. Because most patients cannot change their lifestyle or dietary habits, a pharmaceutical strategy is now required to treat NAFLD. Nonalcoholic fatty liver disease (NAFLD), including nonalcoholic steatohepatitis, is treated with vitamin E. (NASH). Vitamin E is also a powerful antioxidant that has been demonstrated to lower oxidative stress in people with NAFLD. Thymol is a monoterpene phenol with a variety of pharmacological effects, however its anti-fatty liver properties have yet to be investigated. Despite the fact that oxidative stress is thought to have a role in the etiology of nonalcoholic steatohepatitis, antioxidant therapies have not been well studied in the treatment of nonalcoholic steatohepatitis. The goal was to learn more about vitamin E and thymol's biological activities, with a particular emphasis on their therapeutic effectiveness in NAFLD. Four groups of thirty-two adult male rats were formed (healthy control, thymol, Vit E, and fatty liver). For 28 days, rats were given either oral vitamin E (200 mg/kg) or thymol (50 mg/kg) randomly. The levels of ALT, AST, TNF- α, Ferritin, CK-MB enzymes, and MAPK gene expression were then determined in the serum. Based on a random effect model analysis, at the end of 28 days of therapy, ALT (41.43 U/L), AST (47.91 U/L), Ferritin (1.13 pg/dl), CK-MB (251.22 IU/L), TNF-α (95.39 pg/mL) ($p \leq 0.001$), and MAPK gene expression levels ($p \leq 0.05$) significantly reduced in both experimental groups compared with the fatty liver group. Vitamin E and thymol therapy is a safe, affordable, and effective therapeutic option in the fatty liver group. Patients with fatty liver disease should be encouraged to take vitamin E and Thymol supplements, which are both safe and affordable, because more effective new therapeutic options are lacking.

Keywords: steatosis, thymol, vit E, ferritin, MAPK, CK-MB, TNF-α, ALT, AST.

Resumo

Após a epidemia de obesidade, a doença hepática gordurosa não alcoólica (DHGNA) cresceu em prevalência e se tornou a principal causa de morbidade e morte, intimamente ligada a doenças cardiovasculares, como câncer e cirrose. Acredita-se que o fator chave na evolução da DHGNA seja o estresse oxidativo. Como a maioria dos pacientes não pode mudar seu estilo de vida ou hábitos alimentares, uma estratégia farmacêutica para tratar a DHGNA se fez necessária. A doença hepática gordurosa não alcoólica (DHGNA), incluindo esteatohepatite não alcoólica, é tratada com vitamina E. (NASH). A vitamina E também é considerada um poderoso antioxidante que demonstrou reduzir o estresse oxidativo em pessoas com DHGNA. O timol é um fenol monoterpeno com uma variedade de efeitos farmacológicos, porém suas propriedades antigorduras no fígado ainda não foram investigadas. Apesar de se pensar que o estresse oxidativo tem um papel na etiologia da esteatohepatite não alcoólica, as terapias antioxidantes não foram ainda bem estudadas no tratamento da esteatohepatite não alcoólica. O objetivo deste trabalho foi investigar sobre as possíveis atividades biológicas da vitamina E e do timol, com ênfase particular na sua eficácia terapêutica na DHGNA. Foram formados quatro grupos de trinta e dois ratos machos adultos (controle saudável, timol, vitamina E e fígado gorduroso). Durante 28 dias, os ratos receberam vitamina E oral (200 mg/kg) ou timol (50 mg/kg) aleatoriamente. Os níveis de ALT, AST, TNF-α, Ferritina, enzimas CK-MB e expressão do gene MAPK foram então determinados no soro. Com base em uma análise de modelo de efeito aleatório, ao final de 28 dias de terapia, ALT (41,43 U/L), AST (47,91 U/L), Ferritina (1,13 pg/dl), CK-MB (251,22 IU/L), TNF-α (95,39 pg/mL) ($p \leq 0,001$) e os níveis de expressão do gene MAPK ($p \leq 0,05$) reduziram significativamente em ambos os grupos experimentais em comparação com o grupo fígado gorduroso. A terapia com vitamina E e timol é uma opção terapêutica segura, acessível e eficaz no grupo de fígado gorduroso. Pacientes com doença hepática gordurosa

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devem ser encorajados a tomar suplementos de vitamina E e Timol, que são seguros e acessíveis, dado que faltam novas opções terapêuticas mais eficazes.

Palavras-chave: esteatose, timol, vitamina E, ferritina, MAPK, CK-MB, TNF-α, ALT, AST.

1. Introduction

The excessive accumulation of triglycerides in the form of lipid droplets in the liver is known as hepatic steatosis, or fatty liver. Coexistence of hepatic steatosis with other liver illnesses (most notably hepatitis C) has been linked to a poor treatment response and faster progression (Powell et al., 2010). New medications, the majority of which are derived from plants. Thymol, for example, is a dietary monoterpene phenol derivative of cymene found in the oils of thyme and plants like Thymus vulgaris, as well as citrus trees (Archana et al., 2011). The major components of its essential oil are thymol and carvacrol, which include considerable levels of phenols and flavonoids. Zataria multiflora Boiss has been shown to have antioxidative, anti-inflammatory, antidiabetic, hepatoprotective, and insulin sensitivity boosting properties in both in vivo and in vitro investigations (Mohammadi et al., 2014; Sajed et al., 2013). Thymol has various biological properties, including anti-inflammatory (Tsai et al., 2011) and antioxidant (Amiri, 2012) effects in both cellular and in vitro settings. Vitamin E is well recognized as the most important lipid-soluble chain-breaking antioxidant in the human body. Vitamin E molecules have anti-atherogenic and anti-inflammatory effects and antioxidant characteristics (El Hadi et al., 2018). Although the pathophysiology of NAFLD and its development to fibrosis is yet unknown, oxidative stress is thought to play a key role in the fatal hepatocyte damage associated with the disease. The activation of pro-inflammatory cytokines in adipose and liver tissues, such as TNF, has been linked to the development and progression of NAFLD (Kernan and Carcillo, 2017). Ferritin, on the other hand, is a protein that is directly linked to increased liver inflammation and rises significantly in liver necrosis, and as ferritin levels rise, the risk of major liver disease rises as well. Iron buildup is observed in liver fibrosis, and iron accumulation generates inflammatory cytokines and begins oxidative processes, according to the pathophysiological perspective (Du et al., 2017) Ferritin increases MAPK and stimulates NF-B in inflamed hepatocytes in an iron-independent way. According to the goal of this research and the protective effect of vitamin E as an antioxidant, plasma -tocopherol levels are also observed to be lowered in NASH patients, providing a theoretical foundation for its use in treating NASH (Kernan and Carcillo, 2017). On the other hand, because thymol is a natural antioxidant and because there are few human studies on the effect of thymol on NAFLD, the current study compares the effects of vitamin E and thymol as a treatment for NAFLD, noting that they both reduce inflammatory factors, examine the gene expression pathway, and reduce tissue inflammation.

2. Materials and methods

2.1. Animals and diets

Pastor Institute of Iran sold 32 mature male Wistar rats with body weights of 200 g and ages of 140 days. The animals were kept in an air-conditioned room with a constant temperature (22 ± 2 °C) and humidity (40% ± 5%) of 5%. They were kept in steel cages with a light-dark cycle of 12 hours. During the testing time, food and drink were freely accessible. Animal ethics boards approved the procedure for the current investigation. For the induction of fatty liver in animals, 2 mg of high-fat emulsion (400 g corn oil, 15 g sucrose, 80 g whole milk powder, 100 g cholesterol, 10 g sodium deoxycholate, 36 g tween 80, 31 g Propylene glycol1, 2 g multivitamin, 10 g salt, 1 g minerals, 300 mL distilled water) was gavaged for forty days (Figure 1).

2.2. Experimental design and animal treatment protocol

The rats were divided into four groups, each with eight rats: group 1 (normal control), group 2 (fatty liver control), and group 3 (high-fat emulsion (2 mg/kg body weight by gavage). Group 3 got 200 mg/kg/day of vitamin E for 28 days after developing a fatty liver. After having a fatty liver, Group 4 was given 50 mg/kg/day of thymol for 28 days.

2.3. Determination of biochemical parameters

2.3.1. Liver enzymes measurement

The levels of liver enzymes (ALT and AST) were measured using an enzymatic colorimetric approach using

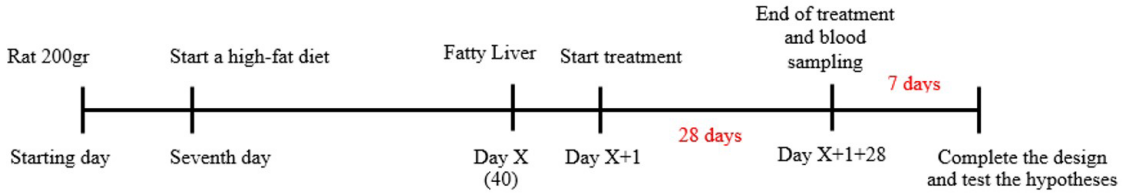

Figure 1. Diet and treatment for this study.

a biochemical Auto Analyzer instrument to assess the degree of liver damage. Iran's Pars Azmoon Co provided the kits. *Measuring plasma levels TNF-α*

Plasma levels TNF-α (Diaclone, France) were measured by ELISA methods (36).

2.3.2. Measuring plasma levels CK-MB

Quantitative detection kits of CK-MB in serum or plasma by photometric method were purchased from Pars Azmoon Co, Iran.

2.3.3. Measuring plasma levels ferritin

The ferritin assay kit was acquired from Pars Azmoon Co in Iran and used in the experiment. On a nutshell, the test was performed in a wells plate. In the non-enzymatic, positive control, and samples wells, add the assay buffer and co-substrate combination. However, other reagents, such as diluted Ferritin, were added in both the positive and negative samples.*MAPK Gene Expression*.

2.3.4. Extraction of total RNA from rat blood

Adult rats were given 2 mg of high-fat emulsion for forty days before being given 50 mg/kg thymol for another 28 days. The RNX Plus technique was used to extract total RNA in this investigation. The following are the stages in this procedure: A sterile syringe needle tip was used to homogenize 1 mL of RNX Plus solution (Qiagen-Germany) into a 2 mL tube containing blood sample. Blood samples were vortexed for 5-10 seconds before incubating for 5 minutes at room temperature. Chloroform (200 l) was added to the solution. To thoroughly blend, shake gently for 15 seconds. Incubate for 5 minutes on ice or at 4 °C. It was centrifuged at 4 °C for 4 minutes at 12,000 rpm. The supernatant's aqueous phase was transferred to a fresh 1.5 mL RNase Free microtube and a corresponding amount of cold isopropanol. Mix well and chill for 15 minutes. To determine the Pellet, the mixture was centrifuged at 12,000 rpm for 15 minutes at 4 °C. The supernatant was removed, and 1 mL of 75 percent cold ethanol was added and agitated gently until Pellet was gone. They were then centrifuged at 4°C for 15 minutes at 7500 rpm. Remove the supernatant and dry the pellets at room temperature. In 50 liters of DEPC water, the Pellet was dissolved. After that, the pellet was put in a water bath at a temperature of 55 to 60 °C. To guarantee the integrity of the total RNA extracted, electrophoresis on a percent 1 agarose gel and observation of strong bands 18s and 28s were done after the extraction processes were completed, and the isolated RNA was kept at -20 °C until further use.

2.3.5. Construction of cDNA from total RNA

The Revert Aid First Strand cDNA Synthesis Kit was used to make the cDNA (Fermentas Co., Germany). The following is how cDNA was made according to Kit's instructions: In the microtubule, 1 liter of Oligo18 (dt), 8 liters of DEPC water, and 3 liters of RNA were combined. The microtubes were heated for 5 minutes at 70°C (ABI thermocycler). The microtubes were placed on the ice as soon as possible. 4 µl of buffer (5X), 1 µl of RNase inhibitor, and 2 µl of dNTP10mM were added to the contents of the microtube. The microtubes were incubated at 37°C for 5 minutes after mixing the contents. The reverse transcriptase enzyme was added to the reaction ingredients in the amount of 1L. The microtubes were then incubated for 60 minutes at 42 °C. Finally, the reaction was halted at 70 °C for 10 minutes. The produced cDNA was kept at -20 °C until it was needed.*Design Primer*

In the rat used in this investigation, a primer was built for the p53MAPK gene, as shown in Table 1. Beacon Designer was used to create this. The mRNA sequences of each gene were aligned after getting the DNA sequences of these genes from NCBI (National Center for Biotechnology Information) and locating the exon and intron sections. The program then verified the primers' accurate binding to the matching sequence. Finally, the NCBI's BLAST software looked at the specificity of primer binding to the target gene (Table 1). To enhance the intron confidence interval, the temperature of the primers was melted close and even at the primer intervals in this investigation. This will stop the gene fragment from replicating on the DNA chain.

2.3.6. RT-PCR technique

One of the most prevalent techniques for DNA replication is polymerase chain reaction (PCR). PCR is used for various purposes, including providing copies of a target gene, analyzing the presence or absence of a gene, detecting prenatal disorders, confirming fetal sex, and discovering the etiology of an unknown condition. MAPK gene was proliferated utilizing RT-PCR methods in this work for cDNA production. In this investigation, the RT-PCR reaction's ultimate volume was considered 50 L (Table 2).

Then, the microtubule including the material is inserted into the thermocyclers device, which is indicated in Table 3.

2.3.7. Real time PCR method

To confirm the right performance of primers, materials, and their accuracy in PCR reactions, all procedures in this research were double-checked using the traditional PCR technique. Steps relating to the Real-time PCR approach were done using SYBR®Premix Ex TaqTM after the

Table 1. Primer sequence of MAPK genes.

Genes	Primer	Primer Sequence	Melting temperature	Proliferous fragment length (bp)
MAPK	F	5′-CCTGCGAGGGCTGAAGTA-3′	60	226
	R	5′-ACGGACCAAATATCCACTGTCT-3′	60	

Table 2. Materials and values used to PCR for the MAPK gene (µL).

Materials	Values
Double Distilled H_2O	36.5
10x-Reaction Buffer	5
dNTP	1
$MgCl_2$	2
Primer F (10 pmol/µL)	1.5
Primer R (10 pmol/µL)	1.5
Taq Polymerase	0.5
cDNA	2
Total Volume	**50**

Table 3. Application of the thermocycler device to perform RT-PCR technique for the MAPK gene.

Cycle	Temperature	Time	Number of cycles
Initialization Step	94	5 min	1
Denaturation Step	94	1 min	35
Annealing Step	60	30 sec	35
Extension/ Elongation Step	72	2 min	35
Final Elongation	72	5 min	1

traditional PCR experiment and optimization (TaKaRa, Dalian, China). The following is a list of the materials required to execute the Real-Time PCR procedure (Table 4).

All materials and primers were examined to optimize and evaluate the Real-time PCR processes. To create a standard curve, a cDNA sample was produced with varied concentrations (6 concentrations, respectively: 1, 1:10, 1: 100, 1: 1000, 1: 10000, 1: 100000) and the expression of IGF-1R, InsR, and GAPDH was assessed at different concentrations. Materials and cDNA were combined according to Table 5, and a Corbett Research Rotor-Gene 6000 real-time DNA analysis instrument was used to record the fluorescence quantity (Corbett Research, Sydney, Australia). The device's settings are also included in the table.

Finally, the expression level of MAPK gene was computed by the following Formula 1:

$$2^{-\Delta\Delta Ct} = 2^{-(\Delta Ct_{sample} - \Delta Ct_{control})} = \frac{2^{-(\Delta Ct)sample}}{2^{-(\Delta Ct)control}} = \frac{2^{-(Ct_{sample} - Ct_{ref})sample}}{2^{-(Ct_{target} - Ct_{ref})control}} \quad (1)$$

where: Ct_{target} = mean Ct for target gene sample = treatment examples; Ct_{ref} = mean Ct for reference gene control = Control examples.

Table 4. Materials Required for Real-Time PCR Technique.

Materials	Values	Final Concentration
Double Distilled H_2O	7.2	1X
SYBR®Premix Ex Taq™	10	0.2 µl
Primer F (10 pmol/µL)	0.4	0.2 µl
Primer R (10 pmol/µL)	0.4	1X
cDNA	2	
Total Volume	**20**	

Table 5. How to set up Real time PCR.

Cycle	Temperature	Time	Number of cycles
Initialization Step	95	30 sec	1
Denaturation Step	95	5 sec	40
Annealing Step	60	15 sec	40
Extension/ Elongation Step	72	30 sec	40
Final Elongation	72	30 sec	1

2.4. Histopathological assays

The tissues were treated and sectioned at a 5 m, after which they were stained with Hematoxylin and Eosin (H&E). After that, the sections were dehydrated, cleaned, and mounted in entellane (Merck Co., Germany), after which they were cover-slipped. Light microscopy (Olympus, Japan) was used to analyze the prepared slides at 4- 40X magnification. **Statistical analysis**

The data are given as a mean and standard deviation (SD). A one-way analysis of variance (ANOVA) was utilized to compare the groups. SPSS was used for the statistical analysis (version 22.0 for windows). Statistical significance was defined as a P-value of less than 0.05.

3. Results and Discussion

The Table 6 below indicates the rate of weight gain, water intake, and food intake during treatment.

3.1. Liver enzymes levels

Plasma AST and ALT levels in the fatty liver group were considerably higher than in the healthy control group, but they were significantly lower in the thymol treated groups (50 mg/kg) and Vitamin E treated groups (200 mg/kg) compared to the fatty liver group (Figure 2 and 3).

3.2. TNF-α levels

Plasma TNF- α levels were considerably higher in the fatty liver group compared to the healthy control group, but

Table 6. The rate of weight gain, water intake, and food intake during treatment.

	Control	Fatty liver	Vitamin E	Thymol
Initial weight	203 ± 1.322	200.875 ± 1.540	200.625 ± 0.754	201.250 ± 1.319
End weight	275.125 ± 2.960	404.750 ± 4.643***	315.750 ± 3.544***	301.625 ± 3.664***
Weight gain*	72.125 ± 2.141	203.875 ± 3.091***	115.125 ± 2.149***	100.375 ± 2.491***
Water intake during treatment	3082.125 ± 131.533	4615.750 ± 89.725***	3972.125 ± 26.253***	3789.5 ± 49.138***
Food intake during treatment	634.125 ± 11.820	1260.250 ± 18.381***	852.750 ± 9.606***	775.625 ± 22.213***

*Weight gain = End weight – Initial weight. *** significance of P<0.01

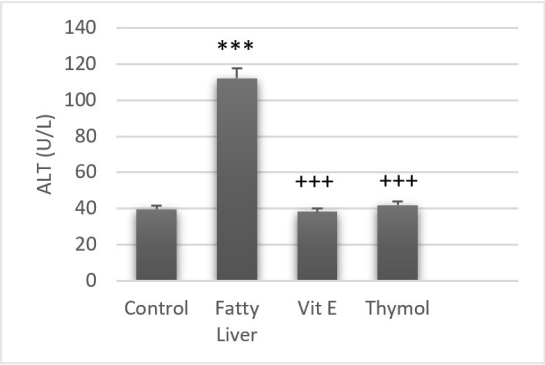

Figure 2. Thymol and Vitamin E Effects on ALT. The fatty liver group got 2 grams of fat emulsion, whereas the experimental groups received 200 mg/kg of vitamin E and 50 mg/kg of thymol. Furthermore, when compared to Fatty liver, the experimental groups demonstrate a positive significant difference. All results are presented as the standard error of the mean, with a significance level of P <0.05. (a one-way ANOVA with post-hoc analysis between groups) ***p <0.001 when compared to normal control and +++p <0.001 when compared to patient control. Vit E = vitamin E.

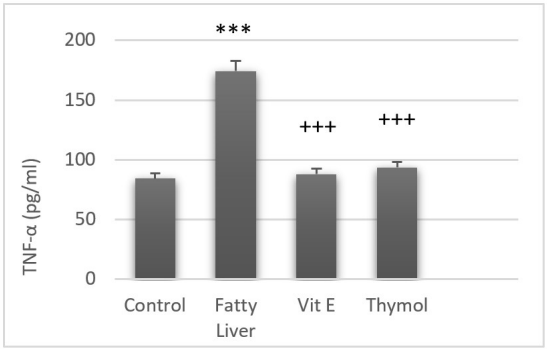

Figure 4. Thymol and Vitamin E Effects on TNF-. The fatty liver group got 2 grams of fat emulsion, whereas the experimental groups received 200 mg/kg of vitamin E and 50 mg/kg of thymol. Furthermore, when compared to Fatty liver, the experimental groups demonstrate a positive significant difference. All results are presented as the standard error of the mean, with a significance level of P <0.05. (A one-way ANOVA with post-hoc analysis between groups) ***p <0.001 when compared to normal control and +++p <0.001 when compared to patient control.

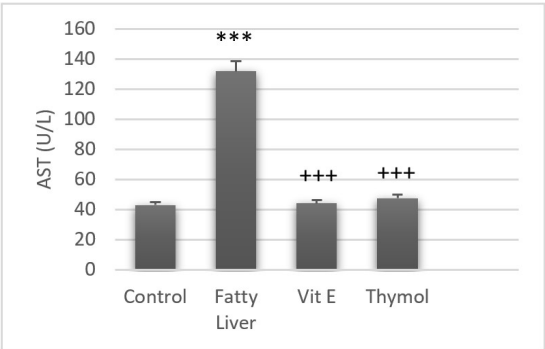

Figure 3. Thymol and Vitamin E Effects on AST. The fatty liver group got 2 grams of fat emulsion, whereas the experimental groups received 200 mg/kg of vitamin E and 50 mg/kg of thymol. Furthermore, when compared to Fatty liver, the experimental groups demonstrate a positive significant difference. All results are presented as the standard error of the mean, with a significance level of P <0.05. (A one-way ANOVA with post-hoc analysis between groups) ***p <0.001 when compared to normal control and +++p <0.001 when compared to patient control. Vit E = vitamin E.

significantly lower in the thymol (50 mg/kg) and Vitamin E (200 mg/kg) treated groups (Figure 4).

3.3. Ferritin levels

Plasma ferritin levels were considerably higher in the fatty liver group than in the healthy control group, but significantly lower in the thymol treated groups (50 mg/kg) and Vitamin E treated groups (200 mg/kg) than in the fatty liver group (Figure 5).

3.4. CK-MB levels

Plasma CK-MB enzyme levels were considerably higher in the fatty liver group compared to the healthy control group, but significantly lower in the thymol (50 mg/kg) and Vitamin E (200 mg/kg) treated groups (Figure 6).

3.5. MAPK gene expression level

The fatty liver group had considerably higher MAPK gene expression than the healthy control group, whereas the thymol treated groups (50 mg/kg) and Vitamin E

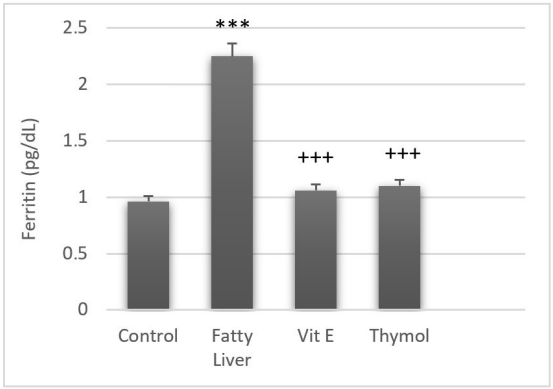

Figure 5. Thymol and Vitamin E Effects on Ferritin. The fatty liver group got 2 grams of fat emulsion, whereas the experimental groups received 200 mg/kg of vitamin E and 50 mg/kg of thymol. Furthermore, when compared to Fatty liver, the experimental groups demonstrate a positive significant difference. All results are presented as the standard error of the mean, with a significance level of P <0.05. (A one-way ANOVA with post-hoc analysis between groups) ***p <0.001 when compared to normal control and +++p <0.001 when compared to patient control. Vit E = vitamin E.

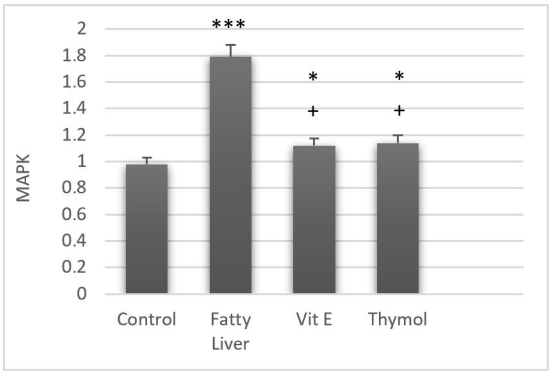

Figure 7. Thymol and Vitamin E Effects on MAPK Gene Expression The fatty liver group got 2 grams of fat emulsion, whereas the experimental groups received 200 mg/kg of vitamin E and 50 mg/kg of thymol. Furthermore, when compared to Fatty liver, the experimental groups demonstrate a positive significant difference. All results are presented as the standard error of the mean, with a significance level of P <0.05. (A one-way ANOVA with post-hoc analysis between groups) *p <0.05 and ***p0.001 vs. normal control and +p <0.05 vs. patient control, respectively. Vit E = vitamin E.

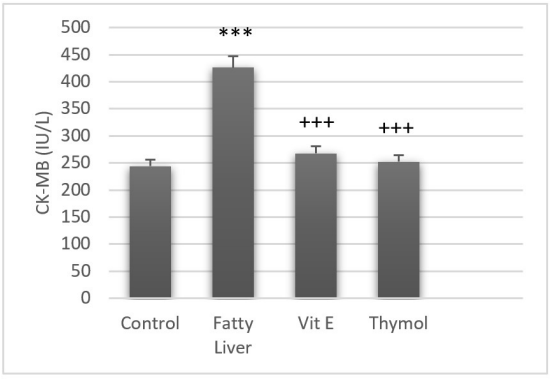

Figure 6. Thymol and Vitamin E Effects on CK-MB. The fatty liver group got 2 grams of fat emulsion, whereas the experimental groups received 200 mg/kg of vitamin E and 50 mg/kg of thymol. Furthermore, when compared to Fatty liver, the experimental groups demonstrate a positive significant difference. All results are presented as the standard error of the mean, with a significance level of P <0.05. (A one-way ANOVA with post-hoc analysis between groups) ***p <0.001 when compared to normal control and +++p <0.001 when compared to patient control. Vit E = vitamin E.

treated groups (200 mg/kg) had significantly lower MAPK gene (Figure 7).

3.6. Histopathological assays

The healthy control group's liver sections showed no histological alterations during the trial, and the hepatocytes had typical radial patterns around the hepatic cords (Figure 8A). The liver in the fatty liver group revealed many abnormalities, including lymphocyte aggregation among hepatocytes, hazy swelling and vacuolization of the cytoplasm, mononuclear inflammatory cell infiltration with congestion and bleeding, hydropic changes, and Kupffer cell hyperplasia (Figure 8B). However, in those given thymol 50 mg/kg and Vitamin E 200 mg/kg, there were fewer degenerative alterations and improved liver architecture (Figure 8C and 8D).

Although the specific process causing NAFLD development and progression is unknown, oxidative stress may be the "second shot" that triggers the switch from steatosis to steatohepatitis, causing liver damage, inflammation, and fibrosis (Dowman et al., 2010; Buzzetti et al., 2016). In a rat model of fatty liver, we tested whether vitamin E or thymol therapy might prevent the development of NASH. In previous research, Vitamin E has shown to improve liver health in animals with fatty liver disease (Pacana and Sanyal, 2012). The advantages of vitamin E in humans have been less firmly defined than in trials with experimental animals. Vitamin E's effectiveness in treating NASH has been evaluated in two major randomized clinical studies (Pacana and Sanyal, 2012). The PIVENS (Pioglitazone vs Vitamin E against Placebo for the Treatment of Non-diabetic

Patients with Non-alcoholic Steatohepatitis) The trial looked at vitamin E's effects (800 IU/day) in non-diabetic, non-cirrhotic people with NASH. Vitamin E substantially improved NASH when compared to placebo, as it decreased steatosis, inflammation, and hepatocellular ballooning but not fibrosis. This result in humans differs from our findings in Pemt/ mice, where vitamin E reduced fibrosis but not steatosis (Lavine et al., 2011). Treatment of the fatty liver group with thymol restores AST and ALT levels to near-control levels, showing that the hepatocytes' functional integrity is preserved. These findings support those of El-Nekeety et al. (2011) and Hamzawy et al. (2012), who investigated the antioxidant activities of Thymus vulgaris oil in male rats exposed to aflatoxin (AFs). The presence

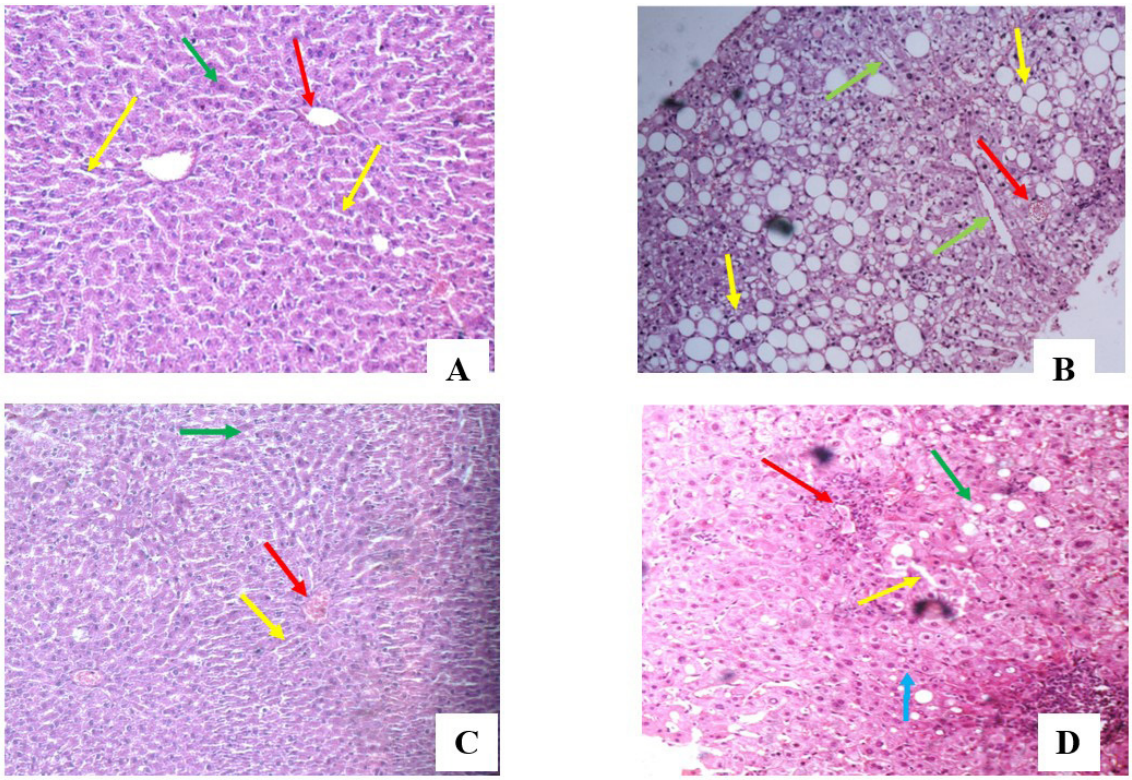

Figure 8. (A) H&E staining and X10 magnification of a portion of the healthy control group's liver tissue (red arrow: Vein Center of the lobule; yellow arrow: sinusoids; green arrow: liver cells); (B) The fatty liver group has histological abnormalities in the liver. The port space and the lobular vein center, hepatocytes, and sinusoids are apparent at this stage. There is extensive vacuolar degeneration. The Kupffer cells are visible and the sinusoids are loose. The nucleus of hepatocytes is conspicuous, and the cytoplasm is acidophilic. Bleeding is associated with blood vessels (Red arrow: Vein center of the lobule; yellow arrow: sinusoids; green arrow: Vacuolar cells); (C) The recipient group's histological abnormalities in the liver are 200 mg/kg vitamin E. The port space and hepatocytes, sinusoids, and kupffer cells are apparent at this time. Hepatocytes have a large nucleus and a somewhat decreased acidophilic cytoplasm. The appearance of kupffer cells is reduced. When compared to the fatty liver group, vacuolar degeneration in the liver cells was dramatically reduced in this group (Red arrow: Vein center of the lobule; yellow arrow: sinusoids; green arrow: Vacuolar cells); (D) The receiving group received 50 mg/kg/day of thymol as shown by histological alterations in the liver. Hepatocytes and sinusoids may be detected alongside kupffer cells at this phase. Vacuolar degeneration is related with fewer cells in this group than in the negative control group (Red arrow: Vein center of the lobule; yellow arrow: sinusoids; green arrow: Vacuolar cells; Blue arrow: kupffer cells).

of fatty degeneration and cellular necrosis in the hepatic tissues of rats after fatty liver induction supported the raised levels of AST and ALT in sera, according to the researchers. Massive centrilobular necrosis, fatty degeneration, and cellular infiltration of the liver have all been linked to the leaking of substantial amounts of serum enzymes into the circulation (Huang et al., 2012). Vitamin E has anti-inflammatory effects as well. The overproduction of proinflammatory cytokines has been demonstrated to be decreased by vitamin E. High-dose vitamin E, on the other hand, has been linked to an increased risk of bleeding. Safaei et al. (2012) reported comparable results after dexamethasone therapy in the same scenario. Thymol supplementation, on the other hand, has been demonstrated to dramatically reduce plasma triglyceride (TG), total cholesterol (TC), free fatty acids (FFAs), low-density lipoprotein (LDL), and raise high-density lipoprotein (HDL) cholesterol when compared to the HFD induced diabetic group. Triglycerides, total cholesterol, free fatty acids, and phospholipids (PL) were all considerably lower in the thymol supplemented groups. According to the findings, thymol may have potential anti-hyperglycemic and anti-hyperlipidemic properties (Saravanan and Pari, 2015). As previously stated, vitamin E and thymol therapy may help NAFLD patients normalize their ALT and AST levels. The development of fatty liver in rats resulted in a considerable rise in TNF- in serum and hepatic tissues, suggesting that fatty liver has a selective effect on macrophage activities. On the other hand, oral treatment of thymol in conjunction with fatty liver dramatically lowered TNF- levels in serum and hepatic tissues. These findings support those of Hamzawy et al. (2012) and Liang et al. (2014) discovered that thymol significantly reduced TNF- α production in lipopolysaccharide-stimulated mouse mammary epithelial cells. The inhibitory action of thymol on the expression of the TNF- α gene may be the mechanism by which it has an anti-inflammatory effect (Karimian et al., 2013). In observational studies

of persons with fatty liver, there is contradictory data about the use of serum ferritin as a prognosticator. It was reported a statistically significant correlation between serum ferritin and NAFLD stage in another prospective study of 50 NAFLD patients, with mean serum ferritin of 309.4 ug/L in a group with NAFLD assessment score of 5, versus mean serum ferritin of 159.5 ug/L in subjects with NAFLD assessment score of <5 ($p \leq 0.001$) (Chandok et al., 2012). Venesection of 198 Italian participants with NAFLD led in a considerable improvement in liver biochemistry and insulin resistance (as determined by Homeostatic Model Assessment) as evidence of concept that iron reserves correspond with disease severity of NAFLD (Marchesini, 2011). According to the research, hyperferritinemia is widespread in individuals with NAFLD; however, rising serum ferritin does not correspond with the progression of fatty liver disease. Despite the absence of a statistically significant relationship, there was a strong trend for serum ferritin to increase as the illness progressed. As a result, serum ferritin might be a valuable measure when used in conjunction with other non-invasive indicators to better predict patients with NAFLD. According to scientific evidence, serum ferritin may be a valuable marker in determining the stage of NAFLD. Although serum ferritin levels are higher in the NAFLD group, the magnitude of the rise does not predict the stage of the underlying liver disease, according to this research. More study is needed to see whether the absence of link is due to an inherent failure in NASH and NAFLD-induced cirrhosis patients' ability to protect their livers from increasing oxidative stress. Hepatocellular carcinoma, alcoholic cirrhosis, and primary biliary cirrhosis all caused slight to moderate increases in total serum CK activity levels in 69 percent, 76.9%, and 51.5 percent of the cases studied, respectively. In contrast, hepatic failure caused a markedly elevated level in 63.2 percent of the cases studied, reaching a maximum total CK activity level of 13.4 I.U./mL in this study. When compared to the healthy control group, the level of CK-MB isoenzyme was considerably higher in the fatty liver group, but significantly lower in the treated groups with thymol and vitamin E. Mitogen-activated protein kinases (MAPKs) play an important function in metabolic regulation in the liver (Manieri and Sabio, 2015). The stress-responsive MAPKs p38 α/β MAPK and JNK1/2 have been identified as important regulators of liver metabolism in both healthy and pathological conditions. Direct dephosphorylation of the MAPKs' regulatory threonine and tyrosine residues, on the other hand, inactivates them (Gehart et al., 2010). While there is substantial evidence that JNK, p38 MAPK, and ERKs play essential roles in liver physiology and disease, the particular intricacies of each of their isoforms' contributions to hepatic metabolism remain a huge knowledge gap. Indeed, hepatic MAPK isoforms seem to work antagonistically in certain circumstances, raising the issue of which MAPK isoforms are crucial in the liver.Although we have a great deal of knowledge on MAPK substrate selectivity, we still don't know the whole repertory of MAPK substrates that the MAPKs in the liver use to regulate metabolism. This knowledge will be crucial to get since it will pave the way for a more detailed mechanistic understanding of the MAPKs' intricacy. In order for MAPKs to attain such high levels of signal specificity, they must be closely controlled (Lawan and Bennett, 2017). According to Wandrer and et al., TNFR1 inhibition decreased liver steatosis and triglyceride levels, as well as the expression and activation of the transcription factor SREBP1 and downstream lipogenesis target genes. Inhibition of TNFR1 also lowered the activity of the MAP kinase MKK7 and its downstream target JNK, which was linked to a considerable reduction in insulin resistance. Anti-TNFR1 medication substantially reduced apoptotic liver damage, NAFLD activity, alanine aminotransferase (ALT) levels, and liver fibrosis compared to control-antibody treatment. As a consequence, our findings point to specific TNFR1 suppression as a possible therapy option for NAFLD (Wandrer et al., 2020). The level of MAPK expression rose dramatically in the fatty liver group in the current investigation, but significantly reduced in the groups treated with thymol and vitamin E as compared to the healthy control group. The study's weakness is that there is no weekly evaluation of the treatment course, and the data are only analyzed after 28 days.

4. Conclusions

The level of MAPK expression rose dramatically in the fatty liver group in the current investigation, but significantly reduced in the groups treated with thymol and vitamin E compared to the healthy control group. The fatty liver group had considerably higher MAPK gene expression than the healthy control group. In contrast, the thymol treated groups (50 mg/kg) and Vitamin E treated groups (200 mg/kg) had significantly lower MAPK gene. This research found that taking 50 mg/kg thymol and 200 mg/kg Vitamin E for 28 days minimized the negative effects of fatty liver. Further research with a bigger sample size and a longer period is suggested.

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References

AMIRI, H., 2012. Essential oils composition and antioxidant properties of three thymus species. *Evidence-Based Complementary and Alternative Medicine*, vol. 2012, pp. 728065. http://dx.doi.org/10.1155/2012/728065. PMid:21876714.

ARCHANA, P.R., RAO, B.N. and RAO, B.S., 2011. In vivo radioprotective potential of thymol, a monoterpene phenol derivative of cymene. *Mutation Research*, vol. 726, no. 2, pp. 136-145. http://dx.doi.org/10.1016/j.mrgentox.2011.08.007. PMid:21933721.

BUZZETTI, E., PINZANI, M. and TSOCHATZIS, E.A., 2016. The multiple-hit pathogenesis of non-alcoholic fatty liver disease (NAFLD). *Metabolism: Clinical and Experimental*, vol. 65, no. 8, pp. 1038-1048. http://dx.doi.org/10.1016/j.metabol.2015.12.012. PMid:26823198.

CHANDOK, N., MINUK, G., WENGIEL, M. and UHANOVA, J., 2012. Serum ferritin levels do not predict the stage of underlying non-alcoholic fatty liver disease. *Journal of Gastrointestinal and Liver Diseases; JGLD*, vol. 21, no. 1, pp. 53-58. PMid:22457860.

DOWMAN, J.K., TOMLINSON, J.W. and NEWSOME, P.N., 2010. Pathogenesis of non-alcoholic fatty liver disease. *QJM*, vol. 103, no. 2, pp. 71-83. http://dx.doi.org/10.1093/qjmed/hcp158. PMid:19914930.

DU, S.X., LU, L.L., GENG, N., VICTOR, D.W., CHEN, L.Z., WANG, C., YUE, H.Y., XIN, Y.N., XUAN, S.Y. and JIN, W.W., 2017. Association of serum ferritin with non-alcoholic fatty liver disease: a meta-analysis. *Lipids in Health and Disease*, vol. 16, no. 1, pp. 228. http://dx.doi.org/10.1186/s12944-017-0613-4. PMid:29197393.

EL-NEKEETY, A.A., MOHAMED, S.R., HATHOUT, A.S., HASSAN, N.S., ALY, S.E. and ABDEL-WAHHAB, M.A., 2011. Antioxidant properties of Thymus vulgaris oil against aflatoxin-induce oxidative stress in male rats. *Toxicon*, vol. 57, no. 7-8, pp. 984-991. http://dx.doi.org/10.1016/j.toxicon.2011.03.021. PMid:21477612.

GEHART, H., KUMPF, S., ITTNER, A. and RICCI, R., 2010. MAPK signalling in cellular metabolism: stress or wellness? *EMBO Reports*, vol. 11, no. 11, pp. 834-840. http://dx.doi.org/10.1038/embor.2010.160. PMid:20930846.

EL HADI, H., VETTOR, R. and ROSSATO, M., 2018. Vitamin E as a treatment for nonalcoholic fatty liver disease: reality or myth? *Antioxidants*, vol. 7, no. 1, pp. 12. http://dx.doi.org/10.3390/antiox7010012. PMid:29337849.

HAMZAWY, M.A., EL-DENSHARY, E.S., HASSAN, N.S., MANAA, F. and ABDEL-WAHHAB, M.A., 2012. Antioxidant and hepatorenoprotective effects of Thyme vulgaris extract in rats during aflatoxicosis. *Global Journal of Pharmacology*, vol. 6, pp. 106-117.

HUANG, G.J., DENG, J.S., HUANG, S.S., SHAO, Y.Y., CHEN, C.C. and KUO, Y.H., 2012. Protective effect of antrosterol from Antrodia camphorata submerged whole broth against carbon tetrachloride-induced acute liver injury in mice. *Food Chemistry*, vol. 132, no. 2, pp. 709-716. http://dx.doi.org/10.1016/j.foodchem.2011.11.004.

KARIMIAN, P., KAVOOSI, G., AMIEGHOFRAN, Z. and KALANTAR, F., 2013. Reduction of NADH oxidase, NO synthase, TNFα, and IL-1β mRNA expression levels on lipopolysacharide-stimulated murine macrophages by Zataria multiflora. *Molecular Biology Research Communications*, vol. 2, no. 3, pp. 89-100.

KERNAN, K.F. and CARCILLO, J.A., 2017. Hyperferritinemia and inflammation. *International Immunology*, vol. 29, no. 9, pp. 401-409. http://dx.doi.org/10.1093/intimm/dxx031. PMid:28541437.

LAVINE, J.E., SCHWIMMER, J.B., VAN NATTA, M.L., MOLLESTON, J.P., MURRAY, K.F., ROSENTHAL, P., ABRAMS, S.H., SCHEIMANN, A.O., SANYAL, A.J., CHALASANI, N., TONASCIA, J., ÜNALP, A., CLARK, J.M., BRUNT, E.M., KLEINER, D.E., HOOFNAGLE, J.H. and ROBUCK, P.R., 2011. Effect of vitamin E or metformin for treatment of nonalcoholic fatty liver disease in children and adolescents: the TONIC randomized controlled trial. *Journal of the American Medical Association*, vol. 305, no. 16, pp. 1659-1668. http://dx.doi.org/10.1001/jama.2011.520. PMid:21521847.

LAWAN, A. and BENNETT, A.M., 2017. Mitogen-activated protein kinase regulation in hepatic metabolism. *Trends in Endocrinology and Metabolism*, vol. 28, no. 12, pp. 868-878. http://dx.doi.org/10.1016/j.tem.2017.10.007. PMid:29128158.

LIANG, D., LI, F., FU, Y., CAO, Y., SONG, X., WANG, T., WANG, W., GUO, M., ZHOU, E., LI, D., YANG, Z. and ZHANG, N., 2014. Thymol inhibits LPS-stimulated inflammatory response via down-regulation of NF-κB and MAPK signaling pathways in mouse mammary epithelial cells. *Inflammation*, vol. 37, no. 1, pp. 214-222. http://dx.doi.org/10.1007/s10753-013-9732-x. PMid:24057926.

MANIERI, E. and SABIO, G., 2015. Stress kinases in the modulation of metabolism and energy balance. *Journal of Molecular Endocrinology*, vol. 55, no. 2, pp. R11-R22. http://dx.doi.org/10.1530/JME-15-0146. PMid:26363062.

MARCHESINI, G. and TARGHER, G., 2011. Commentary: Liver enzymes and the risk of adverse cardiovascular outcomes—the lower, the better? *International Journal of Epidemiology*, vol. 40, no. 6, pp. 1539-1541.

MOHAMMADI, A., GHOLAMHOSEINIAN, A. and FALLAH, H., 2014. Zataria multiflora increases insulin sensitivity and PPARγ gene expression in high fructose fed insulin resistant rats. *Iranian Journal of Basic Medical Sciences.*, vol. 17, no. 4, pp. 263-270. PMid:24904719.

PACANA, T. and SANYAL, A.J., 2012. Vitamin E and non-alcoholic fatty liver disease. *Current Opinion in Clinical Nutrition and Metabolic Care*, vol. 15, no. 6, pp. 641-648. http://dx.doi.org/10.1097/MCO.0b013e328357f747. PMid:23075940.

POWELL, E.E., JONSSON, J.R. and CLOUSTON, A.D., 2010. Metabolic factors and non-alcoholic fatty liver disease as co-factors in other liver diseases. *Digestive Diseases*, vol. 28, no. 1, pp. 186-191. http://dx.doi.org/10.1159/000282084. PMid:20460909.

SAFAEI, N., SHOMALI, T. and TAHERIANFARD, M., 2012. Niacin ameliorates lipid disturbances due to glucocorticoid administration in rats. *Iranian Journal of Basic Medical Sciences.*, vol. 15, no. 4, pp. 997-1002. PMid:23493662.

SAJED, H., SAHEBKAR, A. and IRANSHAHI, M., 2013. *Zataria multiflora* Boiss. (Shirazi thyme): an ancient condiment with modern pharmaceutical uses. *Journal of Ethnopharmacology*, vol. 145, no. 3, pp. 686-698. http://dx.doi.org/10.1016/j.jep.2012.12.018. PMid:23266333.

SARAVANAN, S. and PARI, L., 2015. Role of thymol on hyperglycemia and hyperlipidemia in high fat diet-induced type 2 diabetic C57BL/6J mice. *European Journal of Pharmacology*, vol. 761, pp. 279-287. http://dx.doi.org/10.1016/j.ejphar.2015.05.034. PMid:26007642.

TSAI, M.L., LIN, C.C., LIN, W.C. and YANG, C.H., 2011. Antimicrobial, antioxidant, and anti-inflammatory activities of essential oils from five selected herbs. *Bioscience, Biotechnology, and Biochemistry*, vol. 75, no. 10, pp. 1977-1983. http://dx.doi.org/10.1271/bbb.110377. PMid:21979069.

WANDRER, F., LIEBIG, S., MARHENKE, S., VOGEL, A., JOHN, K., MANNS, M.P., TEUFEL, A., ITZEL, T., LONGERICH, T., MAIER, O., FISCHER, R., KONTERMANN, R.E., PFIZENMAIER, K., SCHULZE-OSTHOFF, K. and BANTEL, H., 2020. TNF-Receptor-1 inhibition reduces liver steatosis, hepatocellular injury and fibrosis in NAFLD mice. *Cell Death & Disease*, vol. 11, no. 3, pp. 212. http://dx.doi.org/10.1038/s41419-020-2411-6. PMid:32235829.