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Research Article

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Hepatoprotective Effects of Myristica Fragrans on NASH Induced by Methionine-Choline Deficient (MCD) Diet

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Abstract

The purpose of this study is to evaluate the hepatoprotective effects of Myristica fragrans extract (MFE) on non-alcoholic steatohepatitis (NASH) by methionine-choline deficient (MCD) diet induced mice model. In HepG2 cells, MTT assay, fatty acid-induced TG content, and the liver damage indicators, AST, ALT, and LDH, were evaluated. C57BL/6 mice were divided into 6 groups; Normal group (normal diet without any treatment), Negative control group (MCD diet only), positive control group (Milk thistle, 200 mg/kg), MFE administration group (100, 200, and 400 mg/kg), After 12 weeks, body weight, serum biochemical parameters for liver function test, absolute and relative liver weight, histopathological changes and ELISA analysis were assessed. In HepG2 cells, MFE reduced the TG accumulation and the expression of biomarkers which are indicators of liver damage. The level of body weight and relative liver weight of all MCD diet groups were significantly lower than that of the normal group. MFE groups promoted marked reductions of biochemical parameters compared to the control group. MFE showed to inhibit fat accumulation or inflammation by suppressing markers of liver diseases progression. The results of this research suggest that MFE extract is considered to be possible candidate for the treatment of NASH and/or liver diseases.

Keywords: Supercritical Fluids (SCF), Non-alcoholic Steatohepatitis (NASH), Myristica Fragrans Extract (MFE), Methionine-Choline Deficient (MCD) Diet, Erythro-(7R,8S)-$\Delta^{8'}$-7-acetoxy-3,3',5'-trimethoxy-8-O-4'-neolignan

1. Introduction

Chronic liver disease is a major cause of mortality and morbidity worldwide. Most chronic liver diseases progress from mild inflammation to more severe inflammation, leading to fibrosis or cirrhosis. This can cause liver dysfunction and portal hypertension, and also increases the risk of liver cancer [1]. The most common cause of liver disease among Koreans is the hepatitis B virus, followed by the hepatitis C virus, alcoholic fatty liver disease, and non-alcoholic steatohepatitis (NASH). NASH refers to cases in which the cause of fatty liver is not due to alcohol, and it is a term that encompasses the entire process from simple hepatic steatosis to steatohepatitis and cirrhosis. In particular, NASH refers to a wide range of liver damage, from simple steatosis to fatty liver disease, bile stasis, advanced fibrosis, and cirrhosis [2]. A high-fat diet combined with excessive fatty acid intake is known to cause obesity, non-alcoholic fatty liver disease (NAFLD), and other metabolic syndromes [3,4].

Myristica fragrans has been widely used as a flavoring agent in food since ancient times, and in traditional Korean medicine, it is used as a carminative for symptoms such as diarrhea, abdominal bloating, vomiting, and loss of appetite. In addition, nutmeg is also known to exhibit activities such as inhibition of Helicobacter pylori growth activation of liver detoxification mechanisms, and antimicrobial effects [1,5-7]. Lignan compounds such as alkyl aryl ether, dibenzylbutane, and tetrahydrofuran found in nutmeg are known to have pharmacological mechanisms including antioxidant effects by inhibiting reactive oxygen species and lipid peroxidation, activation of Nrf-2 expressed by the NFE2L2 (Nuclear factor erythroid-derived 2-like 2) gene, which provides protective effects

against oxidative damage caused by inflammation or toxins, anti-inflammatory effects by inhibiting nitric oxide production, and regulation of PPARα involved in hepatocyte differentiation, growth, and metabolism [8-11]. In this study, we aimed to confirm the efficacy of MFE based on its hepatoprotective pharmacological action in a NASH pathological model, to demonstrate that it can be developed into food products and pharmaceuticals.

2. Methods

2.1. Materials

Nutmeg was purchased from Ipulp Pharmaceutical (Lot No.: EPL19162-1, Seoul, Korea). MCD Diet was purchased from Saeronbio (Seoul, Korea). Milk Thistle was purchased from Panjin Tianyuan Pharmaceutical (Liaoning, China). HepG2 cell was purchased from the American Type Culture Collection (Manassas, VA, USA). Dulbecco's modified Eagle's medium (DMEM), penicillin, streptomycin, and heat-inactivated fetal bovine serum were purchased from Gibco (MA, USA). EnzyChrom™ Triglyceride Assay Kit from BioAssay Systems (CA, USA). Thiazoly blue tetrazolium bromide (MTT), Dimethyl sulfoxide (DMSO), Hydrogen peroxide solution, and Phosphate-buffered saline (PBS) were purchased from Sigma-Aldrich (St. Louis, MO, USA). ALT and AST assay kit was purchased from Asan Pharmaceutical (Whaseong, Korea). Cytotoxicity assay kit was purchased from Doozen Bio (Seoul, Korea), and all other materials and solvents used were of the highest analytical grade.

2.2. MFE Extract

We established the optimal extraction conditions containing a high concentration of Erythro-(7R,8S)-8'-7-acetoxy-3,3',5'-trimethoxy-8-O-4'-neolignan (compound 1), an alkyl aryl ether-type lignan compound, which is one of the bioactive components of Myristica fragrans (Fig. 1). 1 kg of Myristica fragrans was ground and placed in the extraction vessel (capacity: 5L) of a supercritical fluid extractor (5L SCF SYSTEM, POSENTECH, Korea). Carbon dioxide was supplied at a rate of 70 cc/min through a liquefied carbon dioxide supply pump to achieve a supercritical state with extraction vessel temperature at 60℃, separation vessel temperature at 40℃, and a pressure of 250 bar. While supplying an 60% ethanol aqueous solution as a co-solvent at a rate of 15 mL/min using a high-performance liquid chromatography pump (Supercritical Fluid Chromatograph, POSENTECH, Korea), the extract was collected through the separation vessel for 2 hours. The resulting extract was concentrated using a rotary evaporator (EYELA A-3S, Japan) and freeze-dried (BK-10N50, China) to obtain 400 g of MFE.

2.3. Separation and Analysis of Compound 1 by Preparative High-Performance Liquid Chromatography and ^{1}H and ^{13}C-NMR

The extract obtained under conditions of 250 bar and 60% aqueous ethanol was dissolved in the mobile phase and filtered, then separated using a Waters Deltaprep LC300 (Waters, USA) with an injection volume of 10 mL and HPLC conditions of acetonitrile: 0.1% acetic acid solution (50:50 v/v%) [Luna® 5 µm C_{18}(2) 100 Å, LC Column (21.2 x 250 mm, particle size: 5 µm), flow rate: 20 mL/min, detection wavelength: 280 nm]. The collected fraction was freeze-dried using a vacuum freeze dryer (ChemStar 1402N, Welch®, IL, USA) to yield colorless oil-form compound 1.

The ^{1}H and ^{13}C-NMR analyses of compound 1 were conducted using a high-resolution nuclear magnetic resonance spectrometer (600 MHz, AVANCE 600, Bruker, Germany), through which 1H, 13C-NMR, and 2D NMR data (COSY, HSQC, and HMBC) were obtained and analyzed. The LC/MS analysis of compound 1 was performed using an Ultimate3000 system (Thermo Scientific, USA) with HPLC [Waters BEH C_{18} (2.1 x 100 mm, 1.7 µm particle size, flow rate: 0.4 mL/min, detection wavelength: 280 nm, column temperature: 45°C)] under the conditions of A: 0.1% formic acid in water, B: 0.1% formic acid in acetonitrile (0–1 min: 90% A, 1–25 min: 100% B, 25–30 min: 90% B), and the mass spectrometer used was a Triple TOF 5600+ (AB Sciex, USA).

2.4. Cell Lines and Cell Culture

HepG2 Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 100 U/mL penicillin, 100 µg/mL streptomycin, and 10% heat-inactivated fetal bovine serum, under conditions of 37℃ and 5% CO_2 in an incubator (Forma Series II, Thermo Electron Co., MA, USA).

2.5. MTT Assay

Cell viability was measured using the MTT assay. HepG2 cells were seeded in a 96-well plate at a density of 1×10^5 cells/well, and after 24 hours, MFE (50, 100, 300, 500, 1000, and 1500 µg/mL) was administered and cultured for 24 hours. After removing the supernatant, 100 µL of MTT solution dissolved at a concentration of 5 mg/mL in 0.1M PBS (pH 7.0) was added and incubated in a CO_2 incubator for 2 hours. After completely removing the supernatant, 100 µL of DMSO was added to each well and reacted in the dark for 30 minutes, and the resulting formazan was measured at 570 nm using a Microplate reader (BKMPR-1096A, China).

2.6. Intracellular Triglyceride (TG) Assay

HepG2 cells were seeded in a 6-well plate at a density of 1×10^5 cells/well and cultured for 24 hours in DMEM containing 20% oleic acid and 1% bovine serum albumin to induce steatosis. In the experimental groups, DMEM was also supplemented with MFE (50, 100, 300, 500, and 1000 µg/mL). The cells with induced steatosis were collected after trypsin treatment, and the intracellular triglyceride content was measured using the EnzyChrom™ Triglyceride Assay Kit.

2.7. AST, ALT, and LDH Assay

To evaluate the hepatoprotective effect against hydrogen peroxide (H_2O_2), the activities of AST, ALT, and LDH, which are indicator enzymes of liver damage, were measured. HepG2 cells were seeded at 1×10^5 cells/well in a 24-well plate using DMEM medium and cultured for 24 hours in a 5% CO_2 incubator. The cultured cells were pretreated with 100 µg/mL MFE for 1 hour, then treated with 1 mM hydrogen peroxide. After 6 hours, the supernatant was collected, and the activities of AST and ALT were measured using an ALT and AST assay kit, and LDH activity was measured using

a cytotoxicity assay kit.

2.8. Breeding and Diet of Experimental Animals

Experimental animals were purchased from CoaTech using 5-week-old male C57BL/6NHsd mice and acclimated for one week before use. During the experimental period, the temperature was maintained at 23±3°C, relative humidity at 55±15%, ventilation rate at 10 to 20 times/hour, lighting duration at 12 hours (lights on at 8 a.m. to lights off at 8 p.m.), and illumination at 150 to 300 Lux. This animal study was conducted with the approval (19-KE-130) of the Animal Experiment Ethics Committee of Knotus. For the experiment, animals were divided into 6 groups with 9 mice per group: normal group (Normal diet without any treatment), negative control group (MCD diet without any treatment), positive control group (MCD diet and Milk Thistle 200 mg/kg), low-dose group (MCD diet and MFE 100 mg/kg), medium-dose group (MCD diet and MFE 200 mg/kg), and high-dose group (MCD diet and MFE 400 mg/kg). The MCD diet groups were fed the MCD diet for 5 days followed by normal feed for 2 days, allowing them to freely consume the MCD diet and normal feed alternately for 12 weeks (8 weeks' induction and 4 weeks' treatment), and the drugs were administered orally.

- **Body Weight:** Body weight was measured on the day of administration initiation and thereafter once a week and on the day of necropsy.
- **Blood Biomarker Assay:** Blood was collected before the administration of the test substance and at 4 weeks after the administration (on the necropsy day). The separated serum was analyzed using a blood biochemical analyzer (7180 Hitachi, Japan) to examine ALT (alanine transaminase), AST (aspartate transaminase), TG (Triglyceride), and TCHO (Total cholesterol).
- **Necropsy:** Before necropsy, all surviving animals were fasted (water provided) for one night. On the day of necropsy, the animal was anesthetized by inhalation with ether, and if anesthesia was confirmed, blood was drawn using a syringe from the posterior vena cava, and the abdominal aorta and posterior vena cava were cut to die after bloodletting. The blood was injected into a vacutainer tube containing a clot activator and left at room temperature for approximately 15 minutes to coagulate and then centrifuged at 12,000 rpm for 3 minutes to isolate the serum. Serum was stored in a deep freezer set to -70 °C or less until analysis and was used for blood biochemical tests. At the time of necropsy, the liver was removed and weighed, the left lobe of the extracted liver was fixed in a 10% neutral buffered formalin solution for histopathology, and the right lobe was stored in a deep freezer set to -70°C or lower after rapid freezing and ELISA.
- **Histopathological Analysis:** The fixed tissues underwent standard tissue processing procedures, including trimming, dehydration, paraffin embedding, and sectioning, to prepare specimens for histopathological examination. Subsequently, Hematoxylin & Eosin (H&E) staining, Picosirius red staining, and immunohistochemically staining (TGF-β, α-SMA) were performed, and histopathological changes were observed using an optical microscope (Olympus BX53, Japan). Histopathological evaluation was conducted according to SOP standards, and the analysis of Picrosirius red staining and immunohistochemically staining was performed using an image analyzer (Zen 2.3 blue edition, Carl Zeiss, Germany), comparing the stained area relative to the total area for each specimen between groups, as shown in Table 2.
- **ELISA Analysis:** Serum collected on the day of necropsy was used to measure NEFA (Non-esterified fatty acid), and excised liver tissue was used to measure TG, TCHO, and NEFA in the liver tissue.

Position	δH mult. (*J* = Hz)	δC mult.[b]
1	-	129.0 s
2	6.79 brs	111.1 d
3	-	147.8 s
4	-	146.6 s
5	6.72 d (0.8)	115.6 d
6	6.63 brd (7.9)	119.5 d
7	5.68 d (2.8)	76.4 d
8	4.34 dd (5.8, 3.2)	79.6 d
9	1.10 d (6.2)	14.7 q
1’	-	135.8 s
2’ , 6’	6.49 s	106.0 d
3’ , 5’	-	153.4 s
4’	-	133.4 s
7’	3.32 d (6.5)	40.2c t
8’	5.98 m	138.0 d
9’	5.11 brd (17.0)	116.3 t
	5.06 brd (9.8)	
3-OCH3	3.74 s	56.1 q
3’, 5’-OCH3	3.72 s	56.2 q
7-OAc	-	170.0 s
	2.10 s	21.3 q
4-OH	9.00 s	-
[a] Measured at 600 and 150 MHz; obtained DMSO-d_6 with TMS as an internal standard. Data assignment was based on ^{1}H-^{1}H COSY, HSQ[C], and HMBC experiments.		
[b] Carbon multiplicity deduced by 13C and HSQC. c Overlapping signal.		

Table 1: ^{1}H and ^{13}C NMR Spectroscopic Data of Compound 1[a]

2.9. Statistical Analysis

For the results of this experiment, assuming normality of the data, analysis was conducted using parametric multiple comparison procedures or non-parametric multiple comparison procedures. When the result of parametric one-way ANOVA was significant, Dunnett’s multiple comparison test was used for post hoc testing, and when the result of non-parametric Kruskal-Wallis H-test analysis was significant, the Mann-Whitney U test was used for post hoc testing. Statistical analysis was performed using Prism 7.04 (GraphPad Software Inc., San Diego, CA, USA), and p-values <0.05 were considered statistically significant.

3. Results

3.1. ^{1}H and ^{13}C-NMR of Compound 1

The analysis results showed that compound 1 was a colorless oil with a molecular weight measured as 416.46 (439 [M+Na]+) (Table 1 and Figure 1).

Histological feature	**Score**			
	0	**1**	**2**	**3**
Steatosis				
Macrovesicular steatosis	< 5 %	5-33 %	33-66 %	> 66 %
Macrovesicular steatosis	< 5 %	5-33 %	33-66 %	> 66 %
Hypertrophy	< 5 %	5-33 %	33-66 %	> 66 %
Inflammation				
Number of inflammatory foci/field	< 0.5	0.5-1.0	1.0-2.0	> 2.0

Table 2: Histological Feature and Score

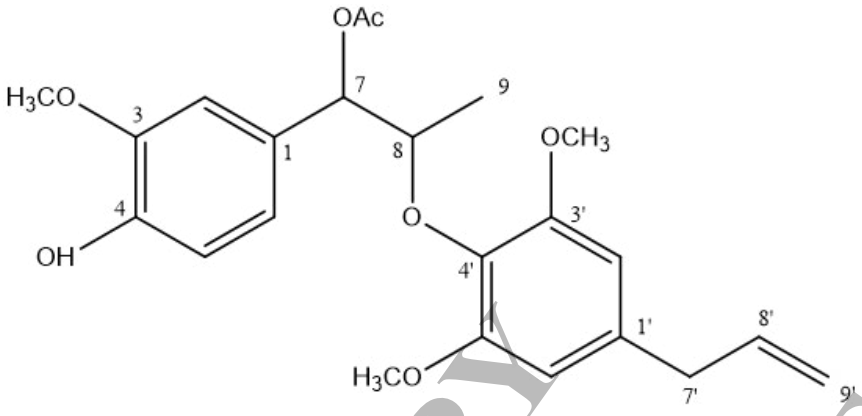

Figure 1: The Structure of Erythro-(7R,8S)-$\Delta^{8'}$-7-acetoxy-3,3',5'-trimethoxy-8-O-4'-neolignan

3.2. MTT Assay

To measure the range of concentrations at which MFE does not exhibit toxicity in HepG2 cells, various concentrations of MFE were applied, and the cell viability of HepG2 cells was measured using the MTT assay. The MTT assay is a method for measuring the concentration of metabolically active and viable cells, and MFE was shown not to affect cell viability at concentrations of 1,500 μg/mL or lower, indicating that MFE has a wide safety range (Figure 2) [12].

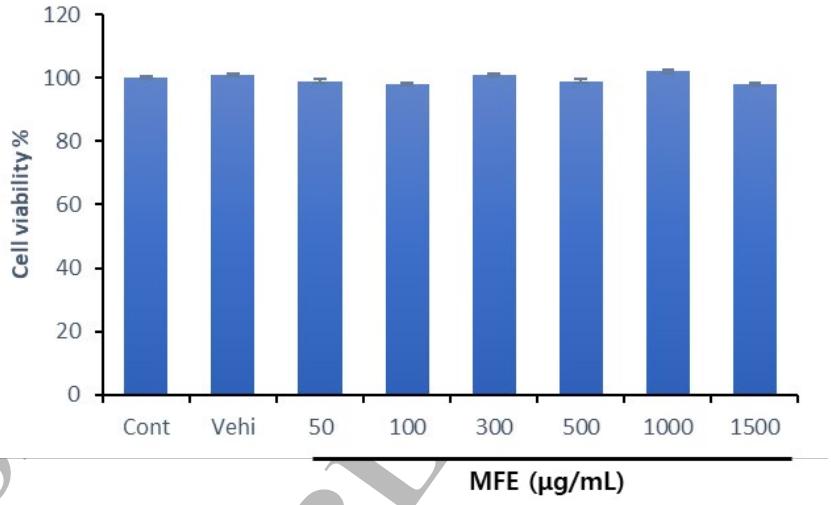

Figure 2: MTT assay. The cells were treated with various concentration of MFE for 24h prior to MTT assay. Values are mean±SD (n=3)

3.3. The Effect of MFE on TG

Oleic acid was applied to HepG2 cells to induce intracellular lipid accumulation, and it was examined whether intracellular lipid accumulation decreased when MFE was administered along with oleic acid. As the concentration of MFE increased, the amount of intracellular TG gradually decreased, and a statistically significant difference was observed when the MFE concentration was 300 μg/mL or higher (Figure 3).

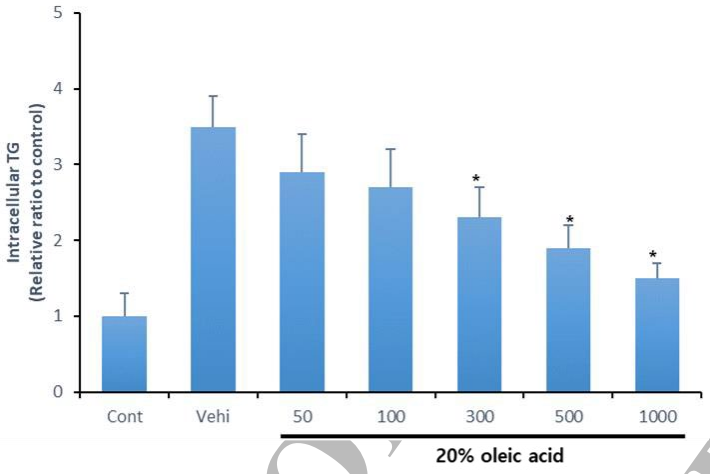

Figure 3: HepG2 cells were treated with oleic acid with various concentration of MFE (50, 100, 300, 500, 1,000 µg/mL) for 24h. Values are mean±SD (n=3). * P<0.05 compared to untreated cells.

3.4. AST, ALT, and LDH Assay

The effect of MFE pretreatment on hydrogen peroxide-induced hepatotoxicity was evaluated. As shown in Fig. 4, compared to the H_2O_2-treated control group, AST and ALT activities were significantly inhibited by NME by 34.9% and 46.6%, respectively, and LDH was also reduced by 43.6%.

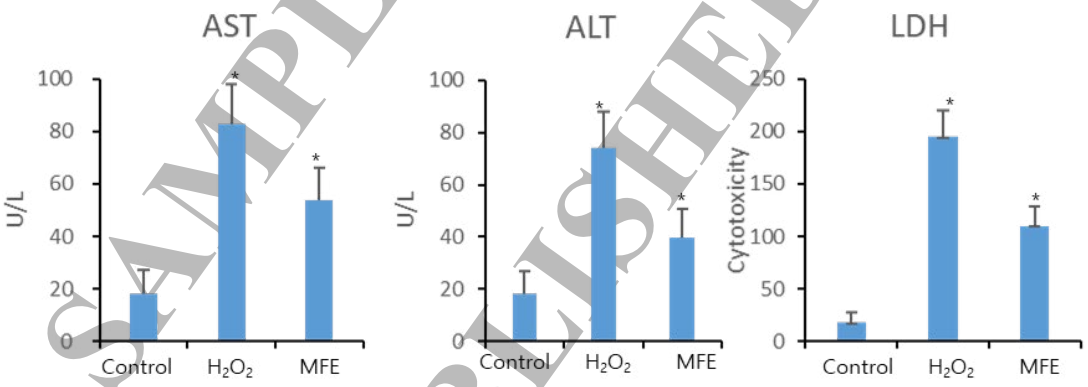

Figure 4: HepG2 cells were pre-treated with MFE for 1h and further exposed to hydroperoxide (H_2O_2) for 6h. Values are mean±SD (n=3). * P < 0.05 compared to only oleic acid treated cells without MFE

3.5. Body Weight

In the normal group, there was little change in body weight, and the body weight levels of all fatty liver-induced groups (G2 to G6) were significantly lower compared to the normal control group (p<0.001), and this pattern persisted until the end of the experiment (Figure 5).

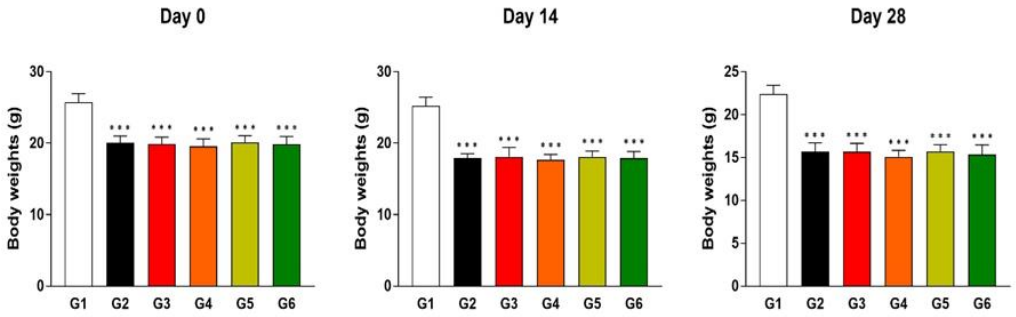

Figure 5: Body weight change of MCD-diet induced mice with/without treatment. Values are mean±SD. The day of first administration was designated day 0. G1: Normal control, G2: Vehicle control, G3: Milk thistle 200 mg/kg, G4: NME 100 mg/kg, G5: NME 200 mg/kg, G6: NME 400 mg/kg. *** p<0.001 compared to the G1

3.6. Blood Biomarker Assay

Before the administration of the test substance, ALT and AST levels in all fatty liver-induced groups (G2 to G6) were significantly higher compared to the normal control group (p<0.001), and the TCHO level in G5 was significantly lower than in G1 (p<0.01). At 4 weeks after the start of test substance administration, ALT and AST levels in all fatty liver-induced groups (G2 to G6) were significantly higher compared to the normal control group (p<0.001), while ALT levels in G3 and G6 were significantly lower than in G2 (p<0.01 and p<0.05), and AST levels in G3, G5, and G6 were significantly lower than in G2 (p<0.001) (Figure 6).

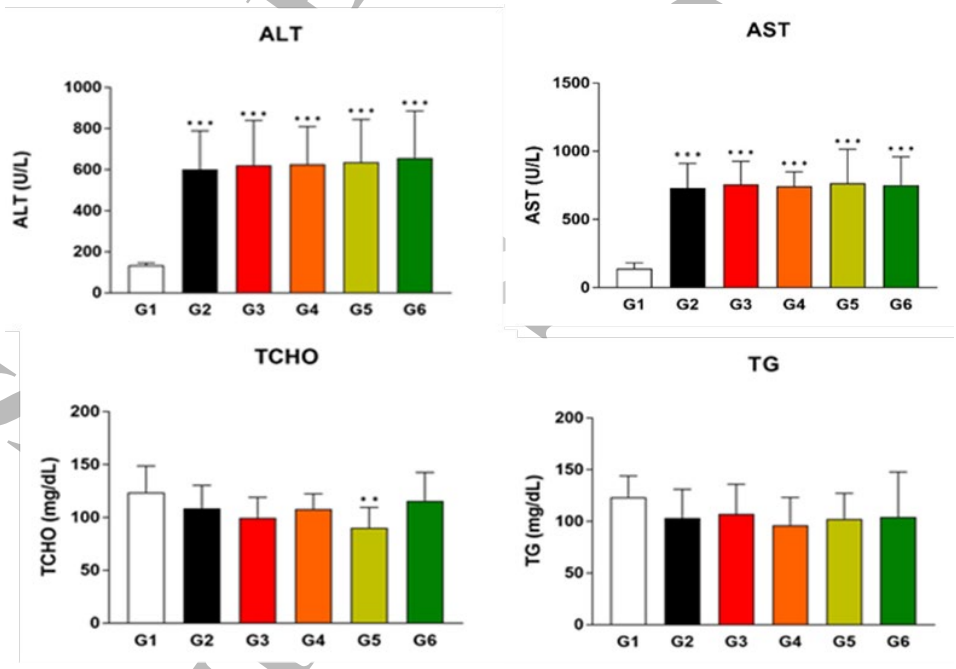

Figure 6: Changes in metabolites in the liver before MFE administration. Values are mean±SD. The week of first administration was designated week 0. G1: Normal control, G2: Vehicle control, G3: Milk thistle 200 mg/kg, G4: NME 100 mg/kg, G5: NME 200 mg/kg, G6: NME 400 mg/kg. ***/**p<0.001/p<0.01 compared to the G1.

At 4 weeks after the start of test substance administration, the TCHO and TG levels of all fatty liver-induced groups (G2 to G6) were significantly lower than those of the normal control group (p<0.001). According to the blood biochemical tests, at 4 weeks after the start of test substance administration, the ALT and AST levels of the test substance-administered groups (G4-G6) tended to be lower compared to the negative control group (Fig. 7). At week 4 after the start of administration of the test substance, the ALT level in G6 was significantly lower compared to G2, and the AST levels in G5 and G6 were significantly lower compared to G2.

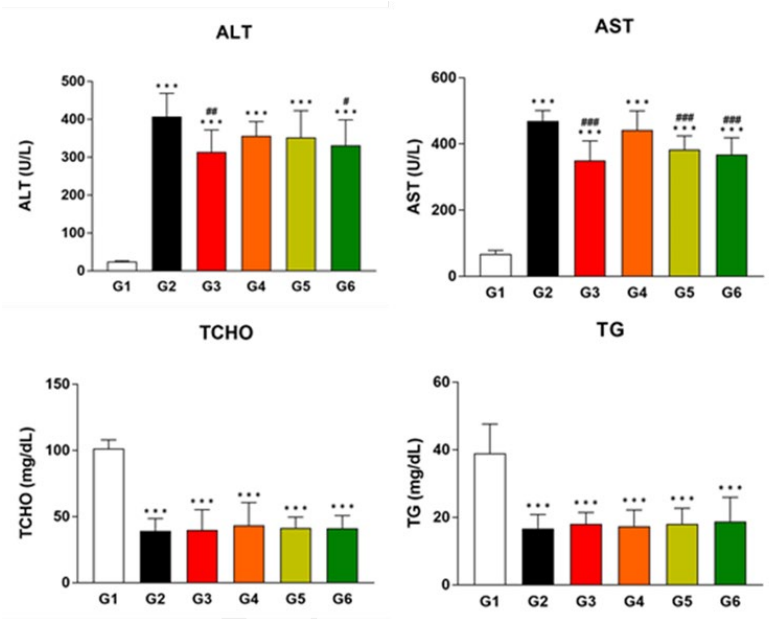

Figure 7b: Changes in metabolites in the liver after 4 weeks. Values are mean±SD. The week of first administration was designated week 4. G1: Normal control, G2: Vehicle control, G3: Milk thistle 200 mg/kg, G4: NME 100 mg/kg, G5: NME 200 mg/kg, G6: NME 400 mg/kg. ***/** p<0.001/p<0.01 compared to the G1.

3.7. Liver Weight

The liver weight levels of G3, G4, G5, and G6 were statistically significantly lower compared to G1 (p<0.001 or p<0.01), and the liver weight levels of G3 and G6 were significantly lower compared to G2 (p<0.01). The relative liver weight levels of all fatty liver-induced groups (G2 to G5) were significantly higher than those of G1 (p<0.001), and the relative liver weight levels of G4 and G5 were statistically significantly lower compared to G2 (p<0.01). Overall, the MFE administration group showed lower relative liver weight levels compared to G2 (Figure 8).

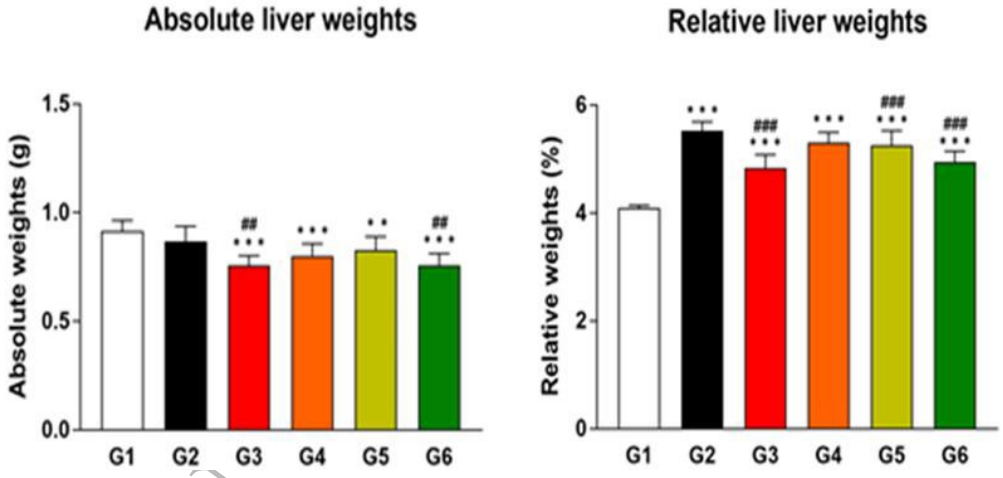

Figure 8: Absolute liver weights & relative liver weights. Values are Mean±SD. G1: Normal control, G2: Vehicle control, G3: Milk thistle 200 mg/kg, G4: NME 100 mg/kg, G5: NME 200 mg/kg, G6: NME 400 mg/kg. ***/** p<0.001/p<0.01 compared to the G1, ###/## p<0.001/p<0.01 compared to the G2

3.8. Histopathological Analysis

The level of macrovesicular steatosis in all fatty liver-inducing groups (G2 to G6) was significantly higher than in G1 (p<0.001), and the level of macrovesicular steatosis in G3, G4, G5, and G6 was significantly lower than in G2 (p<0.01 or p<0.05). The level of microvesicular steatosis in G2, G4, G5, and G6 was significantly higher than in G1 (p<0.001, p<0.01, or p<0.05), and the level of microvesicular steatosis in G3 was significantly lower than in G2 (p<0.01) (Figure 9).

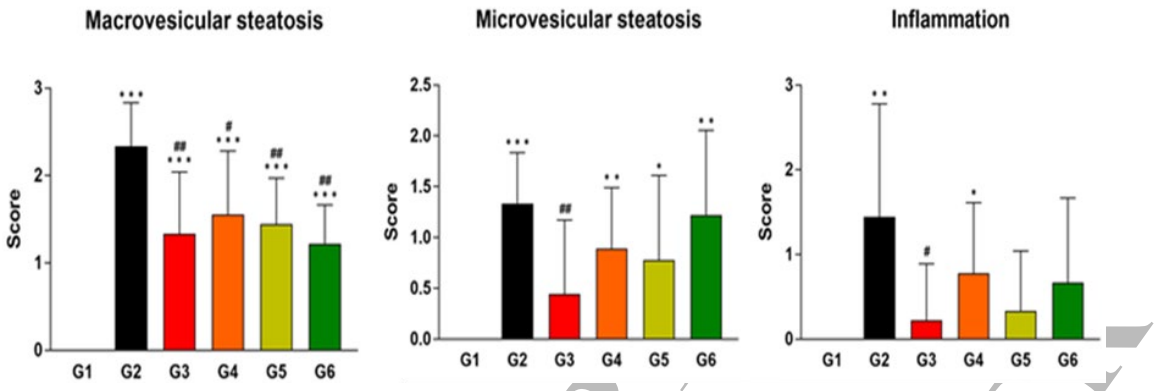

Figure 9: Histological changes of the liver tissue from each group by hematoxylin-eosin staining. Values are mean±SD. G1: Normal control, G2: Vehicle control, G3: Milk thistle 200 mg/kg, G4: NME 100 mg/kg, G5: NME 200 mg/kg, G6: NME 400 mg/kg. ***/**/* p<0.001/p<0.01/p<0.05 compared to the G1, ##/# p<0.01/p<0.05 compared to the G2.

The level of inflammation in G2 and G4 was significantly higher than in G1 (p<0.01 and p<0.05), and the level of inflammation in G3 was significantly lower than in G2 (p<0.05). The analysis results of fibrotic area using Picrosirius red staining showed that the staining area level of G2 was significantly higher than that of G1 (p<0.01), and the staining area level of G6 was significantly lower than that of G2 (p<0.05) (Figure 10).

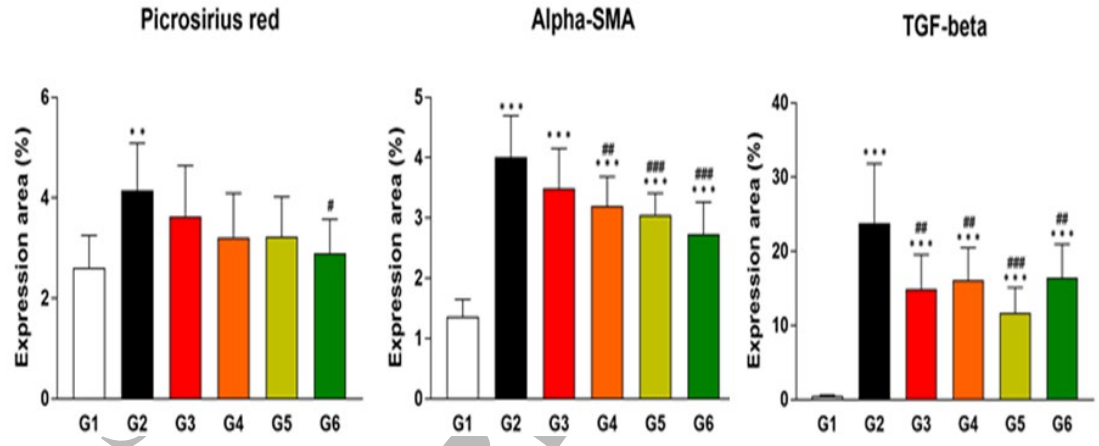

Figure 10: Histological changes of the liver tissue from each group by Picrosirius red staining and Immunostaining. Values are Mean±SD. G1: Normal control, G2: Vehicle control, G3: Milk thistle 200 mg/kg, G4: NME 100 mg/kg, G5: NME 200 mg/kg, G6: NME 400 mg/kg. ***/** p<0.001/p<0.01 compared to the G1, ###/##/# p<0.001/p<0.01/p<0.05 compared to the G2.

As a result of the TGF-β and α-SMA expression area analysis using immunohistochemically staining, the levels of TGF-β and α-SMA expression areas in all fatty liver-induced groups (G2-G6) were significantly higher than in G1 (p<0.001), and the TGF-β expression area levels in the positive control and all test substance-administered groups (G3 to G6) were significantly lower than in G2 (p<0.001 or p<0.01). Additionally, the α-SMA expression area levels in all test substance-administered groups (G4 to G6) were significantly lower than in G2 (p<0.001 or p<0.01). According to the results of histopathological examinations, the level of macro vesicular steatosis in all test substance administration groups (G4 to G6) was significantly lower than that in G2, and although not significant, micro vesicular steatosis and inflammation levels also tended to be lower compared to G2. Additionally, in the analysis of protein expression using immunohistochemically staining, the expression area level of α-SMA, an inflammation- and fibrosis-related factor, showed a dose-dependent decreasing trend with the test substance, and the expression area level of TGF-β decreased significantly in all doses of the test substance compared to G2. Results from fibrosis area analysis using Picrosirius red staining also showed a dose-dependent trend with the test substance and a tendency to decrease in fibrosis area, and the fibrosis area level in G6 was significantly reduced compared to G2. 9. ELISA Analysis

The results of the ELISA analysis showed that TG levels in the liver tissues of G2, G4, and G5 were significantly higher than those in G1 (p<0.001), while TG levels in the liver tissues of G3 and G6 were significantly lower than those in G2 (p<0.001). TCHO

levels in the liver tissues of G2, G4, G5, and G6 were significantly higher than those in G1 ($p<0.001$ or $p<0.05$), and TCHO levels in the liver tissue of G5 were significantly higher than those in G2 ($p<0.01$), whereas TCHO levels in the liver tissues of G3 and G6 were significantly lower than those in G2 ($p<0.001$). Free fatty acid levels in both serum and liver tissues of all fatty liver induction groups (G2 to G6) were significantly higher than those in G1 ($p<0.001$), while serum FFA levels in G3, G4, and G6 and liver tissue FFA levels in G5 and G6 were significantly lower than those in G2 ($p<0.001$, $p<0.01$, or $p<0.05$). ELISA analysis results indicated that TG and TCHO levels in the liver tissue were significantly decreased in G6 compared to G2, and FFA levels in the liver tissues of G5 and G6 and serum FFA levels in G4 and G6 were also significantly decreased (Figure 11).

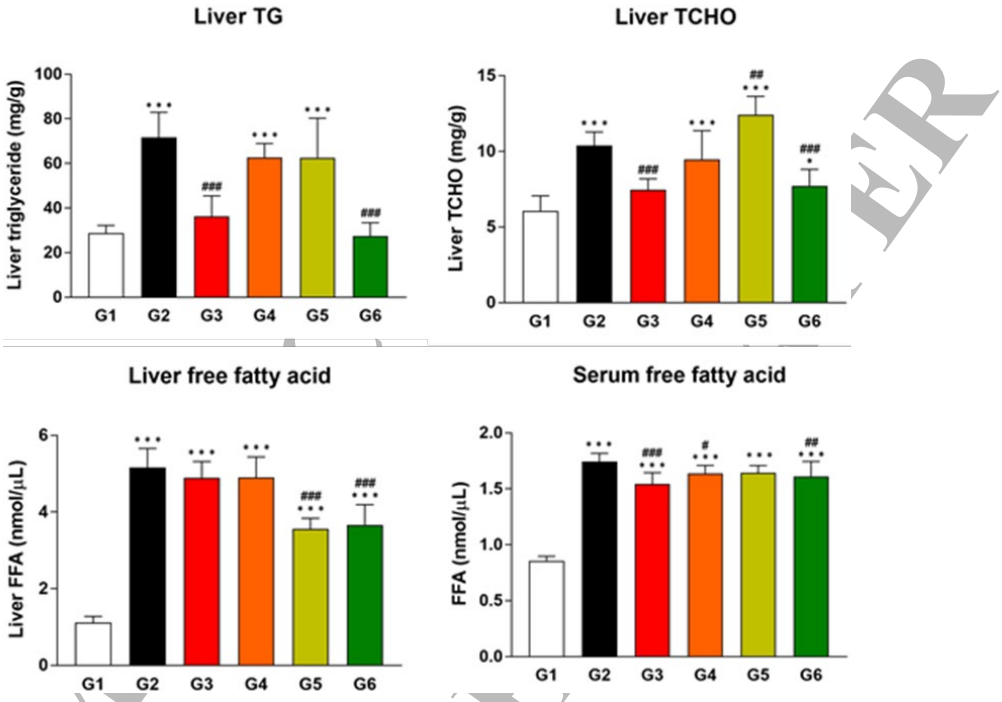

Figure 11: Changes in metabolite levels in liver tissues. Values are Mean±SD. G1: Normal control, G2: Vehicle control, G3: Milk thistle 200 mg/kg, G4: NME 100 mg/kg, G5: NME 200 mg/kg, G6: NME 400 mg/kg. ***/* $p<0.001$/$p<0.05$ compared to the G1, ###/##/# $p<0.001$/$p<0.01$/$p<0.05$ compared to the G2

4. Discussion

The Effect of MFE on TG suggest that MFE may have an effect in reducing steatosis in non-alcoholic fatty liver disease, which is characterized by intracellular fat accumulation. In the AST, ALT, and LDH tests, there was a significant decrease in the group given MFE, suggesting that MFE has an effective hepatoprotective effect. Generally, experiments using the NASH model induced by a high-fat diet are known to significantly increase body weight, but in the MFE-administered group, body weight was found to significantly decrease [13]. In the MCD diet model, continuous body weight loss was also observed in the negative control group, and animal experiments using MFE extract showed the same pattern. This is considered a characteristic of the pathologic model induced by an MCD diet, which is different from the pathologic model induced by fat [14]. As a result, this suggests that MFE can improve body weight and obesity-related symptoms in obese mice. In the analysis of blood biochemistry indicators related to liver function, ALT, AST, THCO and TG, it seems that administering the test substance led to a decrease in liver function-related levels in the fatty liver model induced by the MCD diet. Based on the results of evaluating the effect on liver weight gain, it seems that administering the test substance suppressed liver enlargement caused by fatty liver. Liver fibrosis is a precursor to cirrhosis and occurs due to excessive accumulation of extracellular matrix proteins (EMP), particularly collagen. Generally, when the liver is damaged, extracellular matrix proteins are synthesized in activated stellate cells, which are also called fat- and retinoid-storing cells. The activation of these cells is known to occur not only due to hepatocyte damage caused by free radicals such as nitric oxide, superoxide anion, and peroxynitrite, generated from the failure of detoxification due to glutathione depletion, cytochrome P450 expression, and immune cells infiltrated by Kupffer cells and inflammatory responses, but also through the actions of various cytokines and chemokines [15]. Studies on the effects of MFE in alleviating NAFLD by regulating the gut microbiota, particularly abundant probiotic species, show that it improves the intestinal environment, thereby modulating gut tryptophan metabolism and activating the transcription factor Aryl hydrocarbon receptor (AHR) in the liver through low-molecular-weight compounds [16]. Based on the above histopathological examinations and ELISA analysis results, the administration of the test substance is considered to induce a reduction in the severity of fatty liver lesions, such as fat deposition and inflammation caused

by steatosis, and to induce the suppression of the expression of factors related to inflammation and fibrosis.

5. Conclusion

When the test substance was administered repeatedly for 4 weeks in a Methionine-choline deficient (MCD) diet-induced non-alcoholic steatohepatitis (NASH) C57BL/6 mouse model, the MFE-treated group showed a reduction in liver function-related values in blood biochemical tests compared to the vehicle control group, and a significant decrease in both absolute and relative liver weights was observed. In addition, histopathological examination results showed a dose-dependent trend of change, with alleviation of macro vesicular steatosis and decreased expression levels of inflammation- and fibrosis-related factors. In the high-dose MFE group, in addition to the above results, significant decreases in TG, cholesterol, and FFA levels in the liver tissue as well as FFA levels in the serum were observed. Therefore, repeated administration of MFE for 4 weeks in a MCD diet-induced NASH model is considered to potentially help improve non-alcoholic steatohepatitis.

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