Journal of Experimental Pharmacology Dovepress

Open Access Full Text Article ORIGINAL RESEARCH

Lygodium microphyllum Inhibits *de Novo* Lipogenesis Activity in the Hepatocytes of High-Fat High-Fructose-Induced Rats by Increasing the Levels of SIRT1 and AMPK

Putri Anggreini[1,2,*], Hadi Kuncoro[2,*], Sri Adi Sumiwi[3,*], Jutti Levita[3,*]

[1]Doctoral Program in Pharmacy, Faculty of Pharmacy, Padjadjaran University, Sumedang, West Java, Indonesia; [2]Department of Pharmacognosy and Medicinal Chemistry, Faculty of Pharmacy, Mulawarman University, Samarinda, East Kalimantan, Indonesia; [3]Department of Pharmacology and Clinical Pharmacy, Faculty of Pharmacy, Padjadjaran University, Sumedang, West Java, Indonesia

*These authors contributed equally to this work

Correspondence: Jutti Levita, Department of Pharmacology and Clinical Pharmacy, Faculty of Pharmacy, Padjadjaran University, Sumedang, West Java, 46363, Indonesia, Tel +6222-84288888 Ext 3510, Email jutti.levita@unpad.ac.id

Background: The prevalence of non-alcoholic fatty liver disease (NAFLD) is currently of great concern due to its risk of developing T2DM and cardiovascular disease. The development of NAFLD may be initiated by de novo lipogenesis in the hepatocytes. Sirtuin1 (SIRT1) and adenosine monophosphate-activated protein kinase (AMPK), are responsible for the lipogenesis mechanism. Interestingly, plant sterols, such as beta-sitosterol and stigmasterol, have the potential to lower the LDL-cholesterol in dyslipidemic patients. Beta-sitosterol was present in the ethanol extract of *Lygodium microphyllum* herbs at a concentration of 283.55 µg/g extract. This sterol interacted with the active allosteric-binding site of SIRT1 and AMPK similarly to the proteins' activators.

Purpose: To investigate the anti-lipogenesis activity of the ethanol extract of *L. microphyllum* (ELM) in the liver tissue of rats through the SIRT1 and AMPK levels.

Methods: Forty male Wistar rats were used in this study: (1) normal control group; (2) high-fat high-fructose diet (HFHFD) rats; (3) HFHFD rats treated with metformin; (4) HFHFD rats treated with resveratrol; (5) HFHFD rats treated with beta-sitosterol; (6–8) HFHFD rats treated with ELM doses of 200, 400, and 600 mg/kg BW. Rats in the normal control group were fed regular chow, while other groups of rats were given HFHFD for 35 days. All drugs were given orally on D15 till D35. On D35, the rats were sacrificed, and the liver organs were examined for the liver index, morphology, NAFLD activity score (NAS), and levels of SIRT1 and AMPK.

Results: ELM improves the morphology, the liver index, the steatosis condition, and the NAS of HFHFD-induced NAFLD rats. ELM increases the levels of SIRT1 and AMPK in the liver tissue of HFHFD-induced NAFLD rats.

Conclusion: ELM may have the potential to inhibit de novo lipogenesis by increasing the levels of SIRT1 and AMPK.

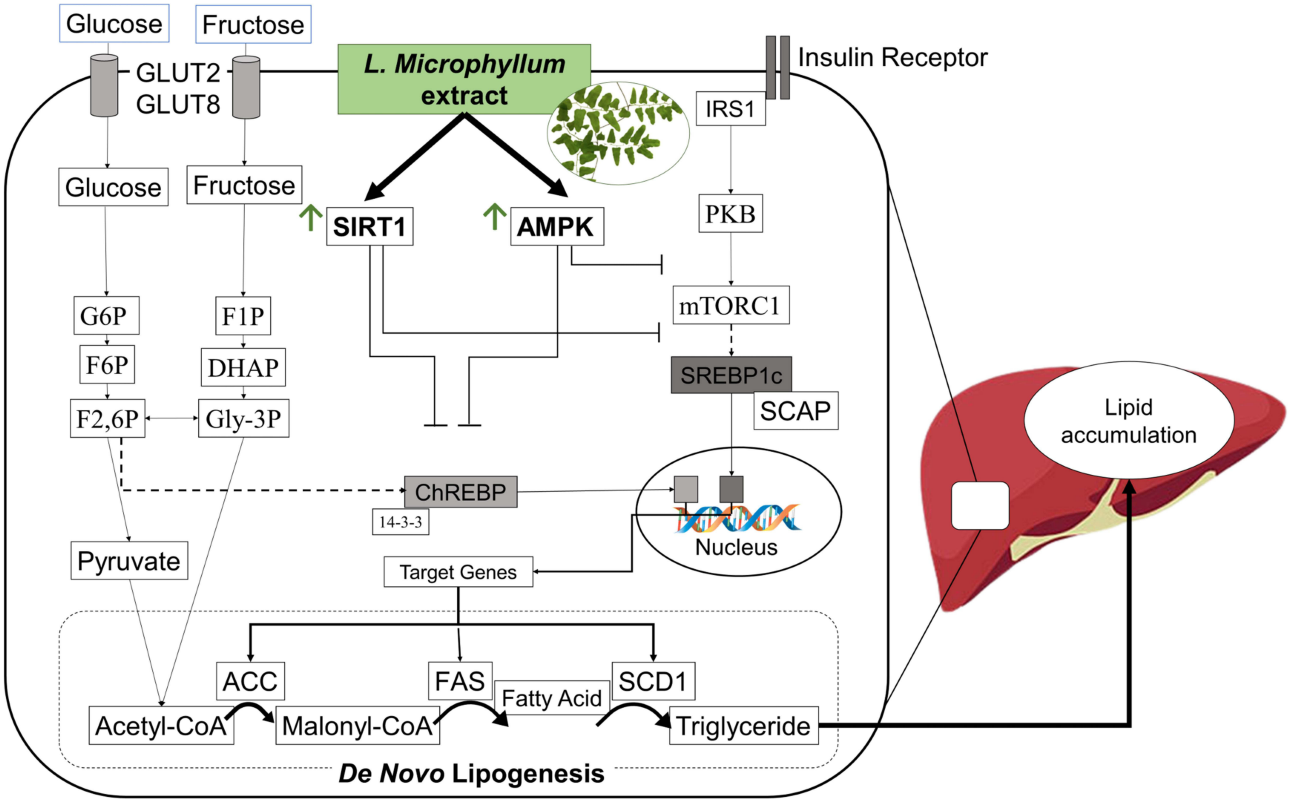
Keywords: beta-sitosterol, *de novo* lipogenesis, Lygodiaceae plants, phytosterols

Introduction

Non-alcoholic liver disease (NAFLD) is a condition of hepatic steatosis (when approximately 5% of hepatocytes contain large lipid droplets or when intrahepatic triglyceride content exceeds 5.6%) that is generated by high-fat or high-sugar dietary intake. The development of NAFLD may be initiated by *de novo* lipogenesis (DNL) in the hepatocytes.[1,2] DNL converts excessive glucose or fructose into fatty acid and triglycerides, thus inhibiting DNL is strongly strived for as a therapeutic target for lipid metabolism-related disease. Numerous proteins are responsible for reducing DNL, including AMP-activated protein kinase (AMPK) and Sirtuin1 (SIRT1).[3] AMPK phosphorylates and inactivates acetyl CoA carboxylase (ACC), the enzyme that catalyzes the first and rate-limiting step in DNL.[4,5] Sirtuin 1 (SIRT1) is also well-known for its role in DNL by regulating hepatocyte lipid metabolism via AMP-activated protein kinase activation. AMPK activation by SIRT1 shelters against the

Received: 13 June 2024
Accepted: 11 September 2024
Published: 9 October 2024

Graphical Abstract



induction of fatty acid synthase and lipid accumulation promoted by high glucose.[6] SIRT1 contributes to the deacetylation and inhibition of SREBP-1C activity in the regulation of hepatic lipid metabolism.[7]

NAFLD affects approximately 20–30% of the global population[8] and reaches 27.4% in Asia.[9] However, there is no specific FDA-approved medication for NAFLD, with several agents being studied in Phase III clinical trials, and others in Phase II clinical trials.[10] Individuals diagnosed with NAFLD are advised to undergo diet and exercise as non-pharmacological therapy, while pharmacological therapy generally involves medications such as antidiabetic agents (metformin, pioglitazone), anti-hyperlipidemic drugs (statins), or anti-obesity drugs (orlistat) depending on the patient's condition.[11,12] These medications are non-specific to NAFLD and may carry significant risks of side effects. Therapies targeting lipogenesis are considered preferable as they specifically inhibit fat formation, however, these drugs are being studied in humans, such as Firsocostat and ASC40,[13] thus indicating that anti-lipogenesis drug discovery remains a challenge. Plant sterols have been FDA-approved as lipid-lowering drugs that work to reduce cholesterols, low-density lipoproteins (LDL), and triglycerides, and increase high-density lipoproteins (HDL) in both human and animal models.[14–18]

The fern plant *Lygodium microphyllum* (Cav). R.Br. (synonym *Lygodium scandens var. microphyllum* (Cav). Luerss., listed in https://powo.science.kew.org/taxon/urn:lsid:ipni.org:names:17143140-1) of family Lygodiaceae, grows in abundance on Kalimantan island. This plant has been reported to possess hepatoprotective activity in carbon tetrachloride-induced hepatotoxicity mice.[19] Several compounds have been identified and isolated from *L. microphyllum*, of which are the phytosterols, namely beta-sitosterol, a major compound in the plant.[20] The ethanol extract of this plant is practically not toxic as evidenced by the high LC_{50} value towards *Artemia salina* larvae (203.704 ppm) and female mice (LD_{50} > 5000 mg/kg BW).[21,22] However, an in vivo study is needed to confirm the effect of *L. microphyllum* on SIRT1 and AMPK. Considering this, our study aims to investigate the anti-lipogenesis activity of the ethanol extract of *L. microphyllum* herbs and beta-sitosterol in the liver tissue of rats through the levels of SIRT1 and AMPK.

Material and Methods

Chemicals

Chemicals, standard drugs, and kit reagents were ethanol 70% technical grade (Bratachem Indonesia), chloroform (CAS No. 67–66-3; Merck Millipore https://www.merckmillipore.com/), beta-Sitosterol (CAS No. 83–46-5; Sigma-Aldrich https://www.sigmaaldrich.com/ID/en/), resveratrol (CAS No. 501–36-0; Sigma-Aldrich https://www.sigmaaldrich.com/ID/en/product/sigma/r5010), metformin 500 mg/tablet (PT Hexpharm Jaya Laboratories Indonesia http://en.hexpharmjayalaboratories.web.indotrading.com/about), regular chow BR II (PT. Wonokoyo Jaya Corporindo Indonesia https://www.wonokoyo.co.id/), home-made beef tallow, Sania superfry palm oil (PT Wilmar Cahaya Indonesia https://www.wilmarcahayaindonesia.com/product-detail/MzQ=), chicken egg yolk and egg white, liquid fructose (Rose Brand Indonesia https://rosebrand.co.id/), D-fructose powder (Pudak Scientific https://www.pudak-scientific.com/), SIRT1 Rabbit Polyclonal Antibody (Catalog No. A17307; Abclonal https://abclonal.com/catalog-antibodies/SIRT1RabbitpAb/A17307), AMPKa1/AMPKa2 Rabbit Polyclonal Antibody (Catalog No. A17289; Abclonal https://abclonal.com/catalog-antibodies/AMPKa1AMPKa2RabbitpAb/A17289), beta-Actin Rabbit Monoclonal Antibody (Catalog No. AC038; Abclonal https://abclonal.com/catalog-antibodies/ActinRabbitmAb/AC038), PRO-PREP™ Protein Extraction Solution (Catalog No. 17081; iNtRON Bio), acrylamide (CAS No. 79–06-1; Sigma-Aldrich https://www.sigmaaldrich.com/ID/en/product/sial/01696), GangNam-STAIN™ Prestained Protein Ladder (iNtRONbio Cat. No. 24052), N,N′-methylenebisacrylamide (CAS No. 110–26-9; Sigma-Aldrich https://www.sigmaaldrich.com/ID/en/product/sigma/m7279), Hematoxylin (CAS No. 517–28-2; Sigma-Aldrich https://www.sigmaaldrich.com/ID/en/product/sigma/h9627), Eosin Y (https://www.sigmaaldrich.id/id_en/e4009-5g-eosin-y-free-acid).

Plant Collection and Preparation of the Extract

The climbing fern herbs were found in abundance in a tropical rainforest in Samarinda, East Kalimantan, Indonesia. The herbs were identified by Dr. Atik Retnowati (Scopus ID 6507457958), a certified botanist at Herbarium Bogoriense, the Research Center for Biology, the Indonesian Institute of Sciences, Indonesia. The herbs were confirmed as *Lygodium microphyllum* (Cav). R.Br. of family Lygodiaceae (voucher specimen number 750IPH101), classified as a Least Concern species with no requirements for approval from the local government for its utilization, despite its correspondence with those described in The IUCN Red List of Threatened Species (https://www.iucnredlist.org/species/194153/8883960).

Extraction was carried out by following a previous method using approximately 3.6 kg of the herbs in 3 L of technical grade ethanol 70% for 3×24 hours at room temperature (26 ± 2 °C), which yielded 15.86% w/w a viscous extract of *L. microphyllum* (abbreviated as ELM).[21]

Animals and Ethical Approval

This animal study was conducted by following the 3Rs (Replacement, Reduction, and Refinement) Principles of the ARRIVE (Animal Research Reporting of In Vivo Experiments) Guidelines (https://arriveguidelines.org/), and was approved by the Research Ethics Committee of Padjadjaran University (No. 376/UN6.KEP/EC/2023), which strictly follows The Guide for the Care and Use of Laboratory Animals (NRC 2011; eighth edition) (https://grants.nih.gov/grants/olaw/guide-for-The-care-and-use-of-laboratory-animals.pdf).[23] Procedures were conducted at the Laboratory of Pharmaceutical Research & Development for Pharmaca Tropicals of Mulawarman University, Samarinda, Indonesia (https://ff.unmul.ac.id/). Forty male Wistar rats (weighing 200 ± 10 g; age of 8 to 12 weeks) were housed in close-house cages, in a room equipped with an air-handling unit, regulated humidity (60 ± 10%), and temperature (25 ± 2 °C), in a 12:12 hour light-dark circle. The rats were acclimatized for 5 days and given standard rodent feed containing 18% protein and free access to drinking water.

HFHFD Preparation and Proximate Analysis

The high-fat high-fructose diet (HFHFD) chow was prepared by utilizing beef tallow (100 g) added to the regular chow BR II (100 g), egg yolk (25 g), egg white (8 g), palm oil (7 mL), and liquid fructose (25 g). The ingredients were mixed

to homogenous, manually formed into pellets, and kept stored at 4–8 °C. Beef tallow was used as the primary fat source, due to its high content of saturated fatty acids (such as palmitic acid and stearic acid) and monounsaturated fatty acids (such as oleic acid).[24] The HFHFD chow was analyzed for its nutritional composition, eg carbohydrate, protein, and fat, by following the Official Methods of Analysis,[25] and resulted in a carbohydrate content of 23.2%, protein content of 21.5%, fat content of 41.8%, ash content of 0.5%, and moisture content of 12.8%.

Animal Modeling for NAFLD

Modeling for NAFLD was carried out on adult Wistar rats (6–8 weeks old; weighing 200–250 g), which were randomly caged into (1) the normal group (n = 3) and (2) the HFHFD group (n = 3), in a controlled environment of 25 ± 2 °C and 12:12 hour light-dark cycle. Rats in the normal group received a standard rodent feed containing 18% protein, while the HFHFD group received the best HFHFD (containing standard rodent feed, mixed with beef tallow 55% and fructose 60%), for 35 consecutive days. On day 15 and day 35, the rats were measured for their serum triglyceride, and on day 35 the rats were sacrificed. The livers were collected for histology analysis, by hematoxylin-eosin (H&E) staining. The NAFLD activity score (NAS) was determined using a Pro-Histo Microscope at 5 fields of view at 400x magnification. Ballooning degeneration, inflammation, and necrosis were scored 0: if absent; scored 1 (minimal): if < 25%; scored 2 (mild): if 25–50%; scored 3 (moderate): if 50–75%; and scored 4 (severe): if >75%.[26]

Experimental Design

Male Wistar rats (n = 40) were randomly divided into 8 groups: (1) normal control group; (2) high-fat high-fructose diet (HFHFD) rats; (3) HFHFD rats treated with metformin (200 mg/kg BW);[27] (4) HFHFD rats treated with resveratrol (50 mg/kg BW);[28] (5) HFHFD rats treated with beta-sitosterol (20 mg/kg BW); (6) HFHFD rats treated with ELM dose of 200 mg/kg BW; (7) HFHFD rats treated with ELM dose of 400 mg/kg BW; and (8) HFHFD rats treated with ELM dose of 600 mg/kg BW. The dose of the standard drugs followed previous studies with modification.[27,28]

Rats in the normal control group (group 1) were fed regular chow composed of ground corn, soybean, a protein source such as fish, and vegetable oil,[29] while other groups of rats were given a high-fat high-fructose diet (HFHFD) for 35 days. All drugs and ELMs were given orally on day 15 (D15) till the last day of the experiment (D35). On the last day (D35), rats were sacrificed using CO_2 euthanasia for 2 minutes by trained personnel. Death was confirmed by determining cardiac and respiratory arrest,[30] and the liver organs were harvested for further examination.

Liver Index and Macroscopic Assessments

The liver index and macroscopic assessments of the liver were conducted following a previous procedure described elsewhere with a few modifications.[31] The livers of the rats were rinsed with saline solution (0.9% sodium chloride), weighed, examined for morphology, and documented by ensuring consistent lighting for each image. The liver index was obtained by calculating the rats' liver and body weight.[32]

Histopathology of the Liver Tissue and NAFLD Activity Score (NAS) Assessment

The liver of each rat was cut in halves, a half portion of the liver was analyzed for its protein content (SIRT1 and AMPK), while the other half portion was histologically analyzed. For histological analysis, the liver was immersed in 10% formalin buffer, then washed with running tap water, dehydrated with ethanol, cleaned with xylene, and embedded in paraffin. The liver was then sliced to a thickness of 5 μm and stained using hematoxylin and eosin. Histological changes were observed using a microscope (Pro Histo Biological Microscope Pro A31). NAFLD activity score (NAS) was conducted based on the previously established methods.[26,33–35] The NAS can range from 0 to 8 and is calculated by the sum of scores of steatosis (0–3), lobular inflammation (0–3), and hepatocyte ballooning degeneration (0–2).[35]

Western Blot Analysis

The half part of the liver was put in a protein extraction solution and homogenized. The lysate was vortexed and the supernatant was collected for protein concentration measurement using the bicinchoninic acid assay (BCA) method, which is based on the reaction of sodium bicinchoninate with the cuprous ion generated by the biuret reaction at basic pH

(pH > 8.0). The BCA assay method is commonly employed because of its simplicity, sensitivity, repeatability, and reproducibility.[36] The protein level was measured using a spectrophotometer until a concentration of 350 ppm was obtained. Subsequently, a total of 5 µL of the protein ladder was loaded into the left well, followed by 10 µL of protein sample into each well. The protein was separated using 12% SDS-PAGE followed by its transfer onto a nitrocellulose membrane. The membrane was then blocked using 1% skim milk for 30 minutes, and incubated with primary AMPKa1/AMPKa2 rabbit polyclonal antibody (1:1000), SIRT1 rabbit polyclonal antibody (1:1000), and beta-actin rabbit monoclonal antibody (1:500) for 24 hours at 4°C. Eventually, the membrane was washed with 1× TBST four times for 15 minutes each on a shaker and then incubated with a secondary antibody donkey anti-rabbit (1:15000) for 90 minutes at room temperature (25 ± 2 °C). The membrane was washed again with 1× TBST four times for 15 minutes each on a shaker and then prepared for detection. Beta-actin was used as a loading control to normalize the levels of protein. Protein bands were visualized, and the membrane was then scanned using a LI-COR Odyssey CLx scanner. Band intensities were measured using ImageJ (https://imagej.net/ij/).

Statistical Analysis

The data is presented in the form of mean ± standard deviation. Statistical analysis using a one-way ANOVA test followed by Tukey's multiple comparison test. The statistical analysis and graph creation were performed using GraphPad Prism version 7 (https://www.graphpad.com/support/prism-7-updates/).

Results

Animal Modeling for NAFLD

Modeling for NAFLD was carried out on adult Wistar rats (6–8 weeks old; weighing 200–250 g), which were randomly caged into (1) the normal group (n = 3) and (2) the HFHFD group (n = 3), in a controlled environment of 25 ± 2 °C and 12:12 hour light-dark cycle. Rats in the normal group received a standard rodent feed containing 18% protein, while the HFHFD group received the best HFHFD (containing standard rodent feed, mixed with beef tallow 55% and fructose 60%), for 35 consecutive days. Rats fed with HFHFD revealed the pathogenesis of NAFLD, characterized by a significant increase in triglyceride levels compared to the normal group (Figure 1). Moreover, a histology examination of the liver tissue showed the presence of ballooning degeneration and necrosis, compared to those in the normal group (Figure 2).

The Effects of ELM on the Liver Index and Morphology of the Liver of HFHFD-Induced NAFLD Rats

The effects of ELM on the morphology of the rat liver are depicted in Figure 3. Macroscopically, the morphology of the livers of all groups reveals no difference, showing a normal wedge shape. The rats in the normal control group exhibited the darkest reddish-brown color compared to those induced with HFHFD, indicating a healthy liver. Treating the rats with ELMs resulted in

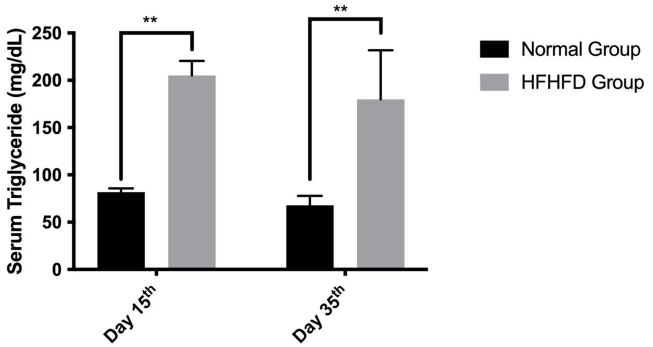


Figure 1 NAFLD optimization in Wistar rats by measuring serum triglyceride levels of HFHFD-induced rats (205.11 ± 15.35 mg/dL at day 15 and 179.98 ± 51.69 mg/dL at day 35) compared to the normal group (81.78 ± 3.88 mg/dL at day 15 and 67.87 ± 9.86 mg/dL at day 35). Data are presented as mean ± SEM of 3 rats. A significant difference is marked by ** indicating $p < 0.01$. Abbreviation: HFHFD, high-fat high-fructose diet; NAFLD, non-alcoholic fatty liver disease; SEM, standard error mean.

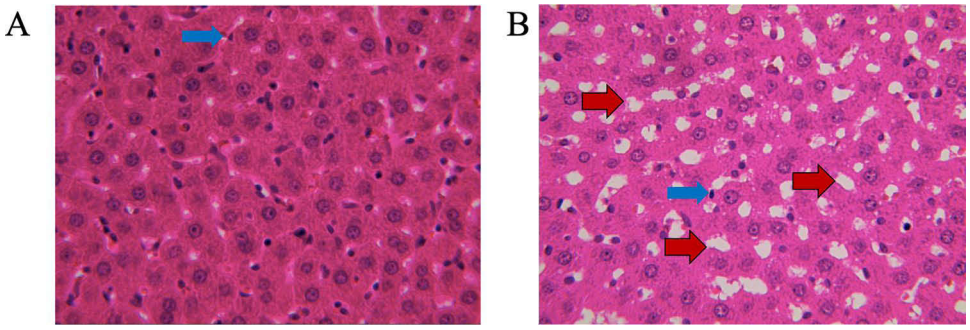


Figure 2 NAFLD optimization in Wistar rats by histology examination of (**A**) the normal group and (**B**) HFHFD-induced rats at day 35 with 400x magnification. Red arrows, ballooning degeneration; blue arrows, Kupffer cell. Abbreviation: NAFLD, non-alcoholic fatty liver disease; HFHFD, high-fat high-fructose diet.

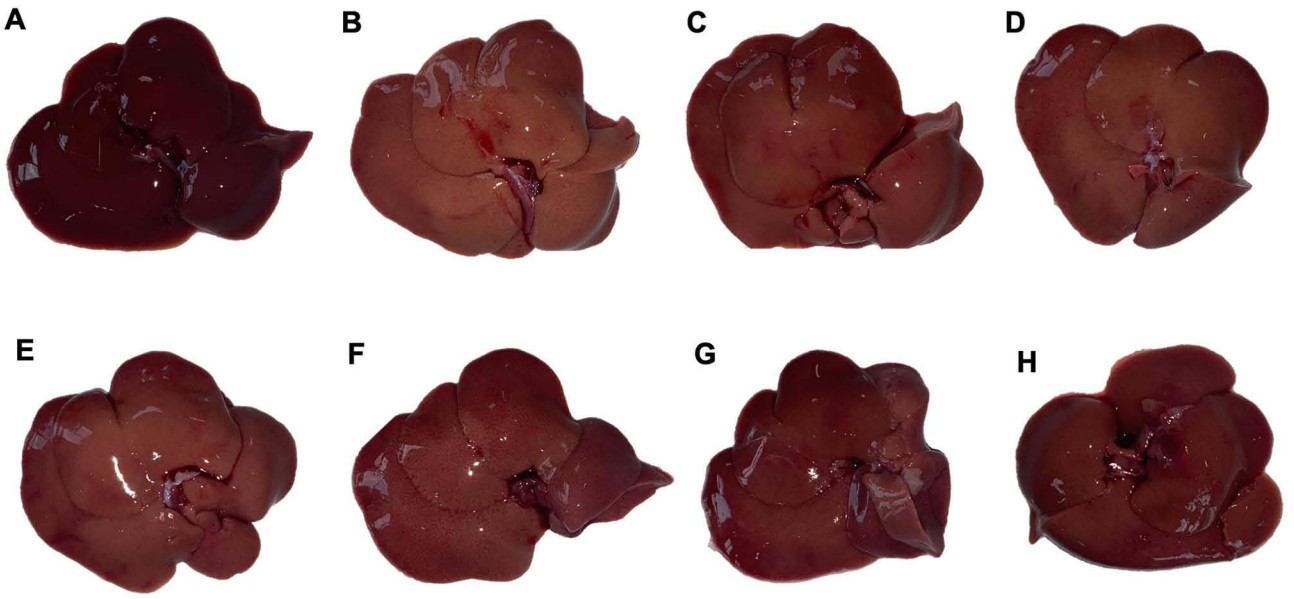


Figure 3 Effects of ELM on the morphology of the liver of HFHFD-induced NAFLD rats: (**A**) the normal control group; (**B**) HFHFD group without treatment: (**C**) HFHFD rats treated with metformin (200 mg/kg BW); (**D**) HFHFD rats treated with resveratrol (50 mg/kg BW); (**E**) HFHFD rats treated with beta-sitosterol (20 mg/kg BW); (**F**) HFHFD rats treated with ELM dose of 200 mg/kg BW; (**G**) HFHFD rats treated with ELM dose of 400 mg/kg BW; (**H**) HFHFD rats treated with ELM dose of 600 mg/kg BW, with 400x magnification. Abbreviation: BW, body weight; ELM, the ethanol extract of *Lygodium microphyllum*; HFHFD, high-fat high-fructose diet; NAFLD, non-alcoholic fatty liver disease.

a darker reddish-brown color compared to that without treatment (the HFHFD group). Similarly, the treatment using metformin, resveratrol, or beta-sitosterol showed a darker reddish-brown color compared to that without treatment. The liver index of the HFHFD group was significantly higher than that of the normal control group. Rats treated with ELM showed a lower liver index than the HFHFD group (Figure 4).

Effects of ELM on the Histopathology and NAS Score of the Liver Tissue of HFHFD-Induced NAFLD Rats

The effects of ELM on the histopathology of the rat liver are depicted in Figure 5. Rats in the normal control group exhibited neatly arranged hepatocyte cells. Meanwhile, the HFHFD group shows ballooning degeneration (indicated by the red arrows) and steatosis (indicated by black arrows). Treating the rats with ELM slightly reduced the ballooning degeneration and steatosis. To determine the extent of liver damage, the NAS score was calculated, and confirmed that ELM significantly reduced the NAS score of the rats compared to the HFHFD group. Similarly, the treatment using metformin or resveratrol, not beta-sitosterol, also significantly reduced the NAS score compared to the HFHFD group (Figure 6).

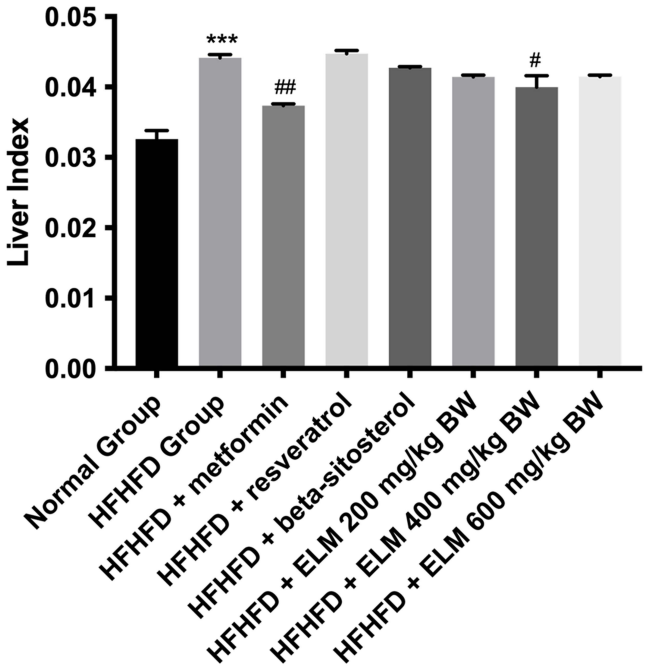


Figure 4 Effects of ELM on the liver index of HFHFD-induced NAFLD rats. The liver index score was analyzed using a one-way ANOVA. Each bar represents the mean ± SEM of 3 rats in a group. *** indicating $p < 0.001$ vs the normal control group; # indicating $p < 0.05$ vs the HFHFD group; ## indicating $p < 0.01$ vs the HFHFD group. Abbreviation: ELM, the ethanol extract of *Lygodium microphyllum*; HFHFD, high-fat high-fructose diet; NAFLD, non-alcoholic fatty liver disease; SEM, standard error mean; BW, body weight.

Effects of ELM on the AMPK/SIRT1 Levels in the Liver Tissue of HFHFD-Induced NAFLD Rats

The two isoforms of AMPK were separated as twin bands at a molecular weight of 62.8 kDa for AMPKα1 and a molecular weight of 62.3 kDa for AMPKα2 as depicted in Figure 7, while the bands of SIRT1 were observed at 110–130 kDa as portrayed in Figure 8.

The effects of ELM on the AMPK/SIRT1 levels in the liver tissue of HFHFD-induced NAFLD rats are depicted in Figures 7 and 8, respectively. Inducing the rats with HFHFD lowered the levels of AMPK and SIRT1 compared to rats in the normal control group. Interestingly, treating the rats with ELM resulted in an increase in the levels of both AMPK and SIRT1 in the liver tissue of HFHFD-induced NAFLD rats. Metformin and resveratrol did not significantly alter AMPK levels, but beta-sitosterol reduced AMPK levels. Metformin, resveratrol, and beta-sitosterol could increase the levels of SIRT1 although not significant.

Discussion

The fern plant *Lygodium microphyllum* (Cav). R.Br. (Lygodiaceae) may have the potential to be utilized as a medicinal plant. In this study we confirm that (1) the ethanol extract of *L. microphyllum* (ELM) improves the morphology and the liver index of HFHFD-induced NAFLD rats comparably to those of metformin, resveratrol, or beta-sitosterol; (2) ELM improves the steatosis condition and NAS score of HFHFD-induced NAFLD rats comparably to those of metformin, resveratrol, or beta-sitosterol; (3) ELM may inhibit *de novo* lipogenesis by increasing the levels of both AMPK and SIRT1 in the liver tissue of HFHFD-induced NAFLD rats, which is better than metformin, resveratrol, or beta-sitosterol.

In a previous study, beta-sitosterol was reported as the major compound in *L. microphyllum*,[20] with total sterol levels of 954.04 µg/g, and the beta-sitosterol level of 283.55 µg/g,[21] thus arising our interest in exploring the anti-lipogenesis activity of this fern plant. As formerly reported, plant sterols reduced cholesterols, low-density lipoproteins, and triglycerides, and increased high-density lipoproteins in both humans and animal models.[14–18]

The inducement with HFHFD was administered for 35 days to stimulate *de novo* lipogenesis (DNL) in the rats. It was delineated by Softic and colleagues (2016) that high-fat high-sugar intake correlates with obesity and the development of

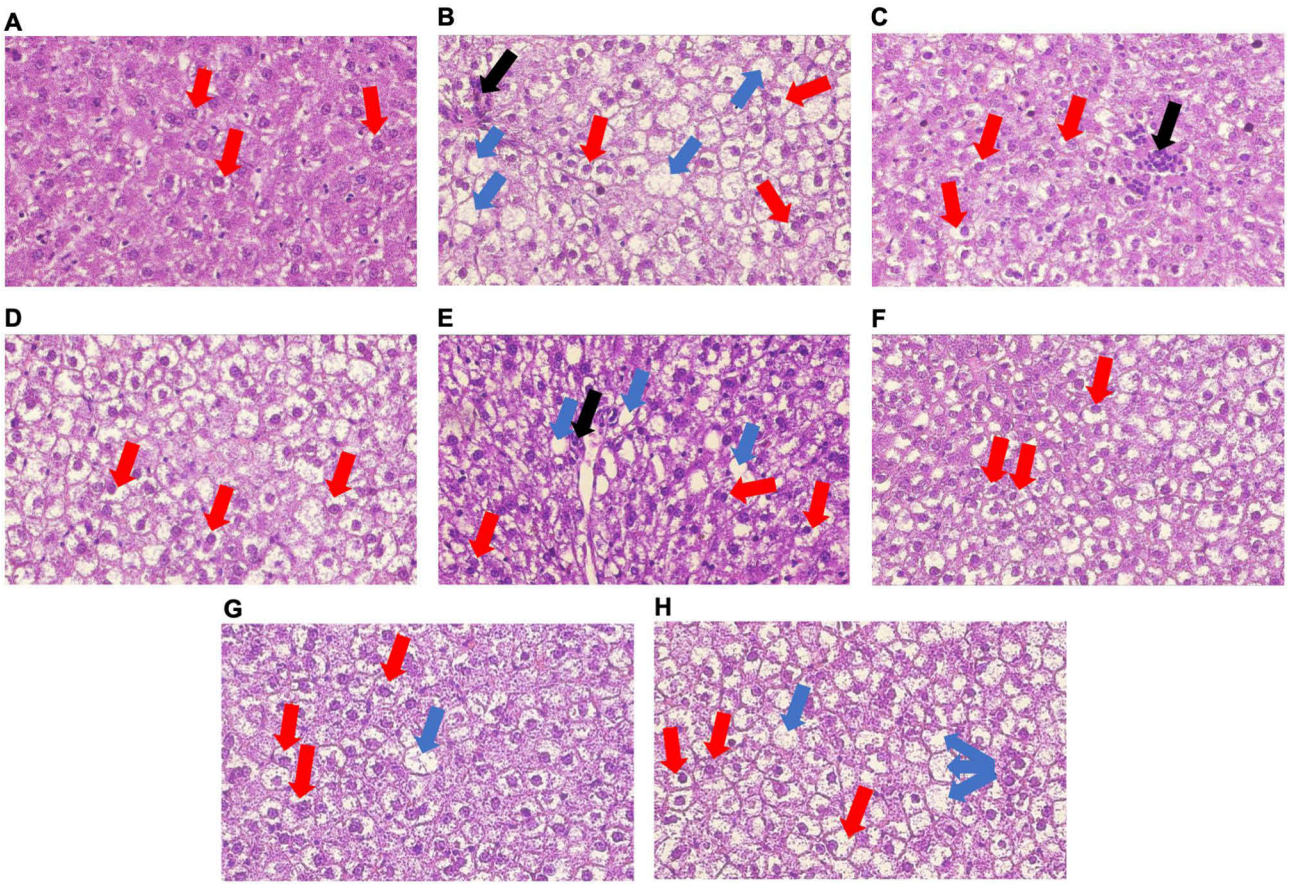


Figure 5 Effects of ELM on the histopathology of the liver tissue of HFHFD-induced NAFLD rats: (**A**) the normal control group; (**B**) HFHFD group without treatment: (**C**) HFHFD rats treated with metformin (200 mg/kg BW); (**D**) HFHFD rats treated with resveratrol (50 mg/kg BW); (**E**) HFHFD rats treated with beta-sitosterol (20 mg/kg BW); (**F**) HFHFD rats treated with ELM dose of 200 mg/kg BW; (**G**) HFHFD rats treated with ELM dose of 400 mg/kg BW; (**H**) HFHFD rats treated with ELM dose of 600 mg/kg BW, with 400x magnificent. The red arrows indicate the ballooning cell, the blue arrows show the steatosis and the black arrows show the inflammation. Abbreviation: BW, body weight; ELM, the ethanol extract of *Lygodium microphyllum*; HFHFD, high-fat high-fructose diet; NAFLD, non-alcoholic fatty liver disease.

NAFLD.[37] When a person consumes sucrose, it will undergo degradation to produce six-carbon monosaccharides, glucose, or fructose, which metabolism converges into glyceraldehyde-3 phosphate and dihydroxyacetone phosphate as intermediate products, and is further converted to pyruvate. Pyruvate will enter mitochondria, where it is changed to acetyl-CoA (Ac-CoA), to be utilized in the tricarboxylic acid (TCA) or citric acid cycle. When energy stores are sufficient, citric acid is transported back into the cytoplasm by the mitochondrial tricarboxylate transport system.[38] Citric acid is eventually converted to acetyl-CoA (Ac-CoA), by the action of adenosine triphosphate citrate lyase (ACL), which is the first step of endogenous fatty acid synthesis. Citric acid is an allosteric activator of cytoplasmic Ac-CoA carboxylase (ACC), which works to transform Ac-CoA to malonyl-CoA, thus initiating *de novo* lipogenesis (DNL).[4,39] Moreover, it was narrated that fructose could rapidly bind into both glycerol and free fatty acids, thus reinforcing DNL, a major contributor to NAFLD pathogenesis.[40]

A study in humans treated with excess intake of carbohydrates for three weeks resulted in an increase of liver fat by 27%, while the increase of total body weight (BW) was only 2%. The same participants were eventually advised to take 24 weeks of a hypocaloric diet, and in consequence, resulted in a loss of 25% liver fat and 4% of BW.[41] High fructose corn syrup (HFCS)-containing drinks were confirmed for their contribution to the development of NAFLD in humans due to their major role in hepatic DNL.[42] In 358 participants with type 2 DM, higher carbohydrate intake correlates with the occurrence of liver steatosis in patients aged < 50 years old.[43]

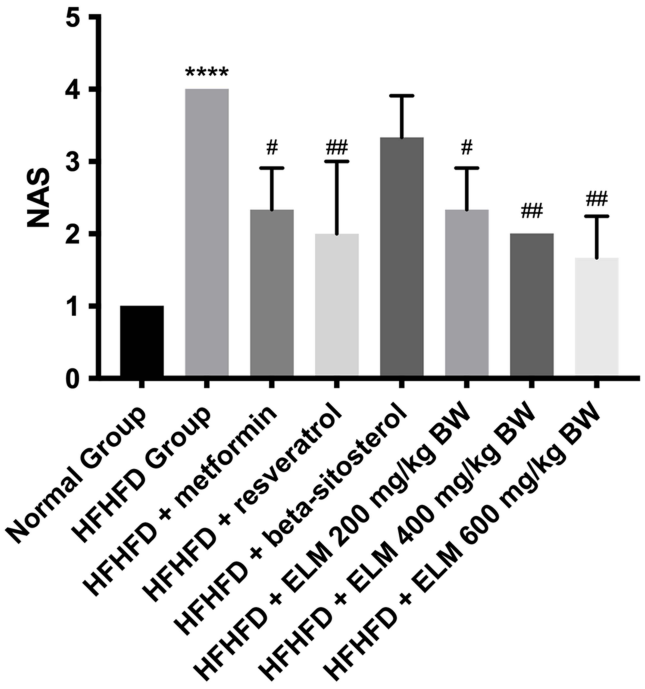


Figure 6 Effects of ELM on the NAS score of the liver tissue of HFHFD-induced NAFLD rats. The NAFLD activity score (NAS) was analyzed using a one-way ANOVA. Each bar represents the mean ± SEM of 3 rats in a group. **** indicating $p < 0.0001$ vs the normal control group; # indicating $p < 0.05$ vs the HFHFD group; ## indicating $p < 0.01$ vs the HFHFD group. Abbreviation: ELM, the ethanol extract of *Lygodium microphyllum*; HFHFD, high-fat high-fructose diet; NAFLD, non-alcoholic fatty liver disease; NAS, NAFLD activity score; BW, body weight; SEM, standard error mean.

In our study, the inducement of the rats with HFHFD resulted in lower hepatic AMPK and SIRT1 levels, while treatment with ELM increased the levels of both proteins in the liver tissue. We compared this result to a former study by Lindholm et al (2013).[44]

Lindholm and colleagues concluded that a high-fat diet (HFD) reduced the activities of both AMPKα1/AMPKα2 in white adipose tissue, heart, and liver of male Sprague–Dawley rats.[45] The effect of an HFD on AMPK has most frequently been studied in a single tissue type, usually in liver tissue,[44–46] heart,[44,47] and/or skeletal muscle.[48,49] The factors that contribute to the activation of AMPK are exercise, caloric restriction, and anti-DM drugs such as metformin and thiazolidinediones.[50–52] AMPK can be activated by SIRT1 which shelters against the induction of fatty acid synthase and lipid accumulation promoted by high glucose.[6] AMPK mediates the metabolic hormones such as leptin, ghrelin, adiponectin, and glucocorticoids, thus activating glycolysis, fatty acid oxidation, and mitochondrial biogenesis, and reducing gluconeogenesis, glycogen, fatty acid, and protein synthesis.[51] Moreover, 2 to 8 weeks of HFD feeding to male ddY mice revealed an alteration of hepatic LKB1-AMPK signaling and SIRT1 expression.[53]

Metformin, an anti-diabetes mellitus biguanide class drug, was reported to activate AMPK in liver cells, thus reducing acetyl-CoA carboxylase (ACC) activity, stimulating fatty acid oxidation, and suppressing the expression of lipogenic enzymes.[54] Metformin effectively decreases blood glucose levels by inhibiting hepatic glucose synthesis, reducing intestinal absorption, and increasing insulin sensitivity. A combination of N-acetyl cysteine and metformin treatment for 48 weeks to 53 male and female patients with non-alcoholic hepatosteatosis showed remarkable improvements in the steatosis degree, ballooning, and NAS score.[55]

Resveratrol (3,5,4′-trihydroxy-trans-stilbene), a polyphenol compound found in abundant levels in grapes (*Vitis vinifera*), can inhibit adenosine triphosphate (ATP) synthase thus activating AMPK without affecting the serine/threonine kinase liver kinase B1 (abbreviated as LKB1, also known as serine/threonine kinase 11, abbreviated as STK11). This polyphenol compound binds to SIRT1 but does not change its catalytic activity.[56] Furthermore, a recent study by Afshari and co-workers (2023) confirmed that a combination of metformin and resveratrol diminishes liver steatosis by stimulating autophagy via the cAMP/AMPK/SIRT1 signaling pathway.[57] Hepatic steatosis is also attenuated by

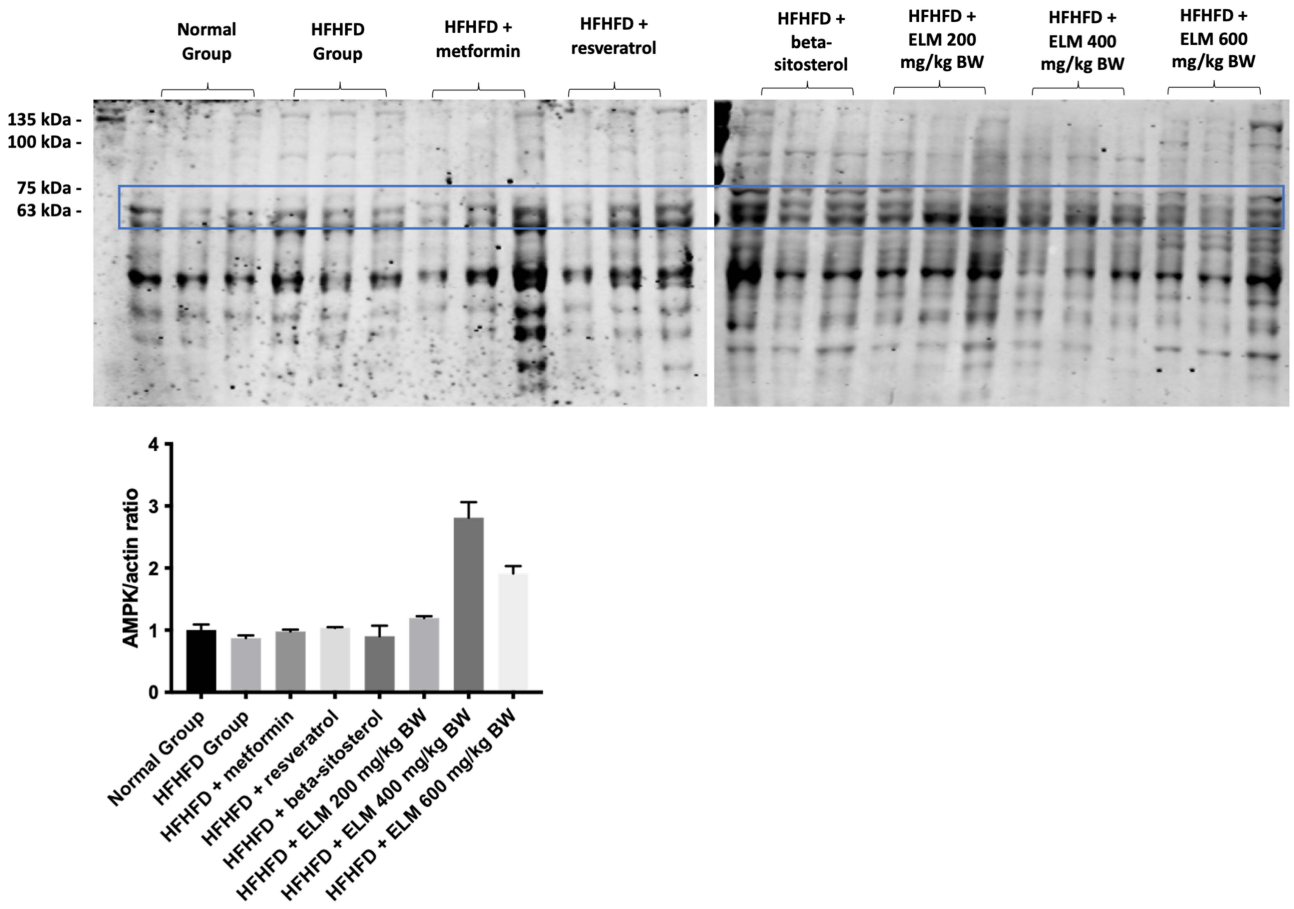


Figure 7 Effects of ELM on the AMPKα1 (observed molecular weight of 62.8 kDa; calculated molecular weight of 64 kDa)/AMPKα2 (observed molecular weight of 62.3 kDa; calculated molecular weight of 65 kDa) levels in the liver tissue of HFHFD-induced NAFLD rats. The upper figure portrays the separated protein bands by Western blotting and the bar diagram of AMPK levels normalized to beta-actin is presented in the lower figure. No significant differences were observed. Higher doses of ELM increased the levels of AMPK in the liver tissue of HFHFD-induced NAFLD rats. The blue box indicates the bands of AMPKα1/AMPKα2 at 62.8 and 62.3 kDa. Abbreviation: AMPK, adenosine monophosphate-activated protein kinase; BW, body weight; ELM, the ethanol extract of *Lygodium microphyllum*; HFHFD, high-fat high-fructose diet; NAFLD, non-alcoholic fatty liver disease.

a combination of metformin and quercetin via the same pathway as reported by Afshari and co-workers.[58] Moreover, resveratrol and metformin could stimulate serum SIRT1 and improve insulin resistance in high fructose-fed animal models.[59]

AMPK activation by phytosterols was limitedly described, thus unlocking further explorations. An article by Jie and co-workers reported that stigmasterol improved neuroinflammation in APP/PS1 mice, and inhibited the inflammatory response of microglia to Aβ42 oligomers via AMPK/NF-kappaB signaling.[60] Conversely, in our study, beta-sitosterol reduces the levels of AMPK in the liver tissue of NAFLD rats, which needs further exploration. It is evidence that beta-sitosterol could lessen the mass of adipose tissue and inhibit the proliferation of preadipocytes as proven by the in vitro study of Awad and colleagues (2000) in 3LT-L1 cells.[61] It is believed that phytosterols may compete with free cholesterol for the binding with the micellar structure, increase cholesterol excretion via the feces, and lower plasma cholesterol levels,[62] thus raising their use as complementary or add-on therapy for patients with obesity and diabetes mellitus.[63]

Although we confirmed that ELM improves the morphology and the liver index of HFHFD-induced NAFLD rats, improves the steatosis condition and NAS score of HFHFD-induced NAFLD rats comparably to those of metformin, resveratrol, or beta-sitosterol, and may inhibit de novo lipogenesis by increasing the levels of both AMPK and SIRT1 in the liver tissue of HFHFD-induced NAFLD rats, our work did not evaluate the levels of the liver enzymes and the lipid profile of the rats., which may be listed as limitations of this study. Nevertheless, this study lays a solid foundation for future investigations into ELM as a promising natural remedy for NAFLD, opening new avenues for the development of

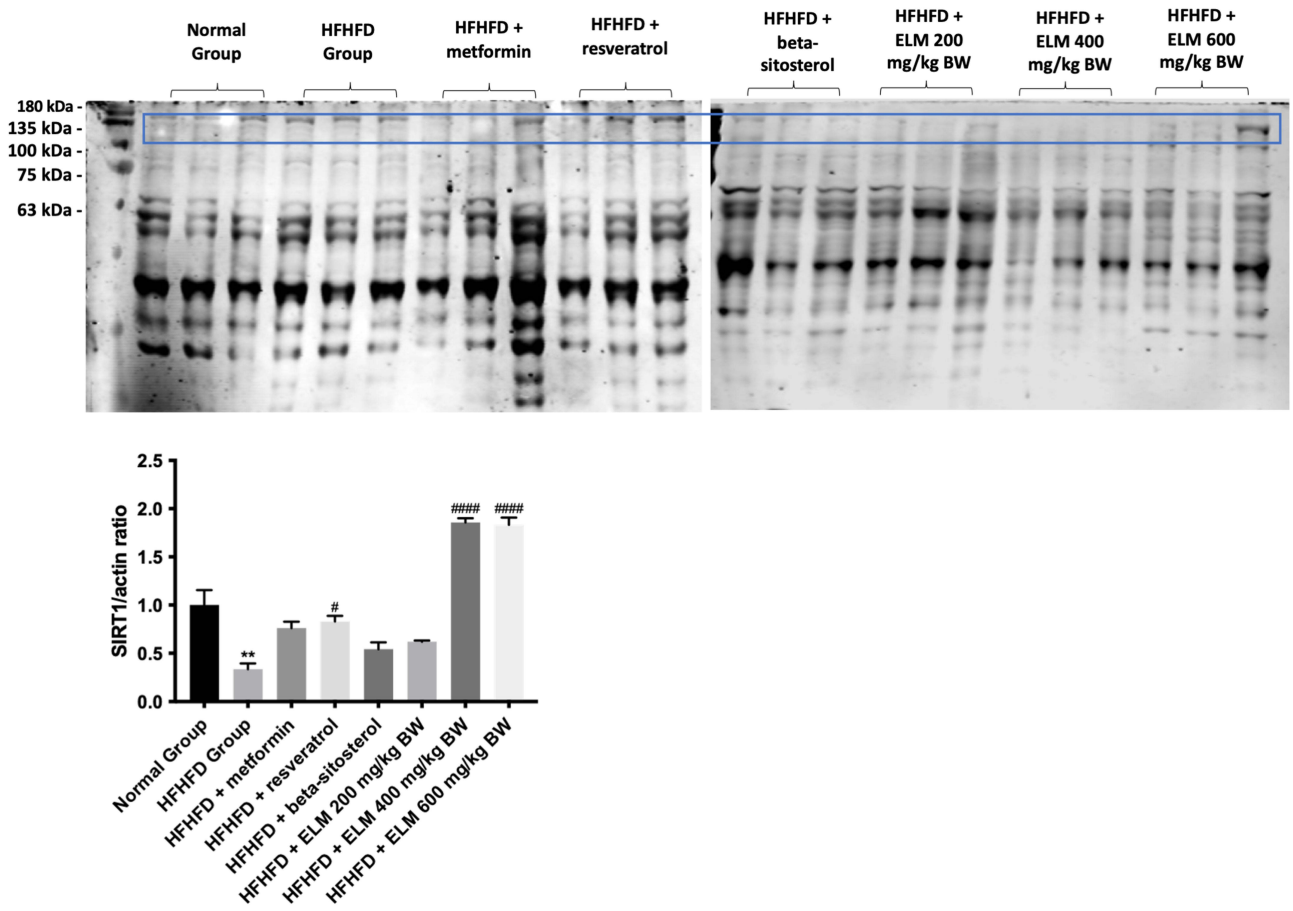


Figure 8 Effects of ELM on the SIRT1 (observed molecular weight of 110–130 kDa; calculated molecular weight of 61/81 kDa) levels in the liver tissue of HFHFD-induced NAFLD rats. The upper figure portrays the separated protein bands by Western blotting and the bar diagram of SIRT levels normalized to beta-actin is presented in the lower figure. Higher doses of ELM significantly increased the levels of SIRT1 in the liver tissue of HFHFD-induced NAFLD rats. ** indicating $p < 0.001$ vs the normal control group; # indicating $p < 0.05$ vs the HFHFD group; ##### indicating $p < 0.0001$ vs the HFHFD group. The blue box indicates the band of SIRT1 at 130 kDa. Abbreviation: BW, body weight; ELM, the ethanol extract of *Lygodium microphyllum*; HFHFD, high-fat high-fructose diet; NAFLD, non-alcoholic fatty liver disease; SIRT1, Sirtuin1.

alternative treatments for this increasingly prevalent liver condition. Understanding the proteins involved in the upstream and downstream pathways of SIRT1 and AMPK is still challenging and may corroborate the whole framework of the mechanism of this plant in inhibiting lipogenesis.

Conclusion

The present work studied the antilipogenesis activity of the ethanol extract of *Lygodium microphyllum* (Cav). R.Br., a fern plant found abundantly in a rainforest of Kalimantan island, Indonesia. Our study revealed that the ethanol extract of *L. microphyllum* improves the morphology, the liver index, the steatosis condition, and NAS score of high-fat high-fructose-induced NAFLD rats comparably to those of metformin, resveratrol, or beta-sitosterol. We confirm that this is the first report on the anti-lipogenesis activity of *L. microphyllum* by significantly increasing the levels of AMPK and SIRT1 in the liver tissue of NAFLD rats, better than that of metformin and resveratrol. It is suggested to further explore *L. microphyllum* for its anti-lipogenesis activity in patients with NAFLD.

Acknowledgments

The authors thank (1) the Directorate of Higher Education of the Ministry of Education and Culture for funding the research; (2) the Faculty of Pharmacy, Mulawarman University for facilitating the laboratories; (3) Ronny Lesmana, dr., M.H., AIFO, Ph.D. at the Central Laboratory of Padjadjaran University for the Western blot analysis

interpretation; and (4) the Rector of Padjadjaran University via the Directorate of Research and Community Engagement for funding the APC.

Funding

This research is funded by the Directorate of Higher Education of the Ministry of Education and Culture via the Doctoral Dissertation Grant no. 044/E5/PG.02.00.PL/2023 and 031/E5/PG.02.00.PL/2023. The APC is funded by Padjadjaran University via the Directorate of Research and Community Engagement.

Disclosure

The authors report no conflicts of interest in this work.

References

1.. Nassir F, Rector RS, Hammoud GM, Ibdah JA. Pathogenesis and prevention of hepatic steatosis. *Gastroenterol Hepatol (NY)*. 2015;11(3):167–175.
2. Ter Horst KW, Serlie MJ. Fructose consumption, lipogenesis, and non-alcoholic fatty liver disease. *Nutrients*. 2017;9(9):981. doi:10.3390/2Fnu9090981
3. Anggreini P, Kuncoro H, Sumiwi SA, Levita J. Role of the AMPK/SIRT1 pathway in non-alcoholic fatty liver disease (Review). *Mol Med Rep*. 2023;27(2):35. doi:10.3892/2Fmmr.2022.12922
4. Fullerton MD, Galic S, Marcinko K, et al. Single phosphorylation sites in Acc1 and Acc2 regulate lipid homeostasis and the insulin-sensitizing effects of metformin. *Nat Med*. 2013;19(12):1649–1654. doi:10.1038/nm.3372
5. von Loeffelholz C, Coldewey SM, Birkenfeld AL. A narrative review on the role of AMPK on de novo lipogenesis in non-alcoholic fatty liver disease: evidence from human studies. *Cells*. 2021;10(7):1822. doi:10.3390/2Fcells10071822
6. Hou X, Xu S, Maitland-Toolan KA, et al. SIRT1 regulates hepatocyte lipid metabolism through activating AMP-activated protein kinase. *J Biol Chem*. 2008;283(29):20015–20026. doi:10.1074/jbc.m802187200
7. Ponugoti B, Kim DH, Xiao Z, et al. SIRT1 deacetylates and inhibits SREBP-1C activity in regulation of hepatic lipid metabolism. *J Biol Chem*. 2010;285(44):33959–33970. doi:10.1074/jbc.m110.122978
8. Sayiner M, Koenig A, Henry L, Younossi ZM. Epidemiology of nonalcoholic fatty liver disease and nonalcoholic steatohepatitis in the United States and the rest of the world. *Clin Liver Dis*. 2016;20(2):205–214. doi:10.1016/j.cld.2015.10.001
9. Iqbal U, Perumpail BJ, Akhtar D, Kim D, Ahmed A. The epidemiology, risk profiling and diagnostic challenges of nonalcoholic fatty liver disease. *Medicines (Basel)*. 2019;6(1):41. doi:10.3390/2Fmedicines6010041
10. Yoo JJ, Kim W, Kim MY, et al. the Korean Association for the Study of the Liver (KASL)-Korea Nonalcoholic fatty liver Study Group (KNSG). Recent research trends and updates on nonalcoholic fatty liver disease. *Clin Mol Hepatol*. 2019;25(1):1–11. doi:10.3350/2Fcmh.2018.0037
11. Arab JP, Candia R, Zapata R, et al. Management of nonalcoholic fatty liver disease: an evidence-based clinical practice review. *World J Gastroenterol*. 2014;20(34):12182–12201. doi:10.3748/2Fwjg.v20.i34.12182
12. Paul J. Recent advances in non-invasive diagnosis and medical management of non-alcoholic fatty liver disease in adult. *Egypt Liver J*. 2020;10(1):37. doi:10.1186/s43066-020-00043-x
13. Prikhodko VA, Bezborodkina NN, Okovityi SV. Pharmacotherapy for non-alcoholic fatty liver disease: emerging targets and drug candidates. *Biomedicines*. 2022;10(2):274. doi:10.3390/biomedicines10020274
14. Brinton EA, Hopkins PN, Hegele RA, et al. The association between hypercholesterolemia and sitosterolemia, and report of a sitosterolemia kindred. *J Clin Lipidol*. 2018;12(1):152–161. doi:10.1016/j.jacl.2017.10.013
15. Ding XQ, Yuan CC, Huang YB, Jiang L, Qian LC. Effects of phytosterol supplementation on growth performance, serum lipid, proinflammatory cytokines, intestinal morphology, and meat quality of white feather broilers. *Poult Sci*. 2021;100(7):101096. doi:10.1016/2Fj.psj.2021.101096
16. Dumolt JH, Radhakrishnan SK, Moghadasian MH, et al. Maternal hypercholesterolemia enhances oxysterol concentration in mothers and newly weaned offspring but is attenuated by maternal phytosterol supplementation. *J Nutr Biochem*. 2018;52:10–17. doi:10.1016/j.jnutbio.2017.09.013
17. Ferguson JJA, Stojanovski E, MacDonald-Wicks L, Garg ML. Curcumin potentiates cholesterol-lowering effects of phytosterols in hypercholesterolaemic individuals. A randomised controlled trial. *Metabolism*. 2018;82:22–35. doi:10.1016/j.metabol.2017.12.009
18. Salehi B, Quispe C, Sharifi-Rad J, et al. Phytosterols: from preclinical evidence to potential clinical applications. *Front Pharmacol*. 2021;11:599959. doi:10.3389/fphar.2020.599959
19. Herman MRHS, Annisa SN, Kuncoro H. Hepatoprotective activity of ethyl acetate fraction from *Lygodium microphyllum* leaves in CCl4-induced damage rats. *AIP Conf Proc*. 2021;2360:040002. doi:10.1063/5.0059476
20. Kuncoro H, Farabi K, Rijai L. Steroids and isoquercetin from *Lygodium microphyllum*. *J Appl Pharm Sci*. 2017;7(11):136–141. doi:10.7324/JAPS.2017.71120
21. Anggreini P, Kuncoro H, Sumiwi SA, Levita J. Molecular docking study of phytosterols in Lygodium microphyllum towards SIRT1 and AMPK, the in vitro brine shrimp toxicity test, and the phenols and sterols levels in the extract. *J Exp Pharmacol*. 2023;15:513–527. doi:10.2147/2FJEP.S438435
22. Kuncoro H, Tappi ASAM, Anggreini P. Toxicity assessment of ethanol extract of Lygodium microphyllum. *Trop J Nat Prod Res*. 2024;8(7):7759–7764. doi:10.26538/tjnpr/v8i7.20
23. Guide for the Care and Use of Laboratory Animals. 2011. *National Research Council of the National Academies*. Eight Edition. The National Academies Press. Washington DC, USA. URL: https://grants.nih.gov/grants/olaw/guide-for-The-care-and-use-of-laboratory-animals.pdf. Accessed October 7, 2024.
24. Zhang F, Li L, Meng X, et al. Feeding strategy to use beef tallow and modify farmed tiger puffer fatty acid composition. *Animals*. 2023;13(19):3037. doi:10.3390/ani13193037

25. Official Methods of Analysis (2023). 22nd ed. Available from https://www.aoac.org/official-methods-of-analysis/. Accessed October 07, 2024.
26. Ryu JE, Jo W, Choi HJ, et al. Evaluation of nonalcoholic fatty liver disease in C57BL/6J mice by using MRI and histopathologic analyses. *Comp Med*. 2015;65(5):409–415.
27. Tehrani SS, Goodarzi G, Panahi G, Zamani-Garmsiri F, Meshkani R. The combination of metformin with morin alleviates hepatic steatosis via modulating hepatic lipid metabolism, hepatic inflammation, brown adipose tissue thermogenesis, and white adipose tissue browning in high-fat diet-fed mice. *Life Sci*. 2023;323:121706. doi:10.1016/j.lfs.2023.121706
28. Ding L, Zhang B, Li J, Yang L, Wang Z. Beneficial effect of resveratrol on α-naphthyl isothiocyanate-induced cholestasis via regulation of the FXR pathway. *Mol Med Rep*. 2018;17(1):1863–1872. doi:10.3892/mmr.2017.8051
29. Warden CH, Fisler JS. Comparisons of diets used in animal models of high-fat feeding. *Cell Metab*. 2008;7(4):277. doi:10.1016/2Fj.cmet.2008.03.014
30. Hickman DL. Minimal exposure times for irreversible euthanasia with carbon dioxide in mice and rats. *J Am Assoc Lab Anim Sci*. 2022;61(3):283–286. doi:10.30802/2FAALAS-JAALAS-21-000113
31. Abdulaziz Bardi D, Halabi MF, Hassandarvish P, et al. *Andrographis paniculata* leaf extract prevents thioacetamide-induced liver cirrhosis in rats. *PLoS One*. 2014;9(10):e109424. doi:10.1371/journal.pone.0109424
32. Wang Z, Li Q, Xiang M, et al. Astragaloside alleviates hepatic fibrosis function via PAR2 signaling pathway in diabetic rats. *Cell Physiol Biochem*. 2017;41(3):1156–1166. doi:10.1159/000464122
33. Brunt EM, Kleiner DE, Wilson LA, Belt P, Neuschwander-Tetri BA. NASH Clinical Research Network (CRN). Nonalcoholic fatty liver disease (NAFLD) activity score and the histopathologic diagnosis in NAFLD: distinct clinicopathologic meanings. *Hepatology*. 2011;53(3):810–820. doi:10.1002/2Fhep.24127
34. Takahashi Y, Fukusato T. Histopathology of nonalcoholic fatty liver disease/nonalcoholic steatohepatitis. *World J Gastroenterol*. 2014;20(42):15539–15548. doi:10.3748/wjg.v20.i42.15539
35. Kleiner DE, Brunt EM, Van Natta M, et al. Nonalcoholic Steatohepatitis Clinical Research Network. Design and validation of a histological scoring system for nonalcoholic fatty liver disease. *Hepatology*. 2005;41(6):1313–1321. doi:10.1002/hep.20701
36. Shen C-H. *Diagnostic Molecular Biology*. 2nd ed. Elsevier Inc; 2023. doi:10.1016/C2020-0-01054-6
37. Softic S, Cohen DE, Kahn CR. Role of Dietary Fructose and Hepatic De Novo Lipogenesis in Fatty Liver Disease. *Dig Dis Sci*. 2016;61(5):1282–1293. doi:10.1007/2Fs10620-016-4054-0
38. Kaplan RS, Mayor JA, Johnston N, Oliveira DL. Purification and characterization of the reconstitutively active tricarboxylate transporter from rat liver mitochondria. *J Biol Chem*. 1990;265(22):13379–13385. [PMID: 2165501]. doi:10.1016/S0021-9258(19)38309-7
39. Brownsey RW, Boone AN, Elliott JE, Kulpa JE, Lee WM. Regulation of acetyl-CoA carboxylase. *Biochem Soc Trans*. 2006;34(Pt 2):223–227. doi:10.1042/bst20060223
40. Chong MF, Fielding BA, Frayn KN. Mechanisms for the acute effect of fructose on postprandial lipemia. *Am J Clin Nutr*. 2007;85(6):1511–1520. doi:10.1093/ajcn/85.6.1511
41. Sevastianova K, Santos A, Kotronen A, et al. Effect of short-term carbohydrate overfeeding and long-term weight loss on liver fat in overweight humans. *Am J Clin Nutr*. 2012;96(4):727–734. doi:10.3945/ajcn.112.038695
42. Basaranoglu M, Basaranoglu G, Bugianesi E. Carbohydrate intake and nonalcoholic fatty liver disease: fructose as a weapon of mass destruction. *Hepatobiliary Surg Nutr*. 2015;4(2):109–116. doi:10.3978/2Fj.issn.2304-3881.2014.11.05
43. Alfadda NA, Aljuraiban GS, Awwad HM, et al. Higher carbohydrate intake in relation to non-alcoholic fatty liver disease in patients with type 2 diabetes. *Front Nutr*. 2022;9:996004. doi:10.3389/2Ffnut.2022.996004
44. Lindholm CR, Ertel RL, Bauwens JD, Schmuck EG, Mulligan JD, Saupe KW. A high-fat diet decreases AMPK activity in multiple tissues in the absence of hyperglycemia or systemic inflammation in rats. *J Physiol Biochem*. 2013;69(2):165–175. doi:10.1007/2Fs13105-012-0199-2
45. Barroso E, Rodríguez-Calvo R, Serrano-Marco L, et al. The PPARβ/δ activator GW501516 prevents the down-regulation of AMPK caused by a high-fat diet in liver and amplifies the PGC-1α-Lipin 1-PPARα pathway leading to increased fatty acid oxidation. *Endocrinology*. 2011;152(5):1848–1859. doi:10.1210/en.2010-1468
46. Ha SK, Kim J, Chae C. Role of AMP-activated protein kinase and adiponectin during development of hepatic steatosis in high-fat diet-induced obesity in rats. *J Comp Pathol*. 2011;145(1):88–94. doi:10.1016/j.jcpa.2010.11.011
47. Ko HJ, Zhang Z, Jung DY, et al. Nutrient stress activates inflammation and reduces glucose metabolism by suppressing AMP-activated protein kinase in the heart. *Diabetes*. 2009;58(11):2536–2546. doi:10.2337/db08-1361
48. Bonnard C, Durand A, Vidal H, Rieusset J. Changes in adiponectin, its receptors and AMPK activity in tissues of diet-induced diabetic mice. *Diabetes Metab*. 2008;34(1):52–61. doi:10.1016/j.diabet.2007.09.006
49. Martin TL, Alquier T, Asakura K, Furukawa N, Preitner F, Kahn BB. Diet-induced obesity alters AMP kinase activity in hypothalamus and skeletal muscle. *J Biol Chem*. 2006;281(28):18933–18941. doi:10.1074/jbc.m512831200
50. Fogarty S, Hardie DG. Development of protein kinase activators: AMPK as a target in metabolic disorders and cancer. *Biochim Biophys Acta*. 2010;1804(3):581–591. doi:10.1016/j.bbapap.2009.09.012
51. Lim CT, Kola B, Korbonits M. AMPK as a mediator of hormonal signalling. *J Mol Endocrinol*. 2010;44(2):87–97. doi:10.1677/jme-09-0063
52. Wong AK, Howie J, Petrie JR, Lang CC. AMP-activated protein kinase pathway: a potential therapeutic target in cardiometabolic disease. *Clin Sci*. 2009;116(8):607–620. doi:10.1042/cs20080066
53. Yoneda M, Guo Y, Ono H, et al. Decreased SIRT1 expression and LKB1 phosphorylation occur with long-term high-fat diet feeding, in addition to AMPK phosphorylation impairment in the early phase. *Obes Res Clin Pract*. 2010;4(3):e201–e207. doi:10.1016/j.orcp.2010.02.002
54. Zhou G, Myers R, Li Y, et al. Role of AMP-activated protein kinase in mechanism of metformin action. *J Clin Invest*. 2001;108(8):1167–1174. doi:10.1172/2FJCI13505
55. Oliveira CP, Cotrim HP, Stefano JT, Siqueira ACG, Salgado ALA, Parise ER. N-acetylcysteine and/or ursodeoxycholic acid associated with metformin in non-alcoholic steatohepatitis: an open-label multicenter randomized controlled trial. *Arq Gastroenterol*. 2019;56(2):184–190. doi:10.1590/s0004-2803.201900000-36
56. Lan F, Weikel KA, Cacicedo JM, Ido Y. Resveratrol-induced AMP-activated protein kinase activation is cell-type dependent: lessons from basic research for clinical application. *Nutrients*. 2017;9(7):751. doi:10.3390/2Fnu9070751

57. Afshari H, Noori S, Zarghi A. A novel combination of metformin and resveratrol alleviates hepatic steatosis by activating autophagy through the cAMP/AMPK/SIRT1 signaling pathway. *Naunyn Schmiedebergs Arch Pharmacol*. 2023;396(11):3135–3148. doi:10.1007/s00210-023-02520-7
58. Afshari H, Noori S, Zarghi A. Hepatic steatosis alleviated by a novel metformin and quercetin combination activating autophagy through the cAMP/AMPK/SIRT1 pathway. *Iran J Pharm Res*. 2023;22(1):e136952. doi:10.5812/ijpr-136952
59. El Agamy DF, Ahmed FED. Resveratrol and/or metformin activates serum sirtuin-1 and decreases insulin resistance in high fructose-fed rats. *Benha Med J*. 2020;37(3):561–577. doi:10.21608/bmfj.2020.30792.1268
60. Jie F, Yang X, Yang B, Liu Y, Wu L, Lu B. Stigmasterol attenuates inflammatory response of microglia via NF-κB and NLRP3 signaling by AMPK activation. *Biomed Pharmacother*. 2022;153:113317. doi:10.1016/j.biopha.2022.113317
61. Awad AB, Begdache LA, Fink CS. Effect of sterols and fatty acids on growth and triglyceride accumulation in 3T3-L1 cells. *J Nutr Biochem*. 2000;11(3):153–158. doi:10.1016/S0955-2863(99)00087-X
62. Trautwein EA, Duchateau GS, Lin Y, Mel'nikov SM, Molhuizen HO, Ntanios FY. Proposed mechanisms of cholesterol-lowering action of plant sterols. *Eur J Lipid Sci Technol*. 2003;105(3–4):171–185. doi:10.1002/ejlt.200390033
63. Vezza T, Canet F, de Marañón AM, Bañuls C, Rocha M, Víctor VM. Phytosterols: nutritional health players in the management of obesity and its related disorders. *Antioxidants*. 2020;9(12):1266. doi:10.3390/antiox9121266