JKKI: Jurnal Kedokteran dan Kesehatan Indonesia
Indonesian Journal of Medicine and Health
Journal homepage: https://journal.uii.ac.id/JKKI
P-ISSN 2085-4145 | E-ISSN 2527-2950

Euphorbia milii and propolis combination tea reduced hepatic steatosis and hepatocyte apoptosis in high-fat diet rat model

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Original Article

ARTICLE INFO

Keywords:
Euphorbia milii and propolis tea,
high-fat diet,
hepatocyte apoptosis,
hepatic steatosis

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DOI: 10.20885/JKKI.Vol15.Iss2.art10

History:
Received: January 29, 2024
Accepted: August 23, 2024
Online: August 27, 2024

ABSTRACT

Background: Dyslipidemia can cause various organ disorders, such as fatty liver disease. Over time, fatty liver disease has become more commonplace worldwide and may cause mortality if the progression worsens. Natural components from *Euphorbia milii* (*E. milii*) and propolis (EMP) have been demonstrated as immunomodulators that reduce total cholesterol levels.

Objective: To prove the effect of EMP tea on inhibiting fatty liver and hepatocyte apoptosis in high-fat diet (HFD)-induced rats.

Methods: This study applied a post-test-only control group design using 18 Wistar rats, which were divided into three groups: K0 (received standard feed), KN (received HFD of 2 g/200 g Body weight (BW) in a day), and P (received HFD of 2 g/200 g BW in a day and EMP of tea 40 mg/100 g BW in a day). The interventions were conducted for 30 days, followed by termination on day 31 for liver tissue collection and analysis. We calculated the hepatic steatosis with the help of hematoxylin-eosin (HE) staining. Hepatocyte apoptosis was also determined with terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining.

Results: The K0 group had a lower hepatic steatosis percentage (17.87 ± 1.81) than KN (63.75 ± 15.88). We also found no hepatocyte apoptosis in the K0 but a high hepatocyte apoptosis index in the KN (3.98 ± 0.29). The combination of EMP tea in HFD-induced rats significantly reduced hepatic steatosis and apoptosis percentage (25.33 ± 1.17 and 0.91 ± 0.61, respectively).

Conclusion: We demonstrated that combining EMP tea reduced hepatic steatosis and hepatocyte apoptotic index in HFD-induced rats, suggesting its potential as a treatment for these conditions.

INTRODUCTION

Fatty liver disease is one of the causes of the silent killer that continues to increase its prevalence by 0.7% annually worldwide from 1991 to 2019. The global incidence of non-alcoholic fatty liver disease (NAFLD) is estimated at 25%, especially in patients with diabetes and obesity.[1,2] Fatty liver disease occurs due to the accumulation of fat in the liver, which can arise due to consumption of a HFD, excessive alcohol consumption, and hepatitis virus infection. Fatty liver can cause obesity, insulin resistance, dyslipidemia, and type 2 diabetes mellitus. It is possible to divide fatty liver into alcoholic fatty liver disease (AFLD) and NAFLD.[3–5]

Non-alcoholic fatty liver is more closely associated with obesity, HFD, and insulin resistance, which could cause disturbances in

peripheral lipolysis, an increase in fatty acids in the liver, and the accumulation of liver fat.[4,6] In general, immunology is one mechanism in the fatty liver through inflammatory processes, which involves the infiltration of immune cells into the liver sinusoid area.[7] The process continues with liver degeneration, where fat droplet accumulation in hepatocyte cells, hepatocytic ballooning, and hepatocyte apoptosis are found. The NAFLD may progress into non-alcoholic steatohepatitis (NASH), a condition of NAFLD with inflammation condition. If the condition progressively worsens, NAFLD causes liver cirrhosis, triggering failure and cancer of the liver.[4,6] The diagnosis of NAFLD is determined by detecting hepatocyte steatosis greater than 5% in individuals without a history of alcohol use.[7,8]

The management of NAFLD includes non-pharmacotherapy such as lifestyle modification, dietary supplementation, physical activity, and pharmacotherapy. The pharmacotherapy includes metformin, thiazolidinedione, glucagon-like peptide-1 (GLP-1), statins, ezetimibe, fibrates, and ursodeoxycholic acid (UDCA).[9] Some of the natural ingredients have proven their benefits in the management of NAFLD, which was associated with the phytochemical content of these natural ingredients. One of the phytochemical contents, which is thought to have a strong potential with antioxidant activity, anti-inflammatory, metabolic disease repair effects, antitumor, and various other effects, can alleviate hepatocyte damage in NAFLD.[10]

Euphorbia milii, or crown of thorns flower, has been used in traditional medicine since 50 BC in various countries. In Indonesia, E. milii is known as "*bunga mahkota duri*", a type of ornamental plant that grows upright on the ground with its stem covered with thorns. E. milii can grow well in tropical environments with high sunlight intensity, so it is easy to find in Indonesia and other tropical countries. It is also a member of the Euphorbiaceae family, similar to *Phylantus niruri* L., which has immunomodulatory potential.[11] Its phytochemicals, including flavonoids, tannin-type saponins, and steroid terpenoids, are similar to those found in Euphorbiaceae family members that are immunomodulators.[12,13] The phytochemicals of E. milii content also have several bioactivities such as antioxidant, anti-inflammatory, anticancer, antiviral, antimicrobial, and others.[14–17]

Propolis or bee glue is derived from bee saliva and contains complex ingredients rich in terpenes and benzoates, cinnamate, caffeic acid, flavonoids, phenolic acids, caffeic acid phenetyl ester (CAPE) which is proven to be an immunomodulator. Propolis has the potential to be anti-bacterial, anti-tumor, anti-inflammatory, antioxidant, and immunostimulant.[17,18] Propolis is also able to reduce fatty liver in hypercholesterolemia.[19] Recent studies have shown that a tea combination of *E. milii* and propolis (EMP) could alleviate total cholesterol levels.[20] The combination in EMP tea is a combination of herbal ingredients from plants (*E.milii* flowers) and animal products (honey bees) which are expected to work synergistically, complementing each other phytochemical components contained therein, increasing the taste sensation in the tea for organoleptic purposes and also to increase the innovation of the herbal product.[21]

In this study, HFD was given along with EMP tea. This concept was adapted from daily human activity, where some people often consume foods with a high-fat content, whether from fast food or other food products. We want to prove the preventive potential of EMP tea, especially in the early development of fatty liver. This study aimed to investigate the potential effect of EMP combination tea in reducing steatosis hepatitis and hepatocyte apoptosis in HFD rats.

METHOD

This study employed a post-test-only control group, using the following resource equation approach:[22]

$$Minimum\ n=10/k + 1$$
$$Maximum\ n=20/k +1$$
$$Minimum\ N=Minimum\ n \times k$$
$$Maximum\ N=Maximum\ n \times k$$

WN was the total number of subjects, n was the sample size of each group, and k was the number of groups. Given the k=3, the minimum n would be 4.3 (rounded up to 5), and the maximum n would be 7.6 (rounded down to 7). Hence, the equation of minimum N was 15, and the maximum N is 21. In conclusion, between 5 and 7 animals per group, or a total of 15 to 21 animals, are required to maintain a degree of freedom within the range of 10 to 20 for the analysis of variance (ANOVA) design. This study used 18 rats,

which were divided into three groups. Sample preparation was conducted at the Integrated Biomedical Laboratory, Faculty of Medicine, Udayana University.

Tea preparation

The tea was made from a combination of *E. milii* and propolis. About 500 gr of red *E. milii* flowers were picked from Ketewel, Bali. *Euphorbia milii* flowers were determined by a botanical expert from Eka Karya Bali Plant Conservation Centre, and their number was 529/IPH.UPT.04/AP/VIII/2012. The flowers were then washed and dried by air-drying. After drying, the flowers were put in an oven at 40°C for a full day. Next, the dried flowers were blended into a fine powder, and about 200 gr of powdered E. milii flower was obtained. Propolis is collected from honeybee nests (Tala), and obtained from honeybee farms in Plaga, Bali. About 600 gr of clean, empty honeybee nests were chopped into tiny bits and processed into a rough powder. After 48 hours of drying at 40°C in an oven, 300 grams of Tala coarse powder (containing Propolis) were obtained, and it was then ready to be packaged with the powdered *E. milii* flower. Each tea bag contained a powder mixture of 2 gr *E. milii* and propolis, with a ratio of the composition of about 2: 3. After steeping a tea bag in 100 milliliters of boiling water, it was chilled before administration to experimental animals. Each experimental animal was given 2 ml daily by nasogastric tube (40 mg).

High-fat diet

The HFD was made from 200 g of lamb fat, 700 g of standard feed, and 100 g of egg yolk. The lamb fat was boiled until melted. The chicken egg yolk was cooked, and the melted lamb fat was then mixed with standard feed. The standard feed was made of 35% K.L.K super®, 15% of rice bran, and 50% of corn.

Experimental animal

This study involved 18 healthy male Wistar rats weighing 150-200 gr and 60-90 days old. These rats were obtained from the Department of Histology, Udayana University. Prior to the experiment, all rats were housed for a week to allow them to become acclimated. Obtained animals were supplemented with food according to their group and water ad libitum with proper cages and environment. Three groups were randomly selected from the Wistar rats, each comprising 6 rats. Group K0 with standard feed (normal control) received no treatment. Group KN (positive control) was given HFD 2 g/200 g BW. Group P (treatment groups) was given HFD 2 g/200 g BW and EMP tea 40 mg/100 g BW. Both EMP tea and HFD were given orally once daily. The animals were then sacrificed on the 31st day of the trial. Hematoxylin-eosin (HE) and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) were used to stain the liver after it had been removed and cut for histopathological examination.

Hepatic steatosis index

The hepatic steatosis index was determined by the percentage of positive steatosis hepatocytes at 400x magnifications in 3 different fields under an Olympus CX300 light microscope, captured by an Optilab Camera, and the counted with Image Raster software. Hepatic steatosis is distinguished by a fat droplet in the hepatocyte cytoplasm on a slide stained with hematoxylin-eosin (HE) (Figure 1).

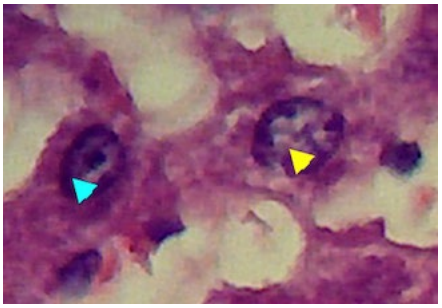

Figure 1. Normal hepatocyte (blue arrow) is characterized by blue nuclei; steatosis hepatocyte (yellow arrow) is characterized by fat droplet/ vacuole in its cytoplasm (Hematoxylin Eosin staining), 400x magnifications under Olympus CX300 light microscope).

Hepatocyte apoptotic index

The hepatocyte apoptotic index was determined by the percentage of apoptotic hepatocytes at 400x magnification in 3 different fields under a light microscope. Hepatocyte apoptosis is characterized by a brown appearance in the TUNEL histological slide (Figure 2), whereas normal hepatocytes will appear blue.

Statistical methods

The quantitative data was analyzed using SPSS 24.00 and shown as the mean ± standard deviation. The data on hepatic steatosis and hepatocyte apoptotic index were examined for homogeneity of variances and normalcy using the Shapiro-Wilk test. The data resulted in non-homogenous variants and non-normally distributed ($p<0.05$). The Kruskal-Wallis test was then used to analyze the data ($p<0.05$), followed by the Mann-Whitney test to determine which group was different compared to the other groups.

Ethics

The Ethical Committee of Udayana University approved the protocol of this study with Ethical clearance number 2167/UN14.2.2.VII.14/LT/2023.

RESULT

Hepatic steatosis index

The steatosis hepatic is an accumulation of lipid droplets in the hepatocytes (Figure 3). The normal control group (K0) showed slight hepatic steatosis, although it received a standard feed. The rats in the K0 group had a good appetite and did not fight over food. Hence, there was a body weight increase and accumulation of lipid droplets in the hepatocyte cells. The negative control groups (KN), which only received HFD, had the highest hepatic steatosis index. By contrast, the hepatic steatosis index in the treatment group (P), which received HFD and EMP combination tea was minimal (Table 1). Thus, it was concluded that HFD could cause

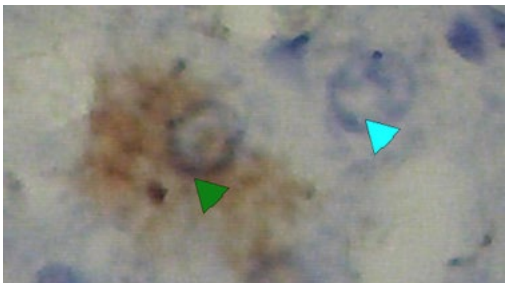

Figure 2. Hepatocytes that undergo apoptosis (green arrow) were characterized by brown color; Hepatocytes that do not undergo apoptosis (blue arrow)) were characterized by blue color (TUNEL staining, 400x magnifications under Olympus CX300 light microscope

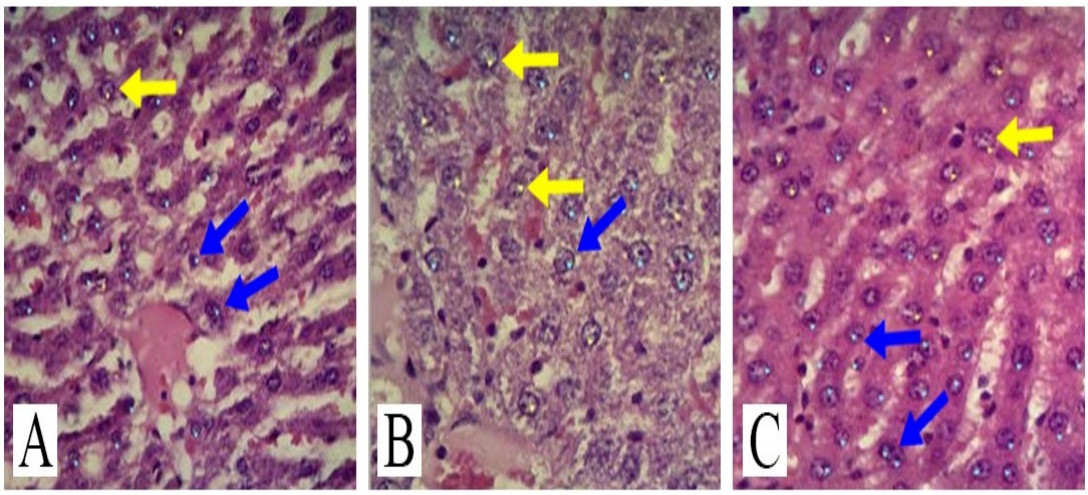

Figure 3. The histopathological examination was done on a Hematoxylin-Eosin staining slide at 400 magnifications. (A) Group K0 (normal control), (B) Group KN (negative control), (C) Group P (treatment groups). Yellow arrow shows steatosis hepatocytes; blue arrow shows normal hepatocytes

more profound hepatic steatosis in the liver and that the administration of EMP combination tea reduced the hepatic steatosis in HFD-induced rats. The mean and standard deviation of hepatic steatosis percentage are represented in Table 1. The result of the Kruskal-Wallis, then followed by the Mann-Whitney test, showed that the hepatic steatosis index among all groups was significantly different ($p<0.05$).

Hepatocyte apoptotic index

No hepatocyte apoptosis was found in group K0, which only received standard feed. However, induction of HFD (KN group) in the rats caused the greatest hepatocyte apoptosis index. This result showed that HFD-model rats caused hepatocytes to undergo apoptosis. The administration of EMP combination tea in HFD-induced rats significantly reduced the hepatocyte apoptotic index (Table 1). We found that diets that consist of high-fat content caused hepatocyte apoptosis, and an EMP combination tea could attenuate hepatocyte apoptosis in HFD-induced rats.

According to the Kruskal-Wallis, followed by the Mann-Whitney test result, there were significant differences ($p<0.05$) in hepatocyte apoptotic index. The mean, standard deviation, and p-value of hepatocyte steatosis are also shown in Table 1.

DISCUSSION

Given the rising incidence of metabolic syndrome, the prevalence of NAFLD has been increasing recently worldwide.[1,2] A consensus describes NAFLD as a condition in which over 5% of hepatocytes had steatosis without a history of alcohol use and other liver disease etiologies.[23] A meta-analytic study by Younossi et al. revealed that NAFLD is associated with some metabolic conditions, including obesity (51.34%), dyslipidemia (69.16%), hypertriglyceridemia (40.74%), diabetes (22.51%), hypertension (39.34%), and metabolic syndrome (42.54%). Diabetes is one of the risk factors for NAFLD patients for progression to NASH, cirrhosis, and death. Nevertheless, poor glycemic control also increases the risk of NASH patients developing cirrhosis.[24]

The percentage of hepatic steatosis index was found in the three groups. The normal control group (K0), which received standard feed, also had steatosis in the liver. Although increased intrahepatic fat content is a risk factor for several

Table 1. Hepatic steatosis and hepatocyte apoptotic index

	Groups (Mean ± SD)			p-value
	K0 (n=6)	KN (n=6)	P (n=6)	
Hepatic steatosis (%)	17.87 ± 1.81	63.75 ± 1.88	25.33 ± 1.17	0.001*
Hepatocyte apoptosis (%)	0.00 ± 0.00	3.98 ± 0.29	0.91 ± 0.61	0.001*

*Kruskal-Wallis $p<0.05$; SD: standard deviation; K0: normal group; KN: HFD-induced; P: HFD-induced and EMP tea.

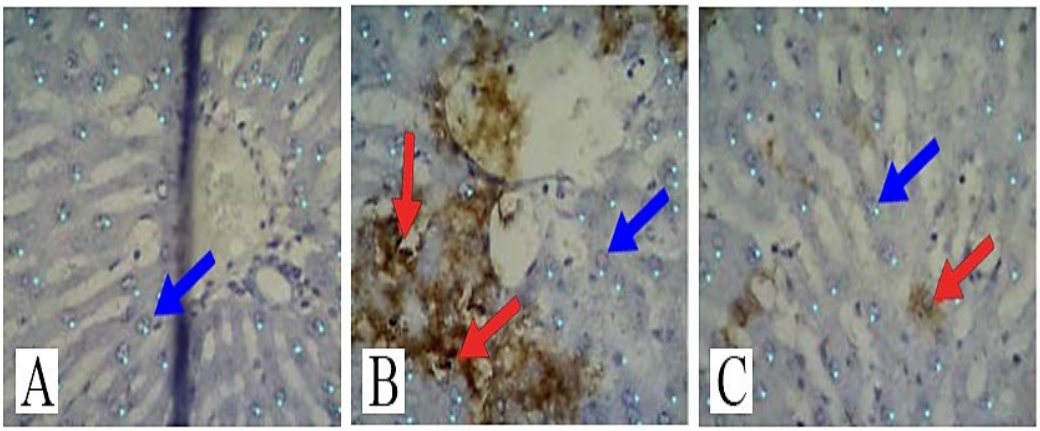

Figure 4. The histopathological examination was done on a TUNEL staining slide at 400 magnifications. Apoptotic hepatocytes can be seen by their brown appearance. (A) Group K0 (normal control), (B) Group KN (positive control), (C) Group P (treatment groups). The red arrow showed apoptosis hepatocytes; the blue arrow showed normal hepatocytes.

metabolic diseases, a small amount of fatty tissue in the rat liver is considered hepatoprotective.8 In this study, the HFD-induced group (KN) demonstrated remarkable hepatic steatosis. This result agrees with a study by Lasker et al., which found that HFD-induced rats significantly increased steatosis in liver histology.[25] Rats that were given HFD have been proven to generate fat accumulation in the liver, lipotoxicity, and cause NAFLD.[25,26] High-fat diet can consist of various regimens with fat content varying between 45-75 kcal.[26] Excessive and continuous consumption of the HFD model may cause obesity, dyslipidemia, insulin resistance, and impaired glucose homeostasis.[3,5]

The hepatic steatosis index in group P, which received HFD and the EMP tea 40 mg/100 gr/g BW per day, was lower than those who did not receive the EMP (KN). Our results showed that the phytochemical content in the EMP inhibited steatosis hepatic in the KN. This same result was also found in the previous study by Soleimani et al., using propolis among NAFLD patients. A 4-month propolis intervention could reduce at least one grade of hepatic steatosis compared to placebo (55.5% vs 18.5%, p=0.008).[27] Another study by Nepali et al. demonstrated that HFD rats treated with Euphorbia supina, one of the Euphorbiaceae family, reduced hepatic steatosis indicated by histological and hepatic biochemical (total triglyceride and total cholesterol).[28] A study by Orsolic et al. also found that propolis intake reduced weight gain and improved lipid profiles in HFD-induced rats. Propolis can regulate cholesterol and triglyceride metabolism. Administration of propolis in HFD-induced rats effectively reduced triglycerides and LDL-c and increased HDL-c serum. HDL extracts the excess cholesterol from peripheral tissues and then delivered to the liver for metabolism and excretion.[29]

Dyslipidemia or hypercholesterolemia is a condition of oxidative stress that can induce inflammation and damage various organs. Dyslipidemia in the liver can cause fatty liver, which is histologically described as fat droplets. It can be followed by liver inflammation and end up in hepatocyte apoptosis.[3] We found that HFD-induced rats showed significantly elevated hepatocyte apoptosis (3.98 ± 0.29%) than the normal control group (0.00 ± 0.00%). The result is similar to Soltis et al., who evaluated the high number of apoptotic hepatocytes in HFD-diet rats (37%) compared to the standard feed (<1%) with TUNEL staining.[5] Excess fatty tissue in the liver may cause lipotoxicity in metabolic and immunological ways, such as endoplasmic reticulum stress, mitochondrial dysregulation, and mitophagy dysfunction. Thus, the buildup of a larger amount of fat in the liver could cause metabolic disorders and eventually disrupt multiorgan systems, including the cardiovascular and the liver.[7,8]

Administration of EMP tea 40 mg/100 gr/gBW daily in HFD model rats reduced the hepatocyte apoptotic index. Our results aligned with those of Radaman et al., who evaluated the histological liver structure in liver damage rats induced with d-galactosamine/lipopolysaccharide. They treated rats with propolis before and immediately after intoxication, which did not show any pathological signs compared with variable degrees of apoptosis for the group that only received intoxication.[30] Orsolic et al. also stated that administration of propolis extract decreased the hepatocyte's caspase 3 expression as a liver apoptosis marker in carbon tetrachloride (CCl4)-induced rats.[29]

Hepatocyte apoptosis may also be related to the production of proinflammatory molecules, such as tumor necrosis factor-α (TNF-α), nuclear factor kappa B (NF-κB), and c-Jun N-terminal kinase (JNK). Moreover, the architecture of the liver in the HFD model rat is disturbed, which may be caused by the leakage of the bile acid. A recent study suggested that these changes may also be associated with and contribute to insulin resistance development.[5] Histologically, the tea combination of EMP showed no toxicity to the liver structure. There was no hepatocyte degeneration and necrosis found in rat's liver in the previous study by Rastika et al.[31] A study by Batool et al., using *Euphorbia dracunculoides* L., one of the Euphorbiaceae family, might reduce the increase in fatty alterations, level of inflammatory, cellular hypertrophy, hepatocytic ballooning, and central vein dilation on CCl4-induced liver damage in rats.[32]

Oxidative damage and inflammation in the liver may cause extracellular matrix deposition and fibrosis.[25] Hepatic stellate cell (HSC), activated by liver injury, is involved in liver regeneration. However, HSC also produces collagen-rich fibrosis and other extracellular matrix proteins, leading to the development of liver fibrotic scar and ceasing

liver regeneration. The liver function gradually gets diminished as the replacement of the functional liver tissue by the fibrotic tissue.[33 31]

The phytochemicals contained in *E. milii* resemble the Euphorbiaceae family, such as Phyllanthus niruri, which has proven to be an immunomodulator.[20] Non-alcoholic fatty liver increases the risk of atherosclerosis and also irreversible liver damage.[34] A previous study also demonstrated that EMP tea alleviated total cholesterol levels and matrix metalloproteinase (MMP)-8 serum, which have a role in atherosclerotic plaque formation and complications.[20] Polymorphonuclear granulocytes and macrophages released the MMP-8 during inflammation. Matrix metalloproteinase-8 is also released by the smooth muscle cells and the endothelial cells in atherosclerotic lesions.[34] In the liver, MMP-8 is associated with leukocyte infiltration in TNF-α induced acute hepatitis.[35]

The main content of propolis is flavonoids. Propolis is also a great resource for nickel, magnesium (Mg), calcium, zinc (Zn), and iron.[36] Propolis improves antioxidant activity by enhancing the antioxidant enzymes, including glutathione (GSH) enzymes, phase II detoxification enzymes, heme oxygenase 1, and superoxide dismutase (SOD).[29,37] Flavonoid content in propolis also has potent antioxidant activity against lipid peroxidation. Propolis has been shown to reduce oxidant molecules such as malondialdehyde (MDA), hydrogen peroxide (H_2O_2), and nitric oxide (NO) in HFD-induced rats.[36] An experimental study by Presetyo et al. showed that ethanol extract of propolis can reduce liver cell injury and fibrosis progression in CCl_4-induced cirrhosis hepatic.[37] Propolis also enhances liver function, as confirmed by the liver function test, like alanine aminotransferase (ALT), lactate dehydrogenase (LDH), aspartate aminotransferase (AST), and alkaline phosphatase (ALP).[29,37]

Quercetin and CAPE in propolis have the potential as an anti-inflammatory agent by inhibiting the NF-κB transcription factor for various inflammatory cytokine mediators. The inhibition of cytokine mediator prevents inflammatory cells from invading the tissue and inhibits cyclooxygenase activity, which prevents the production of prostaglandins involved in inflammation.[19] The inhibition of TNF-α and NF-κB also prevents macrophages from undergoing apoptosis.[36]

A study by Li et al. using CAPE at a dose of 12 mg/kg significantly alleviated the degree of liver fibrosis induced by CCl_4. The effect of CAPE in CCl_4-induced liver fibrosis rats also can reduce ALT, AST, and total bilirubin levels. Li et al. also showed significantly lower MDA concentrations and higher GSH concentrations than the control group. CAPE also prevents catalase and SOD depletion, which are some of the antioxidant enzymes. The concentrations of nuclear factor erythroid 2-related factor 2 (Nrf2) were higher due to CAPE administration. Nuclear factor erythroid 2-related factor 2 (Nrf2) is known as the cellular regulator for resistance against various oxidants.[38]

Euphorbia milii has hepatoprotective activity via antioxidant activity. A high concentration of reactive oxygen species (ROS) in the liver causes several diseases, such as lipid peroxidation and hepatocyte apoptosis. The products of lipid peroxidation have the potential to damage cells by rupturing biological membranes and releasing serum marker enzymes.[30] A study by Babu et al. showed the ethanol extract of *E. milii* can minimize the elevation of some liver biomarkers, such as AST, ALT, ALP, and total serum bilirubin levels in paracetamol-induced hepatotoxicity. They also revealed *E. milii* can either repair or protect against liver necrosis. Moreover, *E. milii* can maintain the structure of normal liver tissue.[39] Another study by Kaur et al. used *E. milii* methanolic extract could scavenge 2,2-diphenyl-1-picrylhydrazyl (DPPH) (19.65 ± 0.545 µg/ml) and H2O2 oxidant (14.66 ± 0.185 µg/ml).[14]

E. milii's antioxidant activity is believed to be because of the plant's active phytoconstituent.[14] Studies have proven that flavonoids can relieve NAFLD by improving oxidative stress, inflammation, metabolism of lipids, and balance of intestinal microbiota related to the liver-gut axis. Several studies also have proven that flavonoids intake was unrelated to NAFLD risk. Flavonoids are natural polyphenolic compounds in all plants, divided into flavones, orange ketones, flavonols, chalcones, isoflavones, anthocyanins, and dihydrogen derivatives. All types of flavonoids, with various mechanisms of action, have been proven to have anti-NAFLD effects. [40,41] The triterpene glycosides and flavonoids in E. milii flower have immunomodulatory potential.[13] The other mechanism of action can be by hepatocyte

lipid metabolism and oxidative stress regulation, one of which is through the Nrf2 pathway, reducing hepatic fat accumulation, regulating the formation of vacuolar-type ATPase (V-ATPase), and various other mechanisms.[40,41] Flavonoids contained in Pueraria lobata have effects as antioxidant and anti-inflammatory. The flavonoid improved NAFLD conditions in obese mice by inducing autophagy by the phosphatidylinositol 3-kinase (PI3K)/ protein kinase B (AKT)/ mammalian target of rapamycin (mTOR) signaling pathway.[42] Total flavonoids from citrus peel can potentially be hepatoprotective and anti-inflammatory in rats with NAFLD by NF-κB and mitogen-activated protein kinase (MAPK) signaling regulation.[43]

β-carotene, also found in *E. milii*, has hepatoprotective activity by reducing steatosis hepatic, inflammation, fibrosis, and cirrhosis in the liver.[44,45] It also regulates the NF-κB pathway and Nrf2 pathway.[45] The antioxidant activity of β-carotene involves the scavenging activity of free radicals and neutralizing singlet oxygen radicals.[44] As a result, the oxidative stress and the inflammatory cytokine released are decreased. By controlling the polarization of macrophages, β-carotene also stops the progression of NASH.[45] Moreover, the administration of β-carotene could prevent and alleviate fat deposition and hemorrhages in monocrotaline-induced steatosis in rat liver. 9-cis β-carotene, as β-carotene isomer, reduces the level of plasma cholesterol level and inhibits inflammation and fat deposition in the liver of HFD-induced rat.[44]

It has been known that vitamins C and E, as well as several minerals, have anti-inflammatory, antiapoptotic, and antioxidant properties. The antiapoptotic activity increases BCL-2 antiapoptotic protein and reduces BCL-2 associated-X (BAX) proapoptotic protein and p53 in NAFLD.[46] The EMP tea contains vitamin C, which can prevent or reduce hepatic steatosis. A study has proven an inverse relationship between serum vitamin C and NAFLD, metabolic dysfunction associated with fatty liver disease (MAFLD), liver fibrosis, or liver cirrhosis. Vitamin C administration has also been shown to reduce inflammation, which contributes to the improvement of NAFLD. Inhibition of the inflammatory response by vitamin C can also be done through decreasing IL-6, TNF-α, and C-reactive protein (CRP), as well as inhibiting de novo lipogenesis.[47] Inhibition or reduction in the initial process of steatosis will prevent the subsequent processes, such as hepatocyte apoptosis or hepatic cirrhosis. Hepatocyte apoptosis can stimulate immune cells and HSC in the liver to develop fibrosis later through the production of inflammasomes and cytokines. Activating caspase, B-cell lymphoma 2 (BCL-2) protein, c-Jun terminal kinase, and TNF-α can induce hepatocyte apoptosis in NAFLD.[48,49]

Magnesium has an anti-inflammatory activity. The first inflammatory reaction insult will decline the intracellular Mg concentration. Lack of Mg puts immune cells in an active state with elevated TNF-α and IL-6 levels. Magnesium in the EMP can regulate inflammation by controlling the proinflammatory cytokines and permitting cells to enter the cell cycle.[50] In cirrhosis model rats, Mg suppressed ROS and inhibited NF-κB transcription factor produced in HSC.[51]

Zinc in the EMP also acts as an independent risk factor for fibrosis in NAFLD. It is a vital mineral that can eliminate ROS and is crucial for the liver's lipid metabolism. It also inhibits the NADPH oxidase enzyme, which plays a role in the formation of O_2. Zinc has been shown to decrease lipid buildup, promote lipolysis, and activate lipophagy through autophagy in the liver. Moreover, Zn supplementation reduces the severity of liver damage and normalizes lipid peroxidation.[52] The micronutrient Fe contained in the EMP can prevent the development of obesity and hepatic steatosis by modulating the mitochondrial signaling pathway.[53]

The combination of phytochemicals and micronutrients in the EMP can inhibit the progression of steatosis and hepatocyte apoptosis in HFD-induced rats.[21] However, this study has various limitations. We did not evaluate the other liver structures and liver biomarkers. We are also aware of the limitations of our study in terms of duration and variations in dose combinations. Further study is required to determine therapeutic and toxic doses for EMP combination tea.

CONCLUSION

Administration of 40 mg/100 g BW EMP combination tea for 30 days ameliorated hepatic steatosis and hepatocyte apoptotic index in HFD-induced rats. There was a strong correlation between hepatocyte steatosis and the hepatocyte apoptotic index. Our studies revealed a potential

use of EMP tea to prevent hepatic steatosis and apoptosis due to HFD consumption.

CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

ACKNOWLEDGEMENTS

We thank LPPM Udayana University for funding through PNBP grants (No. B/1.354/UN14.4A/PT.01.03/2023.

AUTHOR CONTRIBUTIONS

Hepatic steatosis and hepatocytes apoptosis results and analysis were supervised by NML and IGKA. The results were discussed together with the guidance of the research team leader. All members played active roles in compiling and preparing this manuscript.

LIST OF ABBREVIATIONS

AFLD: Alcoholic fatty liver disease; BAX: BCL-2 associated-X; CRP: C-reactive protein; BCL-2: B-cell lymphoma 2; CCl4: carbon tetrachloride; DPPH: 2-diphenyl-1-picrylhydrazyl; CAPE: Caffeic acid phenetyl ester; EMP: Euphorbia milii and propolis; GLP-1: glucagon-like peptide 1; GSH: Glutathione; H2O2 : hydrogen peroxide; HE: hematoxylin and eosin; HFD: High-fat diet; HSC: Hepatic stellate cell; JNK: c-Jun N-terminal kinase; MAPK: mitogen-activated protein kinase; MDA: Malondialdehyde; MMP: Matrix metalloproteinase; NAFLD: Non-alcoholic fatty liver disease; NF-κB: Nuclear factor kappa beta; NASH: Non-alcoholic steatohepatitis; NO: nitric oxide; Nrf2: Nuclear factor erythroid 2-related factor 2; PI3K: phosphatidylinositol 3-kinase/ AKT: protein kinase B/ mTOR: mammalian target of rapamycin; ROS: Reactive oxygen species; SOD: superoxide dismutase; TNF-α : Tumor necrosis factor-α ; TUNEL: Terminal deoxynucleotidyl transferase dUTP nick end labeling; UDCA: ursodeoxycholic acid; Vit: Vitamin; V-ATPase: 2 vacuolar-type ATPase.

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