Effect of *Malus sylvestris* Extract on Histopathological Features of Hypercholesterolemic Wistar Rat (*Rattus norvegicus*) Fatty Liver

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Abstract

Objective: To evaluate the effect of *Malus sylvestris* extract on improving the degree of steatosis and portal inflammation histopathological features of hypercholesterolemic *Rattus norvegicus* strain wistar. **Method:** Forty-two male rats were divided into 6 groups randomly. Hypercholesterolemic fatty liver induced by giving high-fat diet (HFD) for 46 days on groups 1 to 5, while group 6 given standard diet with the same amount of time. Simvastatin was administered in group 2 at a dose of 0.36 mg/day. Groups 3, 4, and 5 were given *Malus sylvestris* extract (MSE) as a treatment at doses of 90 mg/day, 180 mg/day, and 360 mg/day in sequential order. Simvastatin and MSE were administered for 14 days, from day 33 to 46. On day 47, all rats were sacrificed and the liver was removed for histopathological slides preparation with hematoxylin-eosin (HE) staining. Histopathological results were analyzed using Kruskal Wallis test followed by Mann Whitney test. **Result:** Histopathological analysis showed that *Malus sylvestris* extract improved steatosis and portal inflammation features compared to HFD-fed rats in group 1 ($p<0,05$). **Conclusion:** *Malus sylvestris* extract improved the degree of steatosis and portal inflammation histopathological features of hypercholesterolemic rat fatty liver.

Keywords: *Fatty Liver, Histopathological, Hypercholesterolemic, Malus sylvestris, Rat.*

Introduction

Non-alcoholic fatty liver disease (NAFLD) is the accumulation of excess fat in the liver with more than 5% of hepatocytes containing visible lipid vacuoles or steatosis affecting at least 5% of the liver weight without heavy alcohol consumption or other secondary causes[1]. The disease can progress from harmless simple non-alcoholic steatosis (NAS) to non-alcoholic steatohepatitis (NASH), a form of inflammation that damages the liver cells[2]. Liver cell damage then can lead to fibrosis, cirrhosis, and eventually hepatocellular carcinoma (HCC), which require liver transplantation[3]. Although cirrhosis due to hepatitis C is the leading cause of liver transplantation in the United States, NAFLD rank the second. As prevalence continues to increase, NAFLD will become major health problems and the major cause of liver transplantation in the future[4]. This will also increase the demand for liver transplantation with fewer good-quality organs, as more donors have steatotic livers[5].

NAFLD has emerged as the most common chronic liver disease in developed countries. However, the prevalence of NAFLD continues to increase even in developing countries due to worldwide epidemic of obesity and other metabolic syndromes[6]. The prevalence of NAFLD worldwide is approximately 25.24%, with highest prevalence in the Middle East (31.79%) and South America (30.45%)[7]. In Asia, there is almost 10% increase in prevalence from the initial 25.28% in 1999-2005 to 33.90% in 2012-2017[8]. The data have shown that the cases will always increase and become a problem not only in Western countries but also in Asia

due to urbanization. Urbanization will lead to sedentary lifestyle with excessive dietary consumption resulting in obesity, a risk factor for NAFLD[9]. Apart from obesity, NAFLD also accompanied by hyperlipidemia, hypertension, type 2 diabetes, cardiovascular disease, and other metabolic syndromes that may affect not only the liver but also the person's systemic condition[3,7].

Environmental factors play an important role in the development of NAFLD, such as eating habits, daily activities, and socioeconomic factors[10]. Dietary patterns of high sodium and fat with low consumption of fresh fruit have been found in NAFLD patients[11]. By eating fruits and vegetables, especially apples, can reduce the risk of NAFLD due to fiber and chemical contents in apples such as flavonoids, polyphenols, and carotenoids, which have antioxidant and anti-inflammatory effects to prevent and protect the liver from NAFLD[12]. So far, there are only few studies regarding to the effect of apples on treating NAFLD and there are no studies specifically using *Malus sylvestris*. Hence, this study aimed to evaluate the effect of *Malus sylvestris* extract on improving histopathological features of hypercholesterolemic Wistar rat (*Rattus norvegicus*) fatty liver.

Materials and Methods

Malus sylvestris collection and extraction:

Malus sylvestris were collected from apple farm in Junggo Village, Bumiaji, Batu, Indonesia. The apples were washed, drained, and weighed. *Malus sylvestris* with their skins were cut into thin strips and air-dried in the shade. To maximize the drying process, apple slices were roasted at 45ºC for about 48 hours or until completely dried. The dried apple slices were crushed in a blender and sieved into powder. *Malus sylvestris* powder then extracted with 96% ethanol using percolation technique. Extraction process was continued with rotary evaporator to removed the alcohol content and a thick extract will be produced.

Experimental Animal:

Forty-two male Wistar rats around 2-3 months old weighing between 125-200g were used for the study. They were cared for in clean well-ventilated cages with light/dark cycle 12 h respectively and were given water and either a standard or high-fat diet (HFD) *ad libitum*. Before the experiment began, they will be acclimatized for seven days with a standard diet in the Experimental Animal Unit of Pharmacology Laboratory within Faculty of Medicine, Universitas Airlangga, Surabaya, Indonesia.

Experimental design[13]:

Forty-two Wistar rats were randomized and divided into 6 groups each containing 7 rats. Group I received HFD served as negative control rats. Group II received HFD and simvastatin at dose of 0,36 mg/day served as positive control rats. Group III received HFD and *Malus sylvestris* extract at dose of 90 mg/day. Group IV received HFD and *Malus sylvestris* extract at dose of 180 mg/day. Group V received HFD and *Malus sylvestris* extract at dose of 360 mg/day. Group VI received standard diet served as normal control rats. Standard or high-fat diet for the rats was given for 46 days. Simvastatin and *Malus sylvestris* extract (MSE) were administered orally by using oral gavage for 14 days (day 33-46). On day 47, all animals were euthanized using chloroform and sacrificed by cervical dislocation. The liver was removed for histopathological analysis.

Histopathological studies:

All livers were fixed by immersing it in 10% neutral buffer formalin for 24 hours. Histopathological preparations continued with dehydration, clearing, impregnation, and then making paraffin blocks, which were cut by microtome with thickness of 4-5 μm. The tissue slices were attached to glass object and then stained with hematoxylin-eosin (HE). Histopathological slides were observed per field of view under light microscope at 100x magnification to determine the observed area and 400x magnification to observe the cells more clearly. Observation of liver cells used semi quantitative scoring system to evaluate steatosis and portal inflammation features[14].

Statistical Analysis

Statistical analysis was performed using SPSS 20 for windows and the results were represented as mean. All results were analyzed statistically by Kruskal Wallis test, followed by Mann Whitney test to determine the significant difference between groups.

Result

The results were considered to be statistically significant when $p<0.05$. Mean rank value of steatosis and portal inflammation from statistical analysis can be seen in Table 1.

Table 1: Effect of MSE on histopathological features of hypercholesterolemic rats

Histopathology Features	Group I	Group II	Group III	Group IV	Group V	Group VI
Steatosis	33.71a	19.86b	22.64b	18.00b	17.07b	14.29b
Portal Inflammation	34.86a	20.14b	20.14b	18.33b	14.71b	17.43b

Values are represented in mean. Different superscript letters indicate significant difference ($p<0.05$).

Under the microscope, rats in normal control groups showed normal liver histology with minimal steatosis and portal inflammation features (Fig. 1F and 2F). Rats fed with HFD in positive control group showed severe fat vacuoles accumulation (steatosis) in liver cell cytoplasm (Fig. 1A) as well as inflammatory cell infiltration in portal area (Fig. 2A). However, Fig. 1B and 2B showed reduced fatty change and inflammatory cell in the portal area in simvastatin group, this suggest that the drug could alleviate steatosis and portal inflammation. All doses of MSE also showed similar result as simvastatin group by improving steatosis (Fig. 1C, 1D, 1E) and portal inflammation (Fig. 2C, 2D, 2E) features compared to positive control group.

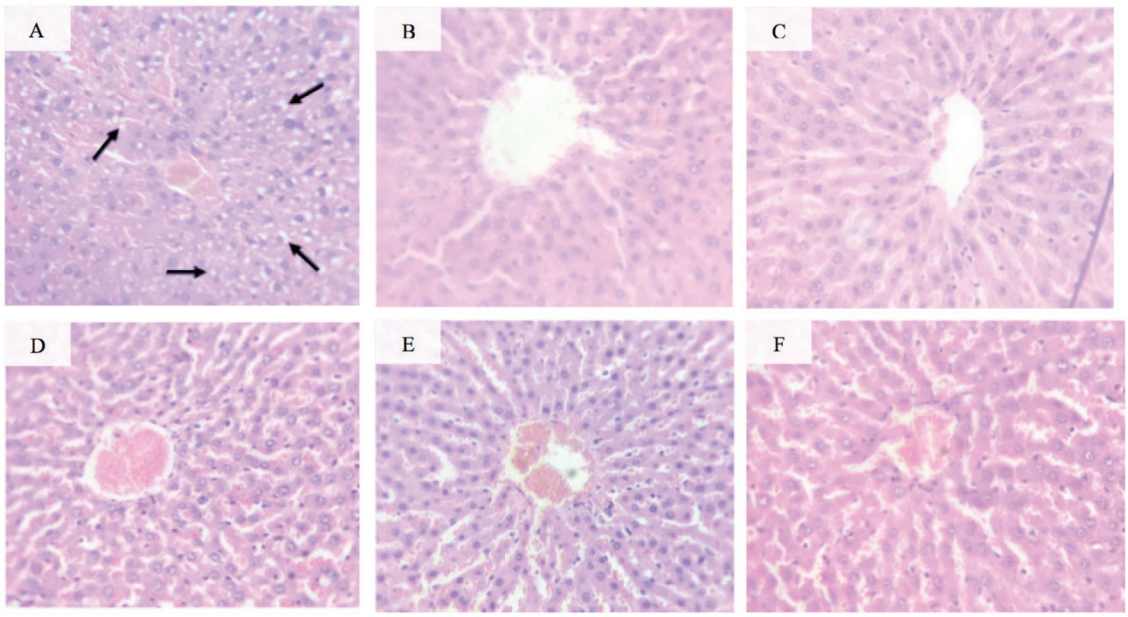

Figure 1. Effect of MSE on improving steatosis features in liver histopathology

(A) Liver tissue of HFD induced rats. Black arrows indicate fatty vacuoles in liver cell cytoplasm (steatosis). (B) Liver tissue of rats treated with simvastatin. (C) Liver tissue of rats treated with MSE at doses of 90 mg/day. (D) Liver tissue of rats treated with MSE at doses of 180 mg/day. (E) Liver tissue of rats treated with MSE at doses of 360 mg/day. (F) Liver tissue of normal control rats.

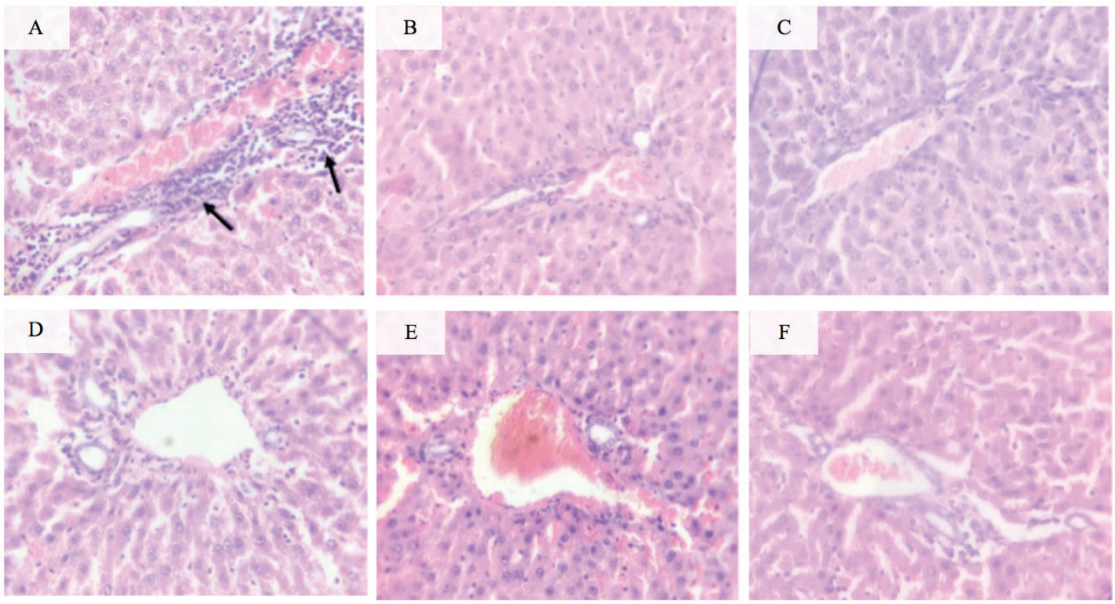

Figure 2. Effect of MSE on improving portal inflammation features in liver histopathology

(A) Liver tissue of HFD induced rats. Black arrows indicate inflammatory cells that fill the portal area. (B) Liver tissue of rats treated with simvastatin. (C) Liver tissue of rats treated with MSE at doses of 90 mg/day. (D) Liver tissue of rats treated with MSE at doses of 180 mg/day. (E) Liver tissue of rats treated with MSE at doses of 360 mg/day. (F) Liver tissue of normal control rats.

Discussion

Accumulation of lipid droplets in liver cells can be macrovesicular or microvesicular, which is rich in triacylglycerol (TAG). Liver does not store TAG in normal conditions, but exposure to stress due to excessive intake of fat or carbohydrates such as HFD consumption may cause fat accumulation in the liver[15]. This is associated with lipotoxicity because it increases mitochondrial stress. Mitochondrial dysfunction increased reactive oxygen species (ROS) production, which reduces antioxidants that act as a defense against oxidative stress in the liver[16]. Ongoing oxidative stress may lead to lipid peroxidation that causes lesions to liver cells leading to degeneration and necrosis[17]. The end products of lipid peroxidation, malondialdehyde, have chemoattractant properties that activate stellate cells as collagen producers and pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF-α) that activates c-Jun N-terminal kinases (JNK) pathway and nuclear factor kappa light chain enhancer of activated B cells (NF-κB), induce the release for more pro-inflammatory cytokines mediating liver inflammation[18]. This may lead to NASH with mixed lesion of necrosis, inflammatory infiltrates, and fibrosis, in addition to steatosis[19].

This study showed that MSE give hepatoprotective effect by improving steatosis and portal inflammation features compared to rats fed with HFD. This might be due to the presence of polyphenols and pectin in *Malus sylvestris*, which have metabolic regulatory, antioxidant, and anti-inflammatory properties. Polyphenols reduce TAG accumulation by various mechanisms, including inhibition of lipogenesis and promotion of fatty acid catabolism by down-regulating sterol regulatory element-binding protein 1c (SREBP-1c), which has a major role in lipogenesis[20]. Dietary fiber has also been shown to reduce the risk of NAFLD. Pectin is one of the dissolved dietary fibers found in apple in high concentrations, which increase the amount of intestinal microbiota that ferments pectin into short chain fatty acids (SCFAs) in large intestine[21]. SCFAs containing 2-5 carbons such as propionate inhibit carbohydrate response element binding protein (ChREBP), acetyl-coenzyme A carboxylase (ACC), and fatty acid synthase (FAS) which plays role in liver lipogenesis[22].

Polyphenols showed anti-inflammatory effect through several signaling pathways, such as by suppressing the activation of the NF-κB pathway, decreasing JNK phosphorylation protein, reducing levels of serum inflammatory cytokines, and increasing antioxidant defenses via the nuclear factor erythroid 2-related factor 2 (Nrf2) pathway[23,24]. In addition to polyphenols, other components in apples, such as pectin and its fermented products (SCFAs), can inhibit the secretion of TNF-α and NF-κB activation that suppress the progression of liver damage[25].

The ability of apple extract on improving histopathological features of fatty liver can also be seen from previous studies with similar results with this study[26,27,28]. In addition, apple extract also showed lower levels of serum total cholesterol, low-density lipoprotein cholesterol (LDL-c), and triglycerides (TG) compared to Western diet (high fat and sugar) group[28]. Further research is needed to investigate certain active ingredient isolates in *Malus sylvestris* extract, which have dominant role on ameliorating NAFLD histopathological features. It is also necessary to examine the effects of *Malus sylvestris* extract other than histopathological studies, as the diagnosis of NAFLD may be made by other tests[29].

Conclusion

Malus sylvestris extract improve the degree of steatosis and portal inflammation histopathological features of hypercholesterolemic rat fatty liver. This shows that *Malus sylvestris* provide hepatoprotective effect that could act as a potential treatment for NAFLD.

Conflict of Interest: The authors declare no conflict of interest.

Ethical Clearance: This study had been approved by Faculty of Medicine, Universitas Airlangga, Surabaya, Indonesia.

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