

Ethanollic Extracts of Mulberry (*Morus alba* Linn) Leaf Prevent Hyperlipidemia and Oxidative Stress-induced Steatohepatitis in Rats

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Abstract

Non Alcoholic steatohepatitis (NASH) is associated with the inflammation of fatty liver due to insulin resistance, dyslipidemia and oxidative stress. Treatment of NASH focused on control metabolic disorder and minimizes oxidative stress. White mulberry (*Morus alba* Linn) leaf has been reported for its anti-cholesterol and antioxidant effect. This study focused on effects of ethanollic extracts of white mulberry (EEM) leaf to total cholesterol, triglycerides and malondialdehyde (MDA) level of a high-fat diet induced steatohepatitis in rats.

The experimental animals, male Wistar strain rats, divided into 5 groups. The rats fed high-fat diet to induced NASH and treated by 90, 180 and 360 mg/bodyweight/day of EEM for 21 successive days. The remaining 2 groups served as control, they fed standard and high-fat diet only. At the end of treatment all subjects were sacrificed using decapitation method. Blood was collected and measured for total cholesterol and triglycerides (Randox). Liver tissue was collected for MDA measurement and histopathologic preparation using haematoxylin-eosin staining. MDA level was measured using thiobarbituric acid reactive-substances (TBARS) method for oxidative stress measurement.

EEM treatment significantly reduce cholesterol, triglycerides, and MDA levels as compared with high-fat diet control group ($p < 0.05$). This result in line with histopathological finding as indicated by relatively mild hepatocytes cellular damage.

It was conclude that EEM might prevent hyperlipidemia and oxidative stress-induced steatohepatitis.

Keywords: *Morus alba* L, steatohepatitis, dyslipidemia, oxidative stress

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is a clinical manifestation of metabolic syndrome. This abnormality comprehends from fat accumulation in hepatocytes, inflammation, necrosis/non-alcoholic steatohepatitis (NASH), and progresses to fibrosis and cirrhosis (Katsiki et al. 2016). NAFLD prevalence globally is 24% and present in 60-70% of diabetes mellitus patient (Xia et al. 2019). Syafitri et al. (2015) show that there are 60-80% of dyslipidemia among NAFLD patient in Padang, while Kasim et al. (2012) shows that 95% of NAFLD patient in Makasar also has obesity. NAFLD incident in children is 10% and increased to 40-70% in obese children (Deeb et al. 2018). The increase of obesity, diabetes and dyslipidemia prevalence lead NASH becomes one of the main etiologies of hepatocellular carcinoma (Kutlu et al., 2018).

NAFLD progress in stages of steatosis, lipotoxicity and inflammation. Insulin resistance is central in causing steatosis by increasing lipogenesis and interfering lipolysis inhibition in adipose tissue which resulting in an increase of fatty acid uptake in the liver. The overabundance of fatty acid burdened the mitochondrial oxidative machinery and caused an increase in reactive oxygen species (ROS) production resulting in an oxidative stress condition. Insulin resistance also induced an increase in inflammatory cytokines production and secretion. Combination of activated inflammatory cytokines and oxidative stress caused fatty liver condition progress to become

steatohepatitis, fibrosis and eventually cirrhosis. ROS caused lipid peroxidation, malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE), which cytotoxicity can cause cellular death (Arab et al. 2016, Buzzetti et al. 2016).

There is no specific treatment for NAFLD. Treatment was focused on metabolic disorder such as diabetes mellitus, hyperlipidemia and the use of antioxidant preparation (Cobbina et al. 2018). White Mulberry (*Morus alba* Linn) plant is popular in Asia since the Silkworm consumed its leaf. *Morus alba* Linn leaf was known for its antioxidant and anti-cholesterol effect (Rodrigues et al. 2019). This study aims to analyze white mulberry leaf ethanolic extract effect to total cholesterol, triglycerides and MDA level of a high-fat diet induced steatohepatitis in rats.

METHODS

This study used a complete randomized design in an experimental study. Mulberry leaf ethanolic extract was feed along with high-fat diet to laboratory rats, and measurement was taken to determined total cholesterol, triglycerides and MDA level. Mulberry leaf procured from Lembang, Bandung and extracted using 95% ethanol and diluted in Na-CMC 1% to form a homogenous solution. The high-fat diet was modified from Lieber et al. (2004) with energy composition maintained at 71%, 18% and 11% from, fat, protein and carbohydrate respectively. Energy composition for standard diet was 35%, 18% and 47% from fat, protein and carbohydrate, respectively was used as a control.

Research subjects are Wistar rats, 2-3-month-old, weighted 150-180 gram procured from Bandung Institute of Technology (ITB) animal breeding facility. The subjects were adapted to laboratory condition for one week before experiment with a 12 hours day and night light cycle and unregulated pellet and water feeding. The subjects were randomly assigned to five intervention group, with each group comprised of five rats. The intervention group were control group with standard diet/no treatment control/NTC (1), High fat diet control (HFDC) group as steatohepatitis group (2), HFD and 90 mg/body weight/day of mulberry leaf ethanolic extract group (3), HFD and 180 mg/body weight/day of mulberry leaf ethanolic extract group (4), and HFD and 360 mg/body weight/day of mulberry leaf ethanolic extract group (5).

Treatment was conducted for three weeks, after which the subjects were sacrificed using decapitation method. Blood was collected and measured for total cholesterol and triglycerides (Randox). Liver tissue was collected for MDA measurement and histopathologic preparation using haematoxylin-eosin staining. MDA level was measured using thiobarbituric acid reactive-substances (TBARS) method for oxidative stress measurement.

Resulting data were presented in mean and standard deviation. Analysis conducted using IBM SPSS statistic 20 software. Normally distributed data were analyzed using the one-way ANOVA test and continued with Duncan test if a significant difference were detected. Abnormally distributed data were tested using the Kruskal-Wallis test and continued with Mann-Whitney test if significance difference were detected. The study determined that significance level of $\alpha=0,05$.

RESULTS

High-fat diet treatment was able to increase total cholesterol, triglycerides and MDA level of HFDC group compared to other treatment groups. The total cholesterol level of HFDC group was significantly increased ($p=0,00$) compared to other groups while no significant difference in total cholesterol detected between NTC group and other treatment groups (fig.1). Kruskal-Wallis test shows a significant difference of triglyceride level between groups ($p=0,02$), further test using Mann-Whitney shows a significant increase of triglyceride level of HFDC group compared to other

treatment groups (fig.2). ANOVA test shows a significant difference of MDA level between all group, further analysis using Duncan test shows that MDA level of HFDC group and group receiving HFD and 90 mg/body weight/day of mulberry leaf ethanolic extract (Ma 90) is significantly increased compared to other groups (fig3).

Liver tissue histopathologic preparation from HFDC group reveal sustained cellular damage of hepatocytes and the presence of fibrotic tissue (100x magnification). Cellular damage such as microsteatosis, necrosis, pyknotic nucleus and lymphocytes infiltration (400x magnification). The findings were consistent with persistent chronic hepatitis. NTC group shows a normal liver lobule structure with an intact cellular membrane (100x magnification) compared to HFDC group, although there were mild microsteatosis and minimal pyknotic nucleus and lymphocytes infiltration (fig 4).

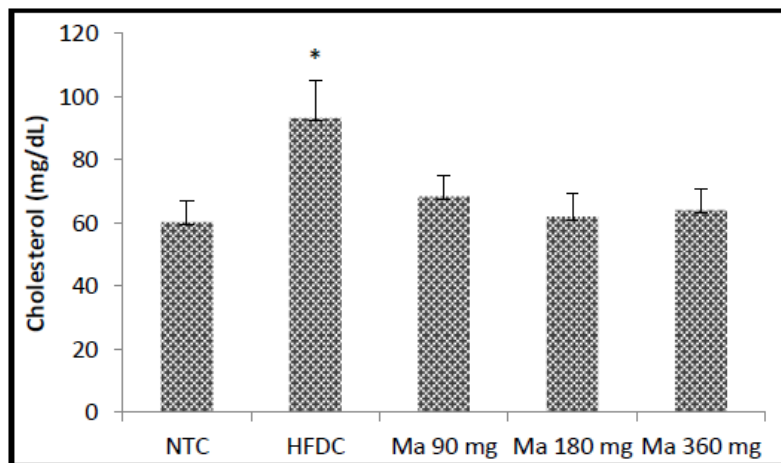


Figure 1. The effect of mulberry leaf ethanolic extract to total cholesterol. Mean \pm SD (n=5).

NTC=Non treatment control, HFDC= High fat diet control, Ma 90 mg, Ma 180 mg, Ma 360 mg is intervention group HFD + 90, 180, and 360 mg/body weight/day of mulberry leaf ethanolic extract respectively. (* $p < 0.05$, compared to other groups)

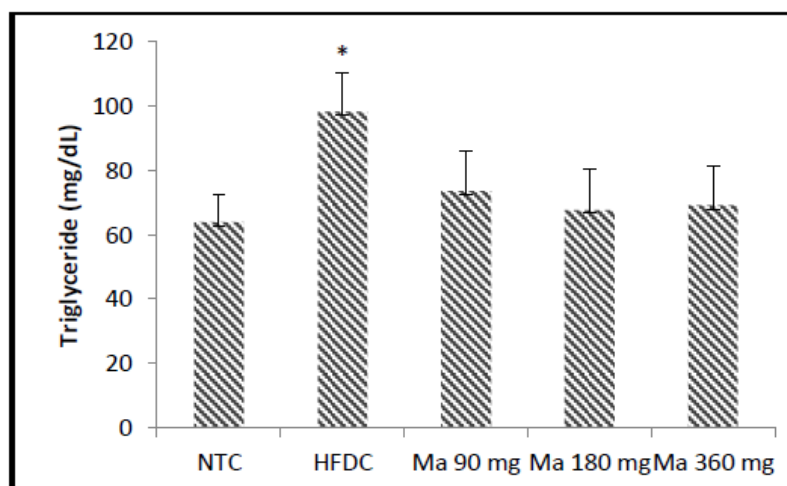


Figure 2. The effect of mulberry leaf ethanolic extract to triglyceride level. Mean \pm SD (n=5).

NTC=Non treatment control, HFDC= High fat diet control, Ma 90 mg, Ma 180 mg, Ma 360 mg is intervention group HFD + 90, 180, and 360 mg/body weight/day of mulberry leaf ethanolic extract respectively. (* $p < 0.05$, compared to other groups)

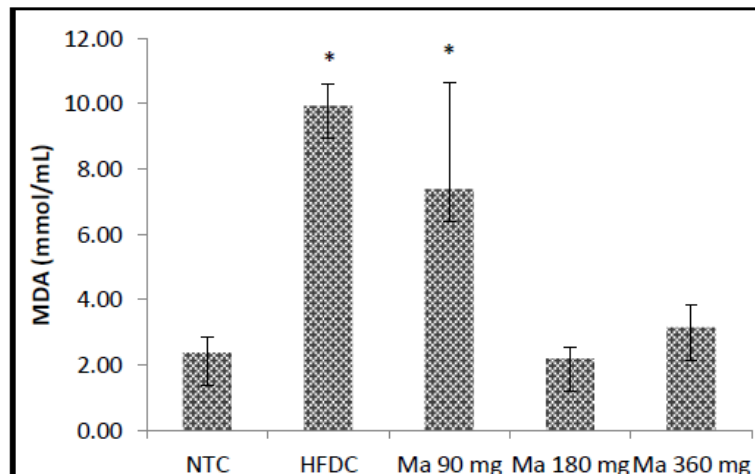


Figure 3. The effect of mulberry leaf ethanolic extract to MDA level. Mean \pm SD (n=5). NTC=Non treatment control, HFDC= High fat diet control, Ma 90 mg, Ma 180 mg, Ma 360 mg is intervention group HFD + 90, 180, and 360 mg/body weight/day of mulberry leaf ethanolic extract respectively. (* $p < 0.05$, compared to other groups)

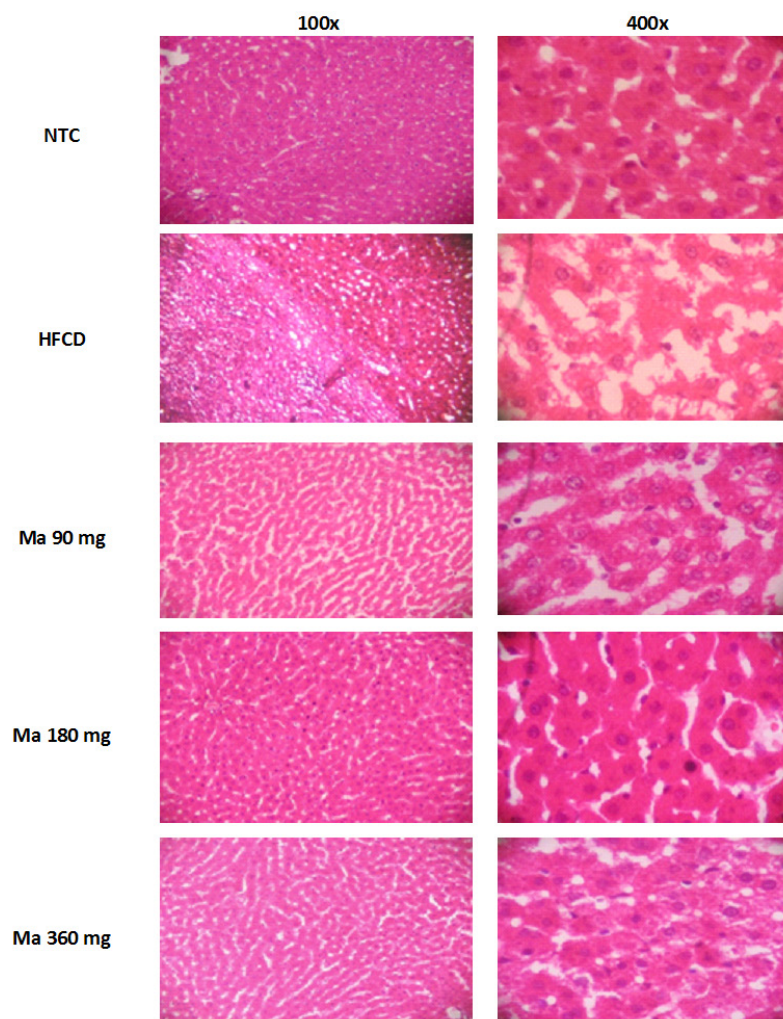


Figure 4. Liver histopathological preparation after three weeks of mulberry leaf ethanolic extract

treatment. NTC=Non treatment control, HFDC= High fat diet control, Ma 90 mg, Ma 180 mg, Ma 360 mg is intervention group HFD + 90, 180, and 360 mg/body weight/day of mulberry leaf ethanolic extract respectively.

DISCUSSION

High fat and high glucose food consumption as a lifestyle caused metabolic disorder such as obesity, hyperlipidemia, and diabetes mellitus (DM). This type of lifestyle was often found in a patient with fatty liver. Intracytoplasmic fat accumulation in hepatocytes, oxidative stress and inflammation progressively caused the fatty liver condition. Treatment for this condition was focused on treating metabolic disorder and on preventing further progressive disease development. Oral antidiabetic for a fatty liver patient with DM or lipid-lowering drug for those with hyperlipidemia can improve laboratory and histological results. Furthermore, an exogenic antioxidant can reduce oxidative stress and improve liver structure and function (Xia et al. 2019, Iqbal et al. 2018, Adiwinata et al. 2015).

Mulberry plant traditionally used to treat inflammation, increase milk lactation, prevent liver and kidney disease, improve joint condition, reduce fever, blood pressure, cholesterol and glucose level. Mulberry leaf tea was popular among east and southeast Asian to treat hypertension. Phytochemical analysis revealed that mulberry leaf extract comprises of the flavonoid rutin, quercetin, and isoquercetin, lipid such as linoleic, palmitic, and oleic acid, kaempferol, sugar-mimicking alkaloids such as 1,4-dideoxy-1,4-imino-D-arabinitol, 1-deoxynojirimycin, and 1,4-dideoxy-1,4-imino-D-ribitol, Mulberrosides A, B, F, phytosterol, vitamin C and minerals (Rodrigues et al. 2019, Chan et al, 2016).

This study evaluated anti-cholesterol and antioxidative effect of the mulberry ethanolic extract on HFD-induced fatty liver of Wistar male rat. Lipid profile measured in this study were total cholesterol and triglycerides level, MDA level and liver tissue preparation.

HFD-induced group shows an increase in total cholesterol and triglycerides level. A significant increase was observed in the HFDC group compared to other groups. Ma 90, Ma 180 and Ma 360 group show no significant difference with the NTC group. Mulberry leaf ethanolic extract prevents an increase of total cholesterol and triglycerides level caused by HFD. These findings are in accordance with Metwally et al. (2016) that shows *Morus alba* ethanolic extract reduce total cholesterol, LDL and triglycerides level of high cholesterol diet-induced obese rat. This effect was suggested caused by phytochemical that suppressed sterol regulation, thus inhibited lipogenic gene expression. These phytochemicals, among others, were mulberrosside A, albanols A and B, also 5,7,2V-trihydroxyflavanone-4V-O-h-glucoside.

Kobayashi et al. (2016) also show that methanolic extract of the mulberry leaf can significantly reduce total cholesterol, plasma and hepatocyte LDL in high cholesterol diet-induced mice compared to hyper cholesterol mice. Quercetin and kaempferol caused a decrease of hydroxymethylglutaryl-CoA reductase (HMG-CoA) gene expression involved in cholesterol biosynthesis. Conversely, lipid metabolic regulator transcription factor PPAR α and γ expression were increased by these phytochemicals.

Morus alba Linn water extract can significantly reduce triglyceride level in hyperlipidemic rat but not total cholesterol and LDL level. This finding shows a specific inhibition of fatty acid biosynthesis (Zeni & Molid, 2010).

This study shows the highest MDA level observed in the HFDC group. High-fat diet burdened mitochondria oxidative machinery and caused the release of reactive oxygen species (ROS). Increase

ROS level induce steatohepatitis condition through lipid peroxidation, cytokine inducement and Fas ligand. Lipid peroxidation produces aldehyde such as MDA and 4-hidrokinonal. This finding is in accordance with several studies that show fatty liver caused by reduced in endogenic antioxidative and an increase in oxidative stress parameter (Ayokanmi & Akinloye, 2019, Yu et al., 2016)

There was no significant difference in MDA level of Ma 180 group compared to the NTC and Ma 360 group. Mulberry leaf was rich in phytochemical with antioxidative activity. Antioxidative component of the extract reduced the effect of oxidative stress as indicated by the lower of MDA level in Ma 180 and 360 groups.

MDA level of Ma 90 group was significantly higher than other groups. This high MDA level was probably caused the inability of Ma 90 group to alleviated the effect of oxidative stress. Metwally et al. (2016) show a significantly reduced hepatic MDA level in obese mice using 250 mg/bodyweight of mulberry leaf extract. Furthermore, Ann et al. (2015) show water extract of the mulberry leaf at 133 and 166 mg/bodyweight reduced, a lipid peroxidation product, 4-HNE level compared to the control group. This result was suggested caused by a higher level of antioxidative heme-oxygenase-1 and glutathione peroxidase in the intervention group.

Steatosis in the current study, were disperse and can be found in all histopathological liver preparation. NTC group shows an intact membrane and nucleus, mild microsteatosis, and pyknotic nucleus. Conversely, HFDC group shows generalized cellular damage such as microsteatosis, macrosteatosis, necrosis and pyknotic nucleus. Fibrotic tissue and lymphocyte infiltration were also found in the HDFC group. These abnormalities resemble histopathological findings of persistent chronic hepatitis. This changes induced by high-fat diet in HFDC group. Lieber et al. (2004) show a similar finding, where high-fat diet caused steatosis with inflamed mononuclear cell over-infiltration.

This study mulberry leaf ethanolic extract at 180 and 360 mg/body weight/day protect hepatocytes from the effect of HFD as indicated by relatively mild liver cell damage. This protective effect also shows by the reduced in MDA, total cholesterol and triglycerides level in these groups suggest an antioxidative and anti-cholesterol activity of the extract. It is possible that the mulberry leaf extract also exerts anti-inflammatory activity. Park et al. (2012) demonstrated that mulberry leaf extract inhibits the release of proinflammatory cytokines from LPS-induced macrophage. Peng et al. (2017) also show that mulberry leaf water extract reduced body weight, hepatic lipid level, increase superoxide dismutase expression, reduce TNF- α level and in general prevent obesity that may induce NAFLD.

CONCLUSION

Mulberry ethanolic extract repair hepatocytes cellular damage caused by high-fat diet through the effects of anti-cholesterol and anti-oxidative stress. This extract could be used as prevention for hyperlipidemia dan oxidative stress-induced steatohepatitis.

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