Combination of *Moringa oleifera* Extract and Fish Albumin can Reduce Inflammatory Cytokine TNFα and IFNγ and Lipid Retention in Steatohepatitis Mice Model

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**Abstract.** Steatohepatitis is a condition where there is accumulation of lipids in the liver. This condition can be caused by alcohol consumption and chemical substances of industries. *Moringa oleifera* is a miracle plant that is capable of hepatoprotective and albumin encourages the process of homeostasis in the body. The aim of this study is to determine the effect of *M. oleifera* and albumin on pro-inflammatory cytokine TNFα and IFNγ and lipid retention on steatohepatitis mice model. The research was conducted using 5 different groups. Normal control was completely untreated. Steatohepatitis control was induced with CCL₄ and alcohol without *M. oleifera* and albumin administration. Three different doses of *M. oleifera* is used in this study with dose 1000mg/kg BW and albumin was 5300 mg/kg BW. The first dose is combination of *M. oleifera* 75% and albumin 25%, next dose is 50% *M. oleifera* and 50% albumin, and the last dose is 25% *M. oleifera* and 75% albumin. After 2 weeks treatment with *M. oleifera* and albumin (except normal and steatohepatitis group), mice were sacrificed and the liver and spleen were isolated. The liver tissues were fixed and embedded in parafin to analyse the histopathology by hematoxylin and eosin staining. The spleen used to isolate CD4 T cells and analyse pro-inflammatory cytokine TNFα and IFNγ by flow cytometry analysis. The results showed the reduction of pro-inflammatory cytokine TNFα and IFNγ. Its reduction was linked to the reduction of lipid retention and it shows a promising effect of combination *M. oleifera* and albumin to treat steatohepatitis.

**Keywords**: Albumin, *M. oleifera*, pro-inflammatory cytokine, steatohepatitis.

1. **Introduction**

Cirrhosis is a pathological condition shows the end stadium of hepatic fibrosis which occurs progressively. This condition marked by liver structure distortion caused by fibrosis which comes from the process of nodule regeneration. Cirrhosis cause complication and able to reduce patient life quality [1]. There are 2.8 million people in the world who suffer from cirrhosis and 1.3 million people died. The high prevalence of this case caused by many factors, such as hepatitis B and C, also alcohol consumption. Asia and Africa are the biggest contributors to cirrhosis in the world [2].

Large alcohol consumption can increase steatosis macrovesicular. Steatosis can be seen as lipid droplet which develops from central vein area to the area of midlobular and vena porta hepatic. The higher activities of lipid peroxidation and stress oxidative the higher chances lead to cirrhosis. Alcohol actively increases lipogenesis activity, reduce adipokine production, increase fatty acids
efflux to adipose tissue, disrupt VLDL secretion, and inhibit lipophagy process and fatty acid oxidation [3].

*Moringa oleifera* has a great bioactive compound and also called by the miracle of the tree because of its benefit on treatment many diseases such as insulin resistance, cardiovascular, hepatic steatosis, and cancer. Reports said this plant has hepatoprotective activity and decrease the plasma lipids of rats fed a high-fat diet, regulate glucose level on diabetic rats, and has a role on cardioprotective by affecting the activities of several enzymes associated in oxidation [4].

*C. micropeltes* also known as Ikan Toman commonly found and well distributed in Kalimantan Indonesia. *C. micropeltes* is albumin-rich compare with other species. Albumin is one of critical marker to identifying steatosis and cirrhosis. Reports said that serum albumin is significantly lower in a patient with intensive hepatic fibrosis compare to those in early stages [5]. The more the liver damage and lost its function, the more albumin will decrease.

Recent years, scientist found several biomarkers for the diagnosis and prediction of stages liver disease such as TNFa and IFNg. TNFa and IFNg are two cytokines originate largely from T-helper Cells and associated with the adaptive immune system. From that, this study aims to know the effect of combination *Moringa oleifera* extract and albumin on TNFa and IFNg on steatohepatitis mice model.

2. Method
2.1 Experimental Design

The study was conducted at the Laboratory of Anatomy, Physiology and Animal Development, Department of Biology, Faculty of Mathematics and Natural Sciences and The Laboratory of Pathology and Anatomy, Faculty of Medicine, Brawijaya University, Indonesia. The study used a completely randomized design which contained 5 the treatment group with each group contained 5 mice. The mice were divided into 5 groups. Group 1 is the normal group (N, n=5) without induction of steatosis and *M. oleifera* and albumin treatment). Group 2 is Steatosis control (S, n=5) without *M. oleifera* and albumin treatment). Group 3 is group of mice induced Steatosis and treatment of *M. oleifera* with different dose, such as dose 1 (S-D1, n=5) 75% *M. oleifera* and 25% albumin, dose 2 (S-D2) 50% *M. oleifera* and 50% albumin, and dose 3 (S-D3) 25% *M. oleifera* and 75% albumin. The absolute dose of *M. oleifera* extract was 1000 mg/kg and albumin 5300 mg/kg.

2.2 Animal Model

The animal model used in this study was female 5-6 week old balb/c mice were obtained from Singosari, Malang, East Java, Indonesia. Mice were fed with standard feed (pellets) and water. Animals were kept in the animal center of the laboratory Anatomy and Physiology, Brawijaya University with 12 hours of light/dark cycle, at a constant temperature (20 ± 1°C) and humidity (50 ± 5%). After 1 week of acclimatization, mice in all groups except the normal group (N) were induced with CCl₄ and alcohol 3 times each week for 3 weeks. The dose of CCl₄ was 0.5 ml/kg BW dissolved in corn oil (1:19) and injected via intraperitoneal [6]. While 50% of alcohol induction was administrated orally at a dose of 12 ml/kg BW [7]. After that, the treatment group mice were treated with a combination of *M. oleifera* and albumin orally for 2 weeks. After the last treatment, mice were sacrificed. Then spleen was taken for lymphocytes isolation before flow cytometry analysis, besides that liver samples were fixed in 4% phosphate-buffered formalin for histopathological observation. All animal conditions and handling were approved by Ethical Clearance KEP-1125-UB year 2019.

2.3 Preparation of *M. oleifera* and albumin extract

*Moringa oleifera* leaves powder was obtained from Materia Medika Batu which was then extracted using subcritical water extraction (SWE) method. The SWE method was carried out because the solubility of the *M. oleifera* leaf compounds was generally low, so it is necessary to
extract with subcritical water method (water with a temperature of 100°C-300°C) or extraction at high temperatures [8]. *M. oleifera* powder was soaked for 15 minutes in subcritical water with a ratio of 1:10 and stirred. After that filtered using filter paper and filtrate was stored in a deep freezer of -20°C then freeze dry.

Albumin was obtained with the trademark "Ivalmin from PT Ismut Medika" that contain a pure extract of skin and scales of *Channa micropellets* which was processed by Freeze Dryer at a temperature of -40°C, -76cmHg and made with water solvent without other additives. The stock of Albumin was prepared by dissolving 13.2 mg ivalmin in 50 ml aquadest. The stock was stored in the refrigerator and warmed before use.

2.4 Antibody Staining and Flow cytometry Analysis

The spleen was taken from sacrificed mice and homogenized with Phosphate Buffer Saline (PBS, Gibco). Homogenate was then centrifuged (HERMLE Z 326 K) 2500 rpm for 5 minutes at 10°C. Pellets were resuspended in 1 mL PBS and 50 µl cell suspension was then stained with antibody CD4 (fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD4 (BioLegend)) for 20 minutes. Cells were added by cytotic (BioLegend) and wash perm (BioLegend) and then centrifuged. Pellet was then stained with PE anti-mouse/rat TNFα (clone: TN3-19.12, BioLegend) and PE/Cy7 anti-mouse IFNγ clone: XMG1.2, BioLegend) 50 µl and incubated for 20 minutes. Cells were added with 400 µl PBS and moved to flow cytometry cuvet tube. Data are recorded in a computer and analyzed with BD CellQuest TM Pro.

2.5 Histopathology Analysis

Liver organs fixed in 4% formalin buffer then embedded in paraffin. Cut and sliced at 4µm thickness. Slices for histopathological were stained with hematoxylin and eosin (HE) [9]. Observation of cells from HE preparations was carried out using an Olympus BX 53 microscope with Standard Version 1.5 CellSens software.

2.6 Statistical Analysis

Statistical analysis was done using SPSS 16.0 Base system for windows (SPSS, Inc., Chicago). The values were presented as mean ± SD. The statistical analysis was performed by using one-way ANOVA test. In this study, $P < 0.05$ was considered statistically significant.

3. Result and Discussion

3.1 Reduction of lipid retention

Histopathological observation in this study shows that the normal liver tissue (Fig. 1a) were free of inflammation and lipid droplets (steatosis), conversely in steatosis control (Fig. 1b). Induction of alcohol and CCL$_4$ can increase the accumulation of lipid in the liver and it shows as the first stage of response in the liver to binge either the alcohol and CCL$_4$. The influx of fatty acids to liver increases due to alcohol exposure. It is also caused de novo lipogenesis as one of the major reason of hepatic steatosis development. Alcohol exposure stimulate sterol regulatory element-binding protein-1c (SREBP-1c) as an essential transcription factor for de novo lipogenesis and its target lipogenesis gene lead to hepatic steatosis [10]. Lipid superoxidation and stress oxidative caused by the accumulation of lipid products such as trygliceride in hepatocytes, resulting apoptosis and inflammation [11]. After treatment with combination of *M. oleifera* and albumin, it shows reduction of lipid droplets relatively. The reduction of lipid droplets are different in each dose. Dose 2 (50% *M. oleifera* and 50% albumin) (Fig. 1d) shows the most effective lipid reduction compare with two other doses (75% *M. oleifera* and 25% albumin (S-D1) & 25% *M. oleifera* and 75% albumin (S-D3)) (Fig. 1c and Fig. 1e). Combination of *M. oleifera* and albumin (50%;50%) shows histology condition close to normal (closely free of lipid droplets). This finding supported by Almatrafi et al (2017) [4], *M. oleifera* inhibit SREBP-1c gene that responsible in lipogenesis. Another research also found, *M. oleifera* compound, chlorogenic acid (CGA), inhibited
the activities of fatty acid synthase, 3-hydroxy-3-methylglutaryl CoA reductase, and acyl-CoA cholesterol acyltransferase significantly [12].

Combination of *M. oleifera* and albumin shows a promising effect on steatosis reduction. It is caused by the benefit of *M. oleifera* that have been mentioned and supported by albumin function. Since level albumin is lower in liver damage, albumin treatment seems very useful. Albumin is one of the most transporter of molecules in body, including flavonoid transporter. Flavonoid enters to the blood and binds with serum albumin before being transported to the organs and tissues. This interaction modulate the bio-availability and influence the bio-activity of flavonoid as antioxidant agent [13].

3.2 Reduction The Number of CD4+IFNγ+ and CD4+TNFα+ cells

Several mechanisms such as stress oxidative, inflammation, and apoptosis are associated with liver damage induced by alcohol and CCL4. Inflammatory cytokine including TNFα and IFNγ are involved in liver injury. Alcohol consumption causes inflammation through an augmented permeability of the intestinal membrane and increases the portal concentration of endotoxin (LPS) in the blood. Kupffer cells was then activated and produced inflammatory cytokine. Moreover, apoptosis and phagocytosis of Kupffer cells leads to hepatocyte injury by TNFα [14]. In addition, CCL4 induce upregulated of *Bax* as specific genes that responsible in cell apoptosis, CCL4 also has role in TLR-4 signaling pathway. TLR-4 recruits MyD88 to initiate down-streaming signaling events toward NfkB phosphorylation, which then induced the release of pro-inflammatory cytokine [15].

This finding supported previous studies that induction of alcohol and CCL4 significantly increases proinflammatory cytokine TNFα and IFNγ (Fig. 2) compare to normal (p<0.05). Interestingly, combination of *M. oleifera* extract and albumin treatment for 2 weeks could reduce significantly the both cytokines, except in 75% *M. oleifera* and 25% albumin (S-D1) (Fig. 2) (p>0.05). Dose 1 (75% *M. oleifera* and 25% albumin (S-D1)) could not reduce the number of CD4+IFNγ+ and CD4+TNFα+ cells since it was assumed that 25% of 5300mg/kg albumin can not transport active compound in 75% of 1000mg/kg *M.oleifera* optimally.

Normally, TNFα is important and required in the proliferation of hepatocyte during liver generation, as it has a multifunctional role in regulating various processes including inflammation, proliferation and apoptosis. TNFα can induce NfkB which has protective effect in normal condition. During liver injury, Intracellular events such as apoptosis and hepatic cells death were accelerated and triggered by TNF. So elevated level of TNFα correlated with a detrimental prognosis [16]. Apoptosis trigerring also told to be caused by the interaction of TNFα and IFNγ. The interation between these two cytokines can induce iNOS gene expression that can lead to the production of oxidizing species and promote the apoptosis of hepatocyte. Expression of iNOS gene can be induced by the activation JAK/STAT pathway by IFNγ potentiated TNFα-induced NfkB binding to DNA and lead to the activation of IRF-1 [17]. Since these two cytokine able to aggravate liver
injury, this study seems has promising effect on liver disease treatment by reducing proinflammatory cytokine TNFα and IFNγ.

Figure 2. Effect of *M. oleifera* reduced CD4+TNFα+ and CD4+IFNγ+ cells on flow cytometry analysis. Data are percentage of cytokine production and mean ± SD (n=5). N. Normal group (untreated group), S. Steatosis group induced by alcohol and CCL4, S-D1. Steatosis induced by alcohol and CCL4 and treated by *M. oleifera* and albumin Dose 1 (75%:25%), S-D2. Steatosis induced by alcohol and CCL4 and treated by *M. oleifera* and albumin Dose 2 (50%:50%), S-D3. Steatosis induced by alcohol and CCL4 and treated by *M. oleifera* and albumin Dose 3 (25%:75%).

4. Conclusion
Alcohol and CCL4 induce steatosis through lipogenesis and lipid accumulation. Accumulation of lipid droplets relatively could be inhibited by supplementation combination of *M. oleifera* and albumin treatment for two weeks. The most effective lipid reduction shows combination ratio of *M. oleifera* and albumin 1:1. Dose 2 with 50% *M. oleifera* and 50% albumin (S-D2) and Dose 3 with 25% *M. oleifera* and 75% albumin (S-D3) also show a promising effect on the reduction of cytokine proinflammatory TNFα and IFNγ significantly. These two cytokine must be suppressed during liver injury because the elevated production of its cytokine correlated with detrimental prognosis.

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