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RESEARCH ARTICLE

Liver Regenerative and Hepatoprotective Effects OF *Moringa Oleifera* Extract in the Liver Fibrosis Animal Model

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Abstract

Objective: This study was conducted to know the liver regenerative and hepatoprotective effects of *Moringa oleifera* (MO) in the animal models of liver fibrosis. Methods: The study was a randomized post-test only controlled group design. Male *Rattus novergicus* of liver fibrosis caused by induction of CCl4 was treated with extract of (MO). Histological of the liver tissue with hematoxylin and eosin (H&E) staining were investigated by a light microscope. We calculate the number of hepatocytes expressing MDA and cytokeratin-7 (CK-7) in liver tissue using the immunohistochemical analysis. Results: Induction of CCL4 for 14 weeks results in liver fibrosis (METAVIR score F-3) and improves by the administration of MO. The number of hepatocytes expressing MDA in liver fibrosis models sevenfold higher than normal models and significantly lower after treated with MO. ALT and AST in the treated models significantly lower than in the liver fibrosis models. On the other hand, the number of hepatocytes expressing CK-7 in both normal and liver fibrosis models was lower. The CK-7 significantly higher after the administration of MO. Many ductular reactions develop in the treated animal models. Conclusions: in our knowledge, this is the first study reported a liver regenerative effect of MO in the liver fibrosis, beyond its hepatoprotective effect. The mechanism of liver regenerative probably through a hepatic progenitor cell proliferation.

Keywords: *Moringa oleifera*, liver fibrosis, liver regenerative, CK7, MDA.

Introduction

Liver fibrosis is the result of a processes series due to chronic inflammation in the liver tissue. It's a resultant of the process of fibrogenesis and regeneration of liver tissue. The balance between the fibrogenesis and regeneration is maintained under normal conditions and as long as there is a source of injury. When an injury occurs continuously, the balance shifts to fibrosis progression, and if the source of injury is absent, the balance shifts towards fibrosis regression [1]. So far, it is known that the cell that plays the most role in the progression and regression of liver fibrosis is HSC.

Quiescent-HSC (q-HSC) was activated into activated-HSC (a-HSC) which is a fibrogenic phenotypically cell producing collagen [2]. Hepatocytes are the most valuable parenchymal cells in liver tissue. Besides metabolism main function, hepatocytes have an important role in the progression and regression of liver fibrosis. Hepatocytes have a high ability to regenerate, so when became the "first victims" of acute injury, these cells are able to repair themselves. Whereas in extensive and chronic injury, as in the liver fibrosis condition, regeneration of

hepatocytes involves hepatic progenitor cells (HPC)/liver progenitor cells (LPCs)/ oval cells activation [3, 4]. Oval cell proliferation was known as the Ductular Reaction (DR), which is characterized by increased expression of CK-7 or CK-19. The oval cells that excrete CK-7 are the forerunners of new cholangiocyte cells. In the event of massive hepatocyte damage, these cells can undergo trans-differentiation into new hepatocytes [5].

Acute or chronic injury to hepatocytes results in hepatocytes apoptosis, then followed by activation of non-parenchymal cells in the liver tissue (HSC and Kupffer cells) which aims to cleanse apoptotic bodies. Activation of non-parenchymal cells also induces more inflammation. Hepatocyte apoptosis produces abundant reactive oxygen species (ROS) that disrupt hemostasis and lead to progressive fibrogenesis [6].

ROS can induce Kupffer cells and inflammatory cells in the circulation to produce profibrotic cytokines. ROS can also directly encourage the activation of HSC into fibrogenic cells [7]. Uncontrolled ROS induce lipid damage through the lipid peroxidation process. One of the products of lipid damage through the lipid peroxidation process is Malondialdehyde (MDA), which is often used in research to determine the level of lipid peroxidation from cellular damage [8].

ROS has an important role in the pathogenesis of liver fibrosis, therefore several studies have examined the effects of antioxidants in liver disease, one of them was *Moringa oleifera* (MO) [9]. MO has flavonoid compounds that are believed to provide hepatoprotective effects through antioxidant and anti-inflammatory mechanisms [10, 11].

MO leaf extract also has the effect of increasing the hepatocyte regeneration and repairing damaged hepatocyte via its antioxidant properties [12, 13]. How the mechanism of hepatocyte regeneration effect of MO is still not well known. This study was conducted to know the hepatoprotective and regenerative effects of MO in rat liver fibrosis models.

Material and Methods

Chemicals

This study used CCl_4 (#820354.0010, MERCK, Schuchardt, Germany), corn oil, NaCl 0.9%, ketamine, formalin 10%, Cytokeratin-7 mouse monoclonal antibody (Santa Cruz Biotechnology, Inc, sc-53263), Rabbit polyclonal Malondialdehyde antibody (Abcam, ab6463) reagen ABX Pentra ALT CP and reagen ABX Pentra AST CP.

Plant Material, Extraction and Analysis of Flavonoid Compounds

Moringa Oleifera (MO) leaves were collected from Batu, East Java, Indonesia in February 2018. Leaves were identified and authenticated at "Unit Pelaksana Teknis" (UPT) Balai Materia Medica, Batu, East Java, Indonesia. MO leaves were air dried and grounded into fine powder. They was extracted with 96% ethanol by maceration. The MO extract was dissolved in aquades for treatment. Liquid Chromatography-Mass Spectrometry (LC-MS) analysis was used to determine the compounds of MO extract.

Animals

Adult male *Rattus novergicus* Wistar strain rat 200g–300g were purchased and certified from the Rattus Breeding Center Singosari, Malang, Indonesia. All rats were housed at Pharmacology Laboratory of Universitas Brawijaya and acclimatized in the laboratory for 1 week prior to experiment. The housing condition were controlled, with a room temperature and a diurnal 12-hour light/ dark cycle. All experimental protocols described in this study were approved by the Health Research Ethics Committee, Faculty of Medicine Universitas Brawijaya (number: 153A/EC/KEPK/06/2018).

Design of Study

This experimental using randomized post-test only controlled group design was conducted at Universitas Brawijaya, Malang, Indonesia. Rats were randomly divided into 5 groups: negative control (NC), positive control (PC), treatment-1 (T-1), treatment-2 (T-2), and treatment-3 (T-3), include 6 rats per group.

Induction of Liver Fibrosis

The method was modified from Li, et al [14]. Liver fibrosis was induced by intraperitoneal injection 10% (in corn oil, 1:9) of CCl_4 1 cc/kg BW twice a week for 12 weeks and continued with 2 cc/kg BW for the last 2 weeks. Injection of CCl_4 was given to all rats except in NC group.

MO Ethanol Extract Administration and Sample Collection

T-1, T-2 and T-3 groups received MO ethanol extract by oral gavage. The dose of MO ethanol extract was 150 mg/kg BW in the treatment-1 group, 300 mg/kg BW in the treatment-2 group, and 600 mg/kg BW in the treatment-3 group. After 14 weeks, the rats were euthanized 48 hours after the last CCl_4 injection by injecting 50 mg/kg BW of ketamine intramuscularly and then the liver and serum were collected.

Histopathological Evaluation

The liver was processed using formalin, then rehydrated in ethanol and embedded in paraffin block. The embedded liver in paraffin block was cut using microtome and mounted on a glass slide. The slide was dewaxed and rehydrated, then washed using distilled water. The slide next was stained using hematoxylin and eosin (H&E).

Microscopic figure was scanned using a light microscope **Olympus BX51**. All sections were investigated by a light microscope. The degree of liver fibrosis was assessed using METAVIR score, where F0: normal tissue, F1: fibrosis is limited to the porta, perisinusoidal, and intralobular areas, F2: fibrosis in several portal areas, fibrous septum formed between the portals, intralobular architecture damage occurs, F3: fibrous portal-central septum occurs, accompanied by damage to intralobular structure, cirrhosis has not been seen, F4: cirrhosis [15].

Immunohistochemical Analysis

MDA and cytokeratin-7 expression in liver tissue was analyzed by immunohistochemical analysis. Slides of liver tissue are depolished and rehydrated in alcohol. Incubation was carried out using Rabbit polyclonal Malondialdehyde antibody (Abcam) and cytokeratin-7 mouse monoclonal antibody (Santa Cruz) at 5 ^{0}C overnight as primary antibody and continued with peroxidase labelling using the UltraTek HRP Anti-Polyvalent (DAB) Staining System (ScyTek Laboratories Inc.) kit. The slides are then counted using Hematoxylin. The slide was then scanned using the Olympus BX51 light microscope. MDA and cytokeratin-7 expression were calculated using the manual counting method at 400x magnification in 20 visual fields using image raster 3.0 software.

AST/ALT Analysis

Optimized UV-test is used in AST / ALT tests based on IFCC (International Federation of Clinical Chemistry) modification methods. Blood from rats that had just been taken from the heart organ was left for 10-20 minutes at room temperature, then centrifuged at a rate of 2000-3000 rpm for 20 minutes then serum was taken. Serum was used for the AST / ALT test using ABX Pentra ALT CP reagents and ABX Pentra AST CP reagents with HORIBA ABX Pentra C200 devices.

Statistical Analysis

IBM SPSS Statistics 22 software was used to carry out a one-way analysis of variance (ANOVA) on obtained data. The analysis was followed by Tukey's post hoc for parametric data or Kruskal Wallis test continued by Mann Whitney for non-parametric data. T-test was used in ALT/AST analysis. Correlation test uses Pearson for parametric data and Spearman for non-parametric data. Significance level used $p<0.05$.

Results

In this study, Liquid Chromatography-Mass Spectrometry analysis was used to find out the flavonoid content in *Moringa oleifera* leaf ethanol extract (Table 1).

Table 1: Flavonoid content of *Moringa oleifera* leaf ethanol extract

S. No	Component
1.	Quercetin-3-Glycoside
2.	Quercetin
3.	Quercitrin
4.	Kaempferol-3-glycoside
5.	Kaempferol glucoronide
6.	Rutin
7.	Naringenin

Histology of Liver Tissue

The histological examination of liver tissue can we see in the **Fig. 1**: a) The NC liver tissue (normal mice) histology: normal hepatic architecture with portal triad and surrounding hepatocyte, sinusoid and no visible fibrosis tissue; b) the PC liver tissue (liver fibrosis models) histology: showing damaged hepatic architecture. Many hepatocytes have cytoplasmic vacuolation, swelling with granular in cytoplasm. There was fibrosis tissue in the portal triad and radiated to many septa (METAVIR-score F-3); c) the T-1 liver tissue histology: The hepatic architecture was almost the same as the PC, METAVIR F-3 with a little hepatocytes containing cytoplasmic vacuolation; d) the T-2 liver tissue histology: There was fibrosis tissue in the triad portal (METAVIR F-2). Showing infiltration of inflammatory cell in fibrotic area. Hepatocytes with cytoplasmic vacuolation still persist but the hepatic architecture was better than T-1.; e) the T-3 liver tissue histology: the hepatic architecture was normal. There was no fibrotic area in the portal triad (F-0).

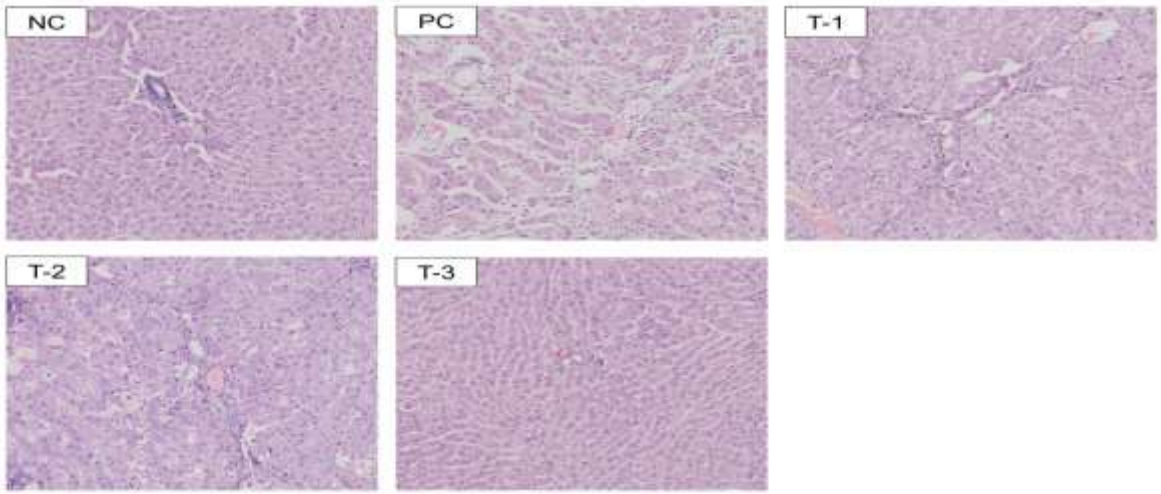

Fig. 1: Histology of liver tissue by Hematoxylin Eosin staining in rats of (a) NC, (b) PC, (c) T-1, (d) T-2, and (e) T-3 groups.

NC, negative control; PC, positive control; T-1, Treatment-1; T-2, Treatment-2; T-3, Treatment-3

MDA Expression in Liver Tissue

Fig. 2 shows the various liver tissue cells that express MDA in each group. MDA expression was identified from the brownish colour in the cytoplasm. The brown colour is formed from the bond between the antigen and the antibodies in immunohistochemical staining. MDA expression was expressed in terms of the number of hepatocytes expressing MDA.

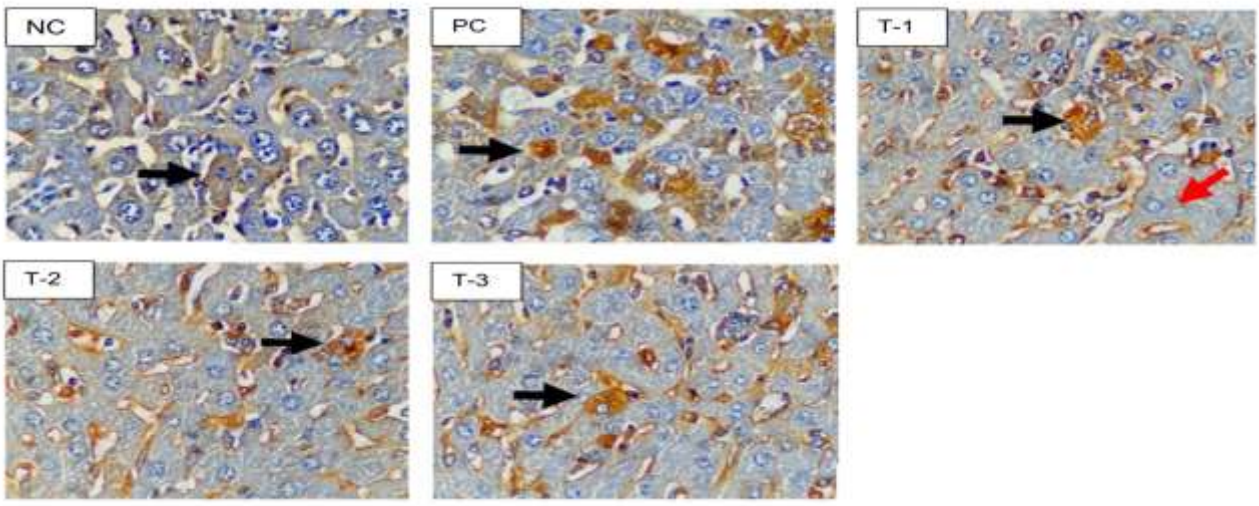

Fig. 2: The hepatocyte expressing MDA in the several groups (400 x magnification)

Black arrows indicate hepatocytes that express MDA. Red arrows indicate normal hepatocytes. MDA, malondialdehyde

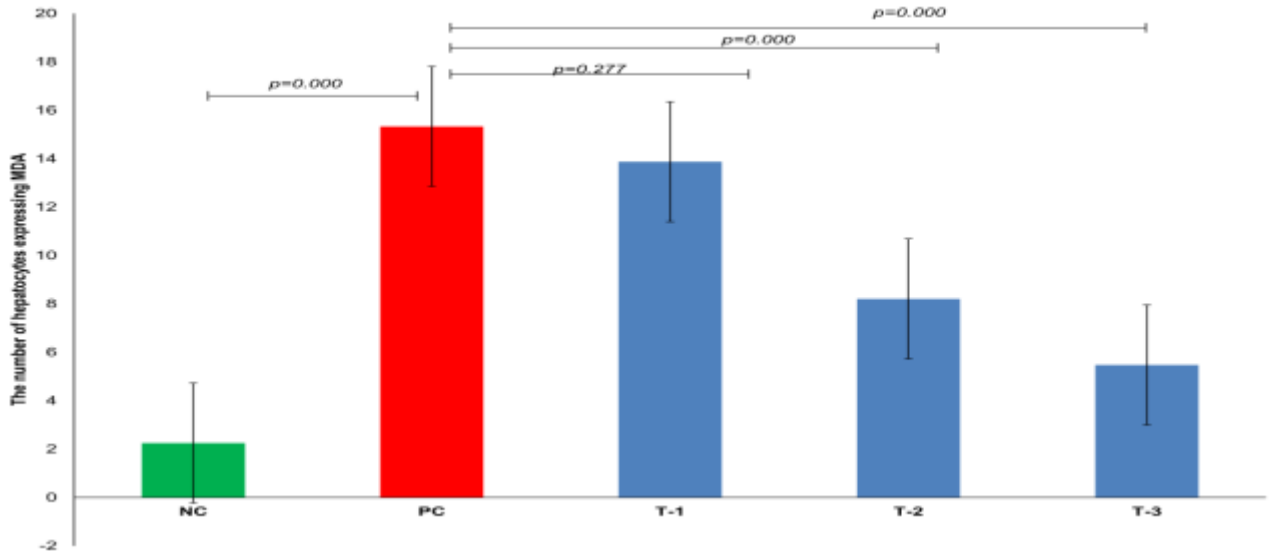

Fig. 3: The mean number of hepatocytes expressing MDA in several groups (20 visual fields). The NC group (normal rat) had a mean number of hepatocytes expressing MDA of 2.25 cells. The mean number of hepatocyte cells in the PC group (rat model of liver fibrosis) was 15.33 cells (it's means increase sevenfold). In the T-1 group, there was a mean of hepatocytes that expressed MDA of 13,875 cells (tend to be lower, p = 0.277). In the T-2 group, there was a significant lower in the number of hepatocytes expressing MDA (8,208 cells; p = 0,000). Whereas the T-3 group had a mean number of hepatocytes expressing MDA of 5.479 cells (the both of treated groups significantly different to the PC group; p = 0,000). MDA, malondialdehyde; NC, negative control; PC, positive control, T-, treatment

Discussion

Liver fibrosis is a wound-healing response to liver injury and hepatic stellate cell (HSC) is the main cellular effector that major producer of the extracellular matrix. The use of animal models for experimental liver fibrosis research has been extensive and crucial for determining mechanisms underlying progression and resolution of liver fibrosis [16, 17].

In this study, induction of CCL4 for 14 weeks results in liver fibrosis which is characterized by accumulation of ECM and damage to the architecture of the liver tissue. Hepatocytes undergo edema accompanied by cytoplasmic vacuolation. There are fibrosis tissues at around the portal triad and septa.

This study was proven that the administration of MO extract can inhibit the progression of fibrogenesis, characterized by improved hepatic architecture, as shown in Fig. 1. This improvement was clearly seen in the T-3 group. Histology of liver tissue in the T-3 group is almost similar to liver tissue in the normal condition (NC). The most of hepatocytes are normal in condition with the sinusoidal neatly followed by rows of hepatocytes.

MDA was known as a biomarker of oxidative stress. Oxidative stress effects are mainly divided into three categories, peroxidation of lipids, protein damage, and DNA damage. MDA was known to be increased specifically in increased peroxidation of lipids. In this study, the expression of MDA was significantly increased (sevenfold) in fibrotic rat (PC) liver compared to healthy rat (NC) liver (Fig. 2).

On histological examination, there are many damaged hepatocytes, characterized by cytoplasmic vacuolation (**Fig. 1,** PC). This showed an increased level of lipid peroxidation, indirectly reflected the oxidative process. The results of this study were in line with Hamza's study, which reported that MO can reduce the degree of fibrosis through its anti-oxidant.

Administration of MO was able to significantly reduce MDA expression and that is probably caused by ability to suppress HSC activation [10]. These results also support previous studies by Itoh et al [18], El-Sayed et al [19], and El-bakry et al [20] who reported that CCl4 exposure can increase MDA levels.

A similar study was carried out by Rocha et al [21], that the administration of CCl_4 significantly increased the immunohistochemical expression of liver tissue MDA when compared to the control group.

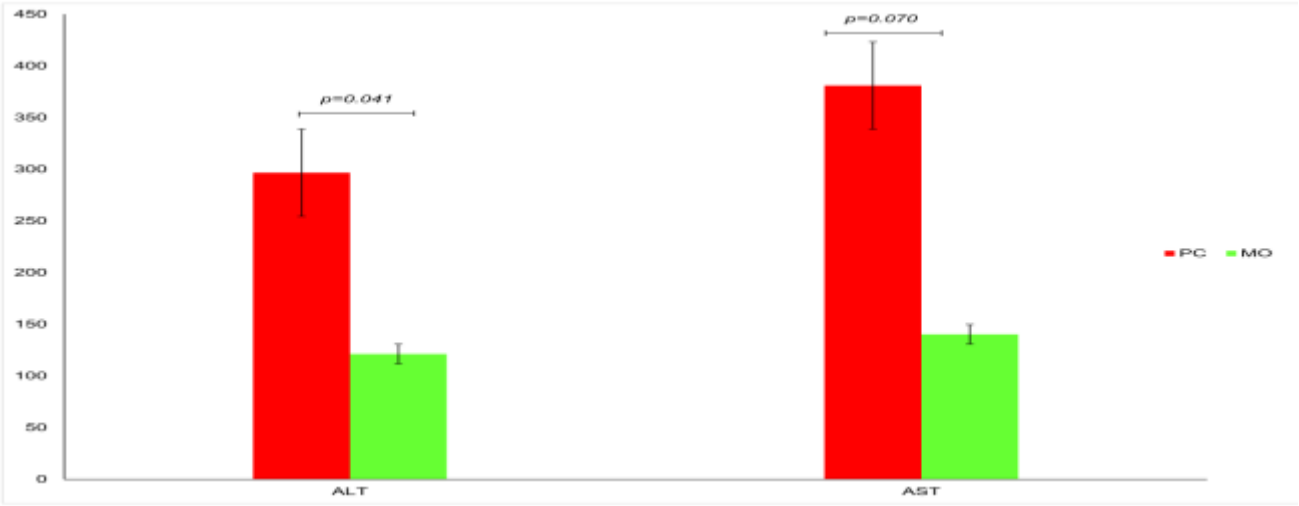

Fig. 4: The comparison of ALT and AST levels between positive controls (liver fibrosis rats) and treated *Moringa oleifera* rats (T-3)

The levels of ALT and AST in the treated groups significantly lower than in the liver fibrosis groups and the level of AST tend to lower than the liver fibrosis group but not signficant. ALT, amino alanine transferase; AST, aspartate transaminase.

Moringa oleifera was shown as natural source of many natural antioxidants such as ascorbic acid, vitamins, and phenolic compounds [22, 23]. In this study, there was lower of MDA expression in the MO treatment groups. The number of hepatocytes expressing MDA has a significant negative correlation (*r = - 0.922; p = 0.000*) to the increased dose of MO (Fig. 5).

The comparation of number hepatocytes expressing MDA between liver fibrosis model without treatment (PC) to T-1 was not significant, but when compared to the T-2 and T-3, showed a significantly difference (Fig. 3). These indicated that the lower of hepatocyte expressing MDA in our study resulted of antioxidative activity in MO, especially quercetin, quercetin-3-Glycoside, kaempferol-3-glycoside and kaempferol glucuronide (Table 1). Hepatoprotective effect of MO was proven by significantly lower of level ALT than PC. Although not significant, the level of AST to lower than PC (Fig. 4). MO provides a decreased effect on the synthesis of glutathione peroxidase in hepatocytes so that it can reduce MDA expression [24]. MO has strong antioxidant activity due to the high content of polyphenols.

Polyphenols are compounds in plants that can reduce damage due to oxidative stress in tissues through the mechanism of scavenging free radicals [25]. The content of phenolic compounds in MO leaves was kaempferol, rhamnetin, quercetin, chlorogenic acid, routine, and apigenin. These phenolic compounds act as reducing agents through a single oxygen scavenger and hydrogen donor so that the used radicals become stable [26].

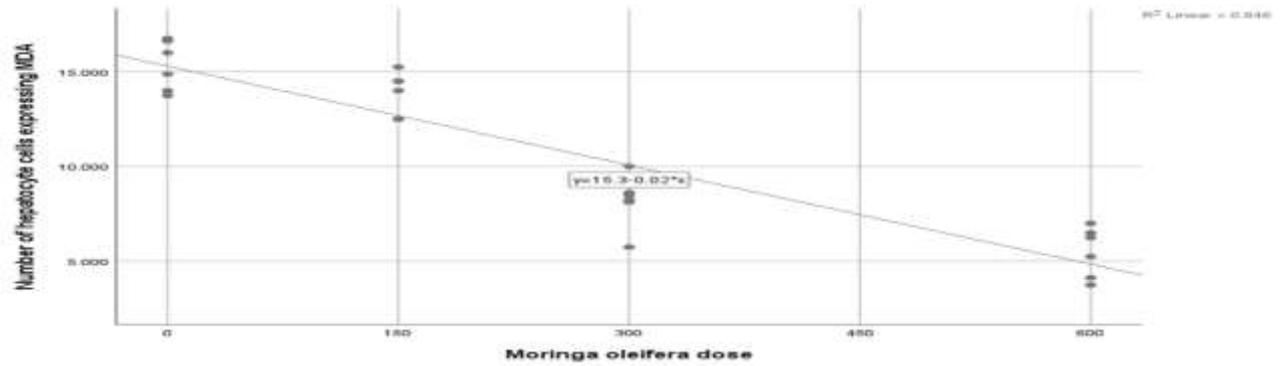

Fig. 5: Scatter-plot of *Moringa oleifera* dose to the number of hepatocytes expressing MDA (Pearson correlation test).

There was negative correlation between the mean number of hepatocytes expressing MDA and MO doses. Increasing the dose of MO provides more protection against hepatocyte damage characterized by lower of MDA expression in the hepatocyte. (Pearson correlation test; r = - 0.922, p = 0.000). MDA, malondialdehyde; MO, Moringa oleifera.

Liver plays an important role in regulating the body's homeostasis including metabolism, biotransformation, synthesis, storage and immunology of cells. These liver functions are mostly played by hepatocytes. Hepatocyte are the most cells in the liver and have a great ability to regenerate. Liver regeneration and repair of damaged hepatocyte related acute injury was taken responsibility by neighbour hepatocyte replication, but liver regeneration in chronic injury was involved of HPC proliferation [4, 27].

In the Fig. 7 clearly showed that there are binucleated hepatocyte in NC group (normal rats). Its mean that in the normal liver, liver regeneration was repaired by replication of hepatocyte than hepatic progenitor cells proliferation. On the other hand, there were many ductular reactions in PC and treatment MO groups. Its mean that liver regeneration in fibrosis condition caused by chronic injury was contributed by hepatic progenitor cells proliferation than hepatocyte replication. When we see the result of histological examination (Fig. 1), there was improvement of liver fibrosis degree in treatment groups. These improvements are the evidence that MO has regenerative effect in the liver fibrogenesis through increasing of hepatic progenitor cells proliferation.

Liver regeneration caused by chronic inflammation was characterized by an increase in CK-7 expression. CK-7 is expressed in hepatic progenitor cells (HPC), which may develop to both hepatocytes and cholangiocytes. Therefore, CK-7 was recognized as a regeneration indicator of liver cell (hepatocyte) or biliary tree (cholangiocyte) [28].

In this study, the number of hepatocytes expressing CK-7 in the NC and PC groups was very low. The number of hepatocytes expressing CK-7 in the two MO treatment groups (dose 150 or 300 mg/kg BW) were higher than PC, but still not significant. The mean number of hepatocytes expressing CK-7 have significant different to PC after the dose of MO was 600 mg/kg BW (Fig. 6). This proves that MO leaf extract has the effect of liver regeneration by increase of CK-7 expression.

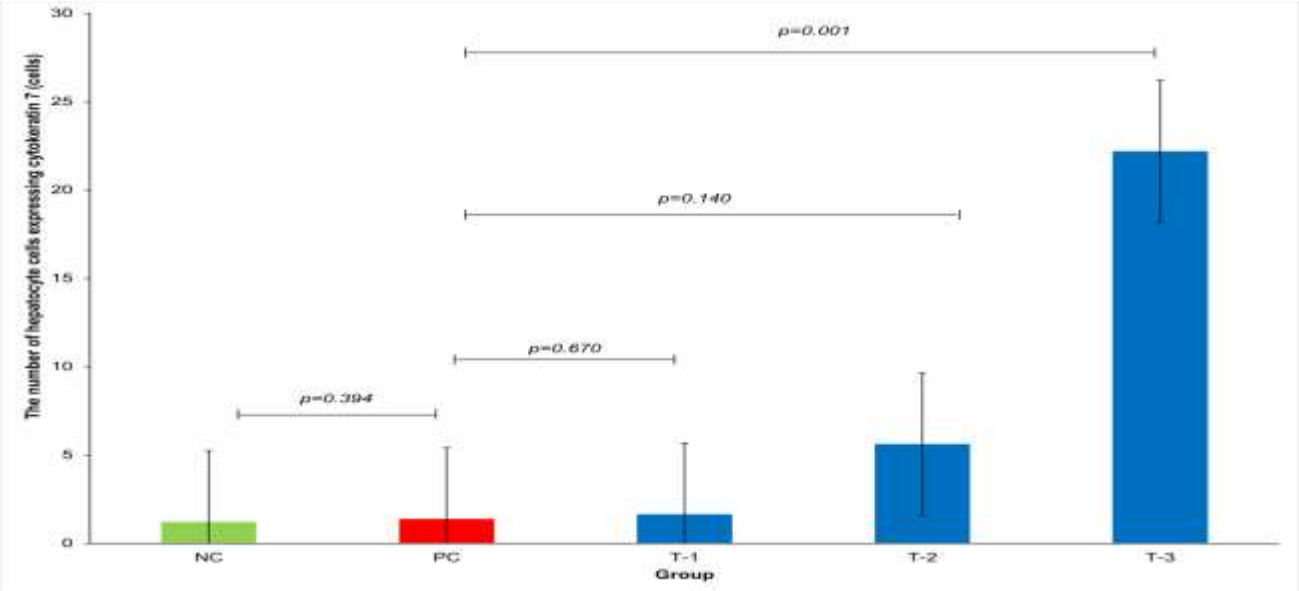

Fig. 6: The mean number of hepatocyte cells expressing cytokeratin 7 (20 visual fields).

In NC, the mean number of hepatocyte cells expressing CK7 in the cytoplasm was 1.21 cells. In PC, the mean number of hepatocyte cells expressing CK7 was 1.37 cells. The low CK7 in the control group indicates that hepatocyte cells are unable to proliferate and regenerate liver tissue. The administration of MO was able to be higher of CK7 expression, it was seen from the mean number of CK7 expressions in T-1 (1.64 cells), although it was not significantly different compared to PC ($p = 0.670$). CK7 expression was higher in T-2 (5.62 cells), but still not significantly different to PC ($p = 0.140$). The number of hepatocytes expressing CK7 take to highest value in T-3 (22.19 cells) and has a significant difference to the PC ($p = 0.000$). CK7, cytokeratin-7; NC, negative control; PC, positive control

Some herbal medicines were known to be used as anti-liver fibrosis. These herbal have potency as anti-fibrotic, anti-hepatotoxic, antioxidant, anti-inflammation and more importantly, should have potency to stimulate hepatocytes regeneration [29]. One study reported that the phenolic content of MO has a regenerative effect on hepatocytes in acute toxicity. The phenolic compound of MO have membrane-stabilizing effect that

can give prevention of membrane hepatocyte rupture [11]. To date, there were no studies have reported the liver regenerative effect of herbal medicine, including *Moringa oleifera*. In our experience, this result may be the first study that reported the effects MO on liver regeneration.

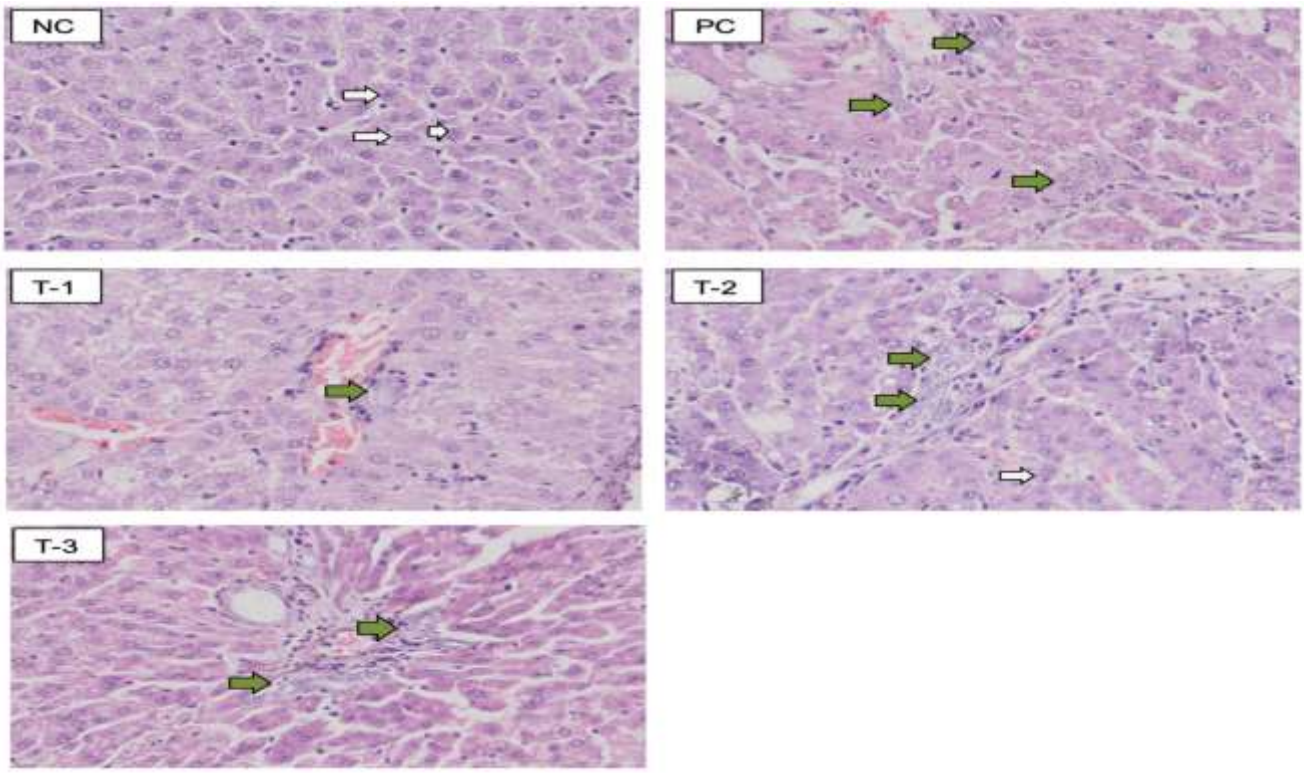

Fig. 7: Regenerative of liver tissue: hepatocyte replication and hepatic progenitor cells proliferation (ductular reaction)

The histological tissue (HE staining) shows that liver regeneration in the negative group occurs due to replication of hepatocytes, and liver regeneration in most of the other group occurs due to oval cell proliferation / ductular reaction (white arrows binucleated hepatocyte that indicate hepatocyte replication, while green arrows was ductular reaction that indicate hepatic progenitor cells proliferation).

The definitive mechanism of MO in inducing liver regeneration is still unclear. *Moringa* active compounds have many pharmacological effects to be used in improving health status, including anti-oxidants, anti-inflammatory, anti-convulsant, anti-tumor, anti-microbial, anti-viral, anti-hyperglycemia, etc. [30].

MO leaf extract contains a lot of phenolics, one of them is ellagic acid (52.7%) which is thought to be an active substance that has a role in liver regeneration, therefore it can repair damaged hepatocyte cells [31]. Ellagic acid itself can also prevent the development of liver cirrhosis by inhibiting the production of ROS and angiogenesis [32].

There were no studies that reported the role of ellagic acid in the chronic liver diseases. The liver regeneration usually was observed in animal models after hepatectomy. One study reported that quercetin has a regenerative effect of hepatocytes on liver tissue after hepatectomy. These effects are thought to be related to the antioxidant, anti-apoptotic and proliferative.

Quercetin probable stimulated mitotic hepatocyte after hepatectomy [33]. Other study showed that rutin can inhibit CCL4 acute toxicity in liver tissue and repairing liver tissue through the IL-6 / STAT3 pathway [34]. In our study, although quercetin and rutin were contained in ethanol extract MO (**Table 1**), but our setting study was in chronic condition. The mechanism of liver regeneration in our study is certainly not the same as the above studies.

Our study concluded that extract ethanol of MO has a hepatoprotective effect by antioxidant property and a liver regeneration effect by inducing HPC proliferation. The hepatoprotective effect of moringa is

indicated by a lower in the number of hepatocytes that express MDA. MO also has a liver regenerative effect that was characterized by increasing number of hepatocytes expressing CK-7. These results need further research to explain the exact mechanism of liver regeneration from MO.

Conclusion

Ethanol extract of MO has a hepatoprotective effect by antioxidant property and a liver regeneration effect by inducing HPC proliferation. The hepatoprotective effect of MO is indicated by a lower in the number of hepatocytes that express MDA. MO also has a liver regenerative effect that was characterized by increasing number of hepatocytes expressing CK-7. These results need further research to explain the exact mechanism of liver regeneration from MO.

Acknowledgments

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