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Moringa oleifera Inhibits Liver Fibrosis Progression by Inhibition of α-Smooth Muscle Actin, Tissue Inhibitors of Metalloproteinases-1, and Collagen-1 in Rat Model Liver Fibrosis

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Abstract

OBJECTIVE: This research was conducted to prove the existence of an anti-fibrosis effect in the ethanol extract of *Moringa oleifera* (MO) leaves on the liver.

MATERIALS AND METHODS: The liver fibrosis rat model was injected with chronic CCl_4. The ethanol extract of MO leaves was divided into three doses (150, 300, and 600 mg/kg body weight) and given through oral gavage.

RESULTS: The results of the histopathological analysis showed that the administration of MO ethanol extract could inhibit liver fibrosis as indicated by lower METAVIR scores than positive control rats. The immunohistochemical expression of collagen-1 in MO-fed rats also showed a decrease. The same thing was seen in the double-immunofluorescence staining analysis of α-SMA and tissue inhibitors of metalloproteinases-1 (TIMP-1), where the administration of MO ethanol extract decreased both markers.

CONCLUSIONS: This study concluded that the increase in collagen-1, α-SMA, and TIMP-1 simultaneously caused a more severe degree of fibrosis (METAVIR score). The ethanol extract of MO leaves was proven to be able to inhibit the progression of liver fibrosis by reducing the expression of all three markers.

Introduction

Liver fibrosis is the resultant process of liver fibrogenesis and fibrosis whose mechanisms are very complex. Liver fibrosis is caused by chronic inflammation as an effort to repair tissue due to prolonged lesions. Repairing the liver tissue results in an increase in the percentage of collagen up to 50% [1]. These collagen tissues (mainly collagen-I and collagen III) are produced by activated hepatic stellate cells (a-HSCs) or myofibroblast. Various types of a-HSC/myofibroblasts produce collagen which is spread throughout the liver tissue replacing normal tissue [2]. The broad distribution of collagen accumulates and leads to fibrotic tissue.

Besides secreting collagen-1, a-HSCs/myofibroblast is also express tissue inhibitors of metalloproteinases-1 (TIMP-1). TIMP-1 inhibits matrix metalloproteinase (MMP) activity in degrading the extracellular matrix. TIMP-1 also inhibits apoptosis of a-HSCs so that these cells survive and continue to produce extracellular matrix. The final result of these complex series is collagen fiber deposition in the Disse space, which forms scar tissue or fibrosis [3], [4]. This raises the suspicion that HSC activation plays a central role in the progression of liver fibrosis. Thus, inhibiting HSC activation or inducing apoptosis of a-HSCs is a preventive measure against the progression of liver fibrosis. The inhibition of HSC activation is thought to increase the fibrinolytic activity and repair liver fibrosis [4], whereas the apoptosis of a-HSCs encourages the resolution of liver fibrosis [5].

It is well known that HSCs activation has an important role in the process of liver fibrogenesis. HSC activation is also characterized by the production of α-smooth muscle actin (α-SMA) immunoreactivity. α-SMA is an actin isoform that stands out among vascular smooth muscle cells, but not much role in skeletal muscle fibrogenesis [6]. In the liver fibrogenesis, the increase in α-SMA occurred through the activation of JNK1 and JNK2 [7]. At the liver

fibrogenesis conditions, α-SMA has an important role in the mobility and contraction of myofibroblasts [8] so that it can reach the area of injury in liver tissue. The recent studies reported that α-SMA does not only have function to myofibroblast contractility but also it can transmit signals to the nucleus to regulate collagen production. α-SMA can transmit signals to the nucleus to regulate collagen production [9].

Clinically, liver fibrosis is an asymptomatic condition, but it needs treatment that prevents it from developing into cirrhosis or liver cancer. Eliminating its etiology is the most effective treatment, but not all etiologies can be eliminated. Therefore, the administration of anti-fibrosis agents that can prevent the progression of liver fibrosis is very necessary [10], [11].

Moringa oleifera (MO) has long been known to have hepatoprotective properties [12] and to provide anti-fibrosis effects [13]. The active compounds contained in MO, especially quercetin and kaempferol, provide antioxidant effects [14]. Quercetin also has the anti-proliferation effect of a-HSCs [15] and can induce apoptosis of a-HSCs [16].

It was known that the survival of a-HSCs has been regulated by collagen and TIMP-1 [3]. Activated HSC expresses collagen and TIMP-1 and both regulate survival of a-HSC for extended life. Hence, this study aims to prove that the administration of the ethanol extract of MO leaves can inhibit liver fibrogenesis by decreasing the expression of collagen-1, TIMP-1, and α-SMA of HSC in fibrosis model rats.

Materials and Methods

This research is a laboratory experimental study with a randomized post-test only controlled group design. The study was conducted at the University of Brawijaya, Indonesia.

Materials

The materials used in this study include CCl_4 (#820354.0010, MERCK, Schuchardt, Germany), corn oil, NaCl 0.9%, ketamine, formalin 10%, anti-α-SMA rabbit monoclonal antibody (M01072-3), TIMP-1 mouse monoclonal antibody (sc-21734), and COL1A1 (sc-25974) goat polyclonal IgG antibody. The materials used in the extraction were MO leaves and 96% ethanol solvent. The MO leaves used were in the powder form, obtained from Materia Medica Batu, Malang, Indonesia. The experimental animal used was the male *Rattus norvegicus* Wistar strain, with a body weight (BW) of 200–300 g obtained from the Rattus Breeding Center Singosari, Malang, Indonesia.

Preparation and manufacture of the ethanol extract of MO leaves

MO leaf powder of 100 g was extracted with 900 mL of 96% ethanol, shaken using an orbital shaker for 30 min, and left overnight at room temperature until it settled. The ethanol extract was filtered using Whatman paper No. 42, and then concentrated using a rotary evaporator. The concentrated MO extract was next dissolved using distilled water and administered orally by gavage to rat models at a dose of 150, 300, or 600 mg/kg BW, according to the treatment group.

Animal model induction of liver fibrosis and MO ethanol extracts administration

Rats were divided into five groups: Negative control (NC), positive control (PC), treatment-1 (T-1), treatment-2 (T-2), and treatment-3 (T-3), with six rats per group. The NC group was given 0.9% NaCl injection twice a week for 14 weeks. The PC group was given 10% (in corn oil, 1:9) CCl_4 intraperitoneal injection at a dose of 1 cc/kg BW for 12 weeks and continued with 2 cc/kg BW for the past 2 weeks. Both positive and NCs were administered daily through gavage. The treatment-1, treatment-2, and treatment-3 groups were given an injection of 10% CCl_4 with the same dose and time as the PC. The difference in treatment lay in the oral administration of the ethanol extract of MO leaves through gavage. The duration of administration of the ethanol extract of MO leaves was the same as the administration of Aquadest to the positive group, which was 14 weeks. 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. The rats were euthanized 48 h after the last CCl_4 injection by injecting 50 mg/kg BW of ketamine intramuscularly and then the liver was isolated. The experimental design of this study is depicted in Figure 1. All experimental animal treatment procedures referred to the institutional and local committee on the care and use of animals of the Brawijaya University School of Medicine, Malang, East Java, Indonesia (number: No 153/EC/KEPK-S3/06/2018).

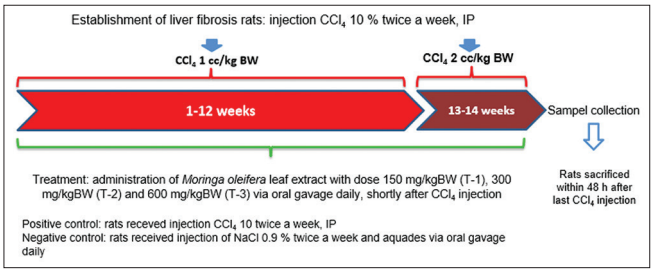

Figure 1: Experimental design

Histopathological, immunohistochemical, and immunofluorescent analyses of liver tissue

The liver was cut in the median lobe. The liver pieces were fixed in 10% neutral formalin and

then rehydrated with multilevel ethanol, embedded in a paraffin block, cut into 5-μm-thick sections using a microtome, and mounted on glass slides. The liver sections were dewaxed in xylene, rehydrated in stratified ethanol for 2–3 min at each concentration, and then washed with distilled water. Next, the slide was stained with Masson's trichrome (MT) reagent following the standard steps in the procedure. Microscopic fields in all sections of the liver were selected randomly for testing using a light microscope Olympus BX51.

The degree of liver fibrosis was analyzed by two anatomic pathologists. A Metavir score was used to assist the fibrosis degree, where F0 is normal tissue; F1: Fibrosis limited to the porta, perisinusoidal, and intralobular areas; F2: Fibrosis in several portal areas, a fibrous septum is formed between the portals, and intralobular architecture damage occurs; F3: A fibrous portal–central septum occurs, accompanied by damage to the intralobular structure, and cirrhosis has not been seen; and F4: cirrhosis [17].

Collagen-1 expression (Santa Cruz Biotechnology, sc-25974) in liver tissue was analyzed by immunohistochemistry. Slides of liver tissue were depolished and rehydrated in alcohol. Incubation was carried out using COL1A1 goat polyclonal antibody (1:50) at 5°C overnight as the primary antibody and continued with peroxidase labeling using the UltraTek HRP anti-polyvalent (DAB) Staining System (ScyTek Laboratories Inc.) kit. The slides were countered using hematoxylin. The slide was then scanned using the Olympus BX51 light microscope. Collagen-1 expression was calculated using the manual counting method at ×400 in ten visual fields, using image raster 3.0 software.

Double-immunofluorescence staining was carried out using TIMP-1 mouse monoclonal antibody (Santa Cruz Biotechnology, sc-21734) and anti-α-SMA rabbit monoclonal antibody (Boster Biological Technology, M01072-3). Slides were pretreated with BSA for 1 h at room temperature and incubated with primary antibodies (1:500) overnight at 4°C. Slides were then incubated with goat anti-mouse IgG rhodamine-conjugated antibody and anti-rabbit IgG FITC-conjugated antibody (Rockland Immunochemicals) as secondary antibodies for 1 h at room temperature. The slides were then countered using 4',6-diamidino-2-phenylindole and washed with phosphate-buffered saline. The observations were made using the FSX-100 microscope and the number of TIMP-1 and α-SMA expressions was counted manually with Image Raster 3.0 software.

Statistical analysis

The results of the study are presented as the mean and standard deviation. Statistical analysis uses one-way ANOVA, followed by Tukey's *post hoc* test for parametric data or the Kruskal–Wallis test, followed by the Mann–Whitney U-test for non-parametric data. Correlations were assessed using Pearson's test for parametric data and Spearman's test for non-parametric data. The processing of research data was analyzed by computer using Statistical Package for the Social Sciences software, IBM SPSS Statistics 22, with a significance level at $p < 0.05$.

Results

Histopathological examination of liver tissue

Histopathological changes in liver tissue were seen based on the results of staining with MT (Figure 2). All rats in the NC group showed F0 fibrosis, whereas all rats in the PC and T-1 groups showed F3 fibrosis (Table 1). On examining the T-2 group, one rat had a degree of F2 fibrosis and the remaining showed a degree of F3 fibrosis. On examining the T-3 group, four rats showed F2 fibrosis and the remaining two rats showed F1 fibrosis. These data correspond to the macroscopic picture of the rat liver, where rats with the highest degree of fibrosis (PC) showed a pale liver color compared to the NC rats and all three treatment groups (Figure 3).

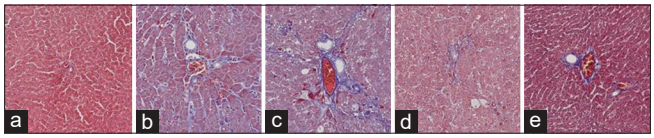

Figure 2: Histology of liver tissue by Masson's trichrome staining in rats of (a) negative control, (b) positive control, (c) treatment-1, (d) treatment-2, and (e) treatment-3 groups

It appears that the Metavir score of the treatment group decreases along with the large dose of MO ethanol extract given.

Table 1: The degree of liver fibrosis (Metavir score)

Group	Degree of fibrosis			
	F0	F1	F2	F3
Negative control	6	-	-	-
Positive control	-	-	-	6
Treatment-1	-	-	-	6
Treatment-2	-	-	1	5
Treatment-3	-	2	4	-

The liver of the PC group appears paler and wrinkled compared to that of the NC group. In the treatment group, T-1 was paler and more wrinkled than T-2 and T-3, whereas T-2 was paler and more wrinkled than T-3.

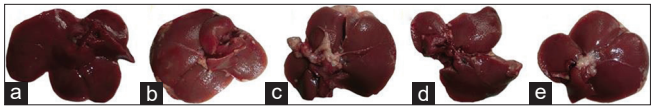

Figure 3: Macroscopic picture of liver in (a) negative control, (b) positive control, (c) treatment-1, (d) treatment-2, and (e) treatment-3 groups

Immunohistochemical examination of collagen-1 expression

The calculation of cells expressing collagen-1 in the liver used the manual counting method on the immunohistochemical preparations by two observers

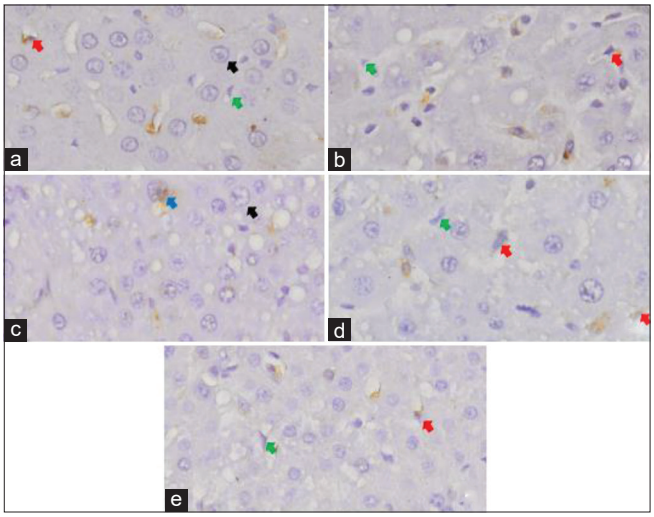

Figure 4: Expression of collagen-1 in liver tissue of (a) negative control, (b) positive control, (c) treatment-1, (d) treatment-2, and (e) treatment-3 groups

independently. The amount of collagen-1 expression was determined based on the ratio between the number of hepatocyte cells and HSCs expressing collagen-1 with the total number of hepatocytes and HSCs in five visual fields with ×400. The final number of cells with collagen-1 expression in liver tissue is the average of the calculation results of the two observers. A description of collagen-1 expression in the liver per group is shown in Figure 4.

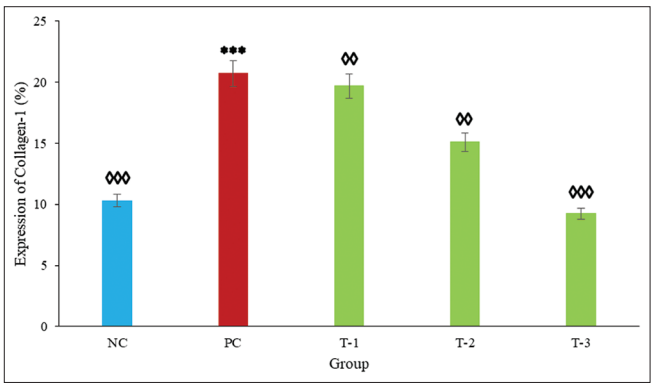

*Figure 5: Collagen-1 expression in the liver tissue of each group. The expression of collagen-1 in the treatment group decreased along with the large dose of MO ethanol extract given. $^{◊◊◊}p < 0.05$ versus positive control (PC); ***$p < 0.05$ versus negative control; $^{◊}p > 0.05$ versus PC*

From Figure 5, it can be seen that CCL_4 induction significantly increases the expression of collagen-1 in the liver ($p = 0.000$) and the administration of MO ethanol extract can reduce the expression of collagen-1. A dose of 150 mg/kg BW (T-1) could reduce the expression of collagen-1 to 19.69% from 20.71%. The dose of 300 mg/kg BW (T-2) was able to reduce the expression of collagen-1 (15.11%) more than T-1, but statistically, it was not significantly different ($p = 0.164$). Significant differences occurred only at a dose of 600 mg/kg BW (T-3), where the mean expression of collagen-1 decreased to 9.24% ($p = 0.000$).

Green arrows indicate liver stellate cells, red arrows indicate liver stellate cells that express collagen-1, black arrows indicate hepatocyte cells, and blue arrows indicate hepatocyte cells that express collagen-1.

Double-immunofluorescence staining of TIMP-1 and α-SMA

TIMP-1 and α-SMA expressions were seen based on the results of the double-immunofluorescence staining of TIMP-1 with α-SMA (Figure 6). TIMP-1 and α-SMA expressions showed the same trend with the results of the analysis of the fibrosis degree in the liver tissue; where both expressions decreased with the increasing dose of MO ethanol extract (Figures 7 and 8).

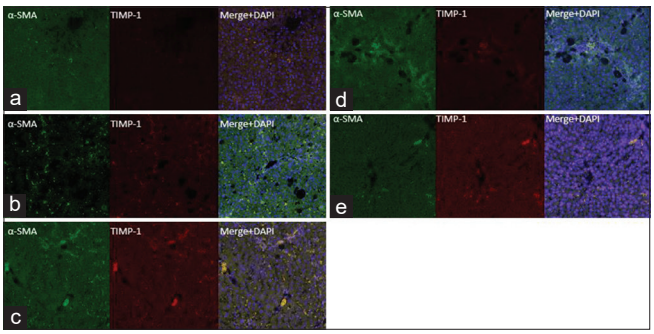

Figure 6: Microscopic features showing α-smooth muscle actin colocalization with tissue inhibitors of metalloproteinases-1 with indirect immunofluorescence staining: (a) Negative control; (b) positive control; (c) treatment-1; (d) treatment-2; and (e) treatment-3

Discussion

CCl_4 is a substance that was often used to induce hepatocyte injury to make liver fibrosis animal models. Chronic induction of CCL_4 results in a progressive loss of liver function caused by chronic inflammation that triggers a prolonged process of fibrogenesis [18]. In this study, CCl_4 induction for 14 weeks resulted in Metavir F3 of liver fibrosis in all animals model (Table 1). Macroscopically, it appears that the liver organ in the control group looks pale and wrinkled compared to the negative group or other groups (Figure 3). CCl_4 is metabolized in the liver by cytochrome P450 to form of trichloromethyl radical (CCl_3). This radical material damage nucleic acids, proteins, and

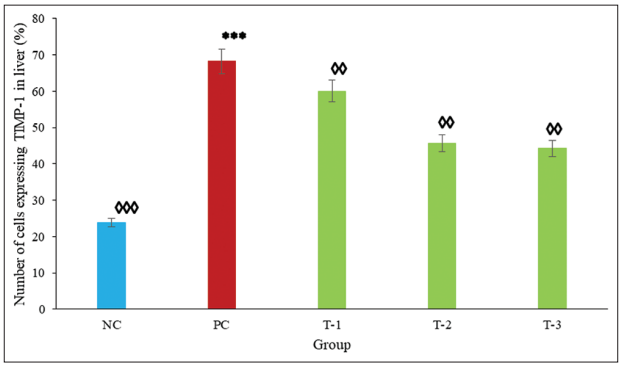

*Figure 7: The average number of cells expressing tissue inhibitors of metalloproteinases-1 in liver tissue: $^{◊◊◊}p < 0.05$ versus positive control (PC); ***$p < 0.05$ versus negative control; $^{◊}p > 0.05$ versus PC (not significant)*

lipids, lead to disruption of cellular processes. Another radical, trichloromethylperoxy radicals (CCl_3OO) induce CCl_3 oxygenation which initiates lipid peroxidation and destruction of unsaturated fatty acids. This mechanism causes a decrease in membrane permeability throughout the cellular compartment and liver damage that is characterized by inflammation and fibrosis tissue [18]. In fact, the mechanism of CCl_4 in causing liver fibrosis is very complex, and requires further research [19].

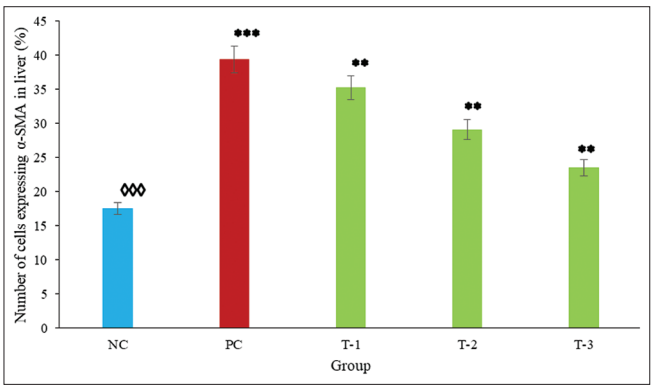

*Figure 8: The average number of cells expressing α-SMA in the liver: ◊◊◊p < 0.05 versus positive control (PC); ***p < 0.05 versus negative control (NC); **p < 0.05 versus NC*

Collagen-1 is the main collagen in liver fibrosis tissue. Collagen is produced by active HSCs (a-HSCs) and is partly responsible for HSC survival. Increased expression of collagen contributes to apoptosis-resistant a-HSC [20]. This study proves that in liver fibrosis conditions (PC), there is an increase in collagen-1 expression. The results of this study are in accordance with the results of previous research, where the induction of CCL_4 can significantly increase the number of cells expressing collagen-1. This previous research shows that the repeated administration of CCL_4 injection to rat liver fibrosis model can increase the expression of collagen-1 [13]. The administration of MO can reduce these expressions (Figure 4). The decrease in the collagen expression was in accordance with the increase in the dose of MO given. Administration of MO with doses 600 mg/kg BW was able to significantly reduce collagen-1 expression compared to the control

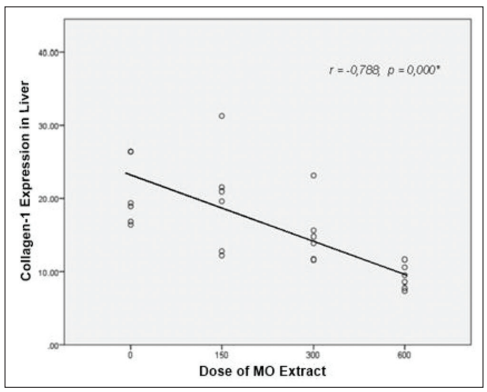

Figure 9: Correlation graph of Moringa oleifera ethanol extract dose versus collagen-1 expression in the liver tissue

group. These MO effect was also the same as the Hamza's research [13].

Liver fibrosis tissue is characterized by extracellular matrix deposition. Collagen and other extracellular material components accumulate and damage the normal structure of the liver parenchyma and vasculature. Thus, one of the mechanisms of anti-fibrosis agents is inactivating the a-HSCs. Inactivation of a-HSCs causes a reduction in collagen-1 production and increased fibrinolysis activity [4]. In our study, there is a significant correlation between dosage and collagen reduction (Figure 9). This indicates that MO has an anti-fibrosis effect which may be associated with decreased collagen expression. This study also showed that in liver fibrosis rats, there was a significant increase in α-SMA (as a marker of a-HSCs), and decreased after MO was given (Figure 8). This provides evidence that one of the anti-fibrosis mechanisms of MO is inactivating a-HSC.

In the process of liver fibrogenesis cannot be separated from the role of TIMP-1, which is expressed by a-HSC. TIMP-1 is a regulator of extracellular matrix protein remodeling. TIMP-1 is thought to be involved in the process of fibrogenesis by preventing collagen degradation due to MMP inhibitory effects and preventing a-HSC apoptosis [21]. In this study, TIMP-1 expression was calculated by double-immunofluorescence staining in cells expressing α-SMA. TIMP-1 expression that colocalized with α-SMA shows that TIMP-1 is expressed by a-HSCs (Figure 6). The number of TIMP-1 expressions in the positive group increased significantly, and then gradually decreased after the ethanol extract of MO leaves was given (Figure 7). When this result was associated with improvement in the degree of fibrosis in the group given MO (Table 1), the reduction of TIMP-1 is additional evidence that MO has a potential mechanism to inhibit liver fibrogenesis through decreasing the amount of a-HSC.

Considering the anti-apoptotic effects of TIMP-1, this study proves that the increased expression of TIMP-1 causes a-HSCs to survive and produce more collagen-1, thereby lead to liver fibrosis. The ethanol extract of MO leaves proved to be able to inhibit fibrogenesis by reducing TIMP-1 expression. The anti-fibrosis mechanism is thought to originate from the antioxidant and anti-inflammatory effects of MO. The compounds in MO, such as quercetin and kaempferol, are compounds that have hydroxyl complexes. The hydroxyl complex easily donates electrons to other unstable atoms (such as free radicals), stabilizing, and neutralizing potential pathological effects. Quercetin is considered a powerful antioxidant because of its ability to capture free radicals and bind metal ions [15], [22]. In this study, we found that the Metavir scores, α-SMA, TIMP-1, and collagen-1 expressions showed the same trend, which decreased with the increasing doses of ethanol extracts of MO leaves.

Conclusion

This study concluded that the ethanol extract of MO leaves has anti-fibrosis properties by inhibiting the progression of liver fibrosis through a decreased expression of collagen-1, TIMP-1, and α-SMA. α-SMA is not only a a-HSC marker but it also has an important role in regulating collagen production. It can be suspected that MO can inhibit the progression of liver fibrogenesis by decreasing the amount of a-HSCs. For that, further research is needed.

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