The curative effect of ethanolic extract of *Moringa oleifera* leaf in an animal model of liver fibrosis

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ABSTRACT

Studies about the prevention effect of *Moringa oleifera* on liver fibrosis has been reported. However, its curative effect has not been reported, yet. This study was conducted to evaluate the curative effect of *M. oleifera* leaf extract on liver fibrosis. It was a laboratory experimental study with a post-test-only control group design. Rats were divided into 5 groups i.e. normal control which received intraperitoneally injections of 1 mL/kg BW of 0.9% NaCl solution twice a wk for 11 wk. Liver fibrosis control which received intraperitoneally injections of 1 mL/kg BW of 10% CCl₄ solution twice a wk for 11 wk. Three *M. oleifera* treatment group which received intraperitoneally injections of 1 mL/kg BW of 10% CCl₄ solution twice a wk for 11 wk continued by *M. oleifera* leaf ethanolic extract at dose of 600 mg/kg BW daily for 3 (MO3), 6 (MO6), and 10 (MO10) wk, respectively. The liver fibrosis level was assessed based on the METAVIR score. Histopathological analysis of liver tissues demonstrated that 11-week CCl₄ induction successfully resulted in liver fibrosis in rats (F3 and F4). The administration of *M. oleifera* leaf ethanolic extract decreased METAVIR scores ranged from F3 to F1. The optimal reduction of the METAVIR scores (F1) was observed in MO3 group after 6 wk administration (p<0.05). It was indicated that *M. oleifera* leaf ethanolic extract ameliorated liver fibrosis. In conclusion, *M. oleifera* leaf ethanolic extract has a curative effect against liver fibrosis.

ABSTRAK

Penelitian tentang efek pencegahan *Moringa oleifera* pada fibrosis hati telah banyak dilaporkan. Namun, penelitian tentang efek kuratifnya belum banyak dilaporkan. Penelitian ini dilakukan untuk mengevaluasi efek kuratif ekstrak daun *M. oleifera* terhadap fibrosis hati. Penelitian ini merupakan penelitian eksperimental laboratoris dengan desain post test only control group. Tikus dibagi menjadi 5 kelompok yaitu kontrol normal yang menerima suntikan intraperitoneal 1 mL/kg BB larutan NaCl 0,9% dua kali seminggu selama 11 minggu. Kontrol fibrosis hati yang menerima suntikan intraperitoneal 1 mL/kg BB larutan CCl₄ 10% dua kali seminggu selama 11 minggu. Tiga kelompok perlakuan *M. oleifera* yang mendapat suntikan intraperitoneal 1 mL/kg BB larutan CCl₄ 10% dua kali seminggu selama 11 minggu dilanjutkan dengan pemberian ekstrak etanol daun *M. oleifera* dengan dosis 600 mg/kg BB setiap hari selama masing-masing 3 (MO3), 6 (MO6), dan 10 (MO10) minggu. Derajat fibrosis hati dinilai berdasarkan skor METAVIR. Analisis histopatologi jaringan hati menunjukkan bahwa induksi CCl₄ selama 11 minggu berhasil menyebabkan fibrosis hati pada tikus (F3 dan F4). Pemberian ekstrak etanol daun *M. oleifera* menurunkan skor METAVIR berkisar antara F3 sampai F1. Penurunan optimal skor METAVIR (F1) diamati pada kelompok MO3 setelah pemberian selama 6 minggu (p<0.05). Diindikasikan bahwa ekstrak etanol daun *M. oleifera* dapat memperbaiki fibrosis hati. Kesimpulannya, ekstrak etanol daun *M. oleifera* mempunyai efek kuratif terhadap fibrosis hati.

Keywords:

- antifibrotic;
- curative effect;
- liver fibrosis;
- *Moringa oleifera*;
- extract

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INTRODUCTION

Liver fibrosis is a condition characterized by an aberrant wound-recovery process reaction that often represents the final stage in the progression of chronic liver disease.\(^1\) As liver disease advances, the progression of fibrosis becomes a crucial factor influencing the prognosis of liver disease and the risk of hepatocellular carcinoma (HCC).\(^2\) Recently, no standard treatment for liver fibrosis is available. Therefore, the management of liver fibrosis usually focuses on eliminating the etiology and complications.\(^3\)

The most effective treatment for liver fibrosis is elimination of the etiology, however it still faces many obstacles. Reversal fibrosis by eliminating of the causative agent occurs too slowly or too rarely to avoid life-threatening complications, especially in advanced fibrosis.\(^4\) The pathogenesis of liver fibrosis is very complex, involving the interaction of various cells and cytokines in liver tissue.\(^5\) Hepatic stellate cells are the cells that play the most role in the progression of liver fibrosis.\(^6\) Therefore, inhibiting HSC activation and inducing a-HSC apoptosis has promise in inhibiting liver fibrosis.

Potent herbal medicines for the management of liver fibrosis have been reported. *Moringa oleifera*, locally name as *kelor*, is one of the herbal medicines that has been used for the treatment various diseases including liver fibrosis. *Moringa oleifera* contains various active constituents such as alkaloids, saponins, tannins, steroids, phenolic acids, terpenes, and flavonoids. The flavonoid of *M. oleifera* includes myricetin, quercetin, kaempferol, isorhamnetin, and rutin.\(^7,9\) A previous study reported that *M. oleifera* leaf extract administration can accelerate liver cell regeneration of rats after CCl\(_4\) induction.\(^10\) *Moringa oleifera* leaf extract was also reported to have hepatoprotective effect on CCl\(_4\)-induced in mice through various pathways and targets including control of oxidative stress, modulation of TLR4/NF-κB, inhibition of α-smooth muscle actin, inhibition of metalloproteases-1, and collagen-1.\(^11-13\) This study aimed to evaluate the curative effect of ethanolic extract of *M. oleifera* leaf on liver fibrosis rats chronically induced by CCl\(_4\).

MATERIAL AND METHODS

Extract and samples preparation

The ethanolic extract of *M. oleifera* was prepared by cold maceration using 96% ethanol as solvent. Briefly, 100 g of dried *M. oleifera* leaves powder was macerated with 900 mL of 96% ethanol. The maceration process was conducted for 24 hr at 37°C with stirred occasionally. The homogenized mixture was filtered using Whatman filter paper and the residue left behind was subjected to two identical maceration. Subsequently, the filtrates obtained from the three macerations were combined and evaporated using a vacuum rotary evaporator until a crude extract was obtained. The extract tested at dose of 600 mg/kg BW was than prepared by diluting the crude extract with aquadest.\(^12,13\)

Experimental design and animal treatment

The curative effect of ethanolic extract of *M. oleifera* leaf in rats model of liver fibrosis was conducted according a method developed by Li *et al.*\(^14\) It was part of a larger study concerning the effects of *M. oleifera* on liver fibrosis of rats conducting at the Laboratory of Pharmacology, Faculty of Medicine, Universitas Brawijaya, Malang. This experimental study used a post-test only control group design conducting on male Wistar rats weighing 150-250 g and 10-12 wk old with 3 rats in each group. The rats were acclimatized to animal house conditions for a period of 7 d before experimental and then intraperitoneal
injected by 0.9% CCl₄ in corn oil (1:9) for 11 wk except normal control rats which intraperitoneal injected 0.9% NaCl. Followed after injection of 0.9% CCl₄, the rats were administered *M. oleifera* leaf extract at dose of 600 mg/kg BW daily for 3 (MO3), 6 (MO6), and 10 (MO10) wk, respectively (TABLE 1). The rats were sacrificed 48 hr following the last NaCl or CCl₄ or *M. oleifera* leaf extract administration. The liver tissue was isolated for subsequent histopathological analysis.

The protocol of the study was approved by the Health Research Ethics Committee of the Faculty of Medicine, Universitas Brawijaya, Malang, Indonesia (No. 09/EC/KEPK/01/2023).

**Liver pathological analysis**

Liver tissue samples were preserved in 10% neutral-balance formalin solution, followed by rehydration through a series of ethanol with different concentrations. Subsequently, the liver tissues were embedded in paraffin blocks and sliced into sections that were approximately 5 µm thick using a microtome. These sections were then placed onto glass slides. The liver sections underwent a dewaxing process using xylene and rehydration through graded ethanol for 2-3 min. After rinsing each of the sections with distilled water, they were subsequently dyed using Masson's trichrome reagent. An Olympus BX51 light microscope was employed to observe the liver tissue images.

The liver fibrosis level was assessed by anatomical pathologist based on the METAVIR score. The liver fibrosis level was categorized as no fibrosis with score of 1 (F0); portal fibrosis without septa with score of 2 (F1); portal fibrosis with a few septa with score of 4 (F2); numerous septa without cirrhosis with score of 8 (F3); and cirrhosis with score of 16 (F4). The ordinal METAVIR score data was presented in ratio data.

### TABLE 1. Treatment of rats

<table>
<thead>
<tr>
<th>Groups</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control</td>
<td>Injection of 0.9 % NaCl (i.p) for 11 wk.</td>
</tr>
<tr>
<td>Fibrosis control</td>
<td>Injection of 10 % CCl₄ (i.p) for 11 wk.</td>
</tr>
<tr>
<td>MO3</td>
<td>Injection of 10 % CCl₄ (i.p) for 11 wk, followed by oral administration of <em>M. oleifera</em> leaf extract at dose of 600 mg/kg BW daily for 3 wk.</td>
</tr>
<tr>
<td>MO6</td>
<td>Injection of 10 % CCl₄ (i.p) for 11 wk, followed by oral administration of <em>M. oleifera</em> leaf extract at dose of 600 mg/kg BW daily for 6 wk.</td>
</tr>
<tr>
<td>MO10</td>
<td>Injection of 10 % CCl₄ (i.p) for 11 wk, followed by oral administration of <em>M. oleifera</em> leaf extract at dose of 600 mg/kg BW daily for 10 wk.</td>
</tr>
</tbody>
</table>
Statistical analysis

Data were presented as the mean ± standard error of the mean (SEM). Comparisons among multiple groups were conducted by one-way analysis of variance (Anova) continued by the post hoc Tukey method using IBM SPSS version 23 software. A p value less than 0.05 was considered as statistically significant.

RESULTS

Characteristic of liver

The general condition of the rats in all groups were good. No cases died were observed or all rats were alive in this study. The liver weight in the fibrosis control group and the treatment groups (MO3, MO6, and MO10) were higher than the normal control. However, it was not statistically significant (p>0.05). The liver volume in the fibrosis control group and the treatment groups, except MO6, were also higher than the normal control. However, it was also not statistically significant (p>0.05) (TABLE 2).

Macroscopically, the color, surface texture, and freshness of the liver organs of each group were different (TABLE 3). The liver of the fibrosis control groups appeared pale with a wrinkled surface texture resembling an orange peel. In contrast, the liver organ in the normal control groups exhibited a fresh and smooth surface. The livers of MO3 and MO10 treatment groups appeared smoother and darker brown than the fibrosis control groups. Meanwhile, the liver of MO6 rat groups displayed a macroscopically glossy and fresh appearance, resembling the liver of the normal control rats.

<table>
<thead>
<tr>
<th>Liver characteristics (mean ± SE)</th>
<th>Normal control</th>
<th>Fibrosis control</th>
<th>MO3</th>
<th>MO6</th>
<th>MO10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight (g)</td>
<td>7.90 ± 0.34</td>
<td>9.13 ± 1.09</td>
<td>10.06 ± 0.77</td>
<td>8.17 ± 0.70</td>
<td>9.37 ± 0.70</td>
</tr>
<tr>
<td>Volume (mL)</td>
<td>7.13 ± 0.55</td>
<td>9.33 ± 120</td>
<td>8.73 ± 0.63</td>
<td>6.10 ± 1.52</td>
<td>9.40 ± 0.70</td>
</tr>
</tbody>
</table>
TABLE 3. The macroscopic appearance of the rat liver organs in the normal control, fibrosis control, MO3, MO6, and MO10 groups.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Liver</th>
<th>Rat 1</th>
<th>Rat 2</th>
<th>Rat 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control</td>
<td></td>
<td><img src="supriono.png" alt="Image" /></td>
<td><img src="supriono.png" alt="Image" /></td>
<td><img src="supriono.png" alt="Image" /></td>
</tr>
<tr>
<td>Fibrosis control</td>
<td></td>
<td><img src="supriono.png" alt="Image" /></td>
<td><img src="supriono.png" alt="Image" /></td>
<td><img src="supriono.png" alt="Image" /></td>
</tr>
<tr>
<td>MO3</td>
<td></td>
<td><img src="supriono.png" alt="Image" /></td>
<td><img src="supriono.png" alt="Image" /></td>
<td><img src="supriono.png" alt="Image" /></td>
</tr>
<tr>
<td>MO6</td>
<td></td>
<td><img src="supriono.png" alt="Image" /></td>
<td><img src="supriono.png" alt="Image" /></td>
<td><img src="supriono.png" alt="Image" /></td>
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<tr>
<td>MO10</td>
<td></td>
<td><img src="supriono.png" alt="Image" /></td>
<td><img src="supriono.png" alt="Image" /></td>
<td><img src="supriono.png" alt="Image" /></td>
</tr>
</tbody>
</table>

TABLE 4. Fibrosis degree based on METAVIR score

<table>
<thead>
<tr>
<th>Groups</th>
<th>n</th>
<th>METAVIR score</th>
<th>Fibrosis score (mean ± SE)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>F0 (1)</td>
<td>F1 (2)</td>
<td>F2 (4)</td>
</tr>
<tr>
<td>Normal control</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>Fibrosis control</td>
<td>3</td>
<td>-</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>MO3 treatment</td>
<td>3</td>
<td>-</td>
<td>-</td>
<td>2</td>
</tr>
<tr>
<td>MO6 treatment</td>
<td>3</td>
<td>-</td>
<td>3</td>
<td>-</td>
</tr>
<tr>
<td>MO10 treatment</td>
<td>3</td>
<td>-</td>
<td>2</td>
<td>1</td>
</tr>
</tbody>
</table>

Note: <sup>a</sup>significantly different vs. normal control; <sup>b</sup>significantly different vs. fibrosis control; <sup>c</sup>significantly different vs. MO3
Histopathological changes in the liver

The histopathological analysis of liver tissue resulted in METAVIR scores, which were transformed into ratio data to calculate the average fibrosis scores, as presented in TABLE 4. The table reveals that the administration of CCl4 for 11 wk resulted in two model rats with liver fibrosis, with METAVIR scores of F4, and one rat with a score of F2. MO administration led to a decrease in METAVIR scores, ranging from F3 to F1. A 6 wk treatment with MO successfully reduced the METAVIR scores to F1 in all rats (p<0.05). It indicates that the 6 wk MO treatment significantly produced fibrosis scores approaching the conditions observed in the control group.

The liver histological examination used Masson’s Trichrome staining (FIGURE 1). This image represents a typical example of the METAVIR scores for each rat group, as presented in Table 2. In the control group, no fibrotic tissue was detected (F0), whereas the Model rats exhibited cirrhosis (F4). In the MO3 group, fibrotic tissue was visible in the portal triad, extending into the central vein and forming portal-central fibrosis (septal fibrosis/F3). The MO10 rats showed fibrotic tissue in several portal triads, resulting in portal fibrosis with a few septa (F2). Fibrotic tissue in the MO6 rats was limited to the portal triad and perisinusoidal regions (portal fibrosis/F1).

FIGURE 1. Histological liver tissue structure by Masson’s Trichrome staining. The images were obtained using an Olympus BX51 microscope with a 400x magnification. The white arrow indicates the fibrosis area.
DISCUSSION

Chemically induction by CCl4 is the most commonly used for animal models of liver fibrosis.17 The CCl4 generates free radicals that cause lipid peroxidation, oxidative stress, membrane integrity loss, and hepatocyte apoptosis/necroptosis.18 It leads to Kupffer cell and hepatic stellate cell activation, resulting in an abundance of inflammatory mediators and collagen production, culminating in long-term liver fibrosis formation.19 In this study, intraperitoneal injections of CCl4 for 11 wk successfully induced fibrosis, even cirrhosis (F4) in the fibrosis control. In addition, severe fibrosis was more observed compared to the normal control. These findings were also supported by the macroscopic appearance of the liver, in which the liver in the fibrosis control appeared pale and wrinkled.

The liver weight and volume were also more remarkable in the fibrosis control than in the normal control, although these differences were not statistically significant (p>0.05). It suggests that liver fibrosis does not significantly affect liver weight and volume. It was consistent with previous study reporting that fibrosis and cirrhosis did not substantially impact liver weight.20 In addition, the correlation between fibrosis and rat's weight and volume might be biased due to the necessity of considering the rat's body weight in this scenario.

The objective of this study is to evaluate the curative effects of M. oleifera against liver fibrosis. Histologically, the liver tissues of rats treated with M. oleifera leaf extracts appeared better than those in the fibrosis control (FIGURE 1). Histological observations revealed that the presence of scattered fibrotic tissue around portal triads and septa of the liver fibrosis rats reduced after administration of M. oleifera leaf extract, especially for 6 and 10 wk administrations (p<0.05). The administration of M. oleifera leaf extract for 3 wk also demonstrated enhancement, although it was not statistically significant (p>0.05). It indicated that 6 wk administration of the M. oleifera leaf extract has a curative effect against rat liver fibrosis. Susanto et al.21 reported that administration of M. oleifera leaf extract for 6 wk significantly decrease in TNF-β expression and prevent hepatocellular carcinoma progression.

The findings of this study supported previous studies in which M. oleifera leaf extract had a preventive effect against rat liver fibrosis. Administration of M. oleifera leaf extract and induction of CCl4 simultaneously, could reduce liber fibrosis degree in rats by inhibiting HSC activation and promoting these a-HSC apoptosis.12-13 Aly et al.22 reported that M. oleifera leaf extract has an hepatoprotective effect by reducing liver enzymes, TNF-α, TNF-β, and degree of liver fibrosis in acetaminophen-induced liver fibrosis in rats.23 These mechanisms12,13,22 are believed can explain the curative effect of M. oleifera leaf extract.

The curative effect of M. oleifera against liver fibrosis is due to its active secondary metabolites especially quercetin and kaempferol. Quercetin was reported ameliorate liver inflammation and fibrosis by regulating hepatic macrophages activation and polarization.23 Furthermore, quercetin reduces hepatic fibrogenesis by inhibiting TGF-β/Smad3 signaling pathway.24 Whereas, kaempferol was reported inhibit a-HSC by regulating miR-26b-5p/Jag1 axis and notch pathway.25 It was also reported ameliorate hepatic fibrosis by suppressing NF-κB pathway and promoting HSC apoptosis.26

The management of liver fibrosis remains a challenge for medical practice due to the unavailability of the standard treatment. Development of alternative treatment from medical plants is urgently needed. Moringa oleifera is
one of the potential medicinal plants for the treatment of liver fibrosis. Several studies in animal model of liver fibrosis have proven the protective and curative effect of M. oleifera leaf against liver fibrosis.\textsuperscript{10-13,22-25} The toxicology was also reported that M. oleifera leaf can be categorized not toxic with a lethal dose 50\% (LD\textsubscript{50}) of 5,585 mg/kg BW.\textsuperscript{27} However, further studies are needed including sub chronic or chronic toxicology, and development of formulation before clinical study conducted.

**CONCLUSION**

In conclusion, the administration of ethanolic extract of M. oleifera leaf at dose of 600 mg/kg BW daily for 6 wk has an effective curative effect against liver fibrosis in rat model fibrosis. Further studies will be conducted to evaluate the chronic toxicity, and to develop the formulation before clinical study conducted.

**ACKNOWLEDGEMENT**

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