Moringa oleifera Prevents In vivo Carbon Tetrachloride-Induced Liver Fibrosis through Targeting Hepatic Stellate Cells

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Abstract

BACKGROUND: Moringa oleifera (MO) exhibits hepatoprotective properties and provides an anti-liver fibrosis effect. However, its mechanism related to the anti-liver fibrosis effect was still unclear.

AIM: The objective of this study was to explain the mechanism of liver fibrosis prevention by MO through hepatic stellate cells (HSCs).

MATERIALS AND METHODS: The liver fibrosis model was induced by the intraperitoneal injection of 10% CCl4 twice a week at a one cc/kg BW dose for 12 weeks and followed by a quantity of 2 cc/kg BW for the past 2 weeks. Ethanolic extract of MO leaves (150, 300, and 600 mg/kg) was orally administered daily. Double immunofluorescence staining and terminal deoxynucleotidyl transferase dUTP nick end labeling analysis were applied to analyze the markers involved in HSCs activation and a-HSC apoptosis.

RESULTS: The results showed that the administration of MO could reduce transforming growth factor-β and nuclear factor-kappa B (NF-κB), increase the expression of tumor necrosis factor-related apoptosis-inducing ligand-receptor 2 and caspase-3, and increase the number of apoptosis a-HSCs.

CONCLUSION: This study showed that the ethanol extract of MO leaves could inhibit liver fibrosis by inhibiting HSCs activation and inducing of a-HSC apoptosis through the extrinsic pathway.

Introduction

The pathogenesis of liver fibrosis is very complex, involving the interaction of various cells and cytokines in the liver tissue. Hepatic stellate cells (HSCs) are the cells that play a major role in the pathogenesis of liver fibrosis [1]. HSC activation promotes the progression of liver fibrosis [2], whereas a-HSC apoptosis leads to regression of liver fibrosis [3]. Therefore, inhibiting HSC activation and inducing a-HSC apoptosis can be the target in inhibiting liver fibrosis [4].

Activation of HSCs both at the initiation and perpetuation stages involves many factors, including transforming growth factor-β (TGF-β), reactive oxygen species (ROS), and nuclear factor-kappa B (NF-κB). TGF-β and ROS induce HSC activation [5], whereas NF-κB inhibits HSCs apoptosis [6]. TGF-β is produced by several liver cells, including a-HSC [5]. Conversely, HSC is also the main target cell for TGF-β [7]. TGF-β promotes the production of ROS in various types of liver cells. Conversely, ROS can also stimulate latent TGF-β activation and induce the expression of the TGF-β gene [8], [9]. It creates a never-ending loop causing continuous HSCs activation. Therefore, targeting TGF-β inhibition can prevent HSC activation.

NF-κB is a protein complex involved in HSC activation. NF-κB inhibits HSCs apoptosis. Therefore, inhibiting NF-κB activation can promote a-HSC apoptosis by reducing the anti-apoptotic function of NF-κB [10].

Spontaneous resolution of liver fibrosis is primarily mediated by a-HSC apoptosis. Apoptosis of a-HSCs reduces inflammation, thereby enabling repair of liver architecture. The elimination of the injury etiology causes the decrease of inflammatory stimulation and the cellular and humoral factors activating HSCs. This phenomenon was followed by the resolution of liver fibrosis. Apoptosis of a-HSC is thought to play an important role in the resolution of liver fibrosis [3].
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Some proteins are involved in the apoptosis of a-HSC, such as tumor necrosis factor-related apoptosis-inducing ligand-receptor 2 (TRAIL-R2) and caspase-3. These proteins were involved in the apoptosis pathway. TRAIL is a protein that may contribute to the apoptotic elimination of a-HSC through binding to its dedicated receptors. TRAIL-R2 is the most expressed by a-HSCs [11]. TRAIL-TRAIL-R2 binding enhances extrinsic pathway apoptosis through caspase 8/10 and leads to increased activation of caspase-3.

Moringa oleifera (MO) is one of the herbs from the Moringaceae family widely grown in Indonesia. Some studies reported the anti-liver fibrotic effect of MO [12], [13], [14], [15], [16]. The mechanism of the action of MO as the anti-liver fibrotic agent is related to antioxidant and anti-inflammatory effects [17]. These studies have not yet clearly elucidated the anti-fibrosis mechanism of MO related to a-HSCs as primary cells in liver fibrosis progression. This study was the first report that revealed the mechanism of MO in the prevention of liver fibrosis by inhibiting HSCs activation and inducing a-HSC apoptosis through an extrinsic pathway.

Materials and Methods

This study was conducted at Brawijaya University, Malang, Indonesia. Materials included CCl4 (MERCK, Schuchardt, Germany), corn oil, 0.9% NaCl, ketamine, and 10% formalin. Primary antibodies such as TGF-β1 mouse monoclonal antibody, NF-κB p65 mouse monoclonal antibody, caspase-3 mouse monoclonal antibody, and DR5/TRAIL-R2 mouse monoclonal antibody were purchased from Santa Cruz Biotechnology, Inc. Anti-alpha smooth muscle actin (α-SMA) rabbit monoclonal antibody was purchased from Boster Biological Technology, and In Situ Cell Death Detection Kit was purchased from Roche. Secondary antibodies using rhodamine-conjugated antibody and IgG FITC-Conjugated Antibody were obtained from Rockland Immunochemicals. MO leaf powder was obtained from Materia Medica Batu, Malang, Indonesia. Ethanol 96% was used as a solvent in the extraction process.

For animals, male rats Rattus norvegicus (200–300 g) of Wistar strain were obtained from Rattus Breeding Center Singsosari, Malang, Indonesia. Cages were labeled, and each cage contained one rat with good ventilation. The cage was placed at room temperature 25°C–28°C and humidity 50%–70%. The rats were adapted for 7 days with a standard diet before being treated. All experimental animal treatment procedures referred to the institutional and local committee on the care and use of animals of Brawijaya University School of Medicine, Malang, East Java, Indonesia, number: No 76/EC/KEPK/03/2018.

MO leaves powder was extracted through a maceration process using 96% ethanol. A total of 100 g of MO leaf powder were immersed in 900 mL 96% ethanol overnight. Next, the liquid was filtered and then concentrated using a rotary evaporator to remove the solvent. Then, the concentrated extract was stored for further use. The extract was analyzed using liquid chromatography–mass spectrophotometry to determine the phytochemical compounds in extract [18]. Then, the extract was dissolved using distilled water with a dose of 150, 300, or 600 mg/kg BW.

This method was designed to describe the use of MO as an anti-fibrosis agent that can prevent liver fibrosis before rats develop into liver fibrosis. This design was intended to represent subjects with risk factors for liver fibrosis. Rats were divided into five groups: The control, model, and three treatment groups (MO150, MO300, and MO600). Each group consisted of six rats. The control group received 0.9% NaCl injection twice a week for 14 weeks. The model group received an injection of 10% CCl4 1 cc/kg BW intraperitoneally twice a week for 12 weeks and continued with a dose of 2 ccs/kg BW for the past 2 weeks. Both the control and model groups received distilled water orally through sonde = gavage needle every day. The MO150, MO300, and MO600 groups received 10% CCI4 injection as in the model group and supplemented orally with MO leaf extract. The dosage of MO leaf extract for the MO150, MO300, and MO600 group was 150 mg/kg, 300 mg/kg, and 600 mg/kg BW, respectively. Graded doses were used to determine the dose-dependent anti-fibrotic effect of MO. Rats were sacrificed 48 h after the last CCI4 injection by intramuscular injection of ketamine 50 mg/kg BW, and then, the liver was collected and stored for slide preparation. The design of the research is presented in Figure 1.

Double immunofluorescence staining and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) analysis was applied to the liver section. The liver was fixed using formalin and then rehydrated in ethanol and embedded in paraffin block. The embedded liver in paraffin block was cut into 5 μm thick sections using microtome and mounted on a glass slide. The liver section was stained using hematoxylin and eosin (H and E) and Masson’s trichrome as previous reports to determine the degree of liver fibrosis using the METAVIR score [18], [19]. Furthermore, the liver section was analyzed using double immunofluorescence...
staining. Briefly, the liver section was fixed with 4% paraformaldehyde for 15 min, washed with PBS 3 times, permeabilized with Triton X-100 2% for 5 minutes, and blocked with blocking solution (2% BSA and 2% FBS in PBS) for 30 minutes. According to standard manuactory procedures, secondary, primary antibodies, and TUNEL reagent were used in specific dilutions. Double immunofluorescence staining was performed using anti-α-SMA rabbit monoclonal antibody and some antibodies primer (caspase-3, TGF-β, NF-κB, and TRAIL-R2) and TUNEL. Primary and secondary antibodies were incubated for 1 h. DAPI staining was used for TUNEL analysis. The images were obtained using a confocal laser scan microscope Olympus FV 1000 and FSX-100 microscope with the appropriate filter combinations. The expression of each marker was analyzed by measuring the fluorescence intensity using ImageJ software.

All data were analyzed using Statistical Product and Service Solution software, IBM Corp. Released 2013. IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp. With a significance level at p < 0.05. The statistical analysis used one-way analysis of variance followed by Tukey's post hoc test for parametric data or the Kruskal–Wallis test and the Mann–Whitney U-test for non-parametric data.

Results

TGF-β, NF-κB, TRAIL-R2, and caspase-3 expressions were analyzed using immunofluorescence staining. The expression of each marker was indicated by fluorescence signal that appeared as a glaring color (vigorous intensity) (Figure 2). In this study, TGF-β, NF-κB, caspase-3, and TRAIL-R2 expressions were explicitly analyzed in a-HSC by double immunofluorescence staining with α-SMA as a-HSC marker to determine their specific role against a-HSC in liver fibrogenesis. The example of double immunofluorescence staining showed in Figure 3. Colocalization of TGF-β with α-SMA showed as merge (dark yellow), indicating the expression of TGF-β in a-HSC. The expression of TGF-β in a-HSC of the model group was 1.5-fold higher than the control group (p = 0.000) (Figure 4a). It indicated that in the condition of liver fibrosis, a significant increase was found in TGF-β expression in a-HSC. The expressions of TGF-β in a-HSC of the treatment groups were significantly lower than the model group (p = 0.000). Although the data trend of the treatment groups fluctuated, the TGF-β expression did not provide a significant difference between the three doses of the treatment groups.

Expression of NF-κB in a-HSC of the model group was 2.5-fold significantly higher than control (p = 0.000). These results proved that in liver fibrosis rats, an increased expression of NF-κB in a-HSC occurred. The expressions of NF-κB in MO150 (p = 0.314), MO300 (p = 0.000), and MO600 (p = 0.000) were lower than in the model group (Figure 4b). This result proved that MO leaves extract could reduce NF-κB expression in a-HSC.

The extrinsic pathway of a-HSC apoptosis was analyzed by immunofluorescence staining of TRAIL-R2 (DR5) death receptor. Expression of TRAIL-R2 in a-HSC was characterized by co-localization between α-SMA (a marker for a-HSC) and TRAIL-R2. The TRAIL-R2 expression in a-HSC of the model group was slightly higher than control (p = 0.894) (Figure 4c). These results indicated that the apoptotic activity of a-HSC through the TRAIL-R2 pathway between no fibrosis rat and fibrosis rat was not much different. The administration of MO leaf extract to MO150, MO300, and MO600 groups caused TRAIL-R2 expression in a-HSC to be significantly higher than model (respectively; p = 0.008, p = 0.033, and p = 0.000). The expression of TRAIL-R2 in a-HSC of the MO300 group was lower than MO150, but it was not significantly different (p = 0.139). Increasing the dosage of MO leaf extract to 600 mg/kg BW in MO600 caused the expression of TRAIL-R2 in a-HSC to be higher than MO300 (p = 0.007). These results indicated that the administration of MO demonstrates a fluctuating effect on the expression of TRAIL-R2 in a-HSC. Furthermore, this phenomenon happened to the expression of TGF-β (Figure 4a). This fluctuating effect forms a pattern that low TGF-β expression caused high TRAIL-R2 expression and vice versa.

Caspase-3 is a marker that is often used to describe the execution process of apoptosis. The expression of caspase-3 in a-HSC of the model was 1.73-fold significantly lower than control (p = 0.000) (Figure 4d). It showed that in liver fibrosis, apoptosis of a-HSC rarely occurred through the extrinsic or the intrinsic pathway. The administration of MO leaf extract caused the expression of caspase-3 in a-HSC to be significantly higher (p = 0.000) than in the model group. These results proved that the a-HSC apoptosis process through the extrinsic pathway significantly increased after MO administration.

Apoptosis of a-HSC was characterized by colocalization between SMA alpha expression and TUNEL (Figure 5). TUNEL value in the model group was significantly 1.24-fold lower than the control group (p = 0.001) (Figure 4e). It showed that the apoptotic activity of a-HSC in liver fibrosis conditions was low so that the fibrogenesis process continued. TUNEL expression of the treatment groups was significantly higher than the model group (p = 0.000). This result showed that the administration of MO caused the increased apoptotic activity of a-HSC. The administration of MO at a dose of 600 mg/kg BW caused the increased apoptotic activity of a-HSC 3-fold compared to the model group.
Discussion

Chronic liver disease is always associated with the abundance of oxidants in the liver tissue [20]. ROS caused hepatocyte apoptosis and triggered inflammatory reaction [21]. TGF-β was recognized as the most critical growth factor in liver fibrogenesis. TGF-β together with ROS strengthened each other for activating q-HSC [22], [23]. TGF-β is a profibrotic cytokine master with pleomorphic abilities, which can increase or decrease the system involved in the liver fibrogenesis process [5]. Activation of the TGF-β pathway in HSC resulted in collagen secretion, whereas activation of the TGF-β pathway in Kupffer cells triggered an inflammatory response [24]. This study may be the first report providing evidence that MO leaves extract can decrease TGF-β expression in a-HSC. The mechanism of MO in decreasing TGF-β expression in a-HSC was still unknown. One study reported that quercetin could inhibit the secretion of TGF-β1 Kupffer cells and a-HSC, as evidenced by reducing serum TGF-β1 mRNA levels [25]. In this study, MO contained quercetin. It was thought to demonstrate an indirect effect on decreasing TGF-β1 expression in a-HSC. Administration of MO caused the decrease of MDA expression [18], and hepatocyte necroptosis inhibited the activity of a-HSC and Kupffer cells in producing TGF-β1. It proved that MO exhibits an inhibitory effect on the activation of quiescent-HSC to activated-HSC.

One of the a-HSC apoptotic stimuli was the activation of death receptors through TRAIL-R2 or TRAIL-R2 [26]. However, in liver fibrosis conditions, a-HSC secreted a lot of TGF-β, causing NK cells to degranulate and decrease the production of IFN-λ. It caused the reducing function of NK cells in inducing...
apoptosis of a-HSC [27]. The administration of MO leaf extract was proven to drive the expression of TRAIL-R2 a-HSC significantly higher than the model group (Figure 4c). This higher apoptotic stimulus for a-HSC was followed by an increase in caspase-3 expression (Figure 4d), an increase in apoptotic activity of a-HSC.
...and a decrease in the number of a-HSC. Increased apoptotic activity of a-HSC and decreased number of a-HSC led to an improvement in the degree of liver fibrosis.

NF-κB demonstrates a key role in liver fibrogenesis by increasing inflammatory mediators, prolonging HSC survival, and inducing HSC activation [28]. The study showed that decreased levels of NF-κB correlated with improvement in the degree of liver fibrosis [29]. Other studies reporting anti-apoptotic effects; a-HSC of NF-κB was due to a decrease in the expression of the Bcl-2 family. The decreasing of NF-κB expression in this was thought to reduce the anti-apoptotic effect of NF-κB and leads to induce apoptosis of α-HSC. However, the path analysis of the result of this study showed that the anti-apoptotic effect of α-HSC through the NF-κB pathway was fragile (data not shown).

Caspase-3 plays as an executor of apoptosis through the extrinsic and intrinsic pathways [30]. Apoptosis of a-HSC through the extrinsic pathway is initiated by ligand receptor TRAIL binding [31] or through inhibition of the NF-κB pathway [32]. This study proved that the expression of caspase-3 in the a-HSC of the model group was significantly lower than in the control group. The administration of MO leaves extract caused the expression of caspase-3 in a-HSC to be significantly higher than in the model group. The expression of caspase-3 in the MO600 group was even closed to caspase-3 expression in the control group (Figure 4d). These results proved that in liver fibrosis conditions, MO could increase the expression of TRAIL-R2 and caspase-3 in a-HSC resulted in inducing apoptosis of a-HSC through the extrinsic pathway. Apoptosis of a-HSC was in line with the high expression of TRAIL-R2 and caspase-3 in the treatment groups. Hence, researchers suspected that the apoptotic induction effect of a-HSC by MO was due to the increased expression of TRAIL-R2 and caspase-3 in a-HSC.

MO contained at least 110 identified active compounds. Some compounds showed positive effects on health. The secondary metabolic of MO exhibits antioxidant, anti-inflammatory, anti-cancer, and other effects derived from flavonoids, glucosides, and glucosinolates [33]. In this study, the secondary metabolic content of MO included quercetin and kaempferol [18]. Quercetin and kaempferol were compounds with antioxidant [34], anti-inflammatory [35], and apoptosis induction effects [36].

So far, no studies exist that reported the role of MO on apoptosis a-HSC. This research was the first study reporting that MO can induce apoptosis of a-HSC through the extrinsic pathway (TRAIL-R2). Researchers suspected that the apoptosis induction mechanism of a-HSC occurred due to the quercetin contained in MO. It was consistent with the previous studies reporting the anti-proliferative effect of quercetin against a-HSC through growth control and induction of apoptosis a-HSC [41].

This study revealed that MO acts as an anti-liver fibrosis agent through multi-target point through inhibition of HSC activation and induction of a-HSC...
apoptosis (Figure 6). The interesting point of this study was the fluctuating trend expression of TGF-β1 and TRAIL-R2 at various doses of MO. These fluctuating effects were common in administering herbs containing several active compounds. The effect depended on the dose, chemical molecular composition, and the amount of active compound [42]. In this study, the analysis of TGF-β1 and TRAIL-R2 expression was carried out specifically in a-HSC. They are influenced by ligand and receptor binding. One study reported that when TGF-β1 bound to the TGFR1 receptor, it caused an increase in intracellular TRAIL-R2 accumulation and decreased TRAIL-R2 expression on the surface of breast cancer cells [43]. The exact mechanism was still unknown, but it might explain the results of this study.

### Conclusion

Ethanol extract of MO leaves inhibited liver fibrosis through inhibition of HSC activation and increased a-HSC apoptosis. Inhibition of HSC activation by MO occurred through an inhibitory mechanism of TGF-β expression in a-HSC. Increased apoptosis of a-HSC occurred through the TRAIL-R2-mediated extrinsic apoptotic pathway.
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References


