

The Effect of *Moringa oleifera* Seed Oil (MOSEIL) on TGF- β Expression in Mice (*Mus musculus*) Model of Liver Fibrosis

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Abstract. Liver fibrosis represents a progressive pathological stage leading to cirrhosis and hepatocellular carcinoma, primarily mediated by the TGF- β /SMAD signaling pathway. This study aimed to evaluate the effect of *Moringa oleifera* Seed Oil (MOSEIL) on TGF- β expression in *Mus musculus* (Balb/c) with CCl₄-induced liver fibrosis. Experimental groups consisted of a normal control (K0), olive oil control (K-), positive control (K+), and three treatment groups (P1–P3) receiving different ratios of CCl₄ : MOSEIL, namely P1 (1:8), P2 (1:16), and P3 (1:32). Immunohistochemical analysis revealed a marked increase in TGF- β expression in the positive control group, while MOSEIL administration resulted in a dose-dependent reduction in expression intensity, with the most prominent effect observed in P2. Histological observations supported these findings, showing decreased necrosis and inflammatory cell infiltration in P1 and P2. In contrast, the P3 group demonstrated a re-elevation of inflammatory activity and fibrotic features, suggesting that excessive dosing may attenuate the protective effects of MOSEIL. The reduced expression of TGF- β in MOSEIL-treated groups indicates that MOSEIL exerts its antifibrotic activity partly through inhibition of this profibrotic cytokine. Therefore, MOSEIL exhibits potential as a natural hepatoprotective and anti-fibrotic agent against chronic liver injury, with an optimal effect observed at a moderate dose.

1 Introduction

Hepatocellular carcinoma (HCC) is the most common primary liver cancer in the world, accounting for 75-85% of cases, followed by Intrahepatic cholangiocarcinoma (ICC) at 10-15% [1]. Major etiological factors include chronic viral infections (hepatitis B and C), Alcohol-related Liver Disease (ALD), Non-Alcoholic Fatty Liver Disease (NAFLD), exposure to hepatotoxic agents such as aflatoxin and carbon tetrachloride (CCl₄), as well as genetic susceptibility [2]. The above risk factors can cause an unresolved inflammatory

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response and leads to the development of fibrosis, cirrhosis, and even liver cancer. In addition, these risk factors also leading to sustained activation of Hepatic Stellate Cells (HSC), Excessive Extracellular Matrix (ECM) deposition, increased type I collagen expression, and progressive fibrotic remodeling of liver tissue .

At the molecular level, liver fibrosis is primarily driven by profibrotic signaling pathways, particularly Transforming Growth Factor- β (TGF- β), which induces HSC activation through the stimulation and phosphorylation. In addition to SMAD signaling, TGF- β activates non-SMAD pathways, including MAPK, ERK, JNK, p38, and PI3K/AKT, which further promote HSC transdifferentiation into myofibroblasts [3]. These TGF- β mediated profibrotic signaling cascades drive the transcription of fibrogenic genes and contribute to the progression of liver fibrosis. Therefore, effective targeting of TGF- β mediated signaling remains a major clinical challenge, given the global burden of liver fibrosis, its link to HCC progression, and limited therapeutic efficacy. Despite advances in understanding the molecular pathogenesis of liver fibrosis, effective pharmacological interventions are still lacking.

Natural products have emerged as promising candidates for antifibrotic therapy. Extracts from *Moringa oleifera* leaves have been reported to attenuate liver fibrosis by reducing TNF- α and p38 MAPK expression, as well as lowering serum ALT and AST levels in CCl₄-induced liver injury models [4-5]. These findings highlight the antifibrotic and anticancer potential of *Moringa oleifera*. However, most previous studies have focused predominantly on leaf extracts, while the therapeutic potential of *Moringa oleifera* seed oil (MOSEIL) remains poorly explored.

MOSEIL contains bioactive compounds, particularly oleic acid, phytosterols, flavonoids, and tocopherols, which are largely absent or poorly extracted in polar leaf extracts. Oleic acid is the predominant unsaturated fatty acid and has been shown to modulate inflammatory signaling pathways and exert anti-inflammatory effects [6]. In addition, bioactive flavonoids such as kaempferol, which have demonstrated the ability to suppress pro-inflammatory cytokines and markers of the MAPK signaling pathway [7]. These bioactive components suggest that MOSEIL may exert antifibrotic effects through modulation of inflammatory and profibrotic signaling pathways.

Unlike previous studies focusing on *Moringa oleifera* leaf extracts, this study evaluates the antifibrotic potential of MOSEIL, which remains relatively underexplored. Therefore, this study aims to investigate the effects of MOSEIL on liver fibrosis in a CCl₄-induced Balb/c *Mus musculus* model, with a particular focus on the TGF- β signaling pathway. We hypothesize that MOSEIL attenuates liver fibrosis primarily by suppressing the expression of TGF- β , thereby limiting downstream profibrotic signaling involved in HSC activation and ECM accumulation.

2 Experimental details

1.1 Sample preparation and ethical clearance

The research sample consisted of *Moringa oleifera* Seed Oil (MOSEIL) Madura obtained from PT. Alami Moringa Sejahtera. The moringa seed extraction process used a maceration method with two solvents: n-hexane and acetone. Ethical clearance was issued by the Animal Care and Use Committee of Brawijaya University under Decree No. 117 KEP-UB-2024 in 2024. This study used 30 male Balb/c mice (*Mus musculus*) aged 8 weeks, weighing between 25-30 grams, obtained from Murhin Farm, Malang, Singosari. The mice were acclimatized for one week before treatment.

1.2 Experimental design and in vivo approach

This study used a Completely Randomized Laboratory Design (CRLD) with a post-test-only control for 12 weeks. The mice were divided into control and treatment groups. Treatment was carried out by intraperitoneal injection at a dose of 2 $\mu\text{L/g}$ body weight (BW), administered three times a week. The study groups consisted of a normal control (K0; untreated), a negative control (K-; olive oil only), a positive control (K+; CCl_4 diluted in olive oil at a ratio of 1:3), and three treatment groups receiving a mixture of CCl_4 and MOSEIL at volume ratios of 1:8 (P1), 1:16 (P2), and 1:32 (P3).

1.3 Dissection and organ collection

Mice treated for 12 weeks were dislocated and dissected. The livers were separated from the other organs, weighed, and documented for morphological observation. The livers were fixed in flasks containing 10% formalin for histological analysis and biomarker expression measurement.

1.4 Hematoxylin-Eosin (H&E) staining

Histochemical measurements with eosin were performed to evaluate tissue morphological changes due to chronic damage, such as fibrosis, nodule regeneration, and hepatocyte necrosis. The liver tissue samples were taken and fixed in formalin, processed into thin sections, and stained using hematoxylin-eosin (H&E). This histological analysis allows identification of abnormal structures, the degree of fibrosis, and cell distribution, which are essential for the diagnosis and assessment of liver fibrosis severity.

1.5 TGF- β measurement with immunohistochemistry

Immunohistochemistry was used to identify the expression of TGF- β , a marker of HSC activation. Preparation of immunohistochemical slides included deparaffinization, antigen retrieval, endogenous peroxidase blocking, non-specific protein blocking, incubation with a primary anti-TGF- β 1 antibody (rabbit polyclonal, Bioss USA, catalog no. bs-0086R, reactive with mouse tissue), followed by secondary antibody incubation, application of diaminobenzidine (DAB) chromogen for positive brown staining, counterstaining using Mayer's hematoxylin, and mounting with positive controls (tissue known to express TGF- β) and negative controls (omission of the primary antibody) processed in parallel.

1.6 Data analysis

Immunohistochemical analysis was performed using Fiji software version 1.54p. Slides were coded prior to imaging. However, image quantification was performed with knowledge of treatment groups. Quantification was conducted by separating the DAB-positive area using the color deconvolution method, with identical analysis parameters applied to all images. Statistical analysis was performed using SPSS version 27.0. Normality testing will be performed using the Kolmogorov–Smirnov test. Intergroup differences will be analyzed using parametric one-way analysis of variance (ANOVA) with the Tukey post-hoc test.

3 Result and discussion

Liver morphology may change following exposure to toxic compounds or experimental treatments; therefore, liver weight was assessed as a macroscopic parameter alongside TGF- β expression. One-way ANOVA showed no significant difference in mean liver weight among groups ($p = 0.216$) (**Fig. 1**). The K0 and K- groups showed comparable liver weights (1.92 g and 1.91 g), while the positive control (K+) exhibited a higher mean liver weight (2.24 g), which may be associated with inflammatory-related hepatomegaly. The P1 and P3 groups showed higher mean liver weights than K+, whereas the P2 group demonstrated a lower mean liver weight. However, liver weight alone cannot be considered a primary or definitive parameter for assessing liver condition, as it does not necessarily reflect underlying histopathological or molecular changes.

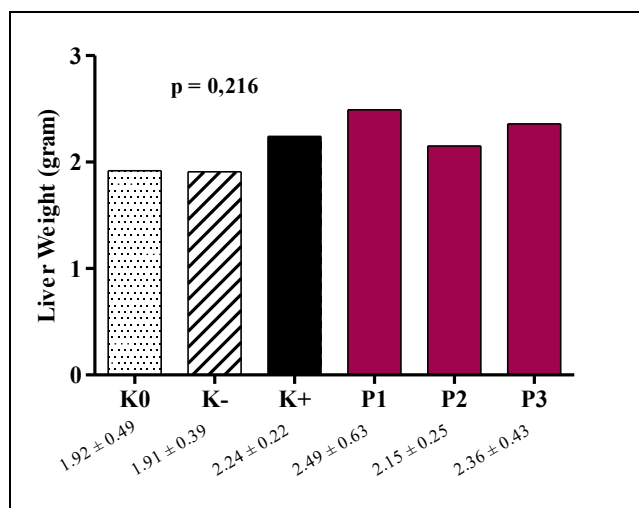


Fig. 1. Graph of average liver weight of mice (gr)

K0 in **Fig. 2**. shows that the liver appeared reddish-brown with a smooth and uniform surface, without discoloration, nodules, or other visible abnormalities. Histologically, normal liver architecture was observed, characterized by clearly identifiable portal triads (Kiernan's trigone) and a well-preserved hepatic lobular structure with cords of polygonal hepatocytes radiating from the central vein and intact sinusoidal architecture, consistent with the features of normal liver histology [8]. Similarly, the K- group, which received olive oil alone, showed a macroscopically normal liver appearance comparable to K0. Histological examination of the K- group revealed intact hepatic lobules with hepatocytes arranged in organized hepatic cords and well-defined sinusoids, without evidence of necrosis, inflammatory cell infiltration, or sinusoidal dilatation.

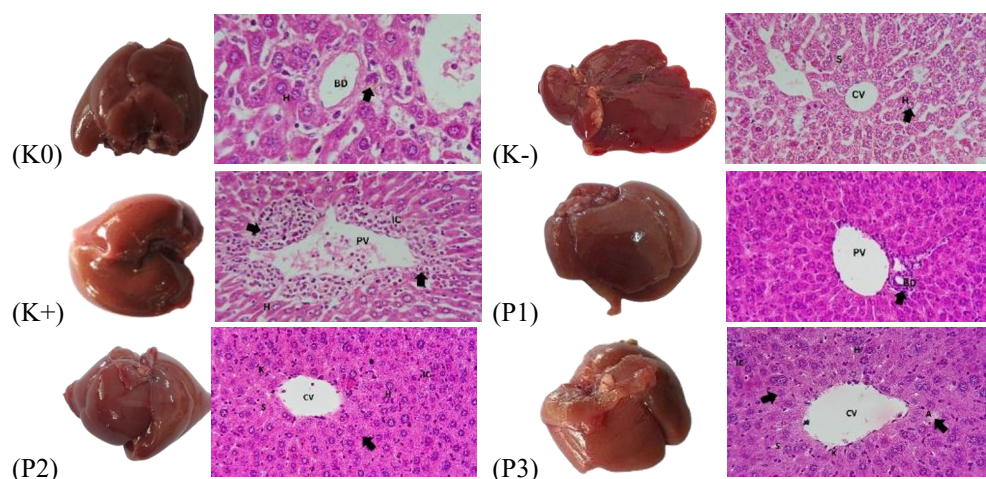


Fig. 2. Liver macroscopy and liver histology of mice with H&E staining at 400x magnification. Histological examination using H&E staining revealed characteristic features of fibrosis, including sinusoidal dilation and infiltration of inflammatory cells.

CCl_4 is known to induce significant liver injury, including hepatocyte necrosis and inflammatory cell infiltration [9]. Consistent with this, the K^+ group exhibited a paler liver with visible surface spots, accompanied histologically by inflammatory cell infiltration around the portal vein and Glisson's capsule, as well as areas of hepatocyte necrosis with disorganized cellular architecture. These findings indicate marked hepatic inflammation due to toxic injury. In contrast, groups receiving combined CCl_4 and MOSEIL showed varying degrees of histological alteration, suggesting differential modulation of liver damage.

Histopathological evaluation showed that the P1 group exhibited smaller necrotic areas and reduced inflammatory cell infiltration compared with the K^+ group, while the P2 group demonstrated the least histopathological damage. In contrast, the P3 group showed increased necrosis and inflammatory cell infiltration in the liver parenchyma and portal triads, indicating aggravated liver injury at a higher MOSEIL ratio. This pattern suggests a dose-dependent response, with protective effects at lower ratios (P1 and P2). In line with the histological alterations observed in P3, previous studies have reported hepatotoxic and genotoxic effects of *Moringa oleifera* at high doses [10]. Conversely, the protective histological features in P1 and P2 are consistent with reports that MOSEIL improves liver histological architecture in CCl_4 -induced hepatotoxicity based on H&E analysis [11].

Areas exhibiting positive TGF- β immunoreactivity, indicated by arrow markers in **Fig. 3.**, represent increased TGF- β protein expression in hepatocytes. This immunoreactivity was predominantly observed in periportal and perivascular regions, which are commonly associated with HSC localization during fibrogenesis. Increased signal intensity reflects higher TGF- β expression, suggesting enhanced profibrotic signaling activity, whereas regions showing minimal immunoreactivity indicate low TGF- β expression and relatively preserved tissue architecture. Consistent with this pattern, the CCl_4 -induced group displayed greater TGF- β immunoreactivity compared with both the normal control and MOSEIL-treated groups.

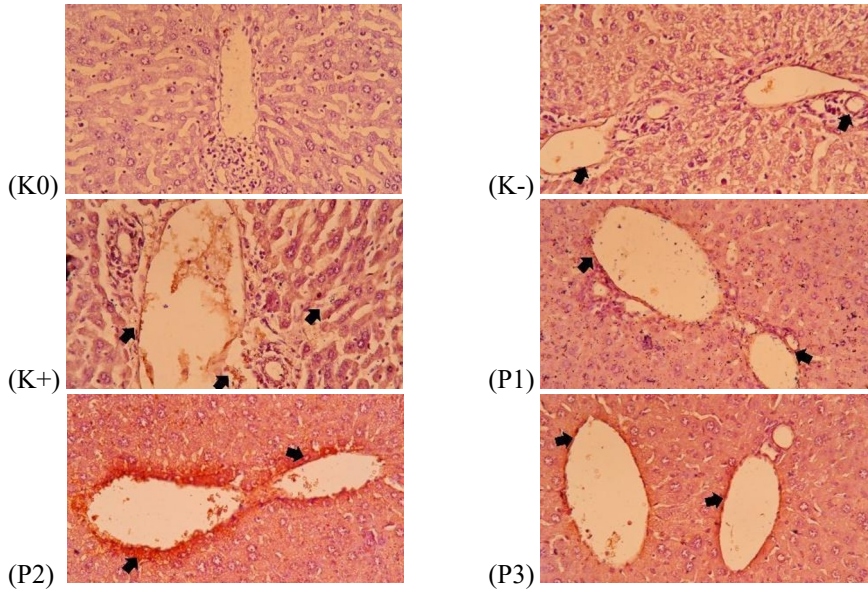


Fig. 3. TGF- β expression with IHC staining at 400x magnification. Immunohistochemical staining revealed positive expression indicated by brown (DAB) coloration in hepatocytes around the portal triad, while areas showing a purple coloration represented negative or weak expression.

Quantitative immunohistochemical analysis using Fiji software (**Fig. 4.**) demonstrated a marked increase in TGF- β expression in the positive control group (K+; 97.876 ± 0.596) compared with the normal control (K0; 55.393 ± 0.452), confirming CCl₄-induced upregulation of this profibrotic protein. Administration of MOSEIL resulted in reduced TGF- β expression across treatment groups, with values of 84.919 ± 0.466 (P1, 1:8), 81.115 ± 0.751 (P2, 1:16), and 87.131 ± 0.714 (P3, 1:32). The greatest reduction was observed in the P2 group, indicating attenuation of CCl₄-induced TGF- β expression by MOSEIL.

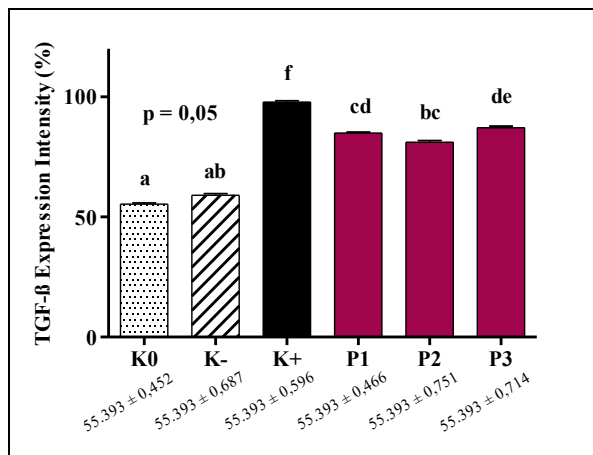


Fig. 4. TGF- β expression intensity (%). Immunohistochemical analysis showed low TGF- β expression in the normal control (K0), a slight increase in the negative control (K-), strong positive expression in the positive control (K+), and a marked reduction in the MOSEIL-treated groups (P1–P3).

The reduction in TGF- β expression observed in MOSEIL-treated groups may be associated with decreased activation of HSC and subsequent limitation of ECM

accumulation, a key event in liver fibrogenesis. This effect is plausibly linked to the antioxidant and anti-inflammatory properties of bioactive compounds present in MOSEIL, including quercetin, kaempferol, and caffeic acid, which enhance antioxidant activity, promote tissue repair, and suppress oxidative stress through modulation of multiple biological targets [12]. Activation of Nrf2 by bioactive compounds from *Moringa* isothiocyanate (MIC-1) has been reported to indirectly suppress TGF- β mediated profibrotic signaling by reducing oxidative stress and inflammatory stimuli that drive TGF- β activation [13]. In addition, phenolic acids have been shown to exert protective effects in liver disease, in part through modulation of oxidative stress and inflammatory processes that contribute to fibrotic signaling pathways [14].

It should be noted that the present study did not evaluate SMAD phosphorylation or downstream fibrogenic gene expression. As a result, although reduced TGF- β immunoreactivity indicates a modulation of profibrotic signaling following MOSEIL administration, the precise involvement of the canonical TGF- β /SMAD pathway cannot be definitively confirmed. Further studies incorporating molecular analyses are therefore required to clarify the underlying mechanisms.

4 Summary

Based on these findings, MOSEIL demonstrates hepatoprotective and anti-fibrotic potential in a CCl₄-induced liver fibrosis model, as evidenced by reduced TGF- β expression and improved histological features. These results support the potential of MOSEIL as a natural therapeutic candidate for mitigating liver fibrosis progression.

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