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Anti Fibrogenesis Effect of Green Materials *Moringa oleifera* Leaf Powder (MOLP) on the Progression of Hepatocellular Carcinoma

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Abstract. Hepatocellular carcinoma (HCC) is cancer with a high prevalence in the world, Asia, and Indonesia. Some HCC can be caused by a viral infection, consuming alcohol for an extended period, and has recently been associated with metabolic syndrome. Liver fibrogenesis is the initial stage of HCC and significantly correlates to the transforming growth factor-beta (TGF-β) expression. TGF-β leads to disease progression stages, from early liver damage through inflammation and fibrosis to cirrhosis and (HCC). The purpose of this study is to investigate the effect of green material *Moringa oleifera* on the expression of TGF-β in the mice model develop to HCC. This study was conducted using a BALB C mice model treated with intraperitoneal injections of green material Moringa oleifera + CCl_4 at a dose of 1 µL/1 g of BW for six weeks. The result shows a significant effect of green material Moringa *oleifera* on the decrease in TGF-β expression. Hence, green material *Moringa oleifera* can be a potential preventative agent for liver fibrogenesis-linked HCC.

INTRODUCTION

Liver fibrosis is a progressive condition of liver disease, which can progress to cirrhosis and become hepatocellular carcinoma (HCC). HCC is the world's second most common cause of death from cancer [1]. In the Asian-Pacific region, HCC is still a prevalent disease with a high number of deaths [2]. In Indonesia, liver cancer diseases and colon have a high prevalence of 22.5% cause of death, especially in men [3]. In several countries in Asia-Africa, HBV is the most common cause of HCC. At the same time, *Hepatitis C* is the most common disease in America, Northern Europe, and Australia [4]. A higher prevalence of HCC, aside from HCV, HBV, and *liver cirrhosis,* is *metabolic syndrome. A metabolic syndrome* due to obesity and diabetes associated with the early incidence of HCC is a *non-alcoholic fatty liver disease* (NAFLD) and *non-alcoholic steatohepatitis* (NASH) [5]. Based on epidemiological evidence, obesity and *type 2 diabetes mellitus* (T2DM) related to NAFLD are independent risk factors of HCC [6]. Previous research shows that 14% of death due to cancer in men and 20% in women are caused by obesity and dyslipidemia [7].

Carbon tetrachloride (CCl_4) is generally adopted to cause a liver injury model in animals since it prompts free radicals and causes a peroxide chain reaction. Cytochrome P450 transforms CCl_4 to trichloroethane radical (CCl_3), which incorporates oxygen (O) to produce in large quantities of active trichloromethyl peroxyl radical (CCl_3O_2) with additional O molecules [8]. Provision of CCl_4, which repeatedly causes *anisonucleosis* can lead to chronic liver injury

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characterized by the variation of nucleus hepatocytes' size. This condition is related to oxidative liver stress [9]. Hepatic stellate cell (HSC), whose primary function is to stock vitamin A and has an essential role in stimulating the immune reaction through cytokines and chemokines excretion, is triggered by liver damage. Once stimulated, HSCs increase the extracellular matrix protein (ECM) production, leading to liver fibrosis. The HSC activation depends on cytokines, especially fibrogenic cytokines, namely TGF-β [10].

In the liver, TGF-β is considered a fundamental molecule, which controls the organ's size and growth by limiting hepatocytes' proliferation. Targeting the signaling pathway of TGF-β are explored to prevent the progression of the liver [11]. *Transforming growth factor-beta* (TGF)-β contributes to all stages of liver disease progression from the early stage of *liver injury* through inflammation and fibrosis to *cirrhosis* and (HCC) [12]. Offering the apoptosis effect is one of the TGF-β function in normal condition. It is crucial to help differentiation and regeneration of cells, but TGF-β at a high level can cause liver cells' death, along with leading to fibrosis and cirrhosis [13]. Loss of TGF-β activity results in hyperproliferative disturbance and cause cancer [14]. Therefore, it is essential to carry out studies to look at the expression of (TGF)-β in the progression towards HCC. A considerable volume of preclinical, experimental data shows that TGF-β is a promising target for drugs in HCC patients [15].

Green material is a variety of plants obtained from nature to be used as a natural or alternative treatment of various health problems; one of them is cancer. Natural medicine has become an essential source of this alternative therapy; one of them is *green material Moringa oleifera* (moringa). Moringa leaves contain high antioxidants and bioactive compounds and have an essential role in their efficacy [16]. Moringa plant has shown potential as an anticancer agent [17], while its leaf extract has several characters, including anticancer, anti-inflammatory, antioxidant, hypotensive, and antifungal activity. There are phytochemical constituents such as carotenoids, fatty acids, niazirin I and II, niazinin, and niazimicin showing potential as wound healing agents [18]. *Moringa oleifera* leaves contain *kaempferol, b-sitosterol, rhamnetin, glucosinolate,* and *isothiocyanate,* which help in anticancer activity [19]. Phenolic acids, including chlorogenic acid (CGA) from the *Moringa oleifera* plant, have been proven to possess antioxidant, anti-inflammatory, and anti-hyperglycemias characters [20]. *Moringa oleifera* has been used traditionally to treat hyperglycemia, inflammation, bacterial or viral infections, and cancer [21]. Therefore, moringa leaves can be used as an anti-fibrosis agent by suppressing excessive TNF-β expression due to exposure of CCl_4.

The high prevalence rate of HCC sufferers can be used as a base for exploring medicinal substances for early cancer prevention. Cancer is a silent killer that hard to detect in its early progression. This results in the difficulty of treating the early progression of cancer in patients. Therefore, this study investigates the prevention in HCC progressive mice model with *green material* MOLP as an effort to prevent fibrosis by looking at TGF-β expression.

EXPERIMENTAL DETAILS

Animal

The experimental animals used were male mice (*Mus musculus*) strains Balb/C, descent from F9 and F8, which were purchased at the Fatma Veterinary Center (PUSVETMA) Surabaya, aged 2 - 3 months with weight 25 ± 2 g. The 30 experimental animals were acclimatized for approximately two weeks. The experimental animals used had obtained ethical standards in the Research Ethics Commission (Animal Care and Use Committee) of Brawijaya University, Indonesia, with the ethical clearance number of 1184-KEP-UB. Food and water were given with procedure ad libitum. The treatment of this research was carried out at the Laboratory of Animal Care, Faculty of Mathematics and Natural Sciences, Department of Biology, State University of Malang, Indonesia.

MOLP Green Materials Preparation

Moringa leaf powder was prepared by the traditional drying technique. Fresh Moringa leaf was washed in underwater flow, hanged, and dried at room temperature for 3 - 4 days., then it was blended and filtered using a 300-mess sieve.

Dosage Making for MOLP Green Materials and CCl_4

For the stock solutions, the CCl_4 was dissolved in corn oil in 1 : 3 ratio, with 1 mL of CCl_4 and 3 mL of corn oil. For the CCl_4 dosage, the provisions of 1 µL/1 g BW in mice was used. The dosage on green materials *Moringa oleifera*, LD50 green materials *Moringa oleifera* in mice, is 2000 mg/kg [22]. The conversion result of LD50 green

materials *Moringa oleifera* into mice became 2240 mg/kg BW. The results of LD50 green materials *Moringa oleifera* to mice used dosage provisions of green materials *Moringa oleifera* of 100, 200, and 400 mg/kg BW.

Experimental Design

In this study, experimental animals were given five levels of treatment with five repetitions each. Placebo, positive control (CCl_4 *ip* injection only), P1 (CCl_4 group + green material *Moringa oleifera* 100 mg/kg BW) ($n = 5$), P2 (CCl_4 + green material *Moringa oleifera* 200 mg/kg BW), and P3 (CCl_4 + green material *Moringa oleifera* 400 mg/kg BW). The liver fibrosis model was injected with CCl_4 intraperitoneal with CCl_4 dosage (1 μL/1 g of BW). The treatment process was carried out in six weeks by giving treatment three times a week. The treatment was carried out simultaneously by injecting 0.1 mL of CCl_4 intraperitoneal taken from the mother liquor and giving green material *Moringa oleifera* of 0.1 mL of green material taken from *Moringa oleifera* solution. After the six weeks of treatment, the mice were dissected, and their liver was taken. Liver samples had to be fixed with 10% formalin at least 7 h before the immunohistochemical process was carried out

Observation of TGF-β Expression and Liver Histology

Hematoxylin-Eosin **(**HE) preparations were investigated using a light microscope. HE preparations were looked for liver cells that had inflammation. Also, we were looking for several cells overrun by immune cells. The preparations were only used for comparison of immunohistochemical preparations. Meanwhile, the observation of immunohistochemical preparations was carried out by taking photos from a light microscope with 100 times magnification. The expression of TGF-β would appear brown in color, which was expressed in the cytoplasm of hepatocytes.

Statistical Analysis

We employed SPSS software to analyze the data in this study. The normality test was carried out by the Kolmogorov – Smirnov test. Primary data in the form of TGF-β expression and secondary data in the form of bodyweight of mice and liver weight were analyzed using parametric one-way analysis of variance (ANOVA) at the 95% confidence level ($\alpha = 0.05$). If the Anova calculation results showed an influence, then it proceeded with the Duncan post hoc test.

RESULTS AND DISCUSSION

The average body and liver weight data of mice are provided in Table 1. The statistical analysis results show no significant difference between the body weight and the liver weight of mice. Bodyweight is commonly being used as a valuable index after the *treatment of* CCl_4 in the efficacy test of CCl_4 related organ impairment [23]. In contrast, the injection of CCl_4 increases the bodyweight up to 33 g [24]. Mice injected with CCl_4 show small nodulation and enlargement of the liver and increased liver weight [25]. A previous study conducted using CCl_4 injection and treatment of extract *blue honeysuckle* (BH) showed that liver weight decreased. The study revealed that BH extract had a hepatoprotective effect [24].

TABLE 1. The Baseline Data of Body Weight and Liver Weight

Groups	Body Weight	Liver Weight
Placebo	26.58 ± 0.27	1.38 ± 0.01
Positive Control	25.89 ± 0.29	1.29 ± 0.07
P1	26.46 ± 2.21	1.46 ± 0.11
P2	25.31 ± 2.39	1.31 ± 0.22
P3	24.99 ± 13.73	1.30 ± 0.14

Data are shown by Mean ± SD with $p < 0.05$).

TGF-β is a cytokine that can be employed as a liver fibrosis marker, and if there is an inhibition in the TGF-β signaling pathway, it will protect the liver from fibrosis [26]. The expression of TGF-β in patients with liver fibrosis shows a high result of the expression, which can be used as a sign that the liver is in working condition [27]. The expression of TGF-β on immunohistochemical preparations can be recognized by the brownish spot/color that appears (Figure 1).

The TGF-β expression is decreased after MOLP treatment and tends to decrease along with the increase of MOLP dosage. The different brown color intensity indicates the significant alteration of TGF-β after MOLP co-treatment. This data imply that green material *Moringa oleifera* can reduce TGF-β levels due to CCl_4 exposure. Moreover, the green material *Moringa oleifera* may suppress HSC activation by inhibiting the TGF-β signaling pathway. Previous studies report that pre-treatment with *Moringa oleifera* demonstrates an anti-proliferative effect that reduces cancer cell growth and induces overall cell death [28]. Also, *Moringa oleifera* leaf extract can reduce hepatocarcinogenesis influenced by *diethylnitrosamine* (DEN) related antioxidant activity to prevent apoptotic [29]. *Moringa oleifera* contains antioxidants and phenolic compounds confirmed to reduce oxidative stress caused by harmful substances [30].

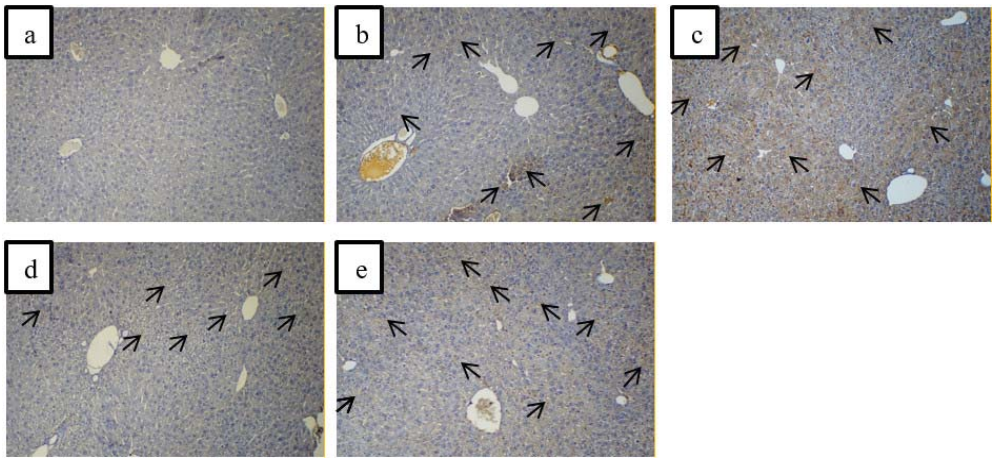

FIGURE 1. Expression of TGF-β of mice Liver in each treatment. a. placebo; b. positive control; c. P1 group; d. P2 group; and e. P3 group. (Black arrow) indicates the presence of TGF-β expression.

The presence of phenolic and flavonoid content of *Moringa oleifera* exerts antioxidant activity to ward off free radicals [31]. *Moringa oleifera* leaves are rich in several bioactive compounds, including beta-carotene, vitamins (B, C, and E), polyphenols, phenolic acids, alkaloids, GLS, ITC, tannins, saponins, oxalates, phytates, and antioxidants [32]. Ethyl acetate in *Moringa oleifera* leaf extract can reduce the production of proinflammatory cytokines, comprising of TNF- α, IL-6, and IL-8 from macrophages derived *monocyte-derived macrophages* (MDM) [33]. *Moringa oleifera* leaves repair and protects the potential against-induced liver damage *thioacetamide* (TAA) [34]. Several compounds within MOLP have been shown to reduce TGF-β levels due to exposure to CCl_4, such as a study showing that phenol can alleviate CCl_4-induced liver fibrosis by suppressing the activation of hepatic stellate cells through inhibition of TGF-β/Smad3 signaling [35].

The histology of the liver with staining *Hematoxylin-Eosin* (HE) is shown in Figure 2. Histology of the liver of mice in each treatment group shows a difference. In this section, some tissues experienced inflammation, which is indicated by the increased number of immune cells. By contrast, mice's liver histology still shows a normal feature in the placebo group without inflammation and or cell damage. Inversely, the positive control group and P1 show mild inflammation characterized by the significant number of immune cells and the presence of sinusoidal dilation. In line with the phenomenon, the previous finding shows that the inflammation tissue increases the immune cell response to injury to liver tissue [36]. In terms of liver inflammation, one of the competent immune cells used as a sign of inflammation is the Kupffer cell. Kupffer cells can be a potential mediator of liver damage [37].

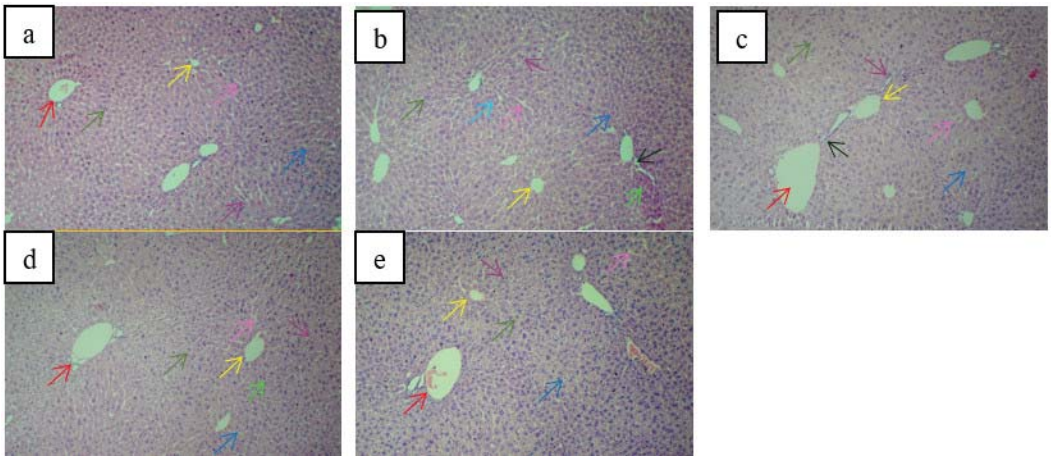

FIGURE 2. Histology of the Liver with *Hematoxylin-Eosin* (HE) staining. a. Placebo; b. positive control. c. P1 group; d. P2 group; and e. P3 group. (Red arrow) shows *vein portal*. (Yellow arrow) shows the *central vein*. (Brown arrow) shows hepatocytes. (Pink arrow) indicates sinusoids. (Green arrows) indicate areas where inflammation is present. (Purple arrow) indicates the presence of a Kupffer cell. (Blue arrow) indicates the presence of immune cells. (Black arrow) indicates sinusoid dilation.

In the P2 group, there are still some immune cells around the *central vein*, which indicate signs of inflammation. This data imply that an increase in the immune cell can sign liver damage [38]. Interestingly, in P3 group do not show the increased number of immune cells and show liver histology results, almost like the Placebo. This preliminary data indicates recovery from the provision of green material *Moringa oleifera*. Several studies suggest that *Moringa oleifera* leaf extract exhibit *antinociceptive* and anti-inflammatory activity in a mouse model of arthritis-induced [39]. Oral administration of Moringa leaf extracts significantly reduces (44-52%) proliferation of HepG2 cells and non-small A549 cell lung cancer cells. These results support the potential of moringa leaf's soluble extract as an oral therapy to treat human liver and lung cancer [40]. MO leaf extract can prevent inflammation through inactivation of NF-kB, inhibiting NF-kB degradation, thereby constraining NF-kB binding to the target DNA. Moringa leaf extracts result in downregulation of proinflammatory mediators, IL-6, TNF- α, and COX-2 [41]. The alteration of TGF-β1, CTGF, and CAT gene expression controlled by treatment using leaf extract *Moringa oleifera* show different antivirus activity in HBV genotypes C and H. This antivirus property is due to the presence of antifibrotic, antioxidant, and the inhibition of IL-6 content [42]. *Moringa oleifera* leaf extract decreases the fibrogenesis through IL-6 and HBsAg secretion in two HBV genotypes.

SUMMARY

The results show that Moringa oleifera co-treatment's green material may alleviate liver fibrogenesis by decreasing the TGF-β expression in the liver. The green material of *Moringa oleifera* leaf powder (MOLP) may reduce hepatic fibrogenesis by suppressing HSC activation, which is associated with reduced oxidative stress and inflammation by inhibition of the TGF-β/Smad3 pathway of signaling. Hence, green material *Moringa oleifera* can be a potential preventative agent for liver fibrogenesis-linked HCC

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