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Hepatoprotective Effects of *Schleichera oleosa* Leaf Extract on TGF-β Expression and Liver Fibrosis in CCl_4-Induced Rat Model

Tias Pramesti Griana[1*], Kholifah Holil[3], Vicki Andrean[1], Aathifah[1], Anik Listiyana[2]

[1]*Anatomy Department, Faculty of Medicine and Health Science, Universitas Islam Negeri Maulana Malik Ibrahim*

[2]*Histology Department, Faculty of Medicine and Health Science, Universitas Islam Negeri Maulana Malik Ibrahim*

[3]*Biology Department, Faculty of Science and Technology, Universitas Islam Negeri Maulana Malik Ibrahim*

[*]*Corresponding author*

Email: tiaspram_esti@kedokteran.uin-malang.ac.id

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Abstract

CCL4 exposure on rat resulted hepatocyte cell necrosis and activated macrophages to produce TGF-β which induced liver fibrosis. Kesambi (Schleichera oleosa (Lour.) Oken) plants have active compounds that affect the inflammatory process. The purpose of this study was to determine the effects of methanol leaf extracts from Kesambi on TGF-β levels and histological feature of liver in rat exposed to CCL4. Thirty male Wistar rat were divided into 5 groups, K- (injected with corn oil i.p), K+ (injected with CCL4 0,5µl/grb.w. i.p, 2x a week), and the treatment group injected with CCL4 0,5µl/grb.w. i.p, 2x a week and administrated methanol leaf extracts from kesambi dose 200mg/kgb.w. (P1), 400mg/kgb.w. (P2), and 600mg/kgb.w. (P3), everyday. After 6 weeks, the rats were dissected. Liver was taken for TGF-β levels measurement by flow cytometry, necrotizing hepatocyte cells calculation and fibrosis score by METAVIR method. TGF-β level of P3 (4,21%) was significantly different (p<0,05) compared to K- (6,34%), K+ (7,21%), P1 (7,80%), and P2 (8,45%). The percentage of ecrosis cells of K+ (54,39%) was significantly different from K- (9,62%), P2 (29,37%), and P3 (18,75%). METAVIR score calculation showed fibrosis in K+ (score 2), P1 (score 2), and P2 (score 1). The administration of methanol leaf extracts from kesambi reduced TGF-β levels and the number of necrotic hepatocyte cells and prevented the formation of liver fibrosis.

1. INTRODUCTION

Chronic liver injury produces an inflammatory response that leads to changes in normal liver structure into regenerative nodules. The most common causes of chronic liver injury are hepatitis C virus infection, excessive alcohol consumption, exposure to chemicals and drugs, and fatty liver due to lipid metabolism disorders known as Non-alcoholic Fatty Liver Diseases (NAFLD) and Non-

alcoholic Steatohepatitis (NASH) [1]. Chronic liver injury is a risk factor for cirrhosis (due to liver tissue fibrosis) and hepatocellular carcinoma. Histopathology of the liver due to chronic injury includes steatosis, hepatocyte necrosis, inflammation, and varying degrees of fibrosis [2]. When liver tissue is injured, macrophages (in this case Kupffer cells) will be recruited to the site of injury mediated by chemokines, namely Monocyte Chemotactic Protein-1 (MCP-1) [3]. Macrophages regulate the inflammatory and fibrosis processes by producing Transforming Growth Factor-β (TGF-β), Interleukin-1β (IL-1β), Interleukin-8 (IL-8), and Platelet Derived Growth Factor (PDGF) [4, 5, 6, 7].

HSC cells are pleiotropic cells that are non-parenchymal cells and fill the perisinusoidal space of Disse. Chronic liver injury activates HSC so that they proliferate and migrate [8]. TGF-β induces the transformation of Hepatic Stellate Cells (HSC) into myofibroblasts and stimulates the synthesis of Extracellular Matrix (ECM) proteins, causing excessive matrix accumulation and producing fibrotic tissue [9].

There are two types of drugs that have been marketed and used as antifibrotics, namely pirfenidone (approved in 2011) and nintedanib (approved in 2014) [10, 11, 12, 13]. In general, the progression of fibrosis can be reduced in patients with antifibrotic administration compared to patients who are not treated with antifibrotics at all. The results of the overall population analysis showed that the antifibrotic therapy that has been used currently cannot increase the survival rate of patients treated with either nintedanib or pirfenidone [14]. The discovery of antifibrotic agents has continued since the last two decades, but until now only two types of antifibrotic drugs have been recognized even though each has its own shortcomings [15]. Therefore, the development of antifibrotic drugs continues to this day.

Various chemical agents are known as carcinogens, used to create animal models of alcoholic and non-alcoholic steatohepatitis and cirrhosis, including carbon tetrachloride (CCl_4) [16]. CCL4 is metabolized in the liver by the cytochrome P450 superfamily of monooxygenases (CYP family) into trichloromethyl radicals (CCL3). Moreover, these radicals interact with nucleic acids, proteins, and lipids, resulting in a disruption of the primary cellular processes in lipid metabolism (fatty degeneration and steatosis) and a decrease in protein. Lipid peroxidation and the destruction of polyunsaturated fatty acids are initiated by the formation of trichloromethylperoxy radicals that result from further oxygenation of CCL3. As a result, membrane permeability in all cellular compartments (mitochondria, endoplasmic reticulum, and plasma membrane) is reduced, resulting in cell necrosis and general liver damage characterized by inflammation, fibrosis and metaplasia. CCl_4 given repeatedly at low doses will induce fibrosis [17]. Liver damage due to CCl_4 is shown by histological images of cell degeneration, necrosis and fat accumulation (steatosis) [18].

Various drugs continue to be developed to overcome chronic liver injury that can prevent fibrosis. Many medicinal plants are studied as one of the sources of bioactive substances that can help prevent damage to liver cells. The Kesambi plant (*Schleichera oleosa* (Lour.) Oken) in Indonesia is one of the plants known as medicine and grows abundantly on the islands of Java, Nusa Tenggara, Bali, Maluku, Sulawesi, Kai Island and Seram Island [19]. Kesambi leaves contain active compounds such as alkaloids, flavonoids, steroids, phenolics and tannins [20]. Kesambi has been shown to inhibit the growth of *Streptococcus aureus* bacteria [21]. Ethanol extract of kesambi has anti-inflammatory and analgesic effects on experimental animals [22]. This study aims to determine whether the administration of methanol extract of Kesambi leaves to Wistar rats exposed to CCL4 intraperitoneally can reduce TGF-β levels and prevent necrosis and fibrosis in the liver

2. MATERIALS AND METHODS

Materials

This research was conducted at the Research and Animal Experiment Laboratory of the Faculty of Medicine and Health Sciences, Universitas Islam Negeri Maulana Malik Ibrahim, Indonesia. Ethical clearance was obtained from the Health Research Ethics Committee of the Faculty of Medicine and Health Sciences with certificate no. 015/EC/KEPK-FKIK/2020. Meanwhile, the Kesambi plant used has been determined by the Herbal Materia Medica Laboratory, with the determination key obtained: 1b-2b-3b-4b-6b-7b-9b-10b-11b-12b-13b-14a-15b-197b-208b-219b-220a-221b-222a-1b-5b-6 which is a species of *Schleichera oleosa* (Lour.) Oken.

Male Wistar rats were obtained from Ratus Breeding Center Malang. The rats feed used was BR-1 brand feed from PT. Wonokoyo Jaya, Indonesia. Kesambi leaf simplicia was obtained from Materia Medica Laboratory, Batu. Methanol 96%, liquid paraffin and entelan from Merck, Germany; CCl4 from Sigma Aldrich, Germany; corn oil from Pietro Coricelli, Italy; 10% formalin and 0.9% NaCl from Otsuka, Indonesia; 95% denatured alcohol, xylol, hematoxylin and eosin from FisherbrandTM, USA; in addition, 0.5% Na-CMC, distilled water, rice husks, aluminum foil, filter paper were used.

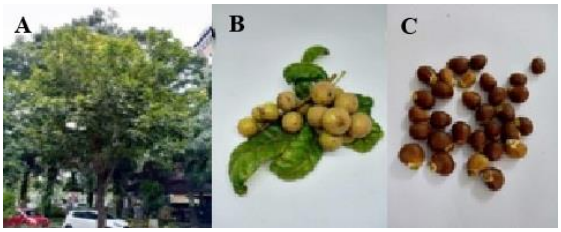


Figure 1. A. *Schleichera oleosa* (Lour.) Oken (Kesambi) tree that the author found in Malang city. B. Kesambi leaves and fruit, C. Kesambi fruit seeds (personal documentation)

Methods

Extraction process

Metabolite compound extraction was carried out using ultrasonic-assisted extraction method with 90% methanol solvent (Merck, Germany). 1 gram of kesambi leaf simplex was put into an erlenmeyer flask, 10 ml of methanol solvent was added with a ratio of simplex: solvent = 1:10. The solution was put into a sonicator (QSonica model Q700 USA) with a frequency of 42 kHz at room temperature for 10-30 minutes. The extraction results were filtered using filter paper and then flowed with nitrogen gas, so that a concentrated extract could be obtained. Before being given to experimental animals, the methanol extract of kesambi leaves was dissolved with 0.5% Na-CMC.

Procedure for Creating Experimental Animal Models for Hepatic Fibosis

Thirty Wistar rats were divided into five groups: a negative control group (K-), a positive control group (K+), and a treatment group that received methanolic leaf extract at varying doses : 200 mg/kgBW (P1), 400 mg/kgBW (P2), and 600 mg/kgBW (P3). The rats were identified and acclimatized for one week, provided with unlimited food and water. Their body weight was measured daily. After acclimatization, the K- group was injected intraperitoneally with corn oil, while the K+, P1, P2, and P3 groups were injected with liquid CCl_4: corn oil (1:4) at a dose of 0.5 µl/grBW, twice weekly for six weeks, to induce liver fibrosis in the rats [23]. Groups P1, P2, and P3 received daily oral methanolic extract of *Schleichera oleosa* leaves, starting on the first day of CCl_4 exposure and continuing for six weeks, using gavage.

Specimen Preparation and Histological Observation of Liver Tissue

The preparation of histological slides involves several steps. The mice were dissected, then the livers were removed and placed in 10% formalin (fixation). The livers were then sliced into small pieces 0.3–0.5 mm thick and arranged into tissue cassettes

(trimming). The tissue cassettes were placed in a special basket and fed into an automated processing machine. The tissue was dehydrated using 70% alcohol for 2 hours, 80% alcohol for 2 hours, 90% alcohol for 2 hours, followed by absolute alcohol for 2 hours, and repeated in a different absolute alcohol solution (dehydration). The basket containing the tissue cassettes was placed in a vacuum press and filled with liquid paraffin at 59–60°C for 30 minutes (vacuuming). The tissue cassettes were then removed and stored at 60°C. Stainless steel molds were heated using a Bunsen burner, and the tissue was placed into the molds and gently pressed (embedding). Liquid paraffin was poured over the tissue until completely submerged, then placed on a cooling plate. After cooling, the paraffin block was removed from the mold (blocking stage).

The paraffin block was then cut using a microtome with a thickness of 3–4 µm (cutting stage). The organ pieces were placed on the surface of warm water in a water bath at 46°C, then placed on a cover glass object that had been smeared with ewith and placed into an incubator at 60°C. The slide was immersed in xylene I, II, and III for 3 minutes each. Then it was dehydrated with ethanol I (100%) and II (95%) for 5 minutes each. The slide was rinsed with running water for 10–15 minutes, then immersed in hematoxylin solution for 15 minutes. After rinsing again with running water, the slide was immersed in eosin for 14 seconds to 2 minutes. Dehydration was repeated using 95% and 100% alcohol for 2–3 minutes, twice each, in separate containers. The slides were then immersed in xylene IV and V for 3 minutes each. Finally, the slides were dried, sealed with Entellan adhesive, and covered with a coverslip (staining stage).

The percentage of necrotic hepatocytes was observed by randomly selecting five fields in the Portal Triad area at 400x magnification. In each field, the number of normal cells, pyknotic cells, karyorectic cells, and karyolytic cells was counted. The percentage of necrotic cells was calculated using the following formula:

$$\text{Percentage of necrotic cells} = \frac{\text{Number of pyknotic cells + karyorrhexis cells + karyolysis cells}}{\text{Total number of cells in 1 field of view}} \times 100\%$$

Scoring was performed to determine the presence of fibrosis using the METAVIR criteria as follows :

Table 1. Scoring the presence of fibrosis using the METAVIR criteria

Categories	Criteria
F0	No fibrosis
F1	Portal fibrosis without septa
F2	Portal fibrosis with rare septa
F3	Numeorus septa without cirrhosis
F4	Cirrhosis

Measurement of TGF-β levels in the liver using the flow cytometry method

TGF-β induces CD4+ T lymphocytes cells to become CD4+CD25+ Tregs [24, 25]. CD4+CD25+ T lymphocytes cells present indicate an inflammation process and response to TGF-beta produced by profibrotic macrophages. A unique population of CD4+ T lymphocytes cells that constitutively express CD25 has been recognized as anergic/suppressor cells [26].

In this study, the number of CD4+CD25+ T lymphocytes cells, which are the result of T lymphocytes cell activation due to binding with TGF-β, was measured. Anti-TGF-β antibodies were used to mark cells whose membrane surfaces bind TGF-β.

The liver organ was crushed and centrifuged. The pellet was isolated, added with 1ml of PBS, and then homogenized. 100uL of pellet solution was taken and placed into a microtube. 500uL of PBS was added and centrifuged again at 2500rpm for 5 minutes at 10°C. 50µL of CD4 cell surface molecule antibody and CD25 cell surface molecule antibody were added, conjugated with phycoerythrin (PE) labels for CD25 and FITC for CD4, respectively. Incubated for 20 minutes at 4°C in a dark room. 300µL of PBS was added and transferred to a cuvette. 50µl of fixative

solution was added to the isolated pellet and incubated for 20 minutes at 4°C in a dark room, 500µl of permeability solution was added, homogenized, and centrifuged at 2500rpm for 5 minutes at 10°C. The centrifuged pellet was added with 50 µl of anti-TGFβ solution and incubated for 20 minutes at 4°C in the dark. The incubation result was added with ±400 µl of PBS and transferred to a cuvette for flow cytometry analysis.

Data analysis

All numerical data are presented as mean±standart deviation (SD), and error bar in figures represent SD. Data normality was evaluated using Shapiro-Wilk test, and homogeneity of variance was assessed using Levene's test. All variables fulfilled the normality and homogeneity criteria ($p>0.05$), allowing analysis using one-way ANOVA. ANOVA results are reported with F values, degrees of freedom (df), and p-values ($F(4.002) = 4$, $p<0.05$). Significant differences between groups were further examined using the LSD post-hoc test.

3. RESULTS

TGF-β levels in the livers of mice exposed to CCL4

Flow cytometry measurements revealed that the lowest mean TGF-β level was in group P3 at 4.21%, significantly different ($p<0.05$) compared to the other groups except group K- ($p>0.05$), as shown in Figure 2. Meanwhile, the mean TGF-β levels in groups K- (6.34%), K+ (7.21%), P1 (7.80%), and P2 (8.45%), although sequentially increasing with the administration of kesambi leaf methanol extract, did not show a significant difference between the groups ($p>0.05$).

These results indicate that administration of kesambi leaf methanol extract at doses of 200 mg/kgBW and 400 mg/kgBW did not affect the mean TGF-β level. However, administration of 600 mg/kgBW of kesambi leaf methanol extract reduced the mean TGF-β level in the livers of mice exposed to CCL4. This shown that only the 600 mg/kgBW dose has a decreasing effect on TGF-β levels.

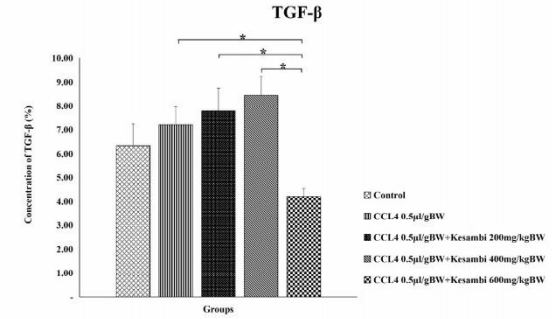


Figure 2. TGF-β levels in the livers of mice exposed to CCL4 with significance *)$p<0.05$

Histological picture of the liver and the number of necrotic hepatocytes

The results of the hepatocyte cell counts showed the highest percentage of necrosis in the K+ group (54.39%). A significant difference was observed between the K+ and K- groups (9.62%) exposed to CCL4 ($p<0.05$), as shown in Figure 3. The K+ group also differed significantly from the P2 group (29.37%) given 400 mg/kgBW of methanolic kesambi leaf extract and the P3 group (18.75%) given 600 mg/kgBW. The number of necrotic hepatocytes in the P3 group was very low, almost the same as in the K- group, as there was no significant difference ($p>0.05$).

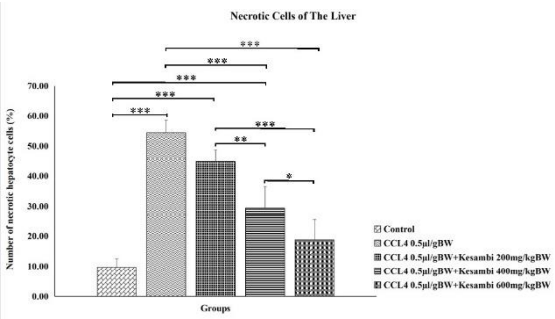


Figure 3. Percentage of the number of necrotic hepatocyte cells with significance *)$p<0.05$, **)$0.05>p>0.01$, ***)$p<0.01$.

These results indicate that CCL4 exposure causes an increase in the number of necrotic hepatocytes. Administration of methanol extract of kesambi leaves at a dose of 200mg/kgBW did not affect the number of necrotic hepatocyte cells, but administration

of doses of 400mg/kgBW and 600mg/kgBW could reduce the number of necrotic hepatocyte cells in the livers of mice exposed to CCL4, even the reduction in the number of necrotic cells reached the same as mice not exposed to CCL4.

In the histological observation of the liver parenchyma shown in Figure 4, pyknotic hepatocytes, karyorrhexis, and karyolysis were found. Infiltration of MN cells and erythrocytes into the sinusoidal spaces was seen. Meanwhile, in the fibrosis image (shown in Figure 4), ECM deposits in the form of hyaline fibers were found in the periportal area and myofibroblasts with spindle nuclei were seen between the hyaline fibers. MN cells infiltrated around the portal vein, bile duct, and hepatic artery from the circulation.

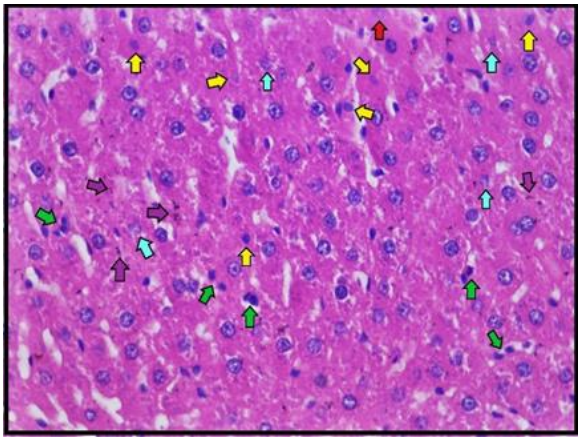


Figure 4. Histological image of the liver parenchyma with HE staining at 400x magnification in the K+ group showing the presence of pyknotic cells (yellow arrows), karyorrhexis cells (blue arrows), karyolysis cells (red arrows), mononuclear (MN) cells green arrows), and erythrocytes (purple arrows)

Fibrosis Score According to the METAVIR Method

The METAVIR score calculation showed no fibrosis in the K- (score value 0) and P3 (score value 0) groups, but fibrosis was found in the K+ (score value 2), P1 (score value 2), and P2 (score value 1), as shown in Table 2. In the K+ and P1 groups, the fibrosis score indicated portal fibrosis with sparse septa, while in P2, the fibrosis score indicated only portal fibrosis without septa.

This illustrates that exposure to CCL4 in experimental animals causes the formation of liver fibrosis in the portal area, which is characterized by septa. Administration of methanol extract of kesambi leaves at a dose of 200 mg/kgBW did not reduce the progression of fibrosis. However, at a dose of 400 mg/kgBW, the severity of fibrosis began to decrease, with fibrosis forming only in the portal area without interlobular septa. Administration of methanol extract of kesambi leaves at a dose of 600 mg/kgBW even prevented fibrosis in mice exposed to CCL4. The image of fibrosis in liver histology is shown in Figure 5.

Table 2. Fibrosis score according METAVIR method.

Groups	METAVIR Score
K-	0
K+	2
P1	2
P2	1
P3	0

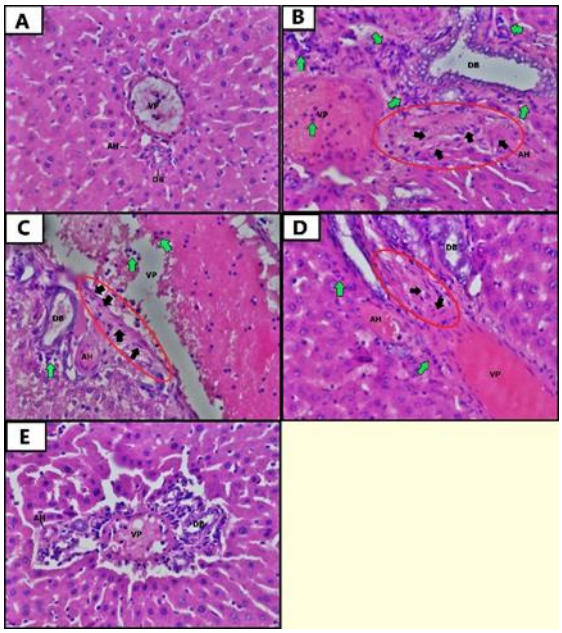


Figure 5. Histological image of the portal triad area of the liver with HE staining at 400x magnification. A. K- group; B. K+ group; C. P1 group; D. P2 group; E. P3 group (VP = portal vein, DB = bile duct, AH = hepatic artery, red

circle = fibrotic area, black arrow = myofibroblast cells, green arrow = MN cells)

4. Discussion

Although extensive exploration of the secondary metabolites of kesambi has been conducted, its use as a treatment for liver disease remains understudied. Goswami et al. (2020) found that the most abundant metabolites in kesambi using thin-layer chromatography were flavonoids, tannins, and phenolics [27]. One of the flavonoids identified was quercetin. Quercetin is a secondary metabolite abundantly found in medicinal plants [28].

The results of metabolite profiling of Schleichera oleosa plants using LC-MS and GC-MS conducted by Vanaja, P., et al. obtained compounds Isorhamnetin 3-glucoside, (1S)-1,5-Anhydro-1-(5,7-dihydroxy4-oxo-2-phenyl-4H-chromen-8-yl)-D-glucitol, and 4-[(Z)-(6-hydroxy-3-oxo-1-benzofuran-2(3H)-ylidene) methyl]phenyl beta-D-glucopyranoside, which are flavonoids [29].

In this study, a decrease in the number of necrotic hepatocyte cells was found in mice exposed to CCL4 and given methanol extract of kesambi leaves at a dose of 400mg/kgBW and a dose of 600mg/kgBW. This is possible because tannins and quercetin have anti-inflammatory and antioxidant effects. In vitro studies, exposure to the tannin fraction from *Terminalia chebula* plant extract inhibited protein denaturation, membrane lysis, and the activity of proteinase and hyaluronidase enzymes [30].

In vitro studies using fluorescence-based high-throughput assays, quercetin was found to inhibit cytochrome P450 (CYP) enzymes [31]. In vivo studies in hamsters exposed to 7,12-dimethylbenz[a]anthracene (DMBA), administration of quercetin caused disruption of Reactive Oxygen Species (ROS) production through down-regulation of CYP1A1 and CYP1B1, and increased regulation of antioxidant defenses. Inhibition of ROS by quercetin will abolish NFkB signaling by preventing phosphorylation and degradation of IkB, translocation of NFkB to the nucleus and transactivation of target genes related to cell proliferation and prevention of apoptosis [32].

The results of this research data also showed a decrease in TGF-β levels in the group of mice exposed to CCL4 and given 600mg/kgBW of methanol extract of kesambi leaves. This is in accordance with research conducted by Domitrović, et. al., where quercetin suppressed TGF-β in Balb/cN mice exposed to CCL4 [33]. Research by Nakamura, et. al. proved that quercetin was able to suppress TGF-β production in NIH3T3 cells and normal human fibroblast cells [34]. Tannin can also inhibit TGF-β production in pulmonary fibrosis model mice exposed to bleomycin [35].

In the group of mice exposed to CCL4 and given 400mg/kgBW of methanol extract of kesambi leaves, a milder decrease in METAVIR fibrosis score was found compared to the positive control group. In fact, no fibrosis was found in mice exposed to CCL4 and given 600mg/kgBW of methanol extract of kesambi leaves. Research by Wang et. al. on tannin exposure in mice with a bleomycin-induced pulmonary fibrosis model showed suppression of alpha-smooth muscle actin protein production, a marker of myofibroblast cells that will actively produce ECM in fibrotic tissue [35]. Ganbolda, et. al. (2019) reported that quercetin administration was able to inhibit the proliferation of HSC-T6 cell lines exposed to TGF-β to induce fibrosis [8]. According to research conducted by Yoshida and Matsuzaki, HSC activation occurs through the TGF-β/Smad pathway [36]. Oral administration of quercetin in NASH model animals resulted in a decrease in steatosis, inflammation, and ballooning in liver histology [37].

5. Conclusion

Administration of methanol extract of kesambi leaves reduced TGF-β levels (at a dose of 600 mg/kgBW) and the number of necrotic hepatocytes (at doses of 400 mg/kgBW and

600 mg/kgBW). Methanol extract of kesambi leaves was also shown to reduce the progression of fibrosis (at a dose of 400 mg/kgBW) and no fibrosis observed histologically (at a dose of 600 mg/kgBW).

6. ACKNOWLEDGEMENTS

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