ORIGINAL ARTICLE

pISSN: 1907-3062 / eISSN: 2407-2230

Hepatoprotective activity of *Averrhoa bilimbi* L. fruit extract on carbon tetrachloride-induced acute liver faiure in Wistar rats

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ABSTRACT

BACKGROUND

Acute liver failure (ALF) is a state of rapid and progressive deterioration of liver function. Continuous exposure to chemicals and viruses can increase reactive oxygen species (ROS) which leads to prolonged inflammation due to the production of tumor necrosis factor-alpha (TNF-α) thus inhibiting the production of platelet-derived growth factor (PDGF). The objective of this study was to evaluate the effect of administration of *Averrhoa bilimbi* L. fruit extract on PDGF levels and TNF-α levels in carbon tetrachloride (CCl4)-induced ALF rats.

METHODS

This study used a post-test-only control group design involving 20 Wistar rats. They were randomized into 4 groups, namely sham, control, T1, and T2. Group T1 was exposed to CCl4 with the administration of *A. bilimbi* fruit extract at a dose of 500mg/kgBW, while, group T2 was exposed to CCl4 and given *A. bilimbi* fruit extract of 750 mg/kgBW. On the 15th day, the serum was analyzed to determine the levels of PDGF and TNF-α using ELISA.

RESULTS

The highest mean PDGF level in the control group was 146.60±15.36 pg/mL, while the highest mean TNF-α level in group T1 was 40.11±4.44 pg/mL. The One-way ANOVA test showed that there were significant differences in TNF-α (p=0.002) and PDGF (p=0.000) levels between the study groups..

CONCLUSION

The administration of *A.bilimbi* L. fruit extract affected PDGF and TNF-α levels in CCl4-induced ALF rats. The present study revealed that *A. bilimbi* fruits have significant hepatoprotective activity in experimental Wistar rats.

Keywords: Acute liver failure, *Averrhoa bilimbi* L., reactive oxygen species, rats

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Date of first submission, September 7, 2022
Date of final revised submission, March 26, 2023
Date of acceptance, April 10, 2023

Cite this article as: Sihaloho RR, Chodijah, Sumarawati T, Wibowo JW, Zulaikhah ST, Putra A, Hidayah N, Ghaissani SS, Nazar MA. Hepatoprotective activity of *Averrhoa bilimbi* L. fruit extract on carbon tetrachloride-induced acute liver faiure in Wistar rats. Univ Med 2023;42:61-9. doi: 10.18051/UnivMed.2023.v42:61-69

DOI: http://dx.doi.org/10.18051/UnivMed.2023.v42:61-69

INTRODUCTION

Acute liver failure (ALF) is manifested by progressive necrosis of the hepatocytes, followed by impaired liver functions and elevated serum transaminase levels.[1–3] Persistent necrosis of hepatocytes induces activation of hepatic stellate cells (HSCs) leading to excessive deposition of extracellular matrix (ECM).[4,5] During hepatic fibrogenesis, there is an increase in inflammatory factors such as tumor necrosis factor-alpha (TNF-α) and excessive accumulation of the hepatic extracellular matrix which is regulated by fibrogenic factors, such as platelet-derived growth factor (PDGF).[6,7] The late stage of chronic progressive fibrosis results in cirrhotic hepatic disease (CHD), which accounts for approximately 55% of the 1.4 million liver-disease-related deaths reported each year worldwide.[8,9] Currently, liver transplantation is the most effective therapy for patients with advanced liver disease, including CHD; however, the limited availability of liver donors and the low survival rates among liver transplant patients remain a serious problem.[5,9–11] Therefore, novel alternative LF treatments that can be used in place of transplantation must be explored. On the other hand, many previous studies have reported that *Averrhoa bilimbi* L. has oxidative radical scavenging and immunosuppression properties which is mainly due to phenolic substances such as terpenoids, phenols, tannins, and flavonoids.[12-14]

Tumor necrosis factor-α (TNF-α) is a pleiotropic cytokine associated with a variety of physiological and pathological conditions, including cytotoxicity, differentiation, growth stimulation, immune modulation, and proinflammatory activity.[5,15,16] Tumor necrosis factor-α exacerbates liver damage following exposure to toxic levels of acetaminophen or carbon tetrachloride (CCl4) and plays a role, as well, in liver repair following CCl4 exposure by stimulating hepatocyte growth factors such as interleukin-6 (IL-6) and transforming growth factor-α (TGFα) and allowing rapid hepatocyte proliferation.[5,6,17] Elevated levels of liver TNFα occur in acute and chronic liver diseases, including fulminant hepatic failure, viral hepatitis, alcohol abuse, metabolic disease, autoimmunity, and biliary obstruction, as well as following chemical-induced hepatotoxicity.[18,19] Chronic liver injury can promote fibrosis, characterized by increased hepatic stellate cell proliferation and their transdifferentiation into myofibroblast-like cells, leading to the extracellular matrix and fibril-forming collagen accumulation.[20,21] Fibroblast proliferation and collagen production are enhanced by growth factors such as TGF-β1 and PDGF.[22,23]

Averrhoa bilimbi L. fruits are rich in phytochemical compounds which exert potent antioxidant activity either by scavenging the reactive molecules or by enhancing antioxidant molecules and enzymes.[14,24] Preliminary phytochemical studies revealed the presence of alkaloids, carbohydrates, phenols, flavonoids, saponins, and tannins.[14,25] A previous study reported that treatment with *Averrhoa bilimbi* L. fruit extract has also optimally maintained the levels of the inflammatory mediators, TNF-α, IL6, and IL 1β, in an acetic acid-induced ulcerative colon rat model by down-regulating the cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (i-NOS) levels in the colon.[12] Another previous report also demonstrated that methanolic extract of *A. bilimbi* leaves showed significant hepatoprotective and antioxidant activity against CCl4-induced hepatotoxicity by increasing the GSH levels and reducing the thiobarbituric acid reactive substances (TBARS) levels.[24] In addition, administration of methanolic extract of *Averrhoa bilimbi* at two different dose levels (250 and 500 mg/kgBW) attenuated the increased levels of the serum enzymes produced by CCl4 and caused a subsequent recovery towards normalization.[24,26] A study to evaluate the hepatoprotective activity of *Averrhoa bilimbi* fruit in Wistar albino rats following acetaminophen intoxication, showed that the liver marker enzymes serum glutamate oxaloacetate transaminase (SGOT), serum glutamate pyruvate transaminase

(SGPT), alkaline phosphatase (ALKP) were found to be two fold increased in the control group, while pre-treatment with *A. bilimbi* inhibited the increase of these enzymes in serum.[27]

However, in vivo studies to provide scientific evidence regarding the potential effect of *Averrhoa bilimbi* L. fruit extract in regulating TNF-α and PDGF levels in ALF need to be explored, such that they may be useful for improving human health. Therefore, this study was designed to investigate the effect of different doses of *Averrhoa bilimbi* L. fruit extract administration on PDGF levels and TNF-α levels in CCl4-induced ALF rats.

METHODS

Research design

This experimental study was of completely randomized design to compare the effects of treatment on the experimental and control groups at the end of the treatment. The research was conducted in June–August 2022 at the Stem Cell Research Laboratory (*Laboratorium Penelitian Sel Punca* - LPSP), Faculty of Medicine, Sultan Agung Islamic University, Semarang.

Maceration and extraction of *Averrhoa bilimbi* L. fruits

The fruits of *A. bilimbi* L. were collected, air dried, and ground into a fine powder by means of mortar and pestle. Extraction from the fruits was done according to the method described by Singh et al.[28] The powder (25 g) was extracted with 250 ml of methanol (95% v/v) in a soxhlet apparatus. The remaining methanol was evaporated using a rotary evaporator. The obtained thick semi-solid crude extract was stored at 2–4ºC for further use.

CCl4 -induced acute liver failure model

The animals were obtained from and certified to be healthy by the Agricultural and Fishery Service of Salatiga under no. 524.3/0211/421. The calculation of the estimated sample size was performed according to the Federer formula: (t-1) (r-1) $\geq$15 (t=number of treatments; r=number of replications); (4-1)(r-1) $\geq$15; 3r–3$\geq$18; 3r$\geq$18; r$\geq$6. Therefore, the minimal replication per group is 6 and the total of 4 groups is 24 animals. Twenty four male Wistar rats (5–6 weeks old and weighing 250–300 g) were fed ad libitum and housed in plastic cages with mesh wire covers, at a room temperature of 24°C, with a 12-h light-dark cycle (laboratory standard). All rats were intra-peritoneally (IP) injected with 0.1 mL/kg CCL4 (Sigma-Aldrich), dissolved in olive oil (1:1), twice weekly, for two weeks, to induce ALF.[15] To evaluate liver function, an assessment of SGOT and SGPT was performed.

Extract of *Averrhoa bilimbi* L. oral administration

After a week of acclimatization, rats were randomly divided using a computer into the following five groups and the treatments were administrated by an animal laboratory operator: untreated (Sham), CCl-4 treated with olive oil/vehicle (Control group), CCL-4 treated with a low dose (500mg/kgBW) of *Averrhoa bilimbi* L. fruit extract (T1), and CCl-4 treated with a high dose (750mg/kgBW) of *Averrhoa bilimbi* L. fruit extract (T2). Each group consisted of five rats. The intervention dose was administered orally daily for up to 14 days. On day 15, all rats were terminated and periorbital blood was collected.

PDGF and TNF-α levels using ELISA

The blood of the rats was harvested via periorbital venous plexus bleeding under general anesthesia on days 0 (pre-treatment), 2, and 7, and the serum was collected by centrifugation at 4^0C. We measured PDGF and TNF-α concentrations by means of enzyme-linked immunosorbent assay (ELISA) kits, based on the manufacturer's instructions (Fine Test, Wuhan, China) and according to a standard curve constructed for each assay. The colorimetric absorbance was recorded at a wavelength of 450 nm.

SGOT and SGPT measurement

SGOT and SGPT were measured to determine liver functions at days 0 (pre-treatment) and 7. Blood samples were collected from periorbital veins under anesthesia using a solution of ketamine + xylazine (100 mg/kgBW + 5 mg/kg) (Alfasan, The Netherlands). The serum was collected by centrifugation at 4ºC. SGOT and SGPT serum levels were measured using an automatic analyzer (BT 3000 PLUS, Italy).

Statistical analysis

Statistical analyses were accomplished with the IBM SPSS Statistics for Windows version 22.0 (IBM Corp, Armonk, NY). All data were presented as mean ± standard deviation (SD). The data obtained were collected, compiled, and tested for normality with the Shapiro-Wilk test and for homogeneity with Levene's test. Data analysis was performed using one-way ANOVA and continued with the Least Significant Difference (LSD) post hoc test using a *p*-value <0.05.

Ethical clearance

The Ethics Committee of Sultan Agung Islamic University approved all procedures performed in this study under number 817/VI/2022/ Komisi Bioetik.

RESULTS

Levels of SGPT and SGOT

The present study revealed that the levels of serum glutamic pyruvic transaminase (SGPT) and serum glutamic-oxaloacetic transaminase (SGOT) increased significantly after CCl4 exposure, indicating considerable hepatocellular injury (Figure 1).

***Averrhoa bilimbi* L. fruit extract decreases TNF-α and PDGF levels in CCL4-induced ALF models**

CCl4 has been shown to induce apoptosis of hepatocytes by increasing the pro-inflammatory factors, such as IL-1, IFN-γ, and TNF-α. The level of TNF-α was measured to examine the effect of *Averrhoa bilimbi* L. fruit extract on the CCl4-induced ALF rat liver tissue. The results of the comparative analysis showed that the TNF-α level was increased after CCl4 induction (control group) (36.12±7.11 pg/mL), and increased further after being treated with the low dose *Averrhoa bilimbi* L. fruit extract (group T1) (40.09±4.86 pg/mL), but was significantly reduced with high dose *Averrhoa bilimbi* L. fruit extract (group T2) (27.09±4.46 pg/mL) (Figure 2A, Tables 1 and 2). These results suggest that the TNF-α level may be regulated by the dose-

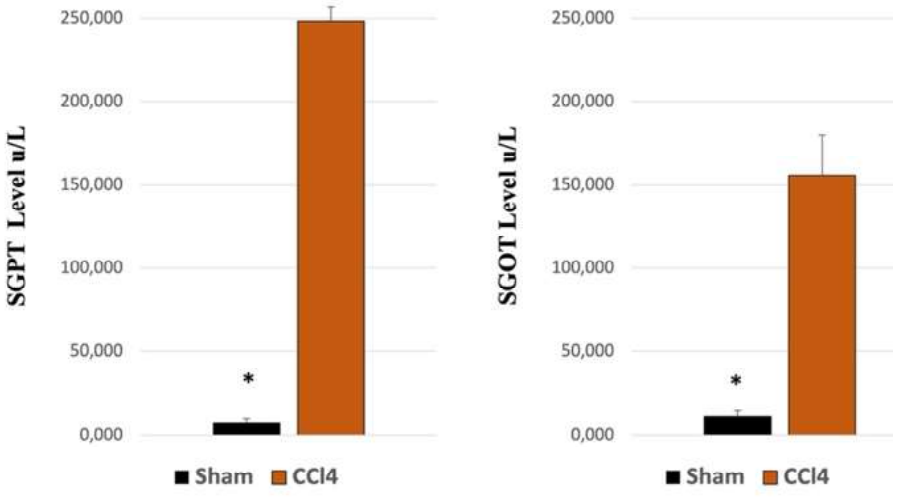

Figure 1. Level of SGPT and SGOT in rats after two weeks of exposure to CCL-4 (n=6 ± SD); Sham=Healthy rats; CCL-4=CCL-4 exposure;*p<0.05

Table 1. Distribution of TNF-α and platelet-derived growth factor by experimental groups

Cytokines	Experimental groups				p value
	Sham (n=6)	Control (n=6)	T1 (n=6)	T2 (n=6)	
TNF-α (pg/mL)	25.18±5.01	36.12±7.11	40.09±4.86	27.09±4.46	0,002*
PDGF (pg/mL)	65.99±5.93	146.3±18.26	41.07±17.53	30.91±3.74	0,000*

Note: Values are mean ± SD, TNF-α: tumor necrosis factor-α; PDGF: platelet-derived growth factor;* Anova test; T1=CCL-4 treated with a low dose of *Averrhoa bilimbi* L. fruit extract, T2= CCl-4 treated with a high dose of *Averrhoa bilimbi* L. fruit extract; *p<0.05

dependent effect of *Averrhoa bilimbi* L. fruit extract.

To determine the role of *Averrhoa bilimbi* L. fruit extract in ALF regeneration, the PDGF levels were measured using ELISA. The levels of PDGF in *Averrhoa bilimbi* L. fruit extract groups, both low dose and high dose, were significantly decreased (T1=41.07±17.53; T2=30.91±3.74) compared with the control group (146.3±18.26) (p<0.05) (Figure 2B, Tables 1 and 2). In addition, the level of PDGF expression in the control group was high due to continued inflammation. The present study had demonstrated the role of hepatoprotective activity of *Averrhoa bilimbi* fruit extract in the experimental animal system. The TNF-α levels and the PDGF levels were decreased in the *A. bilimbi* treated groups, when compared with the control group.

DISCUSSION

The results of this study showed that *Averrhoa bilimbi* L. fruit extract might reduce TNF-α and PDGF levels in CCl4-induced ALF rats. Those results suggest that the hepatoprotective effect of *Averrhoa bilimbi* L. may be produced by reducing the TNF-α and PDGF levels in CCl4-induced ALF rats. Carbon tetrachloride (CCl4) induced toxic liver injury is a well-characterized model of hepatic fibrosis. CCl4 is metabolized by the mitochondrial monooxygenase system (cytochrome P450 2E1) to its active metabolite, an unstable trichloromethyl (CCl3) free radical, and then this active metabolite immediately transforms into trichloromethyl peroxyl (CCl3O2).[17,29,30] These free radicals damage the cell membrane via peroxidative degradation of the fatty acids from phospholipids of the cell membrane and endoplasmic reticulum.[3,5] Subsequently, this process results in the fragmentation of the lipid peroxide radicals, lipid hydroperoxides, and other products, each acting as an active oxidizing agent.[4] Consequently, the structure of the cell membrane and intracellular organelles completely deteriorate, and the structural damage expands.[23] These processes are immediately followed by the infiltration of inflammatory cells and the release of various cytokines and growth factors.[31,32] Moreover, the level of TNF-α consistently rises during the ongoing CCI4-induced liver toxicity.[5] In this study, the decrease in TNF-α level may have been due to the scavenging of free radicals,

Table 2. Post-hoc LSD result

Group	Compared group	TNF-α (p value)	PDGF (p value)
Sham	Control	0.007*	0.000*
	T1	0.001*	0.008*
	T2	0.611	0.000*
Control	T1	0.349	0.000*
	T2	0.020*	0.000*
T1	T2	0.003*	0.105

TNF: tumor necrosis factor; PDGF: platelet-derived growth factor; Sham: healthy rats; Control: CCl-4 treated with olive oil/vehicle, T1=CCL-4 treated with a low dose of *Averrhoa bilimbi* L. fruit extract, T2= CCl-4 treated with a high dose of *Averrhoa bilimbi* L. fruit extract; *p<0.05

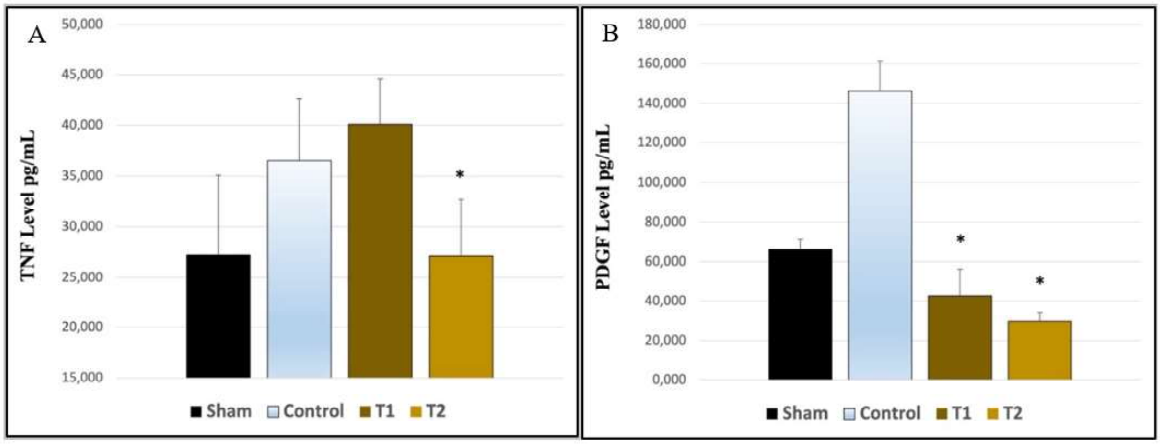

Figure 2. Effect of *Averrhoa bilimbi* L. fruit extract on TNF-α and PDGF level (n=6)). **(A)** Level of TNF-α in each group, **(B)** Level of PDGF in each group. Sham=Healthy rats, Control=CCl-4 treated with olive oil/vehicle, T1=CCL-4 treated with a low dose of *Averrhoa bilimbi* L. fruit extract, T2= CCl-4 treated with a high dose of *Averrhoa bilimbi* L. fruit extract. *p<0.05.

especially nitric oxide (NO). Nitric oxide is one of the abundant free radicals and is highly formed during inflammation, capable of damaging proteins, lipids, and DNA.[33,34] A piece of previous evidence has shown that the antioxidant activity of *Averrhoa bilimbi* L. fruit extract is proven from its high level of NO scavenging activity.[12] Another previous study also revealed that *Averrhoa bilimbi* L. fruit extract has potential antioxidant activity by reducing the NO levels and enhancing the SOD enzyme.[13] Reducing NO levels and increasing SOD may inhibit oxidative stress in microenvironment hepatocytes and reduce immune cell recruitment, which sustainably the pro-inflammatory molecules reduction, primarily TNF-α.[12] This finding is also supported by another study that demonstrated *Averrhoa bilimbi* L. fruit extract also optimally maintained the levels of inflammatory mediators, such as TNF-α, IL-6, and IL-1β in ulcerative colitis rats.[12]

Acute liver failure is also characterized by impairment of the extracellular matrix (ECM) deposition or fibrosis mediated primarily by PDGF. In this study, the decrease in PDGF level may be suggested due to the reducing inflammatory factor and oxidative stress. Platelet-derived growth factor, released and activated after tissue injury, plays an important role in hepatic stellate cell activation and portal fibroblast proliferation, chemotaxis, and migration, thus suppressing its expression is important in fibrosis disease.[22,35] The controlled inflammatory niche may inhibit the activation and differentiation of hepatic stellate cells (HSC) into myofibroblasts, resulting in reducing the HSC stimulator, PDGF.[10] Thus, this process will inhibit release of the profibrogenic factor and reduce impaired ECM deposition.[22] This concept is supported by previous reports revealing that the PDGF signaling pathway plays an important role in the development and prognosis of liver fibrosis and that controlling its level is critical for preventing severe liver disease.[2,23] However, this study did not analyze several potent biomarkers such as NF-κB, IL-6, TGF-β, NO, and SOD after administration of *Averrhoa bilimbi* L. fruit extract in CCl4-induced ALF rats. Overall, the implication of this study is that it may be useful for research on the potential of *Averrhoa bilimbi* L. fruit extract to accelerate and direct target screening through molecular mechanisms to treat ALF. Future studies will be aimed at identifying the optimum dose and the length of *Averrhoa bilimbi* L. fruit extract administration.

CONCLUSION

These findings demonstrated that the dose-dependent effect of *Averrhoa bilimbi* L. fruit

extract administration may reduce the TNF-α and PDGF levels in CCL4-induced ALF rats. This study provides new insights into the benefits of *Averrhoa bilimbi* L. administration in most injured tissues, particularly in the proliferation phase of ALF.

ACKNOWLEDGMENT

The authors would like to thank the Stem Cell and Cancer Research Laboratory for facilitating and technically assisting this research.

FUNDING

None.

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

AUTHOR CONTRIBUTION

R.R.S: conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing; C.C.: supervising, critical reviewing, final approval of the manuscript; T.S.: supervising, and critical reviewing; J.W.W.: supervising, and critical reviewing A.P.: supervising, critical reviewing, final approval of the manuscript; S.T.Z: supervising, data analysis and interpretation, critical reviewing, final approval of the manuscript; N.H.: data analysis and interpretation, manuscript writing; S.S.G.: data analysis and interpretation, manuscript writing; M.A.Z.: data analysis and interpretation, manuscript writing. All authors have read and approved the final manuscript.

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