

Original Research Article

Effect of aqueous leaf extract of *Thunbergia laurifolia* on alcohol-induced liver injury in rats

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Abstract

Purpose: To investigate the antioxidant and anti-inflammatory effects of aqueous leaf extract of *T. laurifolia* against alcoholic liver injury in rats.

Methods: Male Wistar rats were administered either normal saline, ethanol (EtOH), normal saline with low or high dose *T. laurifolia* leaf extract (TL-LD or TL-HD), EtOH with TL-LD or EtOH with TL-HD. Blood biochemical indices: hepatic malondialdehyde (MDA) levels, liver histopathology, hepatic cytochrome P450 2E1 (CYP2E1), nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, and pro-inflammatory cytokines, including interleukin 1 beta (IL-1 β) and tumor necrotic factor alpha (TNF- α) mRNA expressions, were determined using standard methods.

Results: The leaf extract of *T. Laurifolia* decreased hepatic MDA levels, improved liver pathology and down-regulated mRNA expressions of CYP2E1, NADPH oxidase and pro-inflammatory cytokines in ethanol-treated rats.

Conclusion: These results demonstrate that aqueous extract of *T. Laurifolia* exerts hepatoprotective effect against alcoholic liver injury through a mechanism involving inhibition of oxidative stress and inflammation.

Keywords: *Thunbergia laurifolia*, Alcohol, Liver injury, Oxidative stress, inflammation, Protection

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INTRODUCTION

The liver is the primary organ of ethanol metabolism. Chronic alcohol consumption results in alcohol-induced liver disease (ALD). In ALD, the altered capacity of hepatocytes to cope with environmental toxins, and their altered oxidation-reduction potential result in liver injury [1]. The hallmarks of ALD are steatosis, steatohepatitis,

fibrosis, and more severe forms including cirrhosis and hepatocellular carcinoma [2]. Oxidative stress and inflammation are critical factors in etiology of ethanol-induced liver damage [2]. Reactive oxygen species (ROS) are implicated in the progression from hepatic steatosis to steatohepatitis and cirrhosis [3].

Previous studies have reported that ethanol exposure induced the expressions of cytochrome

P450 2E1 (CYP2E1) and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, resulting in the production of ROS and other free radicals [4]. It is known that NADPH oxidase transfers an electron from NADPH to molecular oxygen to produce the superoxide radical ($O_2^{\bullet-}$). A previous study demonstrated the involvement of NADPH oxidase 4 in early alcoholic liver injury and its regulation of the recruitment of inflammatory cells and production of pro-inflammatory cytokines [5].

Excessive alcohol consumption has been shown to increase hepatic translocation of gut-sourced endotoxin/lipopolysaccharide, resulting in activation of innate immune cells such as Kupffer cells and natural killer (NK)/NKT cells, and production of large amounts of ROS, pro-inflammatory cytokines [interleukin-1 (IL-1), tumor necrotic factor- α (TNF- α)] and ultimately, liver injury [6]. Inhibition of pro-inflammatory cytokines has been shown to accentuate recovery from alcoholic hepatitis [7].

Thunbergia laurifolia has long been described in Thai Traditional Medicine. Numerous studies have reported its various pharmacological effects in both *in vitro* and *in vivo* models [8,9]. It has been shown to exert hepatoprotective activity against ethanol-induced liver injury in primary cultures of rat hepatocytes and in rats. However, the mechanisms involved in these effects are still unknown.

Therefore, the present study was carried out to investigate the antioxidant and anti-inflammatory effects of *T. laurifolia* aqueous leaf extract against alcoholic liver injury in male Wistar rats, and the underlying mechanism(s).

EXPERIMENTAL

Preparation of aqueous extract of *T. laurifolia* leaves

Thunbergia laurifolia leaves were collected from a location at Nakhon Si Thammarat province, Thailand. The leaves were washed several times in running water, dried at 60°C for 48 h in a hot air oven, and ground to powder using a blender. The leaf powder (10 g) was extracted with 100 mL of boiling water for 48 min, and the aqueous extract was lyophilized in a freeze dryer (Eyela, Tokyo, Japan). The powder was preserved at -80°C until used.

Experimental animals and treatments

Approval for the animal procedures were received from the Animal Ethics Committee,

Walailak University (certification no. 005/2015). The study was carried out in line with the Guide for the Care and Use of Laboratory Animals from the National Research Council [10].

Twenty-four male Wistar rats (*Rattus norvegicus*) aged 12 weeks were obtained from the Division of Animal House, Faculty of Science, Prince of Songkla University, Thailand. The rats were maintained in stainless steel cages under constant conditions of temperature ($23 \pm 2^\circ\text{C}$), relative humidity (50 – 60 %) and lighting (12 h light/12 h dark cycle). Before the experiment, the rats were provided free access to standard commercial feed and distilled water.

The rats were randomly divided into six groups (4 rats per group): rats gavaged with normal saline once a day for 10 weeks (control group); rats gavaged with ethanol (30 % v/v in saline, 4 g/kg) once a day for 10 weeks (EtOH group); rats gavaged with normal saline once a day for 10 weeks and then gavaged with low dose *T. laurifolia* leaf extract (100 mg/kg) once a day from weeks 5-10 (TL-LD group); rats gavaged with high dose *T. laurifolia* leaf extract (200 mg/kg) once a day from weeks 5 - 10 (TL-HD group); and rats gavaged with low dose *T. laurifolia* leaf extract (100 mg/kg) once a day at weeks 5-10 (EtOH+TL-HD group); rats gavaged with ethanol (30 % v/v in saline, 4 g/kg) once a day for 10 weeks, and rats gavaged with high dose *T. laurifolia* leaf extract (200 mg/kg) once a day at weeks 5 - 10 (EtOH + TL-HD group). The rats were euthanized with thiopental sodium overdose (100 mg/kg body weight) anesthesia. The abdominal cavities were then opened, and the livers were excised. Peripheral blood was taken from the heart of each rat.

Biochemical analysis

The blood samples were centrifuged at 3000 rpm for 5 min. Sera were collected for determination of the levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), triglycerides and total protein using Chemistry Analyzer (Switzerland).

Determination of hepatic MDA levels

A 50 mg/mL liver homogenate was prepared by homogenizing liver sections on ice in phosphate buffered saline containing 1X butylated hydroxytoluene. The homogenate was centrifuged at $10,000 \times g$ for 5 min, and MDA was assayed in the supernatant using OxiSelect™ TBARS Assay Kit (cat no. STA-330, Cell Biolabs, San Diego, CA, USA) in accordance with the manufacturer's protocol.

Histopathology

Fresh liver sections were fixed in 10 % neutral buffered formalin solution, processed for light microscopy and embedded in paraffin. Sections were sliced and subjected to hematoxylin and eosin (H & E) staining in line with standard procedures.

Determination of expressions of hepatic CYP2E1, NADPH oxidase, IL-1 β , and TNF- α mRNA

Hepatic total RNA was extracted using an RNeasy Mini Kit (Qiagen, Germany). The content and purity of the RNA were measured using UV spectrophotometry. Reverse transcription and PCR were performed for amplification of the genes. The thermal cycling conditions were set up with an initial denaturation step at 95 °C for 15 min, and at 94 °C for 1 min. The second step included annealing of primer at 65 °C for 1 min, while the third step involved an extension at 72 °C for 1 min. The final step was elongation at 72 °C for 10 min. The sequences of the primers used are shown in Table 1.

The DNA samples were loaded into 2 % gel agarose. After staining with ethidium bromide, the gel was visualized with an UV transilluminator. The amount of PCR product was detected using GeneTools software with image analysis (Syngene, Frederick, MD, USA).

Statistical analysis

Results are expressed as mean \pm standard error of the mean (SEM). Differences between groups were determined using one-way analysis of variance (ANOVA). Post hoc testing was performed for group comparisons using the Least Significant Difference (LSD) test. Values of $p < 0.05$ were considered significant.

RESULTS

Effect of *T. laurifolia* aqueous leaf extract on liver enzymes, triglycerides, total protein and MDA levels

As shown in Figure 1, rats gavaged with ethanol once a day for 10 weeks (EtOH group) had significantly higher serum levels of AST ($p < 0.05$) and ALT ($p < 0.001$) than rats in the control group. Moreover, the levels of triglycerides and total protein were significantly higher ($p < 0.001$, $p < 0.05$, respectively) in the EtOH group than in the control group. There were no significant differences in plasma levels of liver enzymes, triglycerides and total protein between rats gavaged with 100 mg/kg (TL-LD group) or 200 mg/kg (TL-HD group) of *T. laurifolia* leaf extract once a day from weeks 5 – 10, and rats in the control group. Rats gavaged with ethanol once a day for 10 weeks, and low dose of *T. laurifolia* leaf extract once a day from weeks 5 - 10 (EtOH+TL-LD group) had significantly decreased levels of AST ($p < 0.001$), ALT ($p < 0.001$), triglycerides ($p < 0.05$) and total protein ($p < 0.05$), when compared with rats in the EtOH group.

In contrast, rats gavaged with ethanol and high dose of *T. laurifolia* leaf extract (EtOH + TL-HD group) had significantly decreased levels of AST ($p < 0.001$), ALT ($p < 0.001$), triglycerides ($p < 0.001$) and total protein ($p < 0.05$), relative to rats in the EtOH group. Rats in the EtOH group showed significantly elevated MDA levels, when compared to rats in the control group ($p < 0.05$) (Figure 1 E), while rats in the EtOH+TL-LD and the EtOH+TL-HD groups had significantly decreased levels of MDA ($p < 0.05$), compared to rats in the EtOH group.

Table 1: Primers for CYP2E1, NADPH oxidase, IL-1 β and TNF- α

CYP2E1 [11]	Forward primer	5'-ACTTCTACCTGCTGAGCAC-3'
	Reverse primer	5'-TTCAGGTCTCATGAACGGG-3'
NADPH oxidase [12]	Forward primer	5'-GGAAATAGAAAGTTGACTGGCCC-3'
	Reverse primer	5'-GTATGAGTGCCATCCAGAGCAG-3'
IL-1 β [13]	Forward primer	5'-CCCTGCAGCTGGAGAGTGTGG-3'
	Reverse primer	5'-TGTGCTCTGCTTGAGAGGTGCT-3'
TNF- α [13]	Forward primer	5'-GACCCTCACTCAGATCATCTTCT-3'
	Reverse primer	5'-TGCTACGACGTGGGCTACG-3'
β -actin [14]	Forward primer	5'-TTCTTTGCAGCTCCTTCGTTGCCG-3'
	Reverse primer	5'-TGGATGGCTACGTACATGGCTGGG-3'

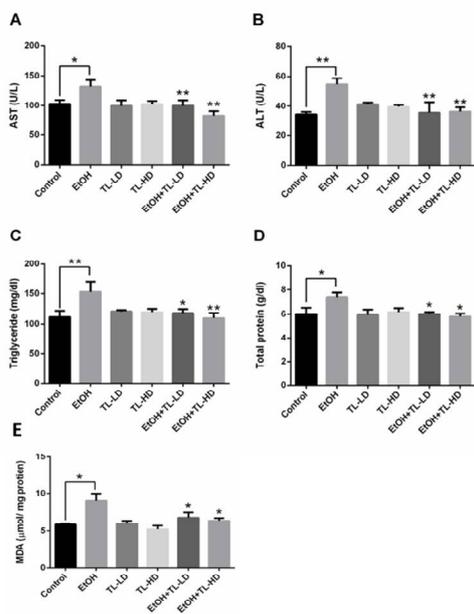


Figure 1: Effect of *T. laurifolia* aqueous leaf extract on liver enzymes, triglycerides, total protein and MDA levels. AST (A), ALT (B), triglycerides (C), total protein (D) and MDA (E) levels of rats in the control, EtOH, TL-LD, TL-HD, EtOH+TL-LD and EtOH+TL-LD groups. Results are expressed as mean \pm SEM (n = 4 per group). *p < 0.05; **p < 0.001

Effect of *T. laurifolia* aqueous leaf extract on histopathological changes in rat liver

Liver tissue from rats in the control group showed normal histology (Figure 2 A). Moreover, liver tissue from rats in the TL-LD and TL-HD groups also had normal histology, indicating that 100 and 200 mg/kg of *T. laurifolia* leaf extract given once a day from weeks 5 -10 did not produce hepatotoxic effects (Figure 2 C and D). The hepatocytes of rats in the EtOH group revealed cell death (Figure 2 B). However, treatments with low dose (EtOH+TL-LD group) and high dose (EtOH+TL-HD group) of *T. laurifolia* leaf extract were able to mitigate alcohol-induced liver pathology, as shown in Figures 2 E and F.

Effect of aqueous leaf extract of *T. laurifolia* on hepatic NADPH oxidase, CYP2E1, IL-1 β and TNF- α gene expressions

Rats in the EtOH group showed significantly up-regulated gene expressions of hepatic CYP2E1, NADPH oxidase, IL-1 β and TNF- α , when compared to rats in the control group (p < 0.001) (Figure 3). In the high and low dose groups of *T. laurifolia* leaf extract (EtOH + TL-LD group and EtOH + TL-HD group), there were significant down-regulations of hepatic CYP2E1 gene expressions (p < 0.05), when compared to the control group (Figure 3 A). Moreover, there was

significant down-regulation of hepatic NADPH oxidase gene expressions in these groups (p < 0.05, p < 0.001, respectively), relative to the control group (Figure 3 B). Rats in the EtOH + TL-LD group had significantly down-regulated gene expression of hepatic TNF- α (p < 0.001), while rats in the EtOH + TL-HD group had significantly down-regulated gene expressions of hepatic IL-1 β (p < 0.05) and TNF- α (p < 0.001), when compared to rats in the EtOH group (Figures 3 C and D). Thus, *T. laurifolia* leaf extract treatment down-regulated gene expressions of hepatic TNF- α and hepatic IL-1 β in response to ethanol treatment.

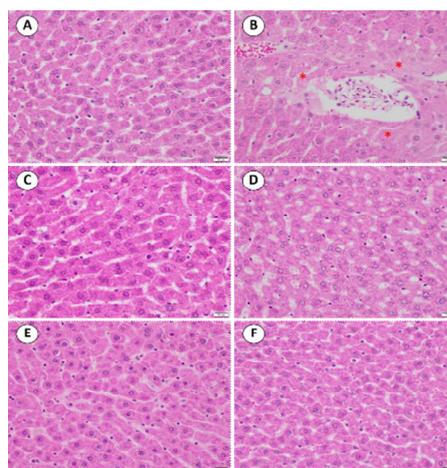


Figure 2: Effect of *T. laurifolia* aqueous leaf extract on liver histopathological changes. Control (A), EtOH (B), TL-LD (C), TL-HD (D), EtOH + TL-LD (E) and EtOH + TL-LD (F) groups. The red asterisks indicate cell death. Scale bar = 20 μ m (H & E staining)

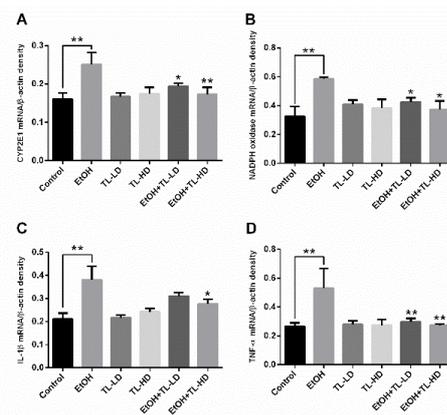


Figure 3: Effect of aqueous leaf extract of *T. laurifolia* on hepatic NADPH oxidase, CYP2E1, IL-1 β and TNF- α gene expressions. Gene expressions of hepatic NADPH oxidase (A), CYP2E1 (B), IL-1 β (C) and TNF- α (D) of control, EtOH, TL-LD, TL-HD, EtOH + TL-LD, and EtOH + TL-LD groups. Results are expressed as mean \pm SEM (n = 4); *p < 0.05; **p < 0.001

DISCUSSION

Normally, serum ALT is considered a more specific biomarker of liver damage than serum AST, because ALT originates primarily from the hepatocytes, whereas AST is also found in extrahepatic tissues [15]. However, increased activities of blood liver enzymes do not necessarily reflect liver cell death [16]. This study has indicated that *T. laurifolia* leaf extract treatment did not induce liver damage. Rather, it normalized blood levels of AST, ALT, triglycerides and total protein in ethanol-treated rats. The effects of *T. laurifolia* leaf extract on ethanol-treated rats are similar to the effects observed with other herbal extract [17].

Oxidative stress is characterized by an imbalance between oxidants and antioxidants. Malondialdehyde (MDA) is a well-known biomarker of oxidative stress. An increase in ROS causes overproduction of MDA. In this study, ethanol increased MDA levels in rats. Interestingly, treatment with *T. laurifolia* leaf extract reversed the ethanol-induced increases in MDA levels. Thus, the antioxidant effect of *T. laurifolia* leaf extract reduces oxidative stress. From previous studies, high performance liquid chromatography analysis of aqueous leaf extract of *T. laurifolia* identified caffeic acid and apigenin as primary constituents, whereas the acetone and ethanol extracts contained primarily chlorophyll a and b, pheophorbide a, pheophytin a, and lutein. The antioxidant properties of plant extracts are due to their phytochemical constituents [18,19].

The present study has demonstrated that ethanol up-regulated gene expressions of hepatic CYP2E1, NADPH oxidase, IL-1 β and TNF- α . It is known that CYP2E1 metabolizes ethanol and generates ROS. Moreover, CYP2E1 overexpression is increasingly recognized as a key factor in hepatic injury [20]. The down-regulation of hepatic CYP2E1 gene expressions and alleviation of ethanol-induced liver damage by *T. laurifolia* leaf extract are similar to previous report [21]. Normally, NADPH oxidase is expressed in hepatocytes, hepatic stellate cells and Kupffer cells in the liver. It is involved in alcoholic liver injury as major source of ROS leading to oxidative stress [5]. In the present study, it was found that *T. laurifolia* leaf extracts at low dose and high dose down-regulated hepatic NADPH oxidase gene expression in response to ethanol intake in rats. Previous studies demonstrated that down-regulation of hepatic NADPH oxidase gene attenuated ALD [5,22].

The pro-inflammatory cytokine IL-1 β is up-regulated in alcoholic liver disease [23]. Another pro-inflammatory cytokine is TNF- α is mainly produced by Kupffer cells in the liver. It is a key mediator in various physiological processes such as inflammation and apoptosis [24]. It has been reported that in alcoholic liver disease patients, the levels of TNF- α were increased, when compared with non-alcoholic fatty liver disease controls [25]. The present study has demonstrated that *T. laurifolia* leaf extract down-regulated gene expressions of hepatic IL-1 β and TNF- α in response to ethanol treatment. These results indicate that *T. laurifolia* possesses anti-inflammatory properties in alcoholic liver injury related to other conditions [9].

CONCLUSION

These findings indicate that the aqueous leaf extract of *T. laurifolia* alleviates alcohol-induced liver injury in male Wistar rats. It does this through reduction in MDA levels, down-regulation of hepatic CYP2E1 and NADPH oxidase gene expressions, and down-regulation of the gene expressions of hepatic IL-1 β and TNF- α . Thus, the extract may potentially be suitable for the treatment of liver injury.

DECLARATIONS

Acknowledgement

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Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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