

Protective Effects of *Glochidion wallichianum* Mull. Arg. on Ethanol-induced Liver Injury in Rats

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Received: 19 April 2021, Revised: 17 June 2021, Accepted: 24 June 2021

Abstract

Chronic ethanol consumption leads to the development of liver disease. In the present study, the effects of *Glochidion wallichianum* Mull. Arg. ethanol extract (GWE) on chronic ethanol-induced liver injury in rats were evaluated. Total phenolic and flavonoid contents and *in vitro* antioxidant activities were determined. In the *in vivo* experiment, male Wistar rats were divided into 5 groups: Control, ethanol, ethanol plus low and high doses of GWE (ethanol+GWE300 and ethanol+GWE600, respectively) and GWE alone (GWE600). All treatments were administered orally for 28 days. Serum biochemical profiles, hepatic malondialdehyde (MDA) levels, antioxidant enzyme activities, cytochrome P450 2E1 (CYP2E1) expression, and liver histopathology were assessed. GWE contained high concentrations of total phenols and total flavonoids and exhibited inhibition of 2,2-diphenyl-1-picrylhydrazyl free radical. The GWE treatment significantly decreased alanine aminotransferase (ALT) levels, reduced hepatic MDA levels, increased catalase (CAT) activity, and improved histopathological alterations. However, compared with the control condition, GWE alone induced a low level of necrosis. The expression of the CYP2E1 mRNA was not different among the groups ($p > 0.05$). Based on these findings, GWE exerts a protective effect on ethanol-induced liver injury in rats through its antioxidant activity.

Keywords: *Glochidion wallichianum*, Ethanol, Liver injury, Antioxidants, CYP2E1

Introduction

Alcoholic liver disease (ALD) is one of the most common causes of liver injury. The spectrum of ALD ranges from simple steatosis to alcoholic steatohepatitis, which eventually progresses to cirrhosis and, finally, to hepatocellular carcinoma [1]. Hepatic damage is mainly caused by acetaldehyde, a major toxic metabolite of alcohol metabolism in hepatic tissues by enzymes such as alcohol dehydrogenase and cytochrome P450 2E1 (CYP2E1) [1]. Acetaldehyde promotes adduct formation, impairing the functions of key proteins, including enzymes, and inducing DNA damage [2]. In addition, the oxidation of CYP2E1 results in the increased production of reactive oxygen species (ROS) such as superoxide radicals, hydroxyl radicals, and hydrogen peroxide that react with cellular macromolecules, such as DNA, proteins, and lipids [3]. ROS inhibit or reduce endogenous enzymatic and nonenzymatic antioxidants and lead to oxidative stress [4]. Various enzymatic and nonenzymatic antioxidants are involved in the mechanism protecting cells from ROS.

In recent years, many natural antioxidants have been reported to exert free radical scavenging and hepatoprotective effects that protect the liver from alcohol-induced injury [5-7]. *Glochidion wallichianum* Mull. Arg. (GW) is an indigenous vegetable belonging to the Phyllanthaceae family that is widely grown in southern Thailand. Young GW leaves are served with Thai fermented rice noodles and Thai curry dishes. The young leaves of this plant are rich sources of β -carotene, lutein, and polyphenols, particularly gallic acid [8]. GW has consistently been shown to display relatively high levels of free radical scavenging activity and reducing capacity *in vitro* [8-11]. However, the antioxidant activity of GWE has not been investigated *in vivo*. The objective of the present study was to evaluate the protective effects of GWE on ethanol-induced liver injury.

Materials and methods

Preparation of the GW ethanol extract (GWE)

GW was collected from Nakhon Si Thammarat, Thailand. After identification, a specimen (01517) was deposited at the Walailak Herbarium, Walailak University. The fresh young leaves were rinsed with distilled water and dried in the oven (UEF700, Memmert, Germany) at 45 °C for 2 days. The dried leaves were pulverized into a powder using a mechanical grinder (SM2,000, Retsch, Germany). The 500 g of powdered GW were extracted 3 times with 5 L of 95 % ethanol by maceration for 3 days at room temperature. The crude extract was filtered through Whatman no. 1 filter paper. The extracts were evaporated under reduced pressure using a rotary evaporator (R-210, Buchi, Switzerland). The crude extracts were weighed and stored at -20 °C until use.

Determination of the total phenolic content

The total phenolic content was determined using the Folin-Ciocalteu method [12]. Briefly, 200 µL of the GWE solution (0.2 mg/mL in 95 % ethanol) were mixed with 100 µL of the Folin-Ciocalteu reagent and 2 mL of 2 % Na₂CO₃. After incubation for 30 min at room temperature, the absorbance was measured at 750 nm using a spectrophotometer (UV-1,800, Shimadzu Scientific Instruments, Japan). Gallic acid was used as the standard for the calibration curve, and the results are reported as mg gallic acid equivalent/g dry weight (DW) of extract.

Determination of the total flavonoid content

The total flavonoid content was determined using a procedure described in the literature [13]. Briefly, 1 mL of the GWE solution (0.4 mg/mL in 95 % ethanol) was mixed with 1 mL of a 2 % AlCl₃ methanol solution. After incubation at room temperature for 15 min, the absorbance was measured at 430 nm using a spectrophotometer (UV-1,800, Shimadzu Scientific Instruments, Japan). Rutin was used as the standard for the calibration curve, and the results are reported as mg rutin equivalent/g DW of extract.

2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging assay

The DPPH radical scavenging activity assay was performed using the method described in the literature [14], in which 100 µL of different concentrations of the extract were mixed with 100 µL of a 0.1 mM DPPH ethanol solution. After incubation for 30 min in the dark at room temperature, the absorbance of the mixed solution was determined at 517 nm using a microplate spectrophotometer (Eon, BioTek Instruments, Inc., USA). Ascorbic acid was used as the positive control. DPPH radical scavenging activity was calculated using the following equation;

$$\text{DPPH scavenging effect (\%)} = \left(\frac{A_1 - A_0}{A_1} \right) \times 100$$

where A₁ is the absorbance of the DPPH solution without sample and A₀ is the absorbance of the sample at various concentrations with DPPH. The antioxidant activity was expressed as EC₅₀ (µg/mL).

Animal experiment

Male Wistar rats weighing 200 - 280 g were purchased from Nomura Siam International Co., Ltd. (Thailand). They were housed under standard environmental conditions (room temperature, 23 ± 1 °C; relative humidity, 50 - 70 %; and 12-h light/dark cycle) and received food and water *ad libitum*. The animal study was approved by the Animal Ethics Committee of Walailak University (certificate no. 004/2019). Thirty rats were randomly divided into 5 groups of 6 rats each. A rat model of ethanol-induced liver injury was established as previously described [15]. The dose of GWE used in this experiment was based on the dose of a plant (*Phyllanthus amarus*) from the Phyllanthaceae family reported in a previous study [16]. The control group received 4 % dimethyl sulfoxide and the GWE group received GWE [600 mg/kg body weight (BW)]. The ethanol group was administered 50 % ethanol (v/v) at a dose of 5 g/kg BW. The ethanol+GWE300 and ethanol+GWE600 groups were treated with GWE at doses of 300 and 600 mg/kg BW, respectively, after ethanol administration. All treatments were administered by oral gavage for 28 days. At the end of the experiment, the rats were anesthetized with thiopental sodium (50 mg/kg BW) by intraperitoneal injection, and blood samples were collected for biochemical analyses using the cardiac puncture technique. The rats were sacrificed with an overdose of thiopental sodium (100 mg/kg BW), and the livers were quickly removed. The left lobe of the liver was fixed with 10 % formaldehyde, and the remaining portion of the liver was stored at -80 °C until use.

Measurement of biochemical parameters in serum

The blood samples were centrifuged at 2,000×g for 15 min, and sera were collected. The serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were measured by kinetic methods. All measurements were performed using ABX Pentra 400 reagents with an automated biochemistry analyzer (ABX Pentra 400, Horiba, France).

Measurement of hepatic malondialdehyde (MDA) levels

Hepatic MDA levels were determined using the procedure described in the literature [17]. Briefly, the liver tissues were homogenized in 1.15 % KCl and centrifuged at 1,600×g for 10 min at 4 °C to collect the supernatant. 200 microliters of each sample were mixed with 0.2 mL of 8.1 % sodium dodecyl sulfate, 1.5 mL of 20 % acetic acid (pH 3.5), and 1.5 mL of 0.8 % thiobarbituric acid. The mixture was heated at 95 °C for 1 h and then cooled in tap water. After centrifugation at 4,000×g for 10 min, the supernatant was collected and measured at 532 nm using a spectrophotometer (UV-1,800, Shimadzu Scientific Instruments, Japan). The hepatic MDA content was determined by comparison with a standard MDA curve and reported as nmol/mg protein.

Determination of superoxide dismutase (SOD) activity

Liver tissues were homogenized in ice-cold 0.1 M Tris/HCl, pH 7.4, containing 0.5 % Triton X-100, 5 mM β-ME, and 0.1 mg/mL PMSF and centrifuged at 14,000×g for 5 min at 4 °C to collect the supernatant. SOD activity was measured using a commercial detection kit (Biovision, USA). SOD analysis was performed based on the ability of SOD to inhibit the reduction of a water-soluble tetrazolium salt (WST-1) to a formazan dye by the xanthine oxidase/xanthine reaction. The results are reported as the inhibition rate (%).

Determination of catalase (CAT) activity

CAT activity was determined using a previously reported procedure [18]. Briefly, liver tissues were homogenized in ice-cold 50 mM potassium phosphate, pH 7.0, containing 1 mM EDTA and were centrifuged at 10,000×g for 15 min at 4 °C to collect the supernatant. The 30 μL of each sample was added to 300 μL of 100 mM H₂O₂ and immediately mixed by inversion. The initial absorbance and that at 1 min after the first reading were recorded at 240 nm using a spectrophotometer (UV-1,800, Shimadzu Scientific Instruments, Japan). CAT activity was reported as U/mg protein.

Quantitative real-time polymerase chain reaction (qRT-PCR) analysis of *CYP2E1* expression

Total RNA was extracted from liver tissues using GENEzol™ reagent (Geneaid, Taiwan) and reverse transcribed into cDNAs using the FIREScript RT cDNA Synthesis Kit (Solis BioDyne, Estonia). Aliquots of cDNAs were amplified using primers for *CYP2E1* (sense, 5'TGTCAAGGAGGTGCTACTGA 3' and antisense, 5'CGCAGCCAATCAGAAATGTG 3') and β-Actin (sense, 5' GACCTCTATGCCAACACAGT 3' and antisense, 5'GGTGTAACCGCAGCTCAGTA 3'). qRT-PCR was performed using 5X HOT FIREPol® EvaGreen® qPCR Mix Plus (ROX) (Solis BioDyne, Estonia) with a QuantStudio™ 3 Real-Time PCR System (Applied Biosystems, USA). The qRT-PCR conditions were as follows: 1 cycle at 95 °C for 12 min to activate the enzyme and 40 cycles of denaturation at 95 °C for 15 s, annealing at 61 °C for 20 s, and extension at 72 °C for 20 s. β-Actin served as endogenous control to normalize the target gene expression levels. The results are reported as the ratio of the target gene to the control gene determined using the 2^{-ΔΔCt} method.

Histopathological analysis

The liver tissues were fixed with 10 % neutral buffered formalin and embedded in paraffin. The tissue paraffin blocks were sliced into 5-μm-thick sections using a manual rotary microtome (RM2235, Leica Biosystems, Germany). The sections were stained with hematoxylin-eosin (H&E). Histological changes were examined in a blinded manner under a light microscope as described in the literature [19]. Briefly, steatosis was scored using the following scale based on the percentage of parenchymal cells containing lipids (micro-or macrosteatosis): < 25 % = 1; 25 to 50 % = 2; 50 to 75 % = 3; and > 75 % = 4. Hepatic inflammation was evaluated using the following scale: No inflammation = 1; occasional foci of inflammatory cells = 2; frequently occurring small foci of inflammatory cells = 3; and frequently occurring large foci of inflammatory cells = 4. Necrosis was assessed using the following scale: Occasional (< 1 %) necrotic hepatocytes = 1; frequent (5 - 10 %) necrotic hepatocytes = 2; small foci of necrotic cells (clusters of > 10 necrotic cells) = 3; and extensive areas of necrosis (> 25 % of the lobular

unit) = 4. The total score was calculated by adding the scores for steatosis, inflammation, and necrosis. Therefore, the total pathology score for a normal liver was equal to 3.

Statistical analysis

Data are presented as means \pm standard deviations (SD). Data were analyzed using parametric tests and one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. Kruskal-Wallis tests were conducted if the data had a non-normal distribution or homogeneity of variance. A p -value < 0.05 was considered significant.

Results and discussion

Total phenolic and flavonoid contents and *in vitro* antioxidant activity

The total phenolic content of GWE was calculated using a linear equation for gallic acid standard ($y = 0.0084x + 0.0176$; $R^2 = 0.9996$) (Figure 1), and the total flavonoid content of GWE was calculated using a linear equation for the rutin standard ($y = 0.0073x + 0.0026$; $R^2 = 0.999$) (Figure 2). Using the equations obtained from the calibration curves, the total phenolic content and total flavonoid content of GWE were 373.93 ± 18.67 mg gallic acid equivalent/g DW and 73.03 ± 0.55 mg rutin equivalent/g DW, respectively.

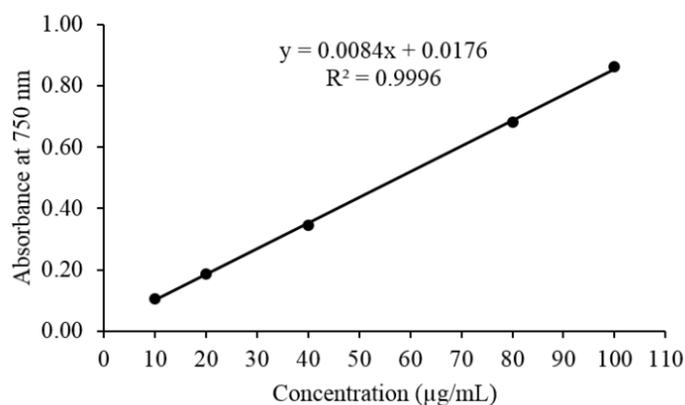


Figure 1 Gallic acid standard calibration curve for the quantification of the total phenolic content.

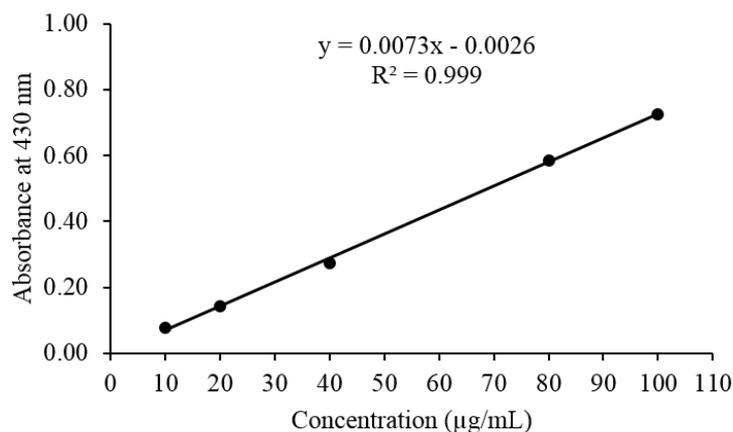


Figure 2 Rutin standard calibration curve for the quantification of the total flavonoid content.

The percentages of the DPPH radical-scavenging activity at different concentrations of GWE and ascorbic acid are shown in Figure 3. The half-maximal effective concentration (EC_{50}) of GWE was 8.32 ± 0.19 $\mu\text{g/mL}$, and the value of ascorbic acid was 7.01 ± 0.16 $\mu\text{g/mL}$.

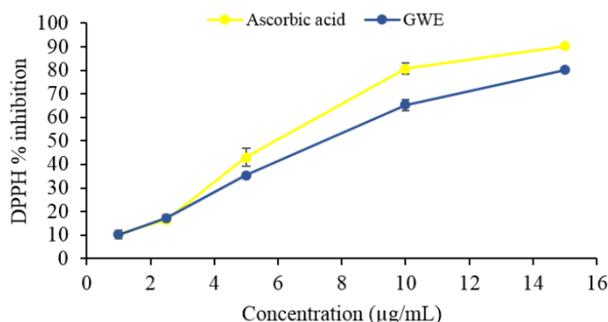


Figure 3 DPPH radical - scavenging activities of GWE and ascorbic acid. Each value represents the mean \pm SD (n = 3).

Effect of GWE on serum ALT and AST levels

Compared with the control condition, ethanol induced an increase in the serum ALT level ($p < 0.05$). Treatment with 600 mg/kg BW GWE reversed the increase in ALT levels induced by the ethanol treatment ($p < 0.05$). The administration of 600 mg/kg BW GWE alone did not produce any significant change in the serum ALT level. A statistically significant difference in serum AST levels was not observed between the groups (Table 1).

Table 1 Effect of GWE on serum ALT and AST levels in different experimental groups of rats.

Group	ALT (U/L)	AST (U/L)
Control	35.33 \pm 2.87	90.33 \pm 9.66
Ethanol	46.00 \pm 5.14 ^a	91.66 \pm 11.99
Ethanol+GWE300	40.00 \pm 8.96	82.00 \pm 8.00
Ethanol+GWE600	37.66 \pm 3.07 ^b	80.83 \pm 15.54
GWE600	35.50 \pm 5.08 ^b	75.83 \pm 12.05

Values are presented as means \pm SD (n = 6). Means in the same column with different superscript letters are significantly different ($p < 0.05$). ^a Compared with the control group; ^b compared with the ethanol group.

Effect of GWE on the hepatic MDA levels

MDA is the end product of lipid peroxidation by ROS and directly induces liver injury. As shown in Figure 4, MDA levels were significantly increased in the ethanol group compared with the control group ($p < 0.05$). The MDA levels of the GWE600 group did not differ from the control group. Treatment with 300 and 600 mg/kg BW GWE significantly reduced the increase in MDA levels induced by the ethanol treatment ($p < 0.05$).

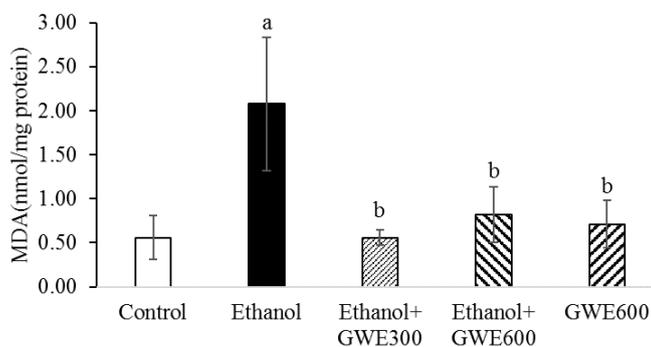


Figure 4 Levels of hepatic MDA in different experimental groups of rats. Values are expressed as the mean \pm SD (n = 6). Different superscript letters are significantly different ($p < 0.05$). ^a Versus the control group, ^b versus the ethanol group.

Effect of GWE on hepatic antioxidant enzyme activities

A significant decrease in CAT activity was observed in the ethanol group compared to the control and GWE600 groups ($p < 0.05$). Treatment with 300 mg/kg BW GWE increased CAT activity ($p < 0.05$) (Figure 5A). A statistically significant difference in SOD activity was not observed between groups (Figure 5B).

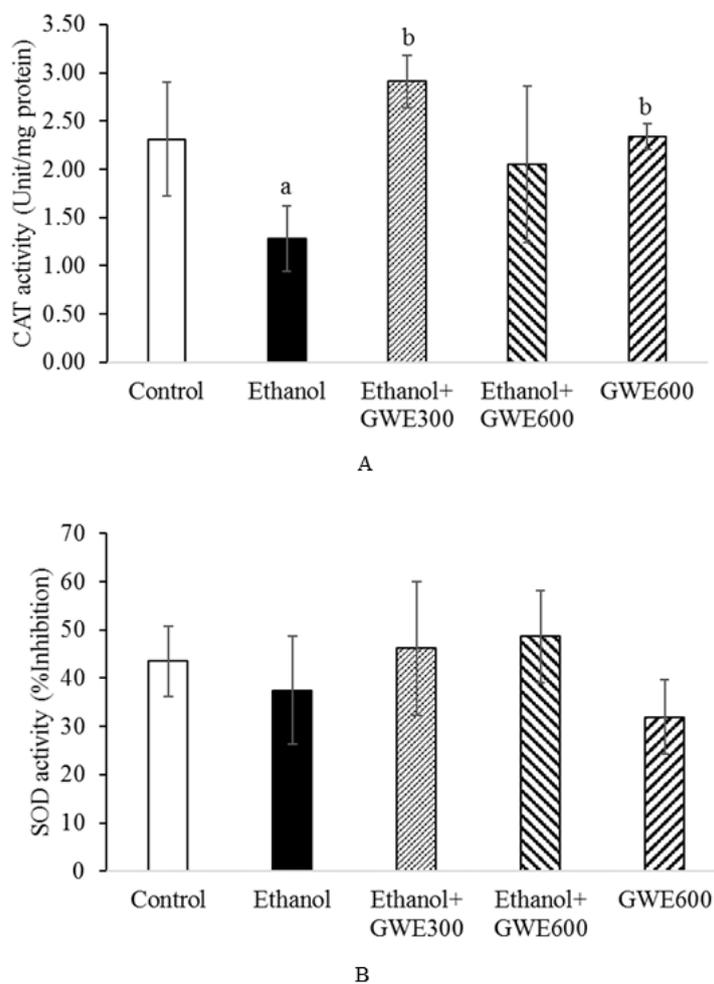


Figure 5 Effect of GWE on CAT activity (A) and SOD activity (B) in different experimental groups of rats. Values are expressed as the mean \pm SD ($n = 6$). Different superscript letters are significantly different ($p < 0.05$). ^a Versus the control group, ^b versus the ethanol group.

Effect of GWE on *CYP2E1* mRNA expression

The expression of the *CYP2E1* mRNA in the liver determined using qRT-PCR is shown in Figure 6. The expression of the *CYP2E1* mRNA in the liver of the ethanol group was not different from the expression observed in the control group ($p > 0.05$). Compared with the ethanol treatment alone, the GWE treatment (300 or 600 mg/kg BW) did not exert a statistically significant effect on the hepatic expression of the *CYP2E1* mRNA. In addition, the expression of the *CYP2E1* mRNA was not altered after treatment with GW600 alone.

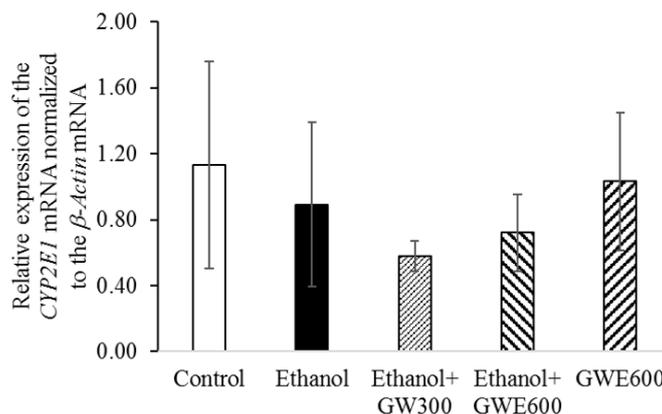


Figure 6 Relative mRNA expression levels of *CYP2E1* in different experimental groups of rats (n = 5).

Effect of GWE on liver histopathology

The H&E stained liver sections from the control group showed a normal morphology of hepatocytes with acidophilic cytoplasm and a normal structure of hepatic sinusoids (**Figure 7A**). In contrast, the liver sections from the ethanol group revealed a marked loss of the normal liver arrangement with a predominance of microvesicular steatosis, cytoplasmic vacuolation and hepatocyte degeneration (**Figure 7B**). The liver sections from the ethanol+GWE300 and ethanol+GWE600 groups showed improvements in hepatic steatosis and necrosis (**Figure 7C** and **D**). However, the liver sections from the GWE600 group showed a slightly vacuolated cytoplasm and slight necrosis (**Figure 7E**).

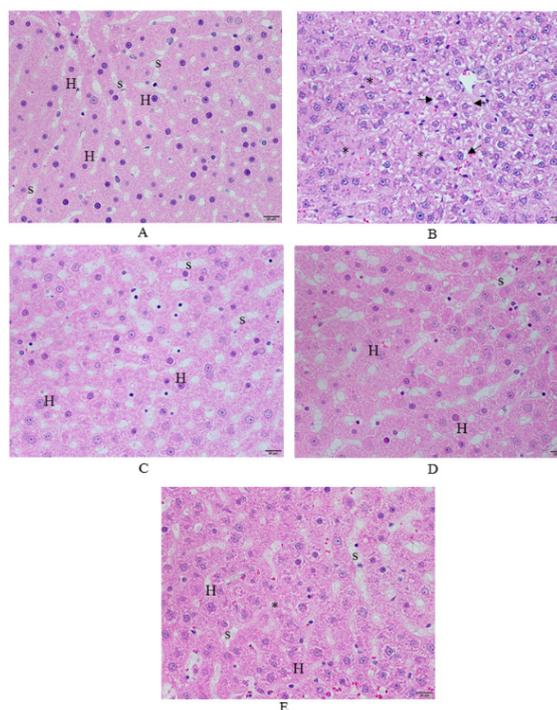


Figure 7 H&E staining of liver tissues; A) tissues from the control group exhibited a normal histological appearance of polygonal hepatocytes, H) radiating from the central vein with rounded vesicular nuclei and acidophilic cytoplasm. Narrow radiating blood sinusoids (s) were observed between liver cords. B) marked microvesicular steatosis, cytoplasmic vacuolation and hepatocyte degeneration were observed in the ethanol group. C) and D) after the GWE treatment (300 and 600 mg/kg BW, respectively), liver histopathology improved. E) the GWE600 group presented mild cytoplasmic vacuolation and slight necrosis. All images were captured at 400 \times magnification. Bar = 20 μ m. H: Hepatocytes; S: Sinusoids; Black arrow: Microvesicular steatosis; Black asterisk: Necrosis.

The results of semiquantitative analysis of histopathological changes in the liver tissues are shown in **Table 2**. The total pathology scores of the ethanol, ethanol+GWE600 and GWE600 groups were significantly higher than the control group ($p < 0.05$). Interestingly, the mean steatosis, necrosis and total pathology scores were significantly decreased in the ethanol+GWE300 group compared with the ethanol group.

Table 2 Summary of the liver pathology scores in different experimental groups of rats.

Group	Steatosis	Inflammation	Necrosis	Total pathology
Control	1.00 ± 0.00	1.00 ± 0.00	1.28 ± 0.08	3.28 ± 0.08
Ethanol	1.20 ± 0.11 ^a	1.00 ± 0.00	2.68 ± 0.59 ^a	4.88 ± 0.58 ^a
Ethanol+GWE300	1.00 ± 0.00 ^b	1.00 ± 0.00	1.15 ± 0.23 ^b	3.15 ± 0.23 ^b
Ethanol+GWE600	1.08 ± 0.20	1.00 ± 0.00	1.87 ± 0.45 ^{ac}	3.95 ± 0.56 ^{ac}
GWE600	1.00 ± 0.00	1.00 ± 0.00	2.53 ± 0.41 ^{ac}	4.53 ± 0.41 ^{ac}

Values are expressed as the mean ± SD (n = 6). Different superscript letters are significantly different ($p < 0.05$). ^a Versus the control group, ^b versus the ethanol group, ^c versus the ethanol+GWE300 group.

Discussion

Alcohol-induced liver injury contributes to the pathogenesis of ALD, which ranges from fatty liver (steatosis) to alcoholic hepatitis, fibrosis, and cirrhosis. In the present study, a rat model that was chronically administered alcohol was established to simulate the early stage of ALD (fatty degeneration and necrosis). As shown in the present study, GWE ameliorates chronic ethanol-induced liver injury. ALT and AST are the enzymes that catalyze the transfer of an amino group from an amino acid to α -ketoglutarate and are mainly localized in the cytosol of hepatocytes. When the liver injury occurs, cell membrane permeability increases and causes a significant increase in serum ALT and AST levels [20]. Therefore, serum ALT and AST levels are biochemical markers of liver injury and potentially indicate early ALD [5,21]. Ethanol metabolism generates ROS which, react with different cellular components, causing the oxidation of lipids and proteins and DNA damage and finally leading to cell death [22]. In the present study, the serum ALT level increased significantly in ethanol-fed rats, indicating hepatocellular damage. Furthermore, chronic ethanol-induced liver injury was further confirmed by histopathological changes in the liver. The GWE treatment markedly decreased the ethanol-induced increase in the ALT level and ameliorated hepatocyte steatosis and necrosis. Our model of ALD, which included 4 weeks of ethanol administration (5 g/kg BW), showed mild liver injury, and thus, a further study should be conducted with a higher dose of ethanol and long-term ethanol treatment for more severe liver damage [23,24].

Several factors and mechanisms are associated with the pathological progression of ethanol-induced liver injury. Ethanol-induced oxidative stress plays a key role in the development of ALD. Ethanol-mediated oxidation in the liver produces many ROS, such as hydroxyl radicals, superoxide radicals, and hydrogen peroxide, leading to an imbalance between oxidants and antioxidant defenses that cause oxidative stress [3]. Prolonged ethanol exposure may result in the excess production of ROS, leading to protein, DNA, and lipid damage that induce necrotic and apoptotic cell death [3,25]. A number of experimental studies have confirmed that ethanol intake increases the formation of lipid peroxidation products, such as MDA, a biomarker of oxidative stress [6,15,21,25]. In our study, increased MDA levels and pathological liver injury were observed in rats after chronic ethanol administration. Treatment with 300 or 600 mg/kg BW GWE reduced MDA levels to a normal level.

SOD and CAT activities were examined in this study to delineate the mechanisms underlying the protective effect of GWE on rats. SOD and CAT provide the first line of defense against ROS. SOD converts superoxide to hydrogen peroxide, and then CAT catalyzes the metabolism of hydrogen peroxide into water and oxygen. Chronic ethanol exposure results in the overproduction of ROS and significantly decreases both SOD and CAT activities in rodent models [26-28]. In the present study, the ethanol treatment significantly decreased CAT activity but did not alter SOD activity. A potential explanation for this finding is the effect of the duration and amount of ethanol consumption on the severity of the liver injury. High doses of ethanol and long-term treatment are associated with increased lipid peroxidation, impaired antioxidant enzyme activities, and severe pathology of the liver [26-28]. Our model showed mild histopathological changes in both steatosis and necrosis in the liver. Therefore, hepatic SOD activity

did not change in the early stage of ethanol-induced liver injury. This result is also consistent with the results from previous studies [29,30]. Treatment with GWE restored CAT activity to a level similar to the CAT activity of the control group, indicating amelioration of the changes induced by oxidative stress and improvements in antioxidant enzyme activities in the liver.

CYP2E1 has been reported to be a major contributor to oxidative stress and the induction of liver injury. Acute and chronic ethanol ingestion increases the expression and activity of *CYP2E1*, which converts ethanol to acetaldehyde and induces ROS production [5,7,31,32]. In the present study, the rats in the ethanol group did not exhibit increased hepatic expression of the *CYP2E1* mRNA compared to the rats in the control group. This result may be attributed to the blood ethanol level and ethanol treatment duration. In this study, 4 weeks of ethanol administration (5 g/kg BW) may not have increased blood alcohol levels to a sufficient extent to induce hepatic *CYP2E1* expression. In contrast, treatment with the Lieber-DeCarli liquid diet for 5 weeks upregulated hepatic *CYP2E1* expression [33]. In that study, the daily intake of ethanol in rats reached 12 - 18 g/kg BW, which was 3 times higher than the intake achieved in our model [34]. Another possible explanation is the duration and amount of alcohol intake. Chronic ethanol exposure (4 - 10 weeks) increases the expression of *CYP2E1* [31,32]. Therefore, in the present study, a 4 - week ethanol gavage model was adopted.

Previous studies have reported that GW contains phenolic compounds, particularly gallic acid, epicatechin gallate, and apigenin [8,35]. The dose of GWE was based on that of *P. amarus* from the Phyllanthaceae family [16], and the major compounds of *P. amarus* are phyllanthin, hypophyllanthin, corilagin, geraniin, ellagic acid, and gallic acid [16,36]. Ethanol extracts of *P. amarus* and GW contain high quantities of gallic acid [16,35].

In the present study, the analyzed GWE had high phenol and flavonoid contents and exerted a strong radical-scavenging effect, suggesting that phenolic compounds are the main components responsible for the antioxidant activity of GWE. This finding was consistent with previous studies showing that GW had the highest antioxidant capacity among southern Thai indigenous vegetables [11]. Our study is the first report of the *in vivo* antioxidant activity of GWE. GWE ameliorates ethanol-induced liver injury. The protective effect of GWE may be attributed to the presence of phenolic compounds, particularly gallic acid and flavonoids. Phenolic compounds act as electron donors, reducing the oxidized intermediates of lipid peroxidation, scavenging free superoxide radicals, and improving antioxidant enzyme function [37]. Based on our results, the GWE treatment attenuated oxidative stress and related damage by increasing the endogenous antioxidant enzyme activity.

Additionally, the hepatoprotective activity of GWE was more potent at the 300 mg/kg BW dose than at the 600 mg/kg BW dose. Treatment with GWE (300 mg/kg BW) improved the necrosis score compared with that obtained with the high dose of GWE (600 mg/kg BW). Moreover, treatment with GWE alone resulted in a low level of necrosis but did not increase the serum aminotransferase levels. Thus, GWE alone might be toxic to the liver. Although the young leaves of GW are excellent sources of antioxidant polyphenols, plant extracts containing concentrated amounts of phytochemicals might be toxic at high doses. Further studies are needed to investigate the toxicity of GW, ensure its safety, identify the active components in GWE, and explore the mechanism of action.

Conclusions

In the present study, the protective effects of GWE on ethanol-induced liver injury were reported for the first time. The protective effects of GWE may be attributed to its ability to reduce the increase in hepatic ALT and MDA levels, increase CAT activity and ameliorate histopathological changes. This potential protective mechanism may be associated with increased radical scavenging and antioxidant activities. GWE alone may be hepatotoxic at a high dose; hence, caution should be exercised when choosing a dose for the management of liver injury.

Acknowledgements

This research was funded by Walailak University, Nakhon Si Thammarat, Thailand (Grant number WU-IRG-62-019).

References

- [1] R Teschke. Alcoholic liver disease: Alcohol metabolism, cascade of molecular mechanisms, cellular targets, and clinical aspects. *Biomedicines* 2018; **6**, 106.
- [2] M Setshedi, JR Wands and SMDL Monte. Acetaldehyde adducts in alcoholic liver disease. *Oxidative Med. Cell. Longevity* 2010; **3**, 178-85.
- [3] MG Moreno and GG Reyes. The role of oxidative stress in the development of alcoholic liver disease. *Revista Gastroenterología México* 2014; **79**, 135-44.
- [4] L Xu, Y Yu, R Sang, J Li, B Ge and X Zhang. Protective effects of taraxasterol against ethanol-induced liver injury by regulating CYP2E1/Nrf2/HO-1 and NF- κ B signaling pathways in mice. *Oxid. Med. Cell. Longev.* 2018; **2018**, 8284107.
- [5] K Maimaitimin, Z Jiang, A Aierken, M Shayibuzhati and X Zhang. Hepatoprotective effect of *Alhagi sparsifolia* against alcoholic liver injury in mice. *Braz. J. Pharm. Sci.* 2018; **54**, e17732.
- [6] I Shukla, L Azmi, SS Gupta, DK Upreti and CV Rao. Amelioration of anti-hepatotoxic effect by *Lichen rangiferinus* against alcohol induced liver damage in rats. *J. Ayurveda. Integr. Med.* 2019; **10**, 171-7.
- [7] Z Jiang, J Wang, H Xue, M Wang, H Jiang, Y Liang, AC Dias, M Gregory, C Chen and X Zhang. Protective effect of wild *Corni fructus* methanolic extract against acute alcoholic liver injury in mice. *Redox Rep.* 2017; **22**, 338-45.
- [8] R Kongkachuichai, R Charoensiri, K Yakoh, A Kringkasemsee and P Insung. Nutrients value and antioxidant content of indigenous vegetables from Southern Thailand. *Food Chem.* 2015; **173**, 838-46.
- [9] P Tangkanakul, G Trakoontivakorn and C Jariyavattanavijit. Extracts of Thai indigenous vegetables as rancid inhibitor in a model system. *Kasetsart J. Nat. Sci.* 2005; **39**, 274-83.
- [10] L Chanudom, P Bhoopong, R Khwanchuea and J Tangpong. Antioxidant and antimicrobial activities of aqueous & ethanol crude extract of 13 Thai traditional plants. *Int. J. Curr. Microbiol. App. Sci.* 2014; **3**, 549-58.
- [11] W Panpipat, W Suttirak and M Chaijan. Free radical scavenging activity and reducing capacity of five Southern Thai indigenous vegetable extracts. *Walailak J. Sci. Tech.* 2011; **7**, 51-60.
- [12] Y Choi, SM Lee, J Chun, HB Lee and J Lee. Influence of heat treatment on the antioxidant activities and polyphenolic compounds of shiitake (*Lentinus edodes*) mushroom. *Food Chem.* 2006; **99**, 381-7.
- [13] A Djeridane, M Yousfi, B Nadjemi, D Boutassouna, P Stocker and N Vidal. Antioxidant activity of some algerian medicinal plants extracts containing phenolic compounds. *Food Chem.* 2006; **97**, 654-60.
- [14] L Jing, H Ma, P Fan, R Gao and Z Jia. Antioxidant potential, total phenolic and total flavonoid contents of *Rhododendron anthopogonoides* and its protective effect on hypoxia-induced injury in PC12 cells. *BMC Compl. Alternative Med.* 2015; **15**, 287.
- [15] S Palipoch, P Koomhin, C Punsawad, P Na-Ek, A Sattayakhom and P Suwannalert. Heme oxygenase-1 alleviates alcoholic liver steatosis: Histopathological study. *J. Toxicol. Pathol.* 2016; **29**, 7-15.
- [16] F Naaz, S Javed and MZ Abdin. Hepatoprotective effect of ethanolic extract of *Phyllanthus amarus* Schum. et Thonn. on aflatoxin B1-induced liver damage in mice. *J. Ethnopharmacol.* 2007; **113**, 503-9.
- [17] H Ohkawa, N Ohishi and K Yagi. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal. Biochem.* 1979; **95**, 351-8.
- [18] RFJ Beers and IW Sizer. A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. *J. Biol. Chem.* 1952; **195**, 133-40.
- [19] MJJ Ronis, A Butura, BP Sampey, K Shankar, RL Prior, S Korourian, E Albano, M Ingelman-Sundberg, DR Petersen and TM Badgera. Effects of N-acetylcysteine on ethanol-induced hepatotoxicity in rats fed via total enteral nutrition. *Free Radic. Biol. Med.* 2005; **39**, 619-30.
- [20] MR Mcgill. The past and present of serum aminotransferases and the future of liver injury biomarkers. *EXCLI J.* 2016; **15**, 817-28.
- [21] T Zhou, YJ Zhang, DP Xu, F Wang, Y Zhou, J Zheng, Y Li, JJ Zhang and HB Li. Protective effects of lemon juice on alcohol-induced liver injury in mice. *Biomed Res. Int.* 2017; **2017**, 7463571.
- [22] ME Toth, L Vigh and M Santha. Alcohol stress, membranes, and chaperones. *Cell Stress Chaperones* 2014; **19**, 299-309.

- [23] W Leelananthakul, D Werawatganon, N Klaikeaw, M Chayanupatkul and P Siriviriyakul. Protective effects of genistein alleviate alcohol-induced liver injury in rats. *Phcog. Mag.* 2019; **15**, 342-7.
- [24] X Wang, K Dong, Y Ma, Q Jin, S Yin and S Wang. Hepatoprotective effects of chamazulene against alcohol-induced liver damage by alleviation of oxidative stress in rat models. *Open Life Sci.* 2020; **15**, 251-8.
- [25] P He, Y Wu, J Shun, Y Liang, M Cheng and Y Wang. Baicalin ameliorates liver injury induced by chronic plus binge ethanol feeding by modulating oxidative stress and inflammation via CYP2E1 and NRF2 in mice. *Oxid. Med. Cell. Longev.* 2017; **2017**, 4820414.
- [26] X Wang, K Dong, Y Ma, Q Jin, S Yin and S Wang. Hepatoprotective effects of chamazulene against alcohol-induced liver damage by alleviation of oxidative stress in rat models. *Open Life Sci.* 2020; **15**, 251-8.
- [27] SA El-Newary, NM Shaffie and EA Omer. The protection of *Thymus vulgaris* leaves alcoholic extract against hepatotoxicity of alcohol in rats. *Asian Pac. J. Trop. Med.* 2017; **10**, 361-71.
- [28] Z Goc, E Kapusta, G Formicki, M Martiniaková and R Omelka. Effect of taurine on ethanol-induced oxidative stress in mouse liver and kidney. *Chin. J. Physiol.* 2019; **62**, 148-56.
- [29] SC Yang, CC Huang, JS Chu and JR Chen. Effects of beta-carotene on cell viability and antioxidant status of hepatocytes from chronically ethanol-fed rats. *Br. J. Nutr.* 2004; **92**, 209-15.
- [30] PF Giriwono, T Hashimoto, Y Ohsaki, H Shirakawa, H Hokazono and M Komai. Extract of fermented barley attenuates chronic alcohol induced liver damage by increasing antioxidative activities. *Food Res. Int.* 2010; **43**, 118-24.
- [31] S Palipoch, P Koomhin and C Punsawad. Effect of aqueous leaf extract of *Thunbergia laurifolia* on alcohol-induced liver injury in rats. *Trop. J. Pharmaceut. Res.* 2019; **18**, 823-8.
- [32] AM Mohamed. Hepatoprotective role of *Solenostemma argel* growing in Egypt on ethanol induced oxidative damage in rats. *Turkish J. Biochem.* 2017; **42**, 419-26.
- [33] K Yamasaki, K Sugamoto, T Arakawa, K Nishiyama and M Yamasaki. Chronic intake of high-dose of blueberry leaf extract does not augment the harmful effects of ethanol in rats. *PeerJ* 2019; **7**, e6989.
- [34] AL Paz, F Hao, LJ Nelson, MT Vázquez, S Canals, MGD Moral, EM Naves, YA Nevzorova and FJ Cubero. Alcoholic liver disease: Utility of animal models. *World J. Gastroenterol.* 2018; **24**, 5063-75.
- [35] AS Yoon and P Sakdiset. Development of microemulsions containing *Glochidion wallichianum* leaf extract and potential for transdermal and topical skin delivery of gallic acid. *Sci. Pharm.* 2020; **88**, 53.
- [36] I Jantan, M Ilankovan, Yuandani and HF Mohamad. Correlation between the major components of *Phyllanthus amarus* and *Phyllanthus urinaria* and their inhibitory effects on phagocytic activity of human neutrophils. *BMC Compl. Alternative. Med.* 2014; **14**, 429.
- [37] NR Perron and JL Brumaghim. A review of the antioxidant mechanisms of polyphenol compounds related to iron binding. *Cell Biochem. Biophys.* 2009; **53**, 75-100.