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The ethanol extraction of *Gardenia stenophylla* Merr fruit mitigates carbon tetrachloride-induced hepatic damage in mice through modulation of oxidative stress and immunosuppression

Nhung, T. T. P. and Quoc, L. P. T.*

Institute of Biotechnology and Food Technology, Industrial University of Ho Chi Minh City, Ho Chi Minh City, Vietnam.

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Abstract The liver is crucial for immune factor production, and xenobiotic metabolism. CCl_4 is a hepatotoxin that oxidative stress, and immune dysfunction. Traditional medicine has reported hepatoprotective effects of various *Gardenia* species. This study evaluated the efficacy of ethanol extract from *Gardenia stenophylla* fruit (EEGS) in mitigating CCl_4-induced liver damage in mice by modulating oxidative stress and immunosuppression. Results indicated that CCl_4 significantly increased MDA and NO levels ($p < 0.05$), indicating oxidative stress and tissue damage. EEGS treatment significantly reduced MDA and NO levels ($p < 0.05$). CCl_4 exposure decreased GSH and TAC levels ($p < 0.05$), whereas EEGS treatment significantly restored these levels ($p < 0.05$). Similarly, CCl_4 inhibited SOD, CAT, and GPx activities ($p < 0.05$), which were significantly increased by EEGS ($p < 0.05$). Immune assessments showed that CCl_4 increased WBC count ($p < 0.05$) and decreased PA, NBT, and TI levels ($p < 0.05$). EEGS significantly improved these immune parameters ($p < 0.05$), with efficacy comparable to silymarin at higher doses ($p > 0.05$). EEGS effectively mitigates CCl_4-induced oxidative stress and immunosuppression in mice, demonstrating its potential as a hepatoprotective agent.

Keywords: Hepatoprotective effects, Immune suppression, Mice model, Oxidative stress

Introduction

The liver plays crucial roles in the anatomy and physiology of mammals, including bile secretion, bilirubin metabolism, nutrient processing, immune factor production, and xenobiotic metabolism related to viruses, drugs, alcohol, and environmental chemicals (Nhung and Quoc, 2024a). Carbon tetrachloride (CCl_4) is a hepatotoxic substance that acts by generating trichloromethyl peroxyl radicals and subsequent oxidative reactions. CCl_4 undergoes metabolism in the endoplasmic reticulum, primarily by CYP450 enzymes, producing trichloromethyl radicals (CCl_3^-) and triggering lipid peroxidation

*Corresponding Author: Quoc, L. P. T.; Email: lephamtanquoc@iuh.edu.vn

(Chen *et al*., 2023). These events contribute to oxidative stress, a crucial factor in liver infection and disease progression (Iqbal *et al*., 2022). CCl_4 also stimulates the production of injurious compounds, causing cellular and tissue damage, thereby affecting immune system function. The uncontrolled generation of free radicals and cytokine production is associated with liver injury, fibrosis, and aging (El-Kot *et al*., 2023). CCl_4 generates free radicals in the liver through metabolism, attacking and oxidizing lipids in cell membranes, leading to lipid peroxidation. Oxidized lipids produce harmful by-products such as malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE), causing damage to liver cells and inflammation. Moreover, oxidative stress stimulates nitric oxide (NO) production, which, when overproduced due to oxidative stress, causes harmful effects such as inflammation and liver cell damage (Allameh *et al*., 2023). Endogenous antioxidants such as glutathione (GSH), total antioxidant capacity (TAC), superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT) counteract free radicals, while exogenous compounds like α-tocopherol, ascorbic acid, carotenoids, and polyphenols neutralize oxidative reactions, protecting liver cells (Johra *et al*., 2023). GSH, GPx, and CAT prevent or minimize lipid peroxidation by removing peroxides and lipid-damaging agents. SOD and CAT reduce NO production under conditions of oxidative stress (Bizon *et al*., 2023). Immune inhibitory substances such as white blood cells (WBC), phagocytic activity (PA), nitroblue tetrazolium (NBT), and total immunoglobulin (TI) are affected by liver damage and oxidative stress induced by CCl_4, resulting in reduced WBC count, decreased PA activity, reduced immunoglobulin production, thereby leading to decreased immune function (Li *et al*., 2021). However, inadequate coping mechanisms against ROS production may lead to cellular damage, emphasizing the need to enhance the antioxidant system (Sharifi-Rad *et al*., 2020). Drugs used in liver treatment may cause liver damage or side effects, including immune suppression, oxidative stress, nephrotoxicity, and carcinogenesis (Zeleke *et al*., 2023). Drug-white blood cell interactions may disrupt immune function, leading to unforeseen consequences (Yonar *et al*., 2011).

The Gardenia genus, comprising over 140 shrub species of the Rubiaceae family, originates from tropical and subtropical regions of Africa, Asia, and the Pacific. Typically, Gardenia species exhibit evergreen leaves arranged opposite or in whorls, tubular white or yellow flowers emitting a strong fragrance, and large, fleshy orange-colored fruits (Lv *et al*., 2018). Gardenia species are utilized medicinally in various ways. For instance, Gardeniae fructus, the fruit of *G. jasminoides*, is used in China and Japan for its diverse properties, including laxative, anti-inflammatory, antipyretic, diuretic, hepatoprotective,

cholagogue, and hemostatic effects. The flowers of *G. jasminoides* are employed in traditional Chinese medicine for contraception. In Malaya, the leaves of this plant are commonly used to treat dyspepsia and nervous disorders. *G. erubescens*, a local medicinal plant in Nigeria, possesses extracts with sedative, analgesic, hypotensive, and diuretic properties. Additionally, compounds derived from the resin of *G. gummifera* and *G. lucida* exhibit antispasmodic, expectorant, diaphoretic, and anthelmintic activities. The root of *G. turgida* is used by the Santal tribe to remedy childhood dyspepsia, and a paste made from mashed roots is applied for headaches. Its fruit treats breast swellings (Wang *et al*., 2024). Furthermore, *G. ternifolia* shows promise as a therapeutic agent against alcohol-induced liver diseases (Sakou and Tietcheu, 2024). In traditional medicine, the root of *G. stenophylla* is used to alleviate fever, diarrhea, urinary problems, skin diseases, eye discomfort, diabetes, joint pain, and headaches. The fruit treats jaundice, conjunctivitis, oral ulcers, toothaches, and bleeding gums (Li-Hua *et al*., 2018).

Liver issues play a critical role in generating a significant number of liver transplant cases and fatalities, driving the increasing demand for the development of novel treatment methods. Recent studies have demonstrated that plant extracts exhibit potent antioxidant properties and safeguard the liver against CCl_4-induced damage by inhibiting lipid peroxidation and enhancing antioxidant enzyme activity.

This study aimed to evaluate the effectiveness of the *G. stenophylla* fruit extract in mitigating liver damage induced by CCl_4 in mice. This was achieved by assessing its impact on oxidative stress and immune suppression

Materials and methods

Chemicals and reagents

Chemicals were procured from Sigma-Aldrich (St. Louis, MO, USA). These chemicals were employed as standard agents throughout the experiments.

Collection of material and preparation of the extract

The fruits of *Gardenia stenophylla* (*G. stenophylla*) were harvested in November 2023 in the Buon Don district, Dak Lak province, Vietnam. The fruits were harvested when nearly ripe, exhibiting a yellow hue due to the presence of gardenin. Subsequently, a meticulous selection process was undertaken to eliminate damaged or diseased fruits. Following this, the fruits were further sorted to ensure uniform ripeness, then detached from the stems,

thoroughly cleaned, sliced into tiny parts, and air-dried for one week. Subsequently, the samples were dried at 60 °C until the moisture was less than 12%. The samples were ground and stored in bags at ambient temperature for use in the subsequent experiments.

The powdered fruit was immersed in 1 L of 96% ethanol for 3 days, with intermittent agitation at room temperature. Following soaking, the mixture was filtered through a muslin cloth and then through filter paper. This filtration process was conducted twice, and filtrates were pooled and concentrated using a rotary evaporator (IKA, Germany) at 40 °C. The ethanol extract derived from *G. stenophylla* fruits was designated as EEGS. The extraction yield was approximately 15%. The extract was stored at 4 °C for future use.

Phytochemical screening of the extract

The extract of *G. stenophylla* fruit has undergone qualitative phytochemical analysis, employing internationally established methods adhering to rigorous standards as delineated by Nhung and Quoc (2024b).

Quantification of phytochemicals in the extract

Total polyphenol quantification: Folin-Ciocalteu assay was used to determine total polyphenol content. A standard curve was generated using gallic acid concentrations ranging from 0 to 200 μg/mL, following the same protocol. The absorbance was measured at 725 nm. The total polyphenol content is reported as milligrams of gallic acid equivalents (GAE) per gram of extract (Nhung and Quoc, 2023a).

Quantification of total flavonoids: This procedure was described according to Nhung and Quoc (2023b). A calibration curve for quercetin was constructed over the concentration range of 0-200 μg/mL. The absorbance was measured at 415 nm and flavonoid content is reported as milligrams of quercetin equivalents (QE) per gram of extract.

Total terpenoid quantification: For this analysis, 200 μL of a 0.1 mg/mL ethanol extract solution was mixed with 1 mL of perchloric acid and 300 μL of a 5% (w/v) vanillin/acetic acid solution. Then, 5 mL of acetic acid was added, and the absorbance was measured at 548 nm. Terpenoid levels were determined based on a standard curve generated from ursolic acid concentrations ranging from 0.0625 to 1 mg/mL (Nhung and Quoc, 2024c).

Experimental animals

Swiss albino mice, averaging 29 ± 2 g in weight and aged 7-8 weeks. Selection criteria for the experimental animals included specific parameters such as body weight, activity levels, and general health. The study was conducted at the animal husbandry facility of the Eastern Agricultural and Food Company, where environmental conditions were controlled at 24–26 °C, 55-60% humidity, and a 12-hour light-dark cycle. The mice were housed in glass cages, each measuring 30 × 30 × 60 cm, and had continuous access to high-quality pellet food and water. Before the experiment, the mice were acclimated to their housing environment for seven days.

Experimental design

Liver injury in mice was induced using a 1:1 mixture of CCl_4 (2 mL/kg body weight) and olive oil, with olive oil serving as the vehicle for administration. The mixture was administered intraperitoneally (ip) over 28 days (Iqbal *et al*., 2022). The mice were randomly assigned to six groups, each consisting of five individuals. The control group received daily oral administration of distilled water (1 mL/kg body weight, po) and intraperitoneal injections of olive oil (2 mL/kg body weight, ip) three times a week. The untreated group was given oral distilled water (1 mL/kg body weight, po) and the CCl_4-olive oil mixture (2 mL/kg body weight, ip) three times weekly. The silymarin group received daily oral doses of silymarin (100 mg/kg body weight, po) in addition to the CCl_4-olive oil mixture (2 mL/kg body weight, ip) three times per week. The experimental groups (EEGS125, EEGS250, and EEGS500) were administered oral doses of EEGS at 125, 250, and 500 mg/kg body weight, respectively, along with the CCl_4-olive oil mixture (2 mL/kg body weight, ip) three times a week. The experimental duration was 28 days. Twenty-four hours after the final treatment, the mice were anesthetized with a Xylazine-Ketamine mixture (16 mg + 60 mg, i.m/i.p.) and euthanized. Blood and tissue samples from the liver, kidneys, and spleen were collected for further analysis.

Assessment of oxidative stress

Preparation of homogenates from liver, kidney, and spleen

Following euthanasia, liver, kidney, and spleen tissues from the mice were excised and immediately immersed in pre-chilled 0.9% NaCl solution at ice temperature. The tissues were then stored at -80 °C until next processing. For analysis, the tissues were homogenized in a 50 mM phosphate buffer (pH 7.0) containing 0.1 mM EDTA, resulting in a 5% homogenate solution for

malondialdehyde (MDA) assessment. The homogenate was centrifuged at 600 × g for 10 minutes at 4 °C to obtain the supernatant, which was used for subsequent analyses following the protocol outlined by Nhung and Quoc (2024c).

Oxidative stress mediators

Estimation of lipid peroxidation (LPO): Lipid peroxidation and oxidative stress in liver, kidney, and spleen tissues were evaluated using a colorimetric assay that quantifies thiobarbituric acid-reactive substances (TBARS). Homogenized tissue samples were mixed with phosphate buffer (PBS), acetic acid (AA), and thiobarbituric acid (0.67% TBA), then subjected to boiling for 30 minutes. The PBS:AA:TBA ratio was 4:5:5 for tissue homogenates and 1:2:2 for serum samples. After cooling, the absorbance of the resulting transparent supernatant was measured at 490 nm. Lipid peroxidation levels were quantified based on the malondialdehyde (MDA) equivalents, as outlined by Johra *et al*. (2023).

Estimation of nitric oxide (NO): The assay measures nitric oxide (NO) levels by its reaction with sulfanilamide to form a diazonium compound, which then couples with N-(1-naphthyl) ethylenediamine hydrochloride (NED). To perform the assay, 20 µL of homogenized tissue sample was added to a 96-well plate and mixed with 0.33% (w/v) sulfanilamide and 0.1% (w/v) NED in a phosphate buffer solution. Absorbance was subsequently recorded at 405 nm. NO concentrations were determined using a standard curve created from serial dilutions of a 1 mM sodium nitrite stock solution, as outlined by Johra *et al*. (2023).

Antioxidants

*Total glutathione (GSH)***:** Homogenized tissue samples were mixed with phosphate buffer solution at a 1:9 ratio. Following this, 100 µL of 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) was added to the mixture and incubated for one hour at 4 °C. The absorbance of the solution was then measured at 450 nm (Johra *et al*., 2023).

*Total antioxidant capacity (TAC)***:** Total antioxidant capacity (TAC) was evaluated using a spectrophotometric method at 532 nm, as described by Tkachenko *et al*. (2014). In this procedure, 0.2 mL of homogenized tissue was combined with 2 mL of 1% Tween 80. For the blank, 0.1 mL of distilled water was used in place of the tissue sample. The mixture was incubated at 37 °C for 48 hours, then cooled. Following this, 1 mL of 40% trichloroacetic acid (TCA) was added, and the mixture was centrifuged at 3,000 × g for 10 minutes. Subsequently, 2 mL of the supernatant was mixed with 2 mL of 0.25%

thiobarbituric acid (TBA) reagent and heated in a water bath at 100 °C for 15 minutes. The absorbance of the resulting solution was measured at 532 nm and compared to the blank sample.

Antioxidant enzymes

Superoxide dismutase (SOD): A 10 µL aliquot of the homogenized tissue sample was added to 90 µL of phosphate buffer solution. Following this, 100 µL of adrenaline was introduced, and the absorbance was immediately recorded at 490 nm. The blank reaction included all components except for the tissue sample. Superoxide dismutase (SOD) activity was assessed by measuring the inhibition of adrenaline auto-oxidation to adrenochrome (Johra *et al*., 2023).

Catalase (CAT): Homogenized tissue samples were dispensed into a 96-well culture plate, and phosphate buffer solution was added. Following this, a solution of hydrogen peroxide at the appropriate concentration was introduced. Absorbance readings were taken at 450 nm, with measurements recorded at three-minute intervals (Johra *et al*., 2023).

Glutathione peroxidase (GPx): Glutathione peroxidase (GPx) activity was assessed at 37 °C using the procedure outlined by Nhung and Quoc (2024c). The reaction mixture consisted of 500 µL of phosphate buffer, 100 µL of 0.01 M reduced glutathione (GSH), 100 µL of 1.5 mM NADPH, and 100 µL of glutathione reductase (GR) (0.24 U). Following the preparation, 100 µL of the tissue extract solution was added to the mixture and incubated at 37 °C for 10 minutes. Subsequently, 50 µL of 12 mM t-butyl hydroperoxide was introduced to 450 µL of the reaction mixture, and absorbance was recorded at 340 nm over 180 seconds using a spectrophotometer (Thermo Fisher Scientific, USA). The enzyme activity was calculated using a molar absorption coefficient of 6.22×10^3 M/cm.

Assessment of immunological

Blood cell (WBC): White blood cell (WBC) quantification was performed using a blood pressure measuring device and Natt-Herrick solution according to the procedure described by Yonar *et al.* (2011). In this procedure, white blood cells were fixed and stained with the Natt-Herrick solution. The WBC count was determined by calculating the white cell population from the red cell count using a Neubauer counting chamber (Germany).

Phagocytic activity (PA): Immediately after processing with heparin, the blood sample was used to evaluate cellular activity. To this end, a suspension of *Staphylococcus* sp. (10^8 cells/mL) in 0.1 mL phosphate buffer solution was

combined with 0.1 mL of the blood sample on a Petri dish and incubated for 30 minutes. Following incubation, the mixture was thoroughly mixed within the well. The Petri dish was then gently agitated, and 0.05 mL of the suspension was transferred onto a glass slide. After allowing the smear to air-dry, it was fixed with ethanol. The cellular activity was then assessed by counting cells and preparing the sample for microscopic examination, per the method described by Yonar *et al*. (2011).

Nitroblue tetrazolium (NBT): The assessment of neutral pH-dependent reactive oxygen species generation in white blood cells was conducted using the nitroblue tetrazolium (NBT) assay. For this procedure, 0.1 mL of blood was placed into each well of a microtiter plate, followed by the addition of 0.2% NBT solution in an equivalent volume. The mixture was incubated at room temperature for 30 minutes. After incubation, 0.05 mL of the NBT-stained cell pellet was transferred to glass tubes containing 1.0 mL of N,N-dimethylformamide. The samples were then centrifuged, and the absorbance was measured at 620 nm using a 1.0 mL cuvette (Yonar *et al.*, 2011).

Total immunoglobulin (TI): Total immune globulin (TI) levels were measured using the method described by Yonar *et al.* (2011). This approach involves assessing the total serum protein concentration by employing standard protein determination techniques. Initially, the serum protein concentration is measured, followed by the precipitation of immune globulin molecules using a 12% polyethylene glycol solution. The difference in protein content before and after precipitation indicates the total immune globulin concentration in the serum.

Statistical analysis

Results are expressed as Mean ± SD. Statistical analysis was conducted to assess the significance between the control and experimental groups using one-way analysis of variance (ANOVA) and the Duncan test, implemented with Statgraphics centurion 19.5.01 software. The significance of the results was determined at $p < 0.05$.

Results

Determination of bioactive compounds in the extract

The ethanol extract from *G. stenophylla* fruit (EEGS) exhibits a diverse chemical composition, including alkaloids, tannins, saponins, polyphenols, terpenoids, steroids, and flavonoids, but lacks cardiac glycosides (Table 1).

Quantitative analysis further reveals that the extract contains substantial amounts of flavonoids, terpenoids, and polyphenols, with specific concentrations of 36.43 ± 1.73 mg QE/g, 71.29 ± 3.37 mg TAE/g, and 64.75 ± 2.24 mg GAE/g, respectively (Table 2). These findings suggested that the EEGS holds significant potential for medicinal applications due to its rich content of bioactive compounds.

Table 1. Phytochemicals in extract from *G. stenophylla* fruit

Phytochemicals	Present in EEGS	Phytochemicals	Present in EEGS
Alkaloids	+	Cardiac glycosides	-
Tannins	+	Steroids	+
Saponins	+	Terpenoids	+
Polyphenols	+	Flavonoids	+

Note: Presence of phytochemicals in EEGS: (+) present and (-) absent.

Table 2. Quantification of flavonoids, polyphenols, and terpenoids in ethanol extract of *G. stenophylla* fruit

Sample	Total flavonoid content (mg QE/g)	Total terpenoid content (mg TAE/g)	Total polyphenol content (mg GAE/g)
EEGS	36.43 ± 1.73	71.29 ± 3.37	64.75 ± 2.24

GAE: Gallic acid equivalents, QE: Quercetin equivalents, TAE: Tannic acid equivalents.

Assessment of oxidative stress

Oxidative stress mediators

Malondialdehyde MDA assessment: The increase in lipid peroxidation, indicated by the production of malondialdehyde (MDA), is a known side effect of elevated reactive oxygen species (ROS) production. Therefore, we quantified MDA levels in treated mice (Figure 1). The results showed that in the untreated group, MDA levels in the liver, kidneys, and spleen increased significantly compared to the normal group ($p < 0.05$), with increases of 0.19, 0.17, and 0.17 nmol/g tissue, respectively. However, treatment with ethanol extract from *G. stenophylla* fruit (EEGS) significantly reduced MDA levels compared to the untreated group ($p < 0.05$), indicating the antioxidant effect of the extract. Notably, the reduction in MDA levels in the groups treated with EEGS at doses of 250 mg/kg and 500 mg/kg was comparable to that observed in the silymarin-treated group ($p > 0.05$), a standard antioxidant. This suggests that EEGS may have antioxidant and tissue-protective potential comparable to known antioxidants such as silymarin.

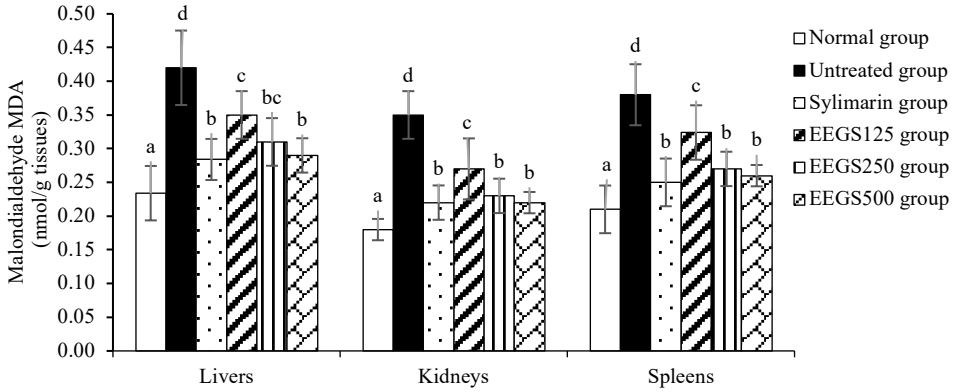

Figure 1. Determination of the effect of ethanol extract of *G. stenophylla* fruit on malondialdehyde (MDA) production in mice with CCl_4-induced hepatic damage. Various letters indicate significant group differences ($p < 0.05$)

Nitric oxide (NO) assessment: Result showed that in the untreated group, NO levels in the liver, kidney, and spleen tissues (1.55 ± 0.17, 1.28 ± 0.12, 1.41 ± 0.15 nmol/g tissues, respectively) were significantly higher compared to the normal group (0.86 ± 0.07, 0.71 ± 0.09, 0.78 ± 0.06 nmol/g tissues, respectively) (Figure 2). It indicated that increased NO production may be a marker of tissue damage or oxidative stress. However, treatment with EEGS significantly reduced NO levels, demonstrating the effectiveness of EEGS in reducing NO production. Notably, at doses of 250 mg/kg and 500 mg/kg, as well as in the silymarin-treated group (100 mg/kg), NO levels in these tissues were comparable, suggesting that EEGS is found as effective as silymarin in controlling NO levels.

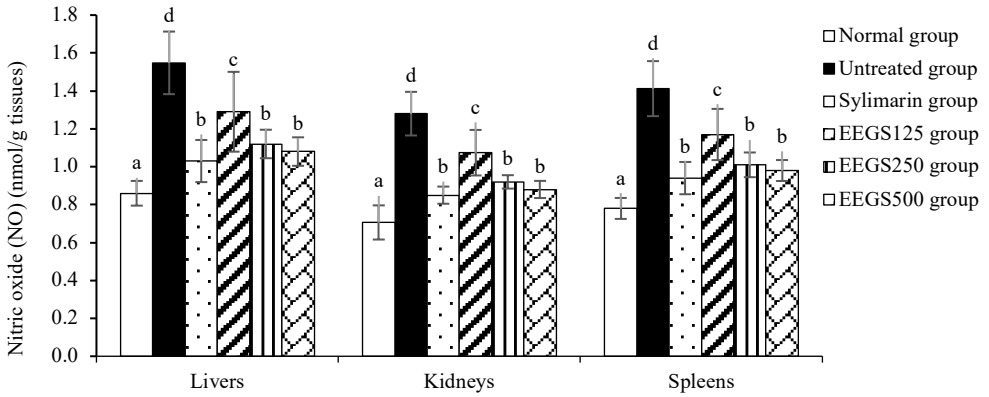

Figure 2. Determination of the effect of ethanol extract of *G. stenophylla* fruit on nitric oxide (NO) production in mice with CCl_4-induced hepatic damage. Various letters indicate significant group differences ($p < 0.05$)

Antioxidants

Total glutathione (GSH) and total antioxidant capacity (TAC) assessment: In this study, the activities of total glutathione (GSH) and total antioxidant capacity (TAC) were measured to investigate the impact of EEGS on oxidative stress in the liver induced by CCl_4 in mice (Table 3). Following CCl_4 treatment, GSH and TAC levels significantly decreased in the livers, kidneys, and spleens of the untreated group compared to the normal group, indicating oxidative liver damage caused by CCl_4 in mice. Interestingly, EEGS treatment significantly increased GSH and TAC levels ($p < 0.05$), suggesting that EEGS effectively mitigates oxidative stress and enhanced antioxidant defenses.

Table 3. Determination of GSH and TAC activity in mice with CCl_4-induced hepatic damage treated with ethanol extract of *G. stenophylla* fruit

Groups	Total glutathione (GSH) (nmol/g tissues)			Total antioxidant capacity (TAC) (nmol/g tissues)		
	Livers	**Kidneys**	**Spleens**	**Livers**	**Kidneys**	**Spleens**
Normal group	2.78 ± 0.08^{f}	2.14 ± 0.04^{f}	2.56 ± 0.06^{f}	37.19 ± 1.14^{e}	28.61 ± 1.08^{e}	34.33 ± 1.13^{e}
Untreated group	1.54 ± 0.04^{a}	1.19 ± 0.03^{a}	1.42 ± 0.03^{a}	20.66 ± 1.03^{a}	15.89 ± 1.05^{a}	19.07 ± 0.98^{a}
Sylimarin group	2.26 ± 0.05^{e}	1.74 ± 0.04^{e}	2.08 ± 0.04^{e}	30.24 ± 1.11^{d}	23.26 ±1.06^{d}	27.91 ± 1.08^{d}
EEGS125 group	1.85 ± 0.04^{b}	1.43 ± 0.03^{b}	1.99 ± 0.03^{b}	24.79 ± 1.01^{b}	22.35 ±1.08^{b}	22.89 ± 1.02^{b}
EEGS250 group	2.09 ± 0.03^{c}	1.61 ± 0.03^{c}	1.93 ± 0.03^{c}	27.96 ± 1.07^{c}	21.51 ± 1.03^{c}	25.82 ± 1.03^{c}
EEGS500 group	2.17 ± 0.04^{d}	1.67 ± 0.03^{d}	1.99 ± 0.03^{d}	29.05 ± 1.09^{cd}	22.35±1.04^{cd}	26.82 ± 1.05^{cd}

Various letters in the same column indicate significant group differences ($p < 0.05$).

Antioxidant enzymes

Superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) assessment: In the context of CCl_4-induced liver injury, SOD, CAT, and GPx are vital antioxidant enzymes that protect liver cells against oxidative stress. Mice treated with CCl_4 exhibited significant inhibition of both superoxide dismutase (SOD) and catalase (CAT) activity ($p < 0.05$) (Table 4) as well as glutathione peroxidase (GPx) activity ($p < 0.05$) (Figure 3) in the untreated group compared to the normal group. This indicates the presence of liver damage or oxidative stress. Conversely, in the groups treated with EEGS and silymarin, the results were reversed (Table 4 and Figure 3). The levels of SOD, CAT, and GPx enzymes increased significantly compared to the

untreated group. It suggested that EEGS can restore the activity of antioxidant enzymes, helping to minimize liver damage caused by oxidative stress. Particularly, the efficacy of EEGS at doses of 250 and 500 mg/kg was equivalent to silymarin ($p > 0.05$), indicating the potential of EEGS in protecting the liver and reducing damage caused by toxic substances.

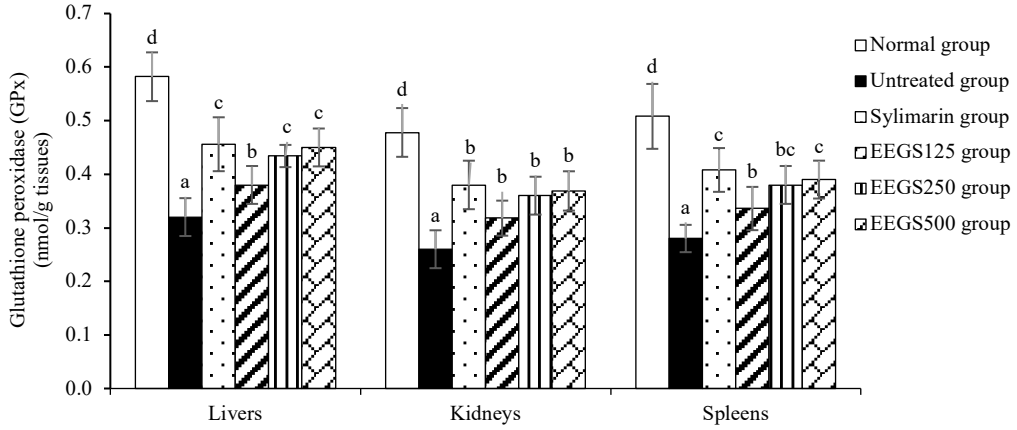

Figure 3. Determination of the effect of ethanol extract of *G. stenophylla* fruit on glutathione peroxidase (GPx) production in mice with CCl_4-induced hepatic damage. Various letters indicate significant group differences ($p < 0.05$)

Table 4. Determination of CAT and SOD activity in mice with CCl_4-induced hepatic damage treated with ethanol extract of *G. stenophylla* fruit

Groups	Catalase (CAT) (nmol/g tissues)			Superoxide dismutase (SOD) (nmol/g tissues)		
	Livers	**Kidneys**	**Spleens**	**Livers**	**Kidneys**	**Spleens**
Normal group	0.21 ± 0.02^{d}	0.17 ± 0.02^{d}	0.19 ± 0.02^{d}	0.85 ± 0.07^{d}	0.69 ± 0.07^{d}	0.72 ± 0.08^{d}
Untreated group	0.11 ± 0.02^{a}	0.09 ± 0.01^{a}	0.11 ± 0.02^{a}	0.47 ± 0.05^{a}	0.38 ± 0.05^{a}	0.41 ± 0.05^{a}
Sylimarin group	0.17 ± 0.02^{c}	0.13 ± 0.02^{c}	0.17 ± 0.02^{c}	0.68 ± 0.08^{c}	0.55 ± 0.05^{c}	0.58 ± 0.06^{c}
EEGS125 group	0.14 ± 0.02^{b}	0.11 ± 0.01^{b}	0.13 ± 0.02^{b}	0.56 ± 0.05^{b}	0.53 ± 0.05^{b}	0.48 ± 0.05^{b}
EEGS250 group	0.15 ± 0.02^{bc}	0.12 ± 0.01^{bc}	0.15 ± 0.02^{bc}	0.64 ± 0.06^{bc}	0.52 ± 0.07^{bc}	0.54 ± 0.06^{bc}
EEGS500 group	0.16 ± 0.02^{bc}	0.12 ± 0.02^{bc}	0.16 ± 0.02^{bc}	0.65 ± 0.08^{bc}	0.53 ± 0.05^{c}	0.56 ± 0.05^{bc}

Various letters in the same column indicate significant group differences ($p < 0.05$).

Assessment of immunological

Blood cell (WBC) and phagocytic activity (PA) assessment: Monitoring white blood cells (WBC) and phagocytic activity (PA) in mice with CCl_4-induced liver injury provides crucial insights into the immune status, response capability, processing, and removal of damaged liver cells. A significant increase in WBC count was observed in the untreated group compared to the normal group ($p < 0.05$). In contrast, the PA count in the untreated group decreased significantly compared to the normal group after CCl_4 treatment ($p < 0.05$) (Table 5). It indicated the detrimental impact of CCl_4 on the immune system. However, using EEGS and silymarin significantly decreased WBC, and increased PA counts ($p < 0.05$), suggesting the potential of these treatment methods in improving immune status and body response to liver injury. Particularly, the significant increased in WBC count in the groups treated with EEGS at doses of 250 and 500 mg/kg was equivalent to the silymarin-treated group ($p > 0.05$) (Table 5), indicating comparable effectiveness of EEGS to silymarin in treating liver injury.

Table 5. Determination of WBC and PA activity in mice with CCl_4-induced hepatic damage treated with ethanol extract of *G. stenophylla* fruit

Parameters	Normal group	Untreated group	Silymarin group	EEGS125 group	EEGS250 group	EEGS500 group
Blood cell (WBC) ($\times 10^3$ cells/mm^3)	4.92 ± 0.17^a	8.86 ± 0.42^e	5.91 ± 0.22^b	7.38 ± 0.29^d	6.39 ± 0.25^c	6.15 ± 0.19^{bc}
Phagocytic activity (PA) (%)	48.08 ± 1.24^f	26,71 ± 0.95^a	40.07 ± 1.18^e	32.05 ± 1.09^b	36.98 ± 1.12^c	38.46 ± 1.17^d

Various letters in the same row indicate significant group differences ($p < 0.05$).

Nitroblue tetrazolium (NBT) and total immunoglobulin (TI): The activity of NBT reflected the efficiency of the phagocytic system and its ability to clear bacteria, and assessing TI provided insights into overall immune competence and the immune system's response to liver injury and oxidative stressors. A significant decreased in NBT and TI concentrations was observed in the untreated group (10.34 ± 0.56, 8.07 ± 0.44 mg/mL, respectively) compared to the normal group (18.61 ± 0.76, 14.53 ± 0.85 mg/mL, respectively) after CCl_4 administration ($p < 0.05$) (Figure 4), indicating the significant impact of CCl_4 on these immune parameters. A significant increase was observed in both NBT and TI concentrations in the groups treated with EEGS and silymarin ($p < 0.05$, Figure 4), wherein the therapeutic efficacy of EEGS at 500 mg/kg (14.89 ±

0.85, 11.62 ± 0.52 mg/mL, respectively) was equivalent to silymarin (15.51 ± 0.68, 12.11 ± 0.47 mg/mL, respectively) ($p < 0.05$), demonstrating the potential of both EEGS and silymarin in improving these immune parameters in the context of CCl_4-induced liver injury.

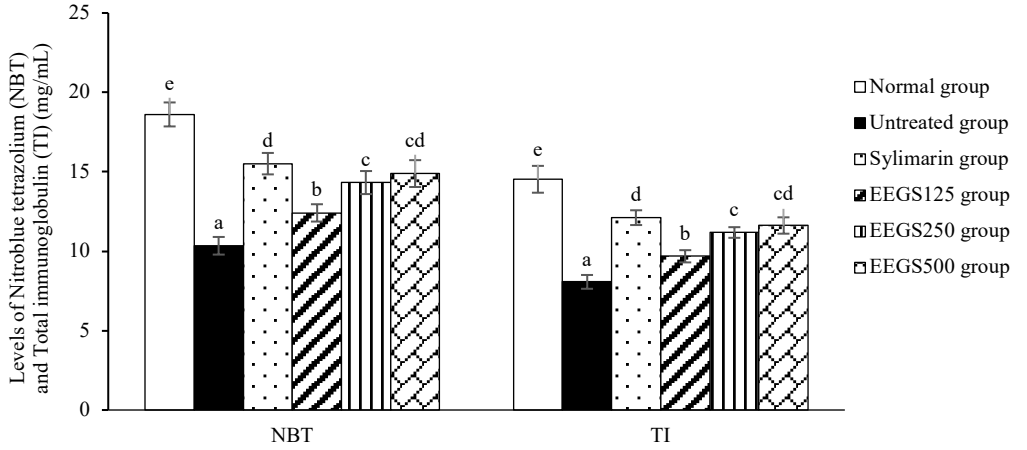

Figure 4. Determination of the effect of ethanol extract of *G. stenophylla* fruit on nitroblue tetrazolium (NBT) and total immunoglobulin (TI) production in mice with CCl_4-induced hepatic damage. Various letters indicate significant group differences ($p < 0.05$)

Discussion

While the nutritional benefits of *G. stenophylla* have been recognized for centuries, recent focus has shifted towards exploring the medicinal applications of its extracts and isolated plant chemicals. Phytochemical analysis reveals that the ethanol extract obtained from *G. stenophylla* fruit (EEGS) contains bioactive compounds with pharmacological effects such as antioxidation, immunomodulation, anti-inflammation, and hepatoprotection. Polyphenols in the extract (36.43 ± 1.73 mg QE/g) are identified as key components known for their potent antioxidative properties and liver-protective effects (Sumaiya *et al*., 2024). Flavonoids found in EEGS (64.75 ± 2.24 mg GAE/g) are reported to protect against liver injury induced by toxic chemicals such as CCl_4, thioacetamide, and alcohol. Terpenoids are emerging as a unique group of plant chemicals (71.29 ± 3.37 mg TAE/g) with multifunctional activities including anti-adipogenesis, anti-inflammatory, analgesic, antipyretic, cardioprotective, anxiolytic, and hepatoprotective effects (Johra *et al*., 2023). Alkaloids, tannins, saponins, and steroids present in EEGS, as demonstrated by phytochemical analysis, have also been shown to have antioxidative, immunomodulatory, anti-

inflammatory, and hepatoprotective effects against hepatic fibrosis (Datta *et al*., 2023).

In this study, the hepatoprotective effects of EEGS were investigated using a CCl_4-induced liver injury mouse model, with comparisons made to standard silymarin. Administration of CCl_4 significantly increased oxidative stress intermediates such as MDA and NO, as well as elevated WBC count in the blood, while concurrently reducing crucial antioxidants including GSH, TAC, SOD, CAT, and GPx, and diminishing immune factors such as PA, NBT, and TI. These findings are consistent with previous studies (Yonar *et al*., 2011), (Algefare *et al*., 2024).

The administration of CCl_4 resulted in increased levels of MDA and NO in the liver, kidneys, and spleen, accompanied by enhanced lipid peroxidation and diminished antioxidant defense mechanisms, leading to hepatic injury. Reactive oxygen species (ROS) are typically generated during physiological and pathological processes, through cellular metabolism, or in response to disturbances in the body's internal environment. Excessive free radicals elevate oxidative stress markers such as MDA and NO. Elevated ROS levels contribute to phospholipid membrane damage and reduce antioxidant levels in affected tissues (Johra *et al*., 2023). In this study, CCl_4-induced liver injury led to elevated levels of stress biomarkers, including MDA and NO, resulting in detrimental consequences for the body. MDA indicates oxidative damage, and its increased levels suggest the extent of this damage. On the other hand, NO acts as a free radical that induces oxidative stress and plays a role in inflammation and cellular damage. The elevation of MDA and NO induced by CCl_4 indicate increased oxidative stress and contributes to liver injury (Algefare *et al*., 2024). Conversely, EEGS and the standard drug silymarin demonstrated a reduction in the levels of stress biomarkers. The decrease in MDA and NO levels due to the action of EEGS is associated with its ability to combat oxidative stress and inhibit their inflammatory processes. The bioactive components in EEGS, such as flavonoids, polyphenols, and terpenoids, are known to reduce oxidative damage and suppress the synthesis of MDA and NO, thereby mitigating hepatic cell damage caused by oxidative stress and inflammation. Additionally, EEGS enhances the activity of the body's natural antioxidant systems, helping to regulate the oxidative balance and reduce hepatic cell damage from oxidative stress indicators such as MDA and NO.

GSH, TAC, SOD, CAT, and GPx are integral components of the body's primary defense system against oxidative stress, working to eliminate reactive oxygen species (ROS) and maintain physiological homeostasis. GSH neutralizes ROS by donating a hydrogen atom from the thiol (–SH) group of cysteine. SOD converts the superoxide anion into oxygen (O_2) and hydrogen

peroxide (H_2O_2). CAT further detoxifies H_2O_2 by breaking it into water (H_2O) and oxygen (O_2). GPx utilizes GSH as a substrate to reduce H_2O_2 into water or corresponding alcohols. TAC measures the cumulative and synergistic interactions of various antioxidants in protecting cells from oxidative damage (Bomble and Nath, 2022). However, when the body experiences oxidative stress induced by CCl_4, it activates glucocorticoid receptors, leading to the rapid consumption of GSH, TAC, SOD, CAT, and GPx to neutralize ROS. This rapid consumption, coupled with an overload and imbalance in the antioxidant system, impairs the synthesis and regeneration of these antioxidants. Additionally, changes in gene expression further contribute to the significant reduction in these antioxidant compounds and enzymes (Zielińska *et al*., 2021). Supplementation with ethanol extract of *G. stenophylla* fruit (EEGS) and silymarin increases the levels of GSH, TAC, SOD, CAT, and GPx by enhancing gene expression and protecting these compounds and enzymes from oxidation. Silymarin and EEGS contain potent antioxidant compounds that directly neutralize free radicals and ROS, thereby reducing oxidative stress on the body's endogenous antioxidant system.

White blood cells (WBC), phagocytic activity (PA), nitroblue tetrazolium (NBT), and total immunoglobulins (TI) serve as crucial indicators for evaluating and modulating the body's immune response. The enumeration of WBC aids in diagnosing infections, inflammation, and immune-related pathologies. PA assessments provide valuable insights into the functional capacity of phagocytes and their ability to combat infections. The NBT reduction assay evaluates the phagocytes' capability to generate reactive oxygen species (ROS) during respiratory bursts, pivotal for pathogen eradication. TI measurements offer a comprehensive evaluation of humoral immune function, reflecting the overall antibody production (Naiff *et al*., 2021). In light of our research, we have observed that CCl_4 induces adverse effects on nonspecific immune responses. This impact is closely tied to the pharmacokinetic profile of CCl_4, encompassing its processes of absorption, distribution, metabolism, and excretion. CCl_4 is extensively absorbed and disseminated throughout the body, with a propensity to accumulate in specific tissues, including immune-related organs, where it can interact with immune cells, thereby modulating immune reactions. We postulate that the immune suppression observed may arise from the accumulation of CCl_4 in organs, with the liver experiencing particularly pronounced effects, leading to hepatic injury (Yonar *et al*., 2011). This inference is supported by the diminished PA, reduced levels of NBT, and decreased TI levels, concomitant with an elevation in WBC count in mice of the untreated group.

Zou *et al*. (2022) illustrated that incorporating *Gardenia* residue into animal feed enhances growth performance, elevates blood metabolite levels, and improves immune and antioxidant indices, along with enhancing meat quality in Xiangcun pigs. Similarly, Kim *et al*. (2021) explored the impact of *Gardenia jasminoides* on the gut microbiota of mice, highlighting its beneficial effects in restoring gut microbial composition. Ma *et al*. (2017) further demonstrated that extracts from *G. jasminoides* stimulate immune responses and boost cognition in adult *Drosophila* models of Alzheimer's disease. Additionally, Park *et al*. (2022) reported that *G. jasminoides* extract alleviates atopic dermatitis induced by DfE in mice by restoring barrier function and modulating T-helper 2 mediated immune responses. In our investigation, the administration of *Gardenia stenophylla* aimed to modulate immune activity in CCl_4-induced liver-injured mice, aligning with previous studies. Our current study results demonstrate that the ethanol extract of *G. stenophylla* fruit (EEGS) has the potential to alleviate CCl_4-induced immune suppression in mice.

In conclusion, the ethanol extract of *G. stenophylla* fruit (EEGS) alleviates CCl_4-induced hepatic damage in mice by modulating oxidative stress and immune suppression. The extract demonstrates antioxidant effects comparable to silymarin (with the most effective being EEGS at 250 mg/kg), reducing lipid peroxidation (as indicated by reduced MDA levels) and controlling NO production (as evidenced by reduced NO levels). Additionally, it enhances antioxidant defenses, resulting in increased GSH and TAC levels, and restores the activity of vital antioxidant enzymes such as SOD, CAT, and GPx, akin to silymarin. Furthermore, the extract improves immune status and response to liver injury, resulting in decreased WBC and increased PA, and enhances immune parameters, resulting in reduced NBT and increased TI levels. These findings suggest the potential therapeutic efficacy of *G. stenophylla* fruit extract against CCl_4-induced liver damage, owing to its antioxidant and immunomodulatory properties.

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