

Protective effects of 1, 8-cineole (eucalyptol) against CCl₄ induced hepatic oxidative damage in rats

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ABSTRACT

Carbon tetrachloride (CCl₄) is a hepatotoxin which induce oxidative stress. Therefore, supplementation with antioxidants may be beneficial in mitigating its hepatotoxic effects. 1,8-Cineole (1,3,3-trimethyl-2-oxabicyclo [2.2.2] octane), commonly referred to as eucalyptol has various therapeutic properties. Hence, this research was conducted to evaluate the protective effects of eucalyptol against CCl₄ induced hepatic oxidative damage in rats. Thirty rats were divided randomly into the following three equal groups (n = 10): healthy control, CCl₄-poisoned group (1 mL/kg BW dissolved in olive oil (1:1 volume) and eucalyptol treated group (100 mg/kg/day). After treatment period (14 days), serum sample was prepared and liver was taken out for measurement of biochemical parameters including liver enzymes such as Alanine transaminase (ALT) and Aspartate transaminase (AST) as well as oxidative stress markers including Malondialdehyde (MDA), Glutathione (GSH), Catalase (CAT), Superoxide dismutase (SOD) and Glutathione Peroxidase (GPX). The results showed that following CCl₄ poisoning, a significant reduction in the levels of GSH, GPX, CAT and SOD was observed in the CCl₄-poisoned group compared to the control, as well as a significant elevation in the levels of AST, ALT and MDA was observed in the CCl₄-poisoned group in comparison with the control ($p < 0.05$). Furthermore, there was a significant increase in the levels of GSH, GPX, CAT and SOD in the eucalyptol treated group compared to the CCl₄-poisoned group. However, there was a significant decrease in the levels of AST and ALT in the eucalyptol-treated group compared to the CCl₄-poisoned group ($p < 0.05$). However, there was no significant in the MDA level in the eucalyptol-treated group compared to the CCl₄-poisoned one ($p > 0.05$). Eucalyptol may be effective in the

prevention of CCl₄-induced liver damage through the reduction of oxidative stress, liver damage caused by elevated liver enzymes, and increasing antioxidant enzymes.

Keywords: Eucalyptol, 1,8-Cineole, Antioxidants, Hepatic damage, CCl₄, Oxidative stress.

Article type: Research Article.

INTRODUCTION

The liver, recognized as the largest metabolic organ in the body, plays a critical role in the biotransformation and elimination of synthetic compounds (El Rabey *et al.* 2021; Parpieva *et al.* 2023). Environmental toxins have the potential to induce liver injury through a multifaceted mechanism involving oxidative stress, inflammation, necrosis, and apoptosis (Xiong *et al.* 2020; Ugochukwu Madu *et al.* 2023; Asadi *et al.* 2024). Previous studies have implicated oxidative stress, inflammation and toxic compounds in the development of hepatocellular damage (Wahid *et al.* 2016; Kabbashi *et al.* 2024). Elevated oxidative stress in the liver is a key factor contributing to the development of hepatic fibrosis (Devi *et al.* 2021). Carbon tetrachloride (CCl₄) is an industrial chemical known for its hepatotoxic properties and remains present as a component in certain insecticides (Vahidi-Eyrisofla *et al.* 2019; Que *et al.* 2022). CCl₄ was extensively utilized as a solvent in the chemical industry; however, its usage has been largely discontinued in most countries over the past decade due to its toxic properties (Teschke 2018; Hashemzadeh *et al.* 2018). Under experimental and laboratory conditions, CCl₄ is one of the most frequently utilized models for studying both acute and chronic liver damage (Kostic *et al.* 2022). Exposure of humans to CCl₄ has been well documented to result in liver fibrosis and hepatocellular injury (Yeh *et al.* 2013). The liver is the primary organ for the metabolism of CCl₄, where it is converted to peroxide radicals. These highly reactive radicals induce oxidative stress, resulting in lipid peroxidation and elevation of liver enzyme levels (Moghadam *et al.* 2017; Di Paola *et al.* 2022). Induction of oxidative stress is a primary mechanism underlying CCl₄ hepatotoxicity. Therefore, supplementation with antioxidants could be beneficial in mitigating its hepatotoxic effects (Kabbashi *et al.* 2024). One of them is 1,8-cineole (1,3,3-trimethyl-2-oxabicyclo [2.2.2] octane), commonly known as eucalyptol. This compound is present in the essential oil fraction of several plant sources, including eucalyptus, rosemary, and camphor (Hoch *et al.* 2023). Eucalyptus essential oil has the highest concentration of eucalyptol, according to Cai *et al.* (2021). Owing to its pleasant taste and aroma, eucalyptol is widely used in making food, perfuming and cosmetic products (Hoch *et al.* 2023). The primary pharmacological properties of eucalyptol are its anti-inflammatory and antioxidant effects. In particular, the production of inflammatory cytokines and reactive oxygen species (ROS) has been shown to be inhibited by eucalyptol (Kennedy-Feitosa *et al.* 2019). As a result, eucalyptol has therapeutic potential for cardiovascular disease, digestive disorders, Alzheimer's disease, and respiratory disorders. In particular, eucalyptol has been used to treat bronchitis, asthma, and chronic obstructive pulmonary disease (COPD; Cai *et al.* 2021). Therefore, this study investigates the protective effects of eucalyptol against hepatic oxidative damage induced by CCl₄ in rats.

MATERIALS AND METHODS

Chemicals and reagents

The materials and reagents used in this investigation included biochemical kits for alanine transaminase (ALT) and aspartate transaminase (AST) provided by Sigma-Aldrich Company, USA, and 1, 8-cineole from Sigma-Aldrich Company, USA, CCl₄, Tris-HCl, thiobarbituric acid (TBA), trichloroacetic acid (TCA), tris-EDTA, dinitrothiocyanobenzene (DTNB) and phosphate-buffered saline (PBS) from Merck Company, Germany.

Animals and experimental design

Thirty adult male Wistar rats (two-month olds; weight 250 to 300 g) were purchased and housed in standard caging (n = 10). The rats were maintained under suitable environmental conditions, which comprised a temperature of 22 °C, a 12-hour light-dark cycle, and unrestricted access to standard laboratory food and tap water. The rats were randomly assigned to three experimental groups, with ten rats in each group:

Group 1: Healthy rats (control) were given water daily for two weeks.

Group 2: Designated as the poisoned group (CCl₄ group), was exposed to CCl₄ (1 mL kg⁻¹ body weight) dissolved in olive oil (1:1 volume) via intraperitoneal (IP) injection twice weekly over a period of two weeks.

Group 3: The rats were given CCl₄ + eucalyptol by oral gavage at a dose of 100 mg kg⁻¹ per day for 14 continuous days.

Rats anesthetized with ketamine (50 mg kg⁻¹) and xylazine (10 mg kg⁻¹) had blood collected from the left heart ventricle and livers dissected for subsequent analyses after 14 days. Serum was subjected to biochemical analysis to determine the levels of AST and ALT. The liver was subjected to biochemical analysis to quantify the levels of malondialdehyde (MDA) and glutathione (GSH) and to assess the activities of catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx; El Rabey *et al.* 2021; Abdollahi *et al.* 2024).

Measurements of biochemical parameters

Determination of liver enzyme activity

Serum ALT and AST levels were quantified using biochemical enzyme kits (Sigma-Aldrich Company, USA) according to the manufacturer's instructions (Babaeenezhad *et al.* 2021).

Homogenization of liver samples

To obtain a liver homogenate, liver samples were minced and homogenized in Tris-HCl buffer (25 mM, pH 7.5) using a homogenizer. Centrifugation at 12,000 rpm for 15 minutes at 4 °C was performed on the resulting 10% (w/v) liver homogenate (Abdollahi *et al.* 2024).

Measurement of oxidative stress markers

Malondialdehyde (MDA)

Malondialdehyde (MDA) levels were quantified using a modified protocol based on Esterbauer and Zollern (Esterbauer & Zollern 1989). The thiobarbituric acid (TBA) test was used to assess hepatic MDA levels. Each sample was assayed in triplicate. Absorbance was measured by spectrophotometry at 532 nm. The absorbance was quantified and reported in units of $\mu\text{mol mg}^{-1}$ of protein malondialdehyde (MDA; Nasiry *et al.* 2017).

Glutathione (GSH)

We implemented a slight modification of the protocol described by Rahman *et al.* (Rahman *et al.* 2007) for the quantification of glutathione (GSH). Specifically, 25 mL of liver homogenate was combined with 140 mL of 0.2 M Tris-EDTA buffer (pH 8), followed by adding 30 mL of 0.1 M DTNB. Absorbance was read at 412 nm and checked against blank. Each sample was assayed in triplicate. GSH levels were finally expressed as $\mu\text{mol L}^{-1} \text{mg}^{-1}$ protein GSH (Ahmadvand *et al.* 2017).

Catalase (CAT)

Catalase (CAT) activity was determined using method modified by Aebi (Aebi 1984). First, 1 mL of 50 mM potassium phosphate buffer (pH 8) was added to 50-mL liver homogenate, followed by the addition of 50-mL H₂O₂. A spectrophotometer was used to measure absorbance at 240 nm in triplicate, with measurements taken at 0, 30, and 60 seconds against a blank. Results were expressed in units per milligram (U mg^{-1} ; Ahmadvand *et al.* 2017).

Glutathione peroxidase (GPx)

Hepatic glutathione peroxidase (GPX) activities were determined by the method of Rotruck and colleagues (Rotruck *et al.* 1973). An ELISA reader was used to read the absorbance at 420 nm. GPX activity was obtained in units of protein (U mg^{-1} ; Babaeenezhad *et al.* 2021).

Superoxide dismutase (SOD)

Superoxide dismutase (SOD) activity was determined by the Lund method, based on the enzyme's ability to inhibit pyrogallol oxidation. The absorbance of the samples was measured immediately and again after 60 seconds. The control was the difference in absorbance between the initial and the 60 s interval. The initial and 60-second absorbance readings were taken. The percentage inhibition of pyrogallol autoxidation was then calculated as follows (Sun *et al.* 1988):

$$\frac{\text{Absorbance of the sample at zero time} - \text{absorbance of the sample after 60 s}}{\text{Absorbance of the control at zero time} - \text{absorbance of the control after 60 s}}$$

The activity of SOD was quantified and expressed as units per milligram (U mg^{-1}) using the following formula:

$$\text{SOD enzyme activity (U mg}^{-1}\text{)} = \frac{\text{Percentage of inhibition of pyrogallol autoxidation}}{50 \%}$$

Statistical analysis

One-way analysis of variance (ANOVA) was used to analyze the experimental groups after verifying the normality of the data using the Shapiro-Wilk test. Tukey's post hoc test was used to compare means between experimental groups. A 5% significant level was maintained throughout all stages of analysis ($\alpha = 0.05$). SPSS version 24 software was used for statistical analysis, and GraphPad Prism version 9 for graphing.

RESULTS

Effect of eucalyptol on the serum level of AST and ALT

As shown in Table 1 and Fig. 1A, the AST serum levels were significantly elevated in CCl_4 -treated rats versus control animals ($p < 0.0001$). Conversely, the serum AST levels were significantly decreased in the eucalyptol-treated group in comparison with the CCl_4 -group ($p < 0.0001$). The data presented in Table 1 and Fig. 1B clearly indicate that the serum ALT levels increased significantly in the CCl_4 -group in comparison with the control rats ($p = 0.0479$). However, no significant changes were observed in the serum ALT levels in the eucalyptol-treated group compared to the CCl_4 group ($p = 0.8946$).

Table 1. Effects of eucalyptol on the serum levels of liver enzymes (AST and ALT) and liver levels of oxidative stress markers (MDA, GSH, GPX, CAT and SOD) in CCl_4 hepatotoxicity in different groups.

Parameter	Group			p-value
	Healthy control	CCl_4 -group	Eucalyptol-treated group	
AST	28.50 ± 2.8	159.2 ± 14.82 ****	99.60 ± 5.1 #####	< 0.0001
ALT	33.50 ± 5.13	44.80 ± 9.53 **	41.50 ± 5.29 *	0.0036
MDA	95.12 ± 23.98	149.1 ± 58.83 *	120.4 ± 50.79	0.0480
GSH	17.28 ± 1.69	8.83 ± 1.12 ****	12.29 ± 1.35#### ****	< 0.0001
GPX	76.91 ± 8.21	46.26 ± 7.32 ****	61.17 ± 10.12## **	< 0.0001
CAT	12.89 ± 1.57	7.88 ± 2.64 ***	9.48 ± 2.69 **	0.0002
SOD	9.95 ± 1.77	3.40 ± 0.97 ****	6.85 ± 1.29#### ****	< 0.0001

* Significant change in comparison with the control group at $p < 0.05$; #Significant change in comparison with the CCl_4 -group at $p < 0.05$.

Effect of Eucalyptol on the level of MDA

The data indicated a statistically significant elevation in hepatic MDA levels in the CCl_4 group compared to the control rats ($p = 0.0480$). Conversely, there was no statistically significant difference in hepatic MDA levels between the eucalyptol-treated and the CCl_4 groups ($p = 0.5482$; Table 1 and Fig. 2A).

Effect of eucalyptol on the level of GSH

Table 1 and Fig. 2B illustrates a statistically significant reduction in hepatic GSH levels in the CCl_4 -treated group relative to the control ones ($p < 0.0001$). Conversely, the eucalyptol treated-group exhibited a significant elevation in hepatic GSH levels than in the CCl_4 -treated group ($p < 0.0001$).

Effect of eucalyptol on the activity of GPX

Exposure to CCl_4 was shown to significantly decrease GPX activity in the CCl_4 -treated group than in the control rats ($p < 0.0001$). In addition, eucalyptol treatment resulted in a significant elevation in GPX activity in the eucalyptol-treated group than in the CCl_4 -treated one ($p = 0.0021$; Table 1 and Fig. 2C).

Effect of eucalyptol on the activity of CAT

The findings demonstrated a significant reduction in hepatic CAT activity in the CCl_4 -treated rats than in the control animals ($p = 0.0002$). Additionally, no significant change in hepatic CAT activity was found between the eucalyptol-treated and CCl_4 -treated groups ($p = 0.4281$; Table 1 and Fig. 2D).

Effect of eucalyptol on the activity of SOD

As shown in Table 1 and Fig. 2E, there was a meaningful decrease in hepatic SOD activity in the CCl_4 -treated animals than in the control rats ($p < 0.0001$). Furthermore, SOD activity was significantly enhanced in the eucalyptol-treated group than in the CCl_4 -treated rats ($p < 0.0001$).

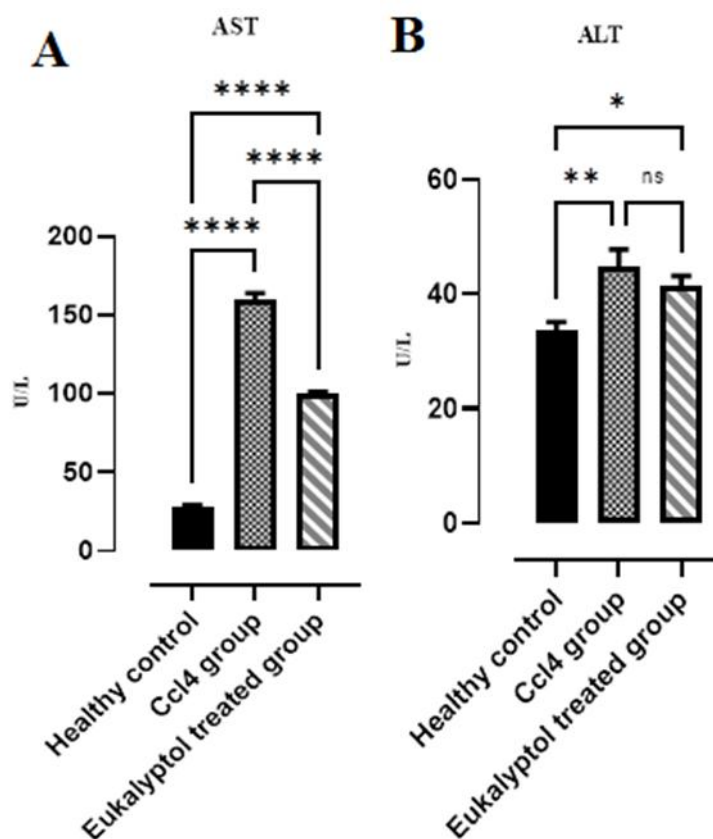


Fig. 1. Effect of eucalyptol on the serum levels of liver enzymes including AST (A) and ALT (B) in CCl₄-induced hepatotoxicity. Data are represented as Mean \pm SD. Comparisons between groups were made using One-Way ANOVA followed by post hoc test. *Significant change at $p < 0.05$.

DISCUSSION

The present study was designed to evaluate the ameliorative properties of eucalyptol against liver oxidative damage caused by CCl₄ in rats. The present study is believed to be the first to demonstrate the ameliorative properties of eucalyptol against liver oxidative damage caused by CCl₄ in rats. The current study concluded that eucalyptol treatment can mitigate the harmful effects of CCl₄ on the rat liver. Most of the various parameters assessed in our experiment were rapidly restored in CCl₄-intoxicated animals after eucalyptol treatment. Our results showed that exposure to CCl₄-induced liver damage as evidenced by the elevated serum AST and ALT levels. Furthermore, exposure to CCl₄ was associated with an elevation in MDA, which serves as a marker of oxidative stress, and a concomitant decrease in the antioxidant parameters such as GSH, GPX, CAT, and SOD. Indeed, CCl₄-induced liver damage has been similarly documented in numerous previous studies (Wahid *et al.* 2016; El Rabey *et al.* 2021). ALT and AST serve as biomarkers to assess liver damage. When the cell membrane integrity is compromised, blood levels of these enzymes increase (Abdollahi *et al.* 2024). The enhanced production of free radicals facilitated by CCl₄ may contribute to the degradation of cell membranes and subsequent liver damage (El Rabey *et al.* 2021). In this study, administration of eucalyptol was shown to reduce serum levels of liver enzymes, particularly AST, confirming its ameliorative effect against liver damage through its antioxidant properties. There are several pieces of evidence that the effectiveness of eucalyptol in preventing the increased ALT and AST levels is dependent on dose (Abdollahi *et al.* 2024). It was also found that the hepatic MDA levels, an important marker of oxidative stress, decreased in treated rats after administration of eucalyptol. Several lines of evidence have shown that eucalyptol can mitigate ROS generation and improve endogenous antioxidant status. Environmental contaminants, such as CCl₄, induce oxidative stress and damage biomolecules in liver cells, resulting in structural and functional changes in the cells (Abdollahi *et al.* 2024). Our results showed an elevation in the antioxidant parameter (GSH) after eucalyptol treatment. In addition, a meaningful recovery in the hepatic activities of antioxidant enzymes, including GPX, CAT, and SOD, was observed after eucalyptol administration. Our research aligns with prior studies, demonstrating that eucalyptol has the potential to mitigate oxidative stress

across various pathological conditions (Ciftci *et al.* 2011; Abdollahi *et al.* 2024). It can be concluded that amelioration of oxidative stress by eucalyptol can be associated with its ROS scavenging activity (Abdollahi *et al.* 2024). Our findings align with those of Ciftci *et al.*, demonstrating that the administration of eucalyptol to rats exposed to 2,3,7,8-tetrachlorodibenzo-p-dioxin enhances the activities of catalase, superoxide dismutase (SOD), and glutathione peroxidase (GPx), as well as the glutathione (GSH) levels, thereby mitigating oxidative stress in the liver (Ciftci *et al.* 2011). Through our biochemical studies, we substantiated the hypothesis regarding the hepatoprotective properties of eucalyptol.

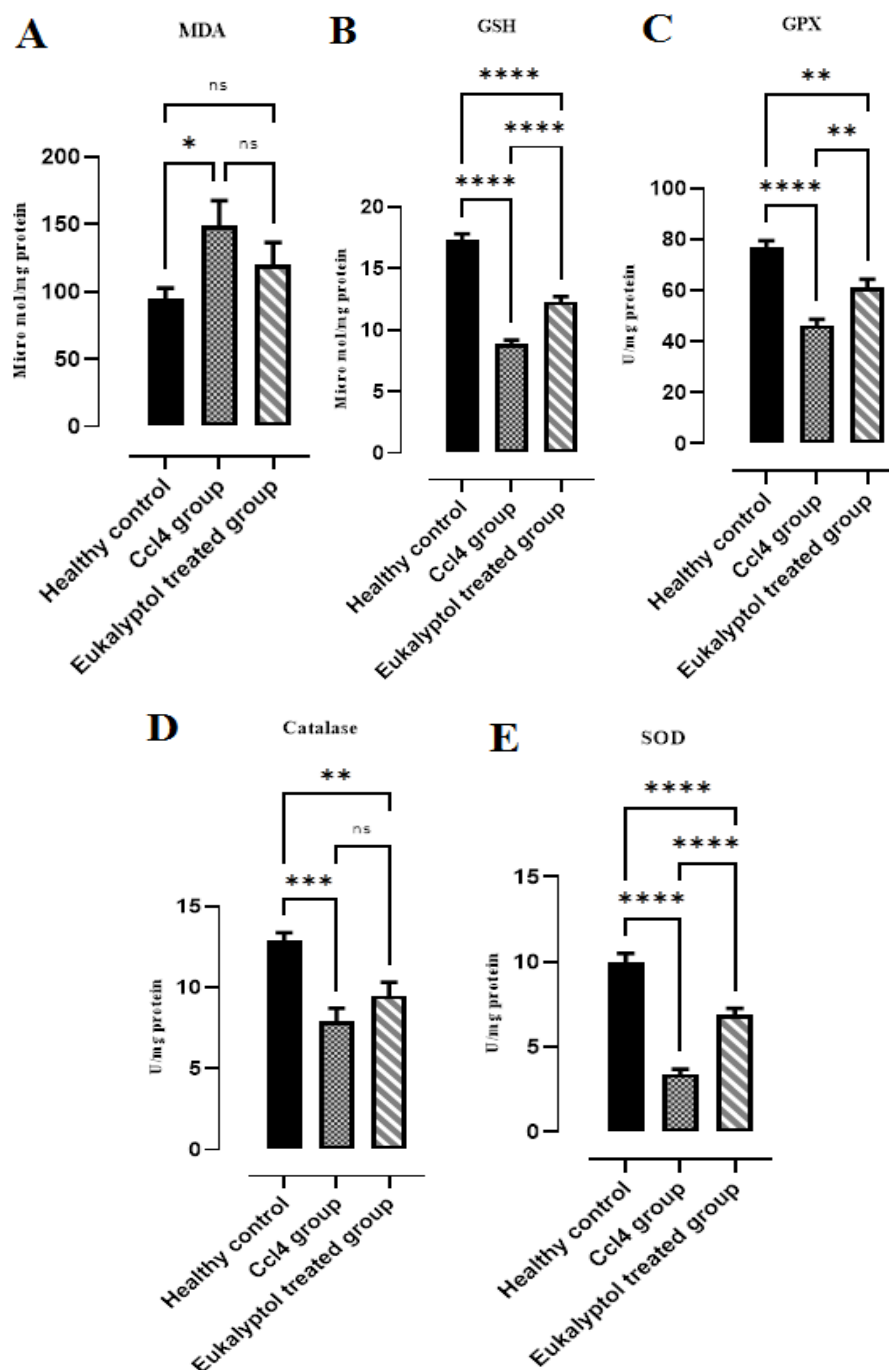


Fig. 2. Effects of eucalyptol on the level of oxidative stress parameters including MDA (A), GSH (B), GPX (C), CAT (D) and SOD (E) in CCl₄-induced hepatotoxicity. Results are represented as Mean \pm SD. Comparisons between groups were made using One-Way ANOVA followed by post hoc test. *Significant change at $p < 0.05$.

CONCLUSION

Our study is the first to demonstrate the efficacy of eucalyptol in mitigating liver damage induced by carbon tetrachloride exposure. The antioxidant properties of eucalyptol significantly contribute to this hepatoprotection by ameliorating liver enzyme dysfunction, reducing oxidative stress and free radical production, as well as enhancing the endogenous antioxidant system.

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