Asian Journal of Green Chemistry 10 (2026) 430-449

Asian Journal of Green Chemistry

Original Research Article View Article Online | View Journal

Green Extraction, Antioxidant, and Hepatoprotective Evaluation of *Calotropis procera* Leaf Extract

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ARTICLE INFORMATION

Submitted: 2025-10-27
Revised: 2025-11-20
Accepted: 2025-12-02
Published: 2025-12-08
Manuscript ID: AJGC-2511-1863
DOI: 10.48309/AJGC.2026.558174.1863

KEYWORDS

Calotropis procera
Cold maceration
Phytochemicals
Antioxidant activity
Hepatoprotection
Paracetamol toxicity

ABSTRACT

The present study investigated the antioxidant and hepatoprotective potential of a methanolic leaf extract of Calotropis procera obtained by cold maceration. Physicochemical evaluation revealed total ash (5.1% w/w), acid-insoluble ash (1.28% w/w), and water-soluble ash (3.81% w/w), serving as diagnostic parameters for standardization. Preliminary phytochemical screening confirmed the presence of flavonoids, phenolics, alkaloids, terpenoids, glycosides, tannins, and saponins, which are known for their pharmacological activities. The extract demonstrated potent *in vitro* antioxidant capacity, with IC_{50} values of 2.88 µg/mL for DPPH ($p < 0.001$) and 5.27 µg/mL for hydrogen peroxide scavenging ($p < 0.001$), significantly lower than those of ascorbic acid (12.60 and 69.22 µg/mL, respectively), while exhibiting a concentration-dependent increase in reducing power up to 100 µg/mL. *In vivo*, paracetamol administration (2 g/kg) induced hepatotoxicity in rats, as evidenced by elevated serum AST (109.33 IU/L), ALT (137.33 IU/L), ALP (152.16 IU/L), bilirubin (1.263 mg/dl), and lipid peroxidation (7.295 M/mg protein), along with depleted GSH (12.71 M/mg protein), SOD (222.83), and CAT (11.80 µM). Treatment with C. procera extract (100 and 200 mg/kg) significantly reversed these changes ($p < 0.001$ for major biochemical markers) in a dose-dependent manner. At 200 mg/kg, AST and ALT levels were reduced to 81.26 and 74.06 IU/L, bilirubin decreased to 1.002 mg/dl, and GSH, SOD, and CAT were restored to 15.99 M/mg protein, 281.07, and 14.17 µM, respectively. Hematological indices including RBC, Hb, PCV, and WBC also improved ($p < 0.01–0.001$). Histological analysis confirmed the attenuation of necrosis and fatty degeneration. These findings established that *C. procera* is a promising hepatoprotective agent with strong antioxidant mechanisms underlying its efficacy.

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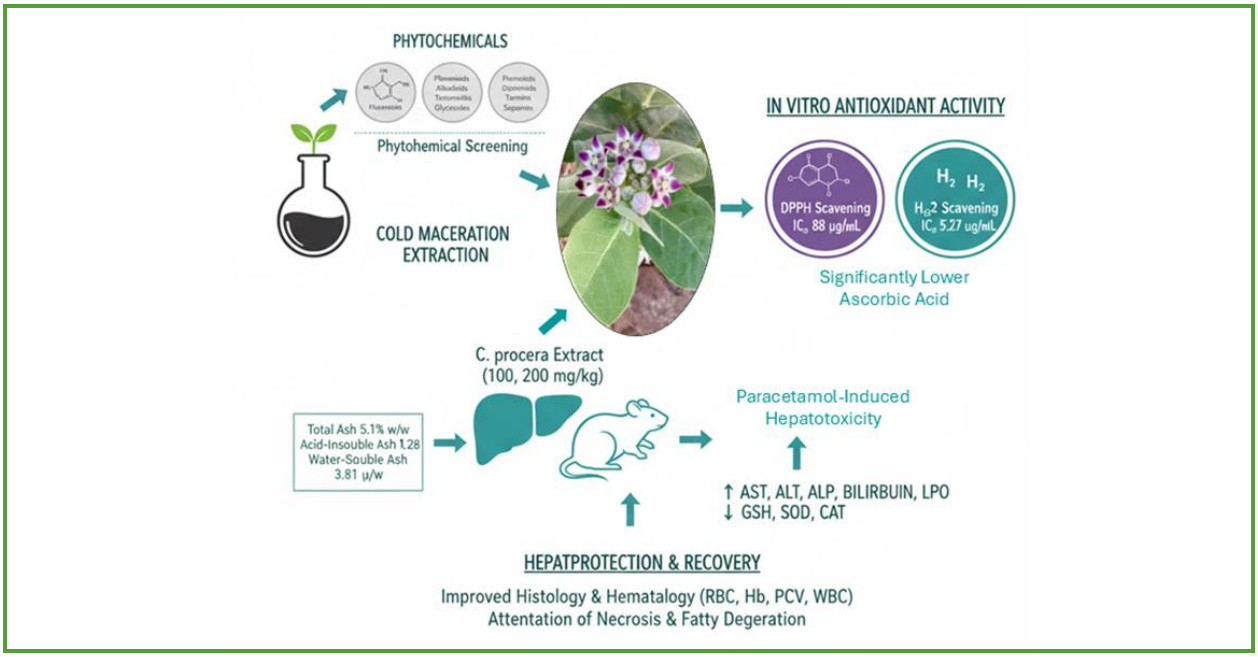
Graphical Abstract



Introduction

The liver is an essential organ associated with several physiological functions, including detoxification, metabolism, and regulation of biochemical pathways essential for life [1,2]. Because of its essential role in xenobiotic metabolism, the liver is frequently exposed to various toxins and drugs, making it highly vulnerable to damage. Paracetamol (acetaminophen) is the most common hepatotoxic substance responsible for drug-induced liver damage. At therapeutic levels, paracetamol is mostly converted into nontoxic glucuronide and sulfate conjugates [3,4]. However, overdose results in the accumulation of the reactive metabolite N-acetyl-p-benzoquinone imine (NAPQI), which depletes glutathione (GSH) and initiates oxidative stress, lipid peroxidation, and hepatocellular necrosis. Despite the availability of N-acetylcysteine as a clinical antidote, the search for natural hepatoprotective agents with potent antioxidant activity continues to be a major focus of modern pharmacological research [5–7].

Medicinal plants are well known for their diverse bioactive constituents, which can mitigate oxidative stress and modulate detoxification systems [8,9]. Traditional systems of medicine, including Ayurveda, Siddha, and Unani, have long relied on natural products to treat liver disorders [10,11]. In recent years, phytochemical investigations and pharmacological studies have validated the hepatoprotective potential of several medicinal plants. In this context, *C. procera* (family Apocynaceae), commonly known as “Swallow-wort” or “Ak,” has attracted attention because of its wide spectrum of ethnomedicinal uses. This plant species is extensively found in tropical and subtropical areas of Asia and Africa has been traditionally used to treat fever, skin ailments, respiratory conditions, and hepatic disorders [12,13].

Phytochemical studies of *C. procera* have revealed the existence of various secondary

metabolites, such as flavonoids, alkaloids, terpenoids, phenolics, tannins, glycosides, and saponins. Flavonoids and phenolic compounds, in particular, are well established for their antioxidant and free radical scavenging capacities. These phytoconstituents are thought to significantly mitigate oxidative stress-mediated liver damage. Preliminary pharmacological studies have indicated that extracts of *C. procera* possess anti-inflammatory, analgesic, antimicrobial, and antioxidant activities, which further support its potential as a hepatoprotective agent [14–17].

Extraction methods significantly influence the yield and bioactivity of the phytoconstituents. Cold maceration is a simple and efficient method that avoids thermal degradation of heat-sensitive compounds, thereby preserving the integrity of bioactive metabolites. This technique is particularly advantageous for extracting phenolics, flavonoids, and other compounds with antioxidant properties [18–20]. By employing cold maceration, the present study ensured the maximum retention of these constituents, thereby enabling a more accurate evaluation of the biological potential of *C. procera*.

Given the traditional claims and phytochemical richness of *C. procera*, it is imperative to provide scientific evidence of its hepatoprotective effects, particularly in the context of paracetamol-induced toxicity, which is closely associated with oxidative stress [21,22]. Therefore, the objectives of the present study were: (i) preparing the leaf extract of *C. procera* through cold maceration, (ii) performing preliminary phytochemical screening, (iii) evaluating *in vitro* antioxidant properties via DPPH radical scavenging, hydrogen peroxide scavenging, and reducing power assays, and (iv) assessing *in vivo* hepatoprotective efficacy against paracetamol-induced hepatotoxicity in Sprague Dawley rats.

The integrated methodology of merging *in vitro* and *in vivo* models offers a thorough understanding of the protective mechanisms of *C. procera*. The results of this study aimed to scientifically validate the ethnomedicinal properties of *C. procera* and emphasize its potential as a natural hepatoprotective and antioxidant agent.

Experimental

Preparation of plant material and extraction by cold maceration

Fresh leaves of *C. procera* were procured in April 2016 from Sonai in Ahmednagar, Maharashtra, India, and verified by the Botanical Survey of India, Pune (voucher specimen no. SMB-02). Leaves were rinsed under running water to eliminate debris and air-dried in the shade at ambient temperature (25–30 °C) with adequate ventilation for 10–14 d until a stable weight was attained. The dry leaves were coarsely crushed using a mechanical grinder and subsequently filtered through a 40–60 mesh screen. The powdered substances were preserved in sealed amber bottles at ambient temperature in a desiccator until extraction.

For cold maceration, 100 g of a dry coarse powder was used. The powdered botanical substance was transported to a sterile, wide-mouth glass container and soaked in analytical-grade methanol (solvent-to-plant material ratio 10:1, w/v; *i.e.,* 1,000 mL methanol for 100 g powder). The container was tightly closed and maintained at room temperature (25 ± 2 °C). The mixture was left to macerate for 72 h with mechanical shaking or manual swirling for 10–15 min every 6–8 h to improve the solvent penetration. After 72 h, the macerate was passed through a muslin cloth and subsequently through Whatman No. 1 filter paper, and the filtrate was collected [18–20,23].

To ensure comprehensive extraction, marc was re-macerated twice using fresh methanol (fresh solvent each time; 48 h per maceration, with intermittent agitation). All filtrates were mixed and concentrated under decreased pressure at 40–50 °C using a rotary evaporator (Buchi/KNF-type) to eliminate methanol. Residual solvent was removed by drying in a vacuum oven at ≤ 40 °C or by desiccation to obtain a dry crude methanolic extract. The extract was weighed, and the percentage yield was calculated as Equation 1:

$$\%\,Yield = \frac{\text{Weight of dry extract (g)}}{\text{Weight of starting plant material (g)}} \times 100 \quad (1)$$

In the present study, the methanolic leaf extract yield of *C. procera* was approximately 17.6% w/w. The dried crude extract was transferred to pre-weighed amber vials, labeled (plant name, solvent, date), and stored at 4 °C until further use (phytochemical screening, *in vitro* antioxidant assays, and *in vivo* studies). Prior to biological testing, the extract was freshly reconstituted in an appropriate vehicle and sonicated to achieve complete dispersion.

Physico-chemical evaluation and preliminary phytochemical screening of C. procera methanolic extract

Powdered leaves and methanolic extracts of *C. procera* were subjected to conventional physicochemical assessment and preliminary phytochemical analysis. Physicochemical characteristics such as moisture content (loss on drying), total ash, acid-insoluble ash, water-soluble ash, and methanol-soluble extractive value were assessed according to the WHO criteria. Moisture content was assessed by oven drying at 105 °C until a consistent weight was achieved; Total ash was derived by incinerating the powdered substance at 450 °C. Acid-insoluble and water-soluble ash values were quantified by boiling total ash with weak hydrochloric acid or distilled water, respectively, followed by filtration and ignition. and Methanol-soluble extractives were estimated by exhaustive maceration of powdered drug in methanol and subsequent evaporation of the filtrate. Qualitative tests were conducted on the methanolic extract for phytochemical screening to identify the principal classes of the phytoconstituents. Alkaloids were detected using Mayer's, Wagner's, Dragendorff's, and Hager's reagents; carbohydrates and reducing sugars were verified by Molisch's, Fehling's, Benedict's, and Barfoed's assays; steroids and triterpenoids were tested by Liebermann–Burchard and Salkowski reactions; saponins were detected by the froth test; tannins and phenolics by ferric chloride and lead acetate tests; and flavonoids by Shinoda and lead acetate reactions. In addition, glycosides were evaluated using Keller–Killiani and Bornträger's tests, while proteins and amino acids were confirmed by Biuret and Ninhydrin reactions. Observations were recorded based on color changes or precipitate formation, and the results were expressed as the presence or absence of each phytoconstituent [24–26].

In vitro antioxidant assay

DPPH Radical scavenging assay

A 0.1 mM DPPH solution was prepared by mixing 3.94 mg of DPPH in 100 mL of methanol and was preserved in the dark at 4 °C until. Stock solutions of the methanolic extract of *C. procera* (1 mg/mL) and ascorbic acid (standard; 1 mg/mL) were prepared in methanol and sonicated to achieve full dissolution. Working doses ranging from 2 to 10 µg/mL were achieved through repeated dilutions.

For this test, 1.0 mL of DPPH solution was combined with 1.0 mL of each extract or standard concentration. A control consisted 1.0

mL of methanol and 1.0 mL of DPPH solution, whereas the sample blank consisted of 1.0 mL of extract and 1.0 mL of methanol. The reaction compositions were incubated in darkness at ambient temperature for 30 min, and the absorbance was measured at 517 nm using a UV-Vis spectrophotometer [27–30].

The percentage inhibition of DPPH radicals was calculated using Equation 2:

$$\%\, inhibition = \frac{Ac-(At-As)}{Ac} \times 100 \qquad (2)$$

Where, Ac = absorbance of the control, At = absorbance of the test, and As = absorbance of the sample blank. All determinations were performed in triplicate, and IC_{50} values were obtained from the regression curve of % inhibition versus concentration.

Hydrogen peroxide (H_2O_2) scavenging activity

The hydrogen peroxide scavenging activity was evaluated using a replacement titration technique. A 0.1 mM solution of H_2O_2 was newly formulated in 0.1 M phosphate buffer at pH 7.4. Stock solutions of the methanolic extract of *C. procera* and ascorbic acid (1 mg/mL) were diluted to operational concentrations ranging from 2 µg/mL to 10 µg/mL.

In the experiment, 1.0 mL of 0.1 mM H_2O_2 solution was combined with 1.0 mL of extract or standard solution. Two drops of 3% ammonium molybdate, 10 mL of 2 M H_2SO_4, and 7.0 mL of 1.8 M KI were added into the mixture, which was subsequently titrated with 5.09 mM sodium thiosulfate until the yellow hue vanished. The volume of sodium thiosulfate utilized by the control (Vc) and the sample (Vs) was documented [31]. The percentage inhibition of hydrogen peroxide was calculated using Equation 3:

$$\%\, inhibition = \frac{Vc-Vs)}{Vc} \times 100 \qquad (3)$$

All experiments were performed in triplicate, and IC_{50} values were determined from the inhibition curves.

Reducing power assay

Several concentrations of the extract and ascorbic acid (10–100 µg/mL) were formulated. For the test, 2.5 mL of extract solution was combined with 2.5 mL of 0.2 M phosphate buffer (pH 6.6) and 2.5 mL of 1% potassium ferricyanide [$K_3Fe(CN)_6$]. The amalgamation was incubated at 50 °C for 20 min. Following incubation, 2.5 mL of 10% trichloroacetic acid was added and the mixture was centrifuged at 3,000 rpm for 10 min. 2.5 mL of the supernatant was extracted and combined with 2.5 mL of distilled water and 0.5 mL of a 0.1% ferric chloride solution. The absorbance was measured at 700 nm. The enhanced absorbance of the reaction mixture signified improved reducing power. All tests were conducted in triplicate, and the results are presented as mean ± standard deviation [32,33].

In vivo hepatoprotective activity

Animals used and acute oral toxicity studies

Sprague Dawley rats weighing between 150 and 200 g were used for this investigation. The subjects were kept at a temperature of 25±2 °C, relative humidity of 45-55%, and under typical ambient conditions with 12 h light and dark cycles. The rats were provided unrestricted access to conventional animal feed and water ad libitum. The Institutional Animal Ethics Committee (IAEC) authorized the study. Acute oral toxicity is defined as the examination of harmful effects resulting from the oral ingestion of a single dose or several doses of a chemical within 24 h [34,35].

Experimental protocol; OECD guideline 423

This study assessed the acute oral toxicity of methanol extracts from the leaves of *C. procera* leaves in accordance with OECD standards 423. This methodology used a multi-step technique to assess the toxicity of leaf extracts from *Calotropis procera*, with each stage using three rats of a single sex. Prior to administration of the dose, 3 to 4 h of fasting (withholding food but not water) was performed on the rats. After the period of abstinence, the animals were weighed, and a dosage of the extract was administered orally, at a concentration of 2,000 mg/kg. Following the administration of the dose, each animal was scrutinized on an individual basis for the first 30 min, then at regular intervals for the first 24 h, with a particular focus on the first 4 h, and finally on a daily basis for the next 14 days. It is well known that there is a fatality rate associated with the aforementioned dosage. As a result, dosages ranging between 100 and 200 mg/kg were chosen for subsequent pharmacological investigation. In accordance with the recommendations of OECD 423, the testing method began with a starting dosage of 2,000 mg/kg.

Acute oral toxicity experiments were conducted in accordance with OECD Standard 423 methodology. This approach was developed to evaluate drugs at fixed dosages, and it provides information that can be used for both the evaluation of hazards and the ranking of substances for the purpose of hazard categorization. After administering the leaf extracts of *C. procera* at an initial starting dose of 2,000 mg/kg body weight in 1% CMC (through the oral route), death was observed due to acute toxicity after 14 days. The effects of the drug on the central nervous system, autonomic nervous system, motor activity, salivation, and coloration of the skin, as well as any other general symptoms of toxicity, were carefully examined and documented. The LD_{50} value of the title compounds was predicted to be more than 2,000 mg/kg body weight and is represented as class 5 (2,000 mg/kg body weight < LD_{50} < 2,500 mg/kg). This is due to the fact that no evidence of toxicity was found in the group of animals at the dosage level of 2,000 mg/kg body weight. The results of the toxicity tests showed that the leaf extracts of *C. procera* were non-toxic at the dosage levels that were examined and were well tolerated by the experimental animals, as their LD_{50} cut-off values were less than 2,000 mg/kg.

Paracetamol-induced hepatotoxicity

Requirements: Plant extracts, acetone, ethanol, silymarin (50 mg/kg), and paracetamol.

Grouping and experimental design

Sprague-Dawley rats were categorized into five groups, each consisting of six animals, as follows:

Group I: Normal Control (NC) administered with 0.5% CMC via the oral route.

Group II: Toxic Control (TC), administered with 0.5% CMC via the oral route and toxicants (paracetamol 2 g/kg p.o. in 0.5% CMC) once a day for 14 days.

Group III: Positive Control administered with toxicants and silymarin (50 mg/kg) in 0.5% CMC and paracetamol 2 g/kg p.o. in 0.5% CMC once a day.

Groups IV and V: Received CPL extracts at 100 and 200 mg/kg in 0.5 CMC, respectively, and paracetamol 2 g/kg p.o. in 0.5% CMC once a day [36–39].

Sprague Dawley rats of both genders (150-200 g) consumed paracetamol at a dosage of 2 g/kg orally in 0.5% CMC once daily for 14 days. On the 15th day, samples were obtained from mice fasted overnight by retro-orbital puncture under general anesthesia. Serum was isolated by centrifugation at 3,000 rpm and freezing (2-4 °C)

for 10 min. The serum biochemical parameters were also evaluated.

Estimation of biochemical parameters

Blood samples were obtained from rats fasted overnight via retro-orbital puncture under general anesthesia. Serum was separated by centrifugation at 3,000 rpm at a temperature of 2-4 °C for 10 min. The hepatoprotective efficacy of CPL was assessed by evaluating various biochemical parameters, including SGOT (aspartate aminotransferase; AST), SGPT (alanine aminotransferase; ALT), alkaline phosphatase (ALP), and bilirubin, as well as *in vivo* antioxidant metrics, such as lipid peroxidation (LPO), reduced glutathione (GSH), superoxide dismutase (SOD), and catalase (CAT) across different groups. All analyses were performed using commercially available kits from Span Diagnostics Ltd., India.

Hematological parameters

A fully automated hematology analyzer (Cell-Dyn® 3700, Abbott Diagnostics, USA) was used to evaluate various hematological parameters, including red blood cell (RBC) count, hemoglobin (Hb) concentration, packed cell volume (PCV), total white blood cell (WBC) count, differential leukocyte count (DLC), and platelet (thrombocyte) counts.

Histological examination

The liver tissues were fixed in 10% phosphate-buffered neutral formalin for histological assessment to preserve structural integrity. The fixed samples were dehydrated using a graded series of ethanol concentrations (50–100%) and then embedded in paraffin wax. Delicate tissue slices were meticulously cut and stained with hematoxylin and eosin (H&E) to elucidate the cellular and structural features under a light microscope. Preliminary evaluation was conducted qualitatively, concentrating on identifying any histological changes or irregularities in the liver structure [40].

Results and Discussion

Physicochemical analysis of crude drug (determination of ash values)

An investigation of the physicochemical properties of powdered leaves of *C. procera* revealed characteristic ash values that reflect the inorganic composition of the crude drug. The overall ash percentage was 5.1% w/w, indicating a significant presence of inorganic materials, which may encompass both physiological ash from plant tissues and non-physiological ash, including soil, sand, or foreign substances [41]. The acid-insoluble ash was 1.28% w/w, indicating a relatively low proportion of silica and other acid-insoluble components, and thereby indicating minimal contamination with earthy materials. The water-soluble ash was 3.81% w/w, demonstrating the existence of water-soluble inorganic salts such as carbonates, phosphates, and silicates. These values serve as important diagnostic parameters for assessing the quality, purity, and identity of the crude drug, and provide baseline data for the standardization of *C. procera* leaf powder.

Preliminary phytochemical analysis

Several phytochemical compounds have been discovered in leaf extracts, with the assistance of research specifically designed for this purpose. According to the qualitative analysis, the primary constituents found in CPL extracts were carbohydrates, alkaloids, cardiac and anthraquinone glycosides, terpenoids, flavonoids, tannins, phenolic compounds, steroids, sterols, proteins, and amino acids. Other constituents include flavonoids, tannins,

phenolic compounds, steroids, and sterols. Given the availability of such a diverse array of chemicals, it is plausible that a vast variety of pharmacological effects can be achieved. Since ancient times, alkaloids and flavonoids have been known to be capable of treating various diseases. The results of qualitative phytochemical screening are presented in Table 1.

In vitro antioxidant assay

DPPH radical scavenging assay

CPL was subjected to the DPPH test, which used an ascorbic acid solution for comparison. A plot was constructed of the absorbance values versus the selected concentration. The proportion of DPPH inhibition curves for DPPH was constructed for ascorbic acid and leaf extract, and the IC_{50} values for the proportion of DPPH inhibition by ascorbic acid and leaf extract were derived using a regression equation. Figure 1 illustrates the slope of the regression line for the ascorbic acid and CPL extracts. Table 2 shows the breakdown of the percentages of free radicals scavenged and absorbed by ascorbic acid and CPL.

According to the research, the IC_{50} values for DPPH were 12.60 µg/mL for ascorbic acid (a reference chemical), and 2.88 µg/mL for CPL, respectively. This was proven in a way that was both clear and persuasive. Based on the findings presented here, it seems that CPL may convert free radicals into the equivalent hydrazine when these radicals are in contact with the hydrogen donors included in antioxidant principles. According to the findings of the current study, the CPL extract has a higher degree of scavenging action than ascorbic acid, which suggests that it has a high concentration of polyphenols and tocopherols. According to the findings of this experiment, an extract of CPL displayed radical scavenging activity by either transferring electrons or donating hydrogen. There was a strong correlation between the overall quantity of polyphenols and their antioxidant activity.

Hydrogen peroxide (H_2O_2) scavenging activity

In the conventional method, the ability of the plant extracts to scavenge H_2O_2 was evaluated by measuring the rate at which H_2O_2 disappeared at a wavelength of 230 nm. The objective of this study was to assess the efficacy of the plant extracts. The hydrogen peroxide radical test is a technique that can determine the level of antioxidant activity in a sample in a very short

Table 1. Preliminary phytochemical profile of the methanolic extract of *C. procera*

Sr./ No.	Phytochemical category	*C. procera*
1	Carbohydrate	Present
2	Alkaloids	Present
3	Cardiac glycosides	Present
4	Anthraquinone glycosides	Present
5	Terpenoids	Present
6	Fats and oils	Absent
7	Flavonoids	Present
8	Saponin	Present
9	Tannin and phenolic compounds	Present
10	Steroids & Sterol	Present
11	Protein and amino acid	Absent

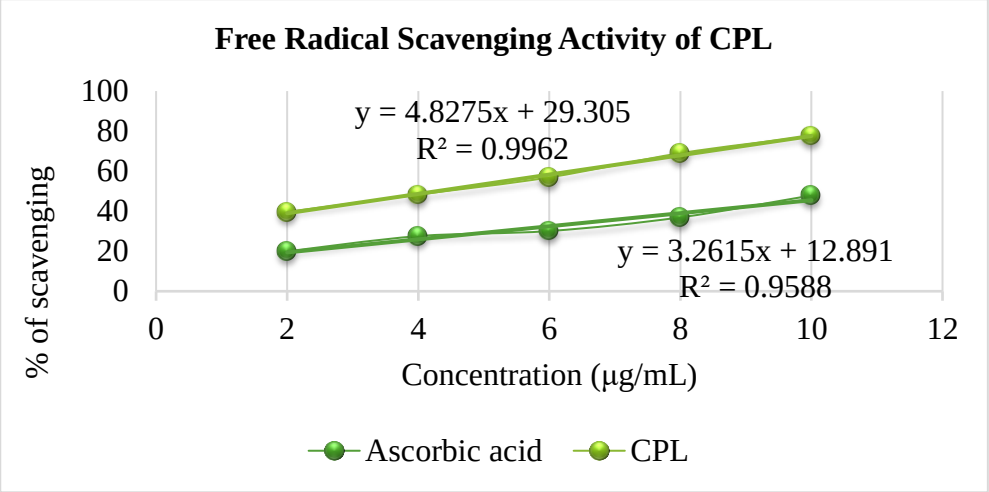


Figure 1. The regression curve of ascorbic acid and CPL extract in the DPPH assay. IC_{50} values were obtained from the regression curve

Table 2. Comparative DPPH radical scavenging activity showing % scavenging and absorbance values of Ascorbic acid vs. CPL

Concentration (µg/mL)	Absorbance of test (At) Ascorbic acid	% of Scavenging	Absorbance of test (At) CPL	% of Scavenging
2	0.410	19.92	0.035	39.65
4	0.371	27.54	0.03	48.27
6	0.358	30.08	0.025	56.89
8	0.323	36.91	0.018	68.96
10	0.267	47.85	0.013	77.58

Table 3. Comparative hydrogen peroxide scavenging activity showing % scavenging and absorbance values of ascorbic acid vs. CPL

Concentration (µg/mL)	Absorbance of test (At) Ascorbic acid	Scavenging (%)	Absorbance of test (At) CPL	Scavenging (%)
2	0.340	46.70	0.044	24.13
4	0.324	49.21	0.039	32.75
6	0.299	53.13	0.031	46.55
8	0.276	56.74	0.023	60.34
10	0.249	60.97	0.015	74.13

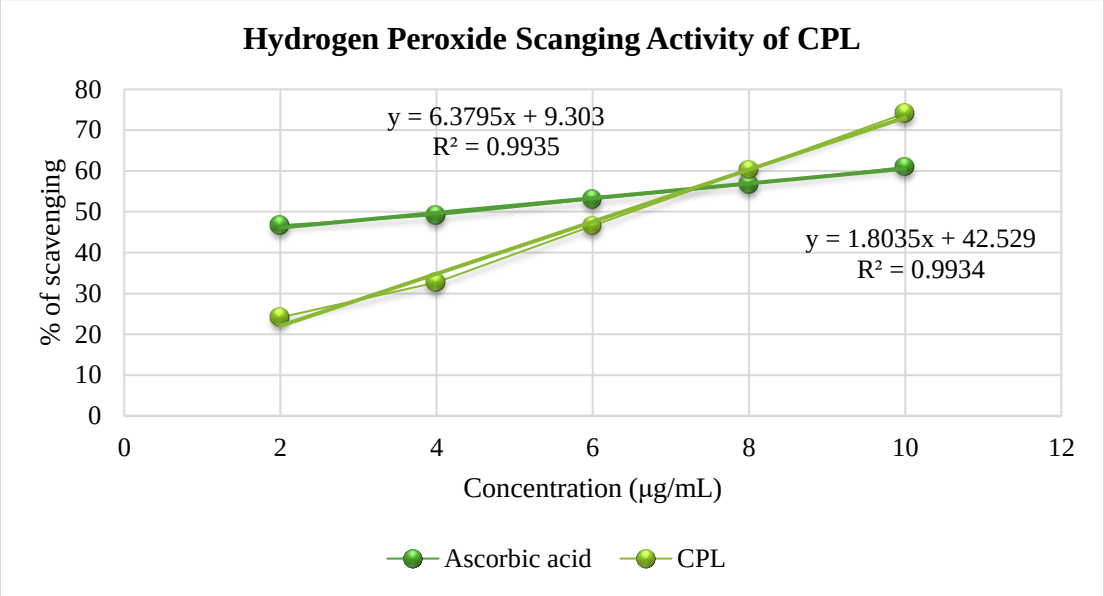


Figure 2. The percentage scavenging activity of CPL in the hydrogen peroxide scavenging assay. IC_{50} values were determined from inhibition curves

period of time. The hydrogen peroxide radical scavenging ability of CPL was calculated using ascorbic acid solution as the benchmark. A comparison was made between the absorbance data and the selected concentration. The regression line equation from the standard curves for H_2O_2 radical scavenging of ascorbic acid and the inhibition curve of leaf extract were used to calculate the IC_{50} values of the percent inhibition of hydrogen peroxide radical scavenging by leaf extract and ascorbic acid. These values were used to determine the IC_{50} values of ascorbic acid and leaf extract. Information on ability of CPL to scavenge hydrogen peroxide is listed in Table 3, and Figure 2 depicts the data in the form of a graph.

The effect of CPL on scavenging hydrogen peroxide was dependent on its concentration (2–10 µg/mL). The IC_{50} value for the H_2O_2 scavenging activity of CPL was determined to be 5.27 µg/mL, whereas the IC_{50} value for ascorbic acid was established at 69.22 µg/mL. The amount of hydrogen peroxide that is created naturally in the environment (including air, water, human body, plants, and bacteria), in addition to the levels found in food, is relatively modest. Rapidly disintegrating into oxygen (O_2) and water (H_2O), this process has the potential to create hydroxyl radicals (OH), which may damage DNA and trigger off the lipid peroxidation process. Because CPL contains phenolic groups, it was able to successfully remove hydrogen peroxide from the atmosphere, which may be related to the fact that this removal was effective. The ability of these groups to donate electrons to hydrogen peroxide, resulting in its conversion to water, was demonstrated.

Reducing power assay

The inclusion of electron-donating chemicals in this experiment led to the reduction of ferricyanide, which was measured as Fe^{3+}, to ferrous oxide, which was measured as Fe^{2+}. This

transformation was observed to have occurred (ferrous iron). The findings are shown in Figure 3 and the absorbance statistics are shown in Table 4. The reducing potential of the extracts suggested a general increase in activity when they were examined at concentrations up to 100 µg/mL, which was shown to be the case when the concentration was elevated. The absorbance of CPL was measured at 700 nm, which demonstrated that it is capable of reducing the impact of free radicals to the greatest extent feasible. CPL indicated a better potential to minimize the impact of free radicals as the concentration of the extract increased.

In vivo hepatoprotective activity

Acute toxicity study

The primary objective of the acute toxicity study was to determine the therapeutic index and ensure *in vivo* safety of the test

Table 4. Absorbance recorded in reducing power assay of CPL and ascorbic acid

Concentration (µg/mL)	Absorbance at 700 nm	
	Ascorbic acid	CPL
10	0.008	0.019
20	0.02	0.021
30	0.033	0.036
40	0.036	0.035
50	0.042	0.039
60	0.057	0.056
70	0.068	0.06
80	0.078	0.061
90	0.097	0.071
100	0.105	0.074

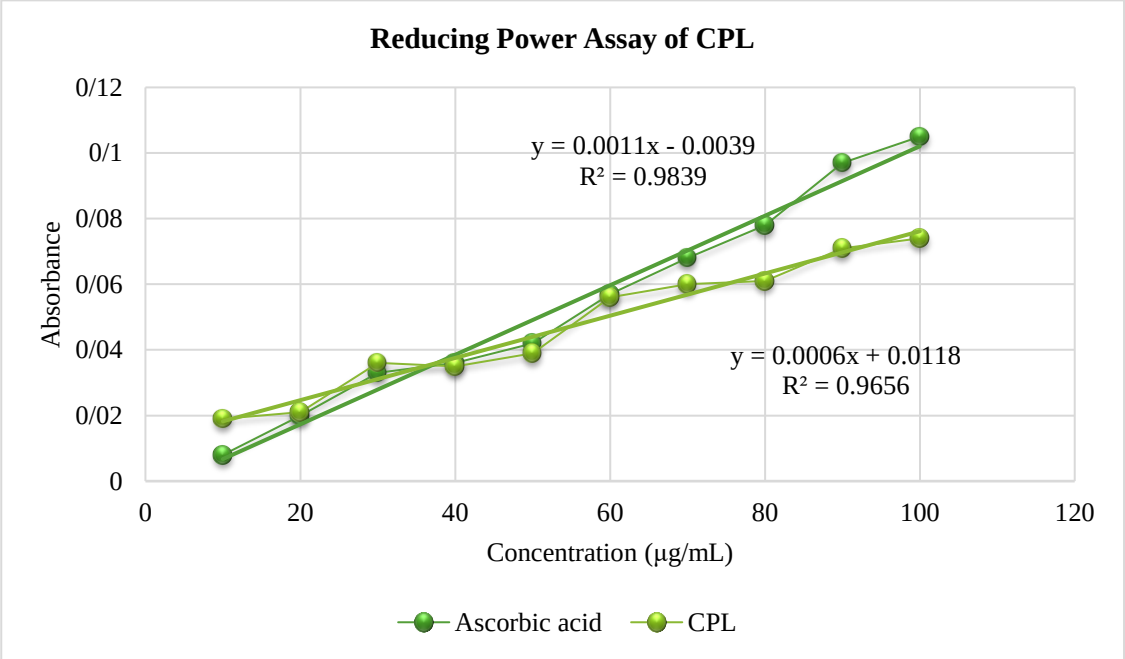


Figure 3. Reducing power assay of CPL

substance. Typically, studies are conducted to estimate the median lethal dose (LD) in experimental animals. In the present study, the methanolic leaf extract of *C. procera* was evaluated for acute oral toxicity following the OECD Guideline 423 protocol. The results indicated that no mortality or visible signs of toxicity were observed in any of the animals during the 14-day observation period, even at the limit dose of 2,000 mg/kg body weight. These findings suggest that the extract is relatively safe for animal use. Based on these results, two doses, 200 mg/kg (one-tenth of the maximum dose) and 100 mg/kg (one-twentieth of the maximum dose), were selected for subsequent hepatoprotective studies.

Paracetamol-induced hepatotoxicity model

Estimation of biochemical parameters

The hepatoprotective potential of CPL was evaluated by analyzing key biochemical markers, including SGOT, SGPT, ALP, and bilirubin, and in vivo antioxidant parameters, such as LPO, GSH, SOD, and CAT, in various animal groups subjected to a paracetamol-induced hepatic injury model. The comparative outcomes of CPL treatment on these parameters are presented in Tables 5–7, while the corresponding graphical representations are illustrated in Figures 4–6. Collectively, these findings indicate that CPL exhibits a protective effect against paracetamol-induced hepatic damage by restoring altered biochemical and antioxidant levels to normal levels.

AST (SGOT) and ALT (SGPT)

Paracetamol overdose produced a marked elevation in serum AST and ALT compared with normal controls (AST: 109.33 vs. 60.33 IU/L; ALT: 137.33 vs. 47.67 IU/L), consistent with hepatocellular injury and leakage of cytosolic transaminases into the circulation. Treatment with silymarin substantially attenuated these increases (AST 70.33 IU/L; ALT 61.17 IU/L), confirming assay sensitivity and expected hepatoprotective effect. The *C. procera* extract decreased transaminase levels in a dose-dependent manner: CPL-100 (AST 83.50, ALT 107.23 IU/L) and CPL-200 (AST 81.26, ALT 74.06 IU/L). The reduction in ALT was particularly notable at 200 mg/kg, indicating that the extract limited the paracetamol-induced hepatocellular membrane damage and enzyme leakage. These findings indicate that *C. procera* conferred significant hepatoprotection, as reflected by the normalization of serum transaminase levels toward control values.

Table 5. SGOT (AST) and SGPT (ALT) of paracetamol-induced model

Group	Treatment	AST (IU/L)	ALT (IU/L)
I	Normal control	60.33 ± 1.493	47.67 ± 2.092
II	Toxic control (paracetamol)	109.33 ± 1.358	137.33 ± 2.231
III	Standard (Silymarin)	70.33 ± 1.453***	61.17 ± 2.104***
IV	*CPL-100mg*	83.501± 1.58***	107.23± 4.08***
V	*CPL-200mg*	81.256±2.23***	74.06±3.15***

*All values are shown as the mean ±SEM (n=6), *P<0.05, **P<0.01, and ***P<0.001 vs. the toxic control group. #P<0.05, ##P<0.01, and ###P<0.001 compared with the normal control.*

Table 6. ALP, bilirubin, and LPO of paracetamol-induced model

Group	Treatment	ALP (IU/L)	Bilirubin (mg/dl)	LPO (M/mg protein)
I	Normal control	117.16 ± 2.442	0.855 ± 0.0152	4.567 ± 0.325
II	Toxic control (paracetamol)	152.16 ± 1.424	1.263 ± 0.0176	7.295 ± 0.222
III	Standard (Silymarin)	123.17 ± 1.302***	0.906 ± 0.0234***	4.983 ± 0.122***
IV	*CPL-100mg*	132.78± 1.98***	1.017± 0.011**	5.983± 0.071**
V	*CPL-200mg*	127.63±2.21***	1.002±0.021**	5.700±0.11***

*All values are shown as the mean ±SEM (n=6), *P<0.05, **P<0.01, and ***P<0.001 vs. the toxic control group. #P<0.05, ##P<0.01, and ###P<0.001 compared with the normal control.*

Table 7. GSH, SOD, and catalase of paracetamol-induced model

Group	Treatment	GSH (M/mg protein)	SOD (% inhibition of NBT)	Catalase (µM)
I	Normal control	19.424 ± 0.620	314.66 ± 3.242	19.348 ± 0.644
II	Toxic control (paracetamol)	12.711 ± 0.575	222.83 ± 3.516	11.805 ± 0.204
III	Standard (Silymarin)	18.052 ± 0.561***	297.66 ± 3.844***	18.250 ± 0.418***
IV	*CPL-100mg*	14.865± 1.71**	267.5± 12.781***	12.23± 1.31*
V	*CPL-200mg*	15.987±0.98***	281.07±11.68***	14.17±1.8**

*All values are shown as the mean ±SEM (n=6), *P<0.05, **P<0.01, and ***P<0.001 vs. the toxic control group. #P<0.05, ##P<0.01, and ###P<0.001 compared with the normal control.*

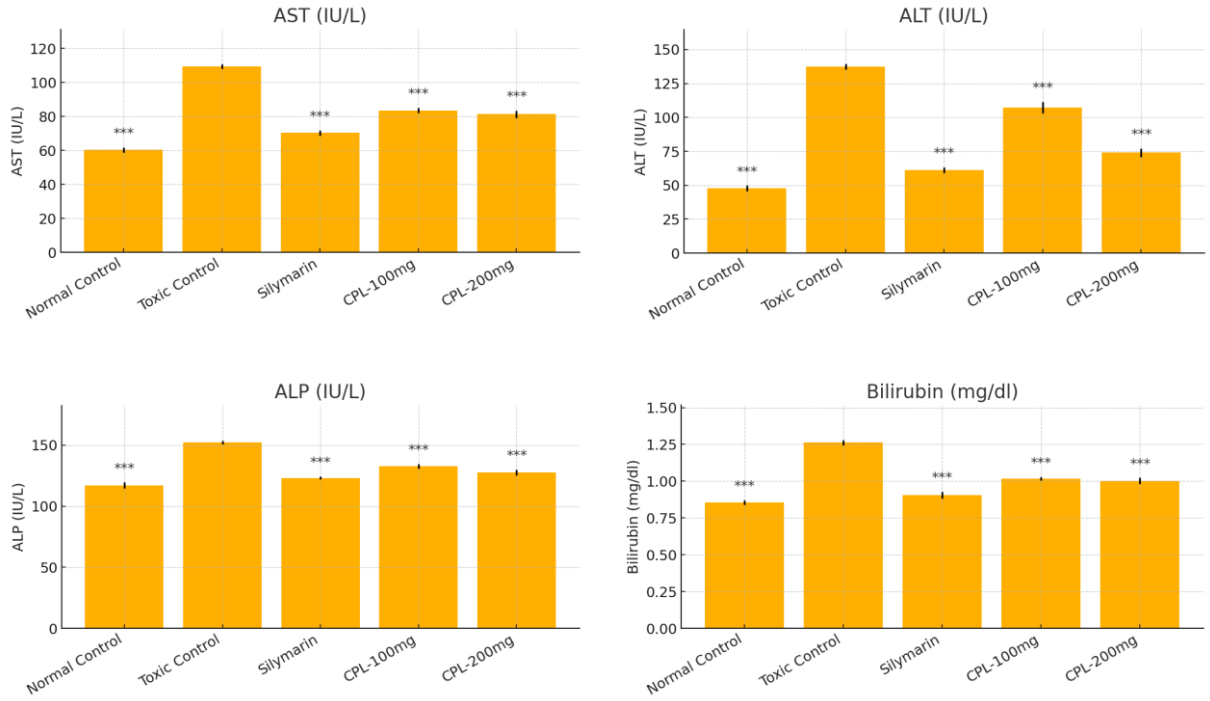


Figure 4. Liver enzymes -effects of *C. procera* extract (100 and 200 mg/kg) on liver enzymes in paracetamol-induced hepatotoxicity model. Data are expressed as mean ± SEM (n=6). Statistical significance markers indicate comparison with toxic control: *p<0.05, **p<0.01, and ***p<0.001

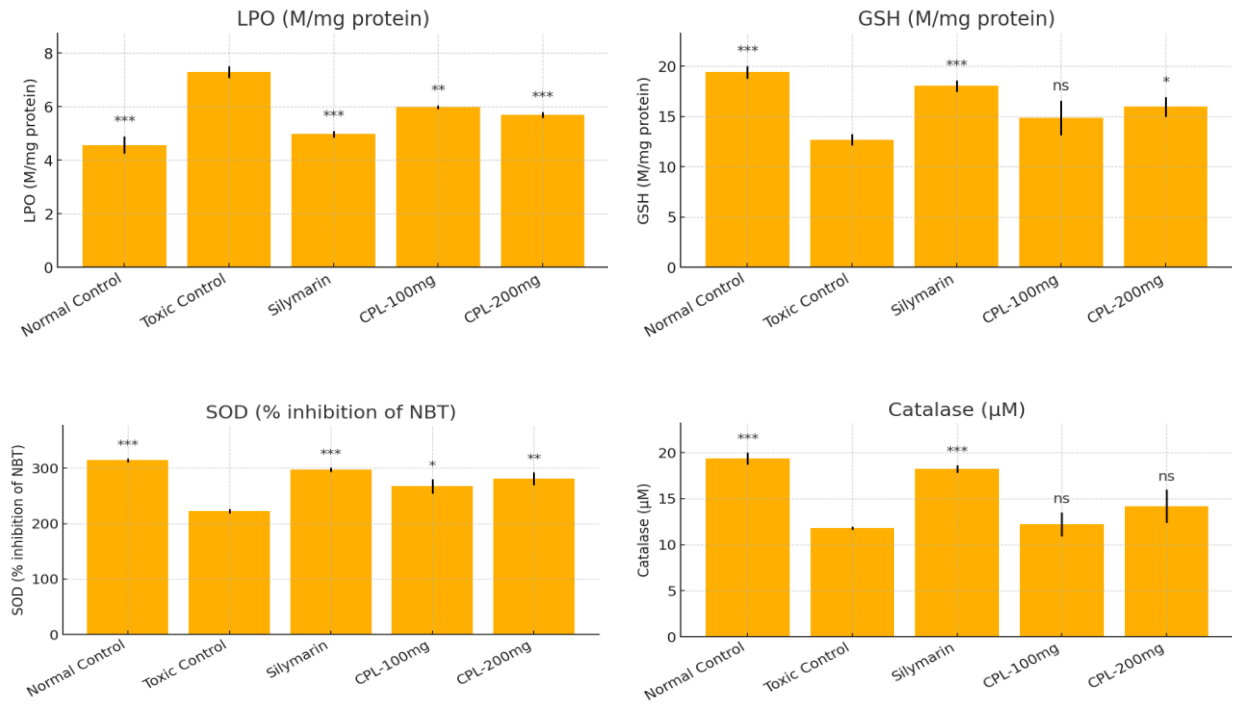


Figure 5. Oxidative stress indicators - Effects of *C. procera* extract (100 and 200 mg/kg) on oxidative stress indicators in a paracetamol-induced hepatotoxicity model. Data are presented as mean ± SEM (n=6). Statistical significance indicators denote comparison with toxic control: *p<0.05, **p<0.01, and ***p<0.001

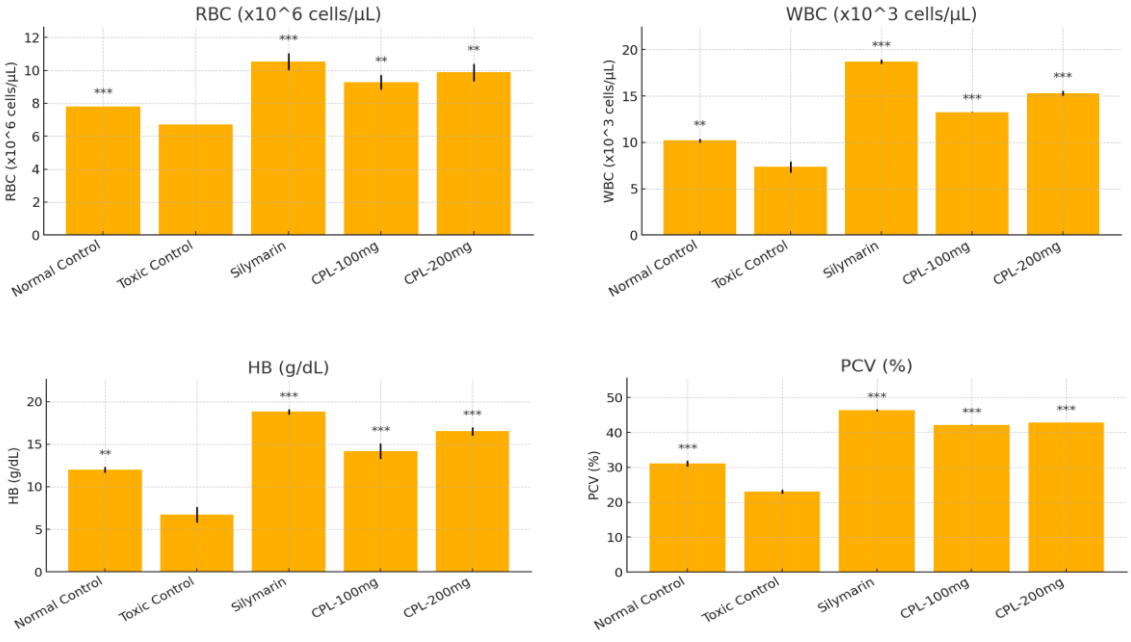


Figure 6. Hematological parameters — Effects of *C. procera* extract (100 and 200 mg/kg) on hematological parameters in paracetamol-induced hepatotoxicity model. Data are expressed as mean ± SEM (n=6). Statistical significance markers indicate comparison with toxic control: *p<0.05, **p<0.01, and ***p<0.001

Alkaline phosphatase (ALP) and total bilirubin

ALP and bilirubin are markers of cholestasis and impaired hepatic excretory function, respectively. Paracetamol treatment modestly elevated ALP and bilirubin (ALP 152.16 IU/L, bilirubin 1.263 mg/dl compared with normal animals (ALP 117.16, bilirubin 0.855). Silymarin restored these parameters to nearly normal levels (ALP 123.17; bilirubin 0.906), whereas CPL at both doses resulted in significant reductions (CPL-100: ALP 132.78, bilirubin 1.017; CPL-200: ALP 127.63, bilirubin 1.002). The decreases observed with CPL-200 were closer to the standard, suggesting that the extract preserved the hepatic excretory function and reduced bile duct/hepatocyte dysfunction caused by paracetamol. The magnitude of bilirubin reduction, although partial, supports the mitigation of bilirubin overproduction or improved conjugation/excretion.

Lipid peroxidation (LPO)

LPO (marker: MDA or related units) levels increased significantly after paracetamol challenge (7.295 vs. 4.567 in controls), reflecting enhanced free radical-mediated membrane lipid damage. Silymarin limited this increase (4.983) and CPL at 100 and 200 mg/kg significantly decreased LPO (5.983 and 5.700, respectively). The reduction in LPO by *C. procera* indicated attenuation of oxidative membrane damage, likely due to scavenging of reactive species or inhibition of lipid peroxidation chain reactions, which is consistent with the *in vitro* antioxidant activity of the extract.

Reduced glutathione (GSH), superoxide dismutase (SOD) and catalase (CAT)

Paracetamol significantly decreased endogenous antioxidant defences: GSH dropped from 19.424 to 12.711 M/mg protein, SOD from 314.66 to 222.83 (% inhibition readout), and CAT from 19.348 to 11.805 µM. Silymarin restored GSH, SOD, and CAT levels to near the control levels, confirming its antioxidant/hepatoprotective action. CPL treatment restored these parameters in a dose-dependent manner: CPL-100 increased GSH to 14.865, SOD to 267.5, and CAT to 12.23; CPL-200 further improved GSH to 15.987, SOD to 281.07, and CAT to 14.17. These results suggest that *C. procera* preserves and/or replenishes endogenous antioxidant systems, preventing oxidative stress amplification during paracetamol metabolism (likely through free radical scavenging and by sparing GSH).

Estimation of hematologial parameters (RBC, WBC, Hb, PCV, and MCH)

The hematological parameters were estimated in all treatment groups, and the results are tabulated in Table 8, with its graphical presentation is depicted in Figure 6. Paracetamol caused declines in several hematological indices (RBC 6.72×10^{6}/µL, Hb 6.71 g/dL, and PCV 23.03%), indicating systemic toxicity and possible bone marrow or hemolytic effects secondary to oxidative stress. Both silymarin and CPL treatments improved hematological profiles: silymarin produced values above normal (*e.g.,* RBC 10.53 and Hb 18.83), whereas CPL-100 and CPL-200 restored RBC, Hb, and PCV toward or above baseline (CPL-200: RBC 9.89, Hb 16.52, and PCV 42.83). WBC (immune response) decreased with paracetamol (7.35×10^{3}/µL) but increased markedly with silymarin and CPL, suggesting recovery of general health and immune competence. Improvement in hematological indices likely reflects reduced systemic oxidative damage and improved hepatic synthetic/metabolic function following treatment.

Table 8. The hematological parameters estimated in paracetamol-induced model

Group	Treatment	RBC (x10^6 cells/uL)	WBC (x10^3 cells/uL)	HB (g/dL)	PCV (%)	MCH (pg)
I	Control	7.8±0.001	10.2±0.19	12.02±0.36	31.12±0.79	12.03±0.49
II	Toxic Control (paracetamol)	6.72±0.01$^{\#\#}$	7.35±0.61$^{\#\#}$	6.71±0.92$^{\#\#}$	23.03±0.58$^{\#\#}$	9.56±0.03$^{\#\#}$
III	Standard (Silymarin)	10.53±0.52**	18.71±0.23**	18.83±0.32**	46.35±0.24**	18.25±0.21**
IV	CPL-100 mg	9.29±0.45**	13.25±0.03**	14.18±0.91**	42.17±0.03**	16.36±0.46**
V	CPL-200 mg	9.89±0.53**	15.32±0.27**	16.52±0.47**	42.83±0.02**	16.95±0.53**

*All values are shown as the mean ±SEM (n=6), *P<0.05, **P<0.01 and ***P<0.001 vs. the toxic control group. #P<0.05, ##P<0.01, and ###P<0.001 compared with the normal control.*

Histological examination

Histology confirmed the following biochemical findings: the toxic control group exhibited necrosis, fatty degeneration, hemorrhage, and disordered hepatic architecture. Silymarin preserved normal parenchymal architecture. CPL-treated livers displayed milder pathological changes (mild inflammation, mild degeneration, and mild fatty change), with the 200 mg/kg dose showing greater protection than the 100 mg/kg dose. These morphological observations corroborated the biochemical and antioxidant data, supporting the overall hepatoprotective effects of *C. procera*. Figure 7 depicts the histopathic pictures of the liver produced by the paracetamol-induced model.

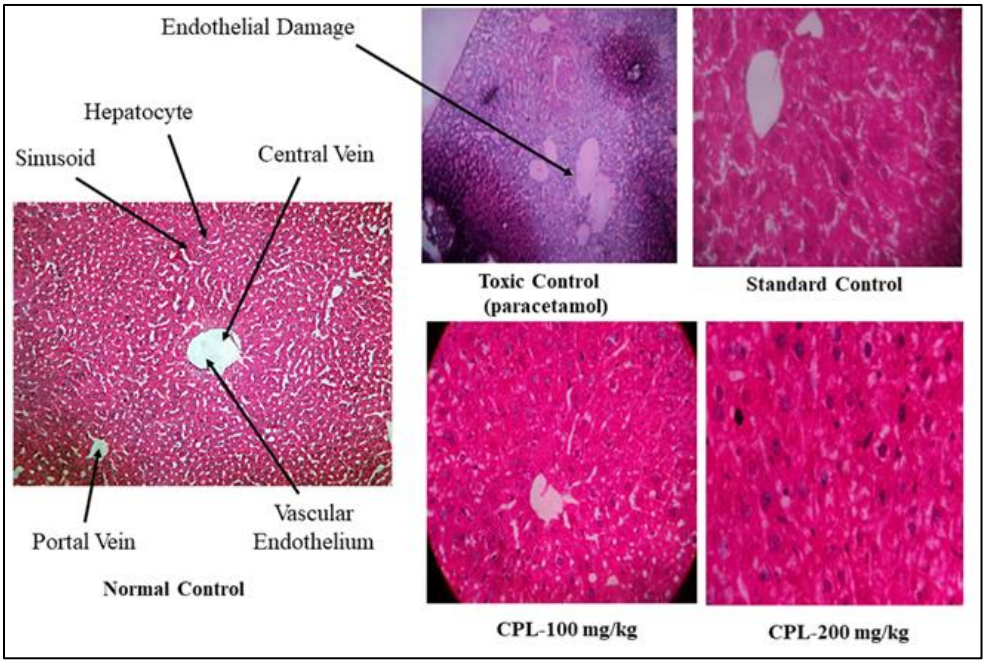


Figure 7. The histological images of liver of paracetamol-induced model

Collectively, the biochemical, antioxidant, hematological, and histological data demonstrated that the methanolic extract of *C. procera* significantly ameliorated paracetamol-induced hepatotoxicity in rats with dose-dependent efficacy and effects similar to those of silymarin. Hepatoprotection correlated closely with the restoration of endogenous antioxidant defences and reduction of lipid peroxidation, indicating that antioxidant mechanisms (free radical scavenging and GSH preservation) likely underpinned the protective action. These findings align with the *in vitro* antioxidant profile of the extract and the presence of polyphenols and flavonoids identified in phytochemical screening.

Conclusion

The methanolic leaf extract of *C. procera*, prepared by cold maceration, exhibited significant antioxidant and hepatoprotective activities. The extract demonstrated superior radical scavenging efficiency compared to ascorbic acid in *in vitro* assays, and provided robust protection against paracetamol-induced hepatotoxicity in rats. Improvement of biochemical markers (AST, ALT, ALP, and bilirubin), restoration of endogenous antioxidant defences (GSH, SOD, and CAT), reduction in lipid peroxidation, and normalization of hematological parameters confirmed its protective efficacy. Histopathological observations further supported these biochemical outcomes, indicating a dose-dependent amelioration of hepatic injury. These effects can be attributed to the presence of flavonoids, phenolics, and other bioactive compounds in the extracts. This study not only validates the ethnomedicinal use of *C. procera*, but also highlights its potential as a natural source of hepatoprotective agents. Further studies are warranted to isolate the bioactive compounds and elucidate the molecular mechanisms underlying their hepatoprotective activity, thereby strengthening their therapeutic applicability.

Acknowledgments

None.

Disclosure Statement

The authors declared that no conflicts of interest were existed regarding the publication of this work.

Statistical Analysis

Results are expressed as mean ± standard deviation. Data were analyzed using analysis of variance (ANOVA) followed by Bonferroni's post hoc test. Statistical significance was set at $p \leq 0.05$. All statistical analyses were conducted using the GraphPad Prism 7 software. All data are shown as the mean ± SEM (n=6), with significance levels indicated as *P<0.05, **P<0.01, and ***P<0.001 compared to the toxic control group. $P < 0.05$, $P < 0.01$, and $P < 0.001$ compared to the standard control.

Ethical Approval

This study assessed the acute oral toxicity of methanol extracts from the leaves of *C. procera* in accordance with OECD standards 423 after approval from the Animal Ethical Committee (Approval No. 1211/PO/Re/S/08/CPCSEA/IAEC/MES/1-2/18).

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HOW TO CITE THIS MANUSCRIPT
S.M. Bairagi, J.G. Wagh, A.R. Thanage, P. Venkata Narasimha Raju, P. Erukulla, K. Vamsi Kandimalla, A. Manukonda, R. Hari Babu. Green Extraction, Antioxidant, and Hepatoprotective Evaluation of *Calotropis procera* Leaf Extract. *Asian Journal of Green Chemistry*, 10 (3) 2026, 430-449.
DOI: https://doi.org/10.48309/ajgc.2026.558174.1863
URL: https://www.ajgreenchem.com/article_235780.html