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Phytochemical Screening, GCMS Profiling, In Vitro Antioxidant, In Vivo Acute Toxicity, and Hepatoprotective Activity of *Cleome simplicifolia* Bioactive Metabolites against Paracetamol-Intoxicated Wister Albino Rats

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Abstract: The current study aimed to appraise extracts of *Cleome simplicifolia* (Cambess.) Hook. f. and Thomson leaves for chemical integrant and biological activities. In this study, different extracts of leaves were estimated for qualitative phytochemical screening, identification of functional groups, mineral content, and phyto-compounds and assessed for in vitro antioxidant and in vivo acute toxicity and hepatoprotective activity antagonistic toward paracetamol-intoxicated Wister albino rats. The results of the qualitative phytochemical assessment of the leaf extracts (acetone, methanol, and distilled water) exhibited the occurrence of useful metabolites. A Fourier transform infrared analysis confirmed the occurrence of O-H, N-H, C=C, S=O, C-O, C-N, C-Cl, and C-Br at 3367.14, 2920.79, 2850.32, 1631.04, 1384.59, 1168.64, 1063.78, 824.78, and 615.25 cm^{-1} wavelengths, whereas energy-dispersive X-ray showed the existence of carbon, oxygen, magnesium, aluminum, silicon, phosphorus, sulfur, chlorine, potassium, and calcium elements in the leaf, respectively. Thereafter, a gas chromatography–mass spectroscopy analysis unveiled the diverse volatile compounds in the methanolic leaf extracts, namely *n*-Heptyl acrylate—18.87%, undecane—17.49%, 2-Propenoic acid, 3-(3,4-dimethoxyphenyl)-, (E)—11.40%, Neophytadiene—11.02%, *n*-Hexadecanoic acid—10.78%, Glafenin—10.09%, Decane—7.45%, Phytol—6.0%, Benzene, (1-methyldodecyl)—3.48%, and 4-Cyclohexyl-1-butanol—3.41%, respectively. An analysis of in vitro antioxidant activity using the 2,2-diphenyl-1-picrylhydrazyl assay of methanolic leaf extract revealed elevated levels of antioxidant ability at 20 (46.18%), 40 (53.83%), 60 (66.64%), 80 (74.03%), and 100 (85.05%) µg/mL. In addition, in vivo acute toxicity determination proved that the methanolic leaf extract was innocuous and caused no mortality at 72 mg, 78 mg, or 82 mg/kg b.wt. doses. The methanolic leaf extracts' in vivo hepatoprotective activity against paracetamol revealed significant efficacy at 50 and 100 mg/kg b.wt. via reduction of aspartate transaminase, alanine transaminase, alkaline phosphatase, bilirubin, and cholesterol (serum blood biochemical markers) followed by an enhancement in superoxide dismutase, catalase, and glutathione (liver antioxidants) with lipid peroxidation depletion compared with the normal group rats. From the investigated study, it was concluded that the *C. simplicifolia* leaf are a potential source for the isolation of biologically active phyto-compounds and have the ability to prevent liver damage by paracetamol induction, where the hepatic restoration ability is indexed to its in vivo and in vitro antioxidant ability, which might be the result of its chemical constituents.

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1. Introduction

Nature is the best maintainer of all the creatures on the earth and balances life by providing wild natural beneficiary sources such as plants. Wild herbal plants have been the core topic of many research studies, as these wild plants are utilized as staple foods, with adequate concentrations of vitamins, minerals [1], carbohydrates, fats and oil, proteins, and natural antioxidants, in tribal and rural communities. Contrary to this, plants produce phytochemicals (primary and secondary metabolites) that prevent the complications associated with harmful diseases by exhibiting medicinal properties, like antihelmintic, neuroprotective, antitumor, antiosteoporotic, immunomodulatory, hepatoprotective, antiatherosclerotic, anti-inflammatory, antifungal, antibacterial, diuretic, and aphrodisiac properties [2,3]. Phyto-compounds are in demand due to their negligible side effects and are safer to use. Beyond their beneficial contribution, plants may contain some antinutritional and toxic substances that could negative implications on health and diets [4]. Hence, it is necessary to analyze wild plants for their nutritional, antinutritional, and phytochemical contents and biological activities before utilization and supplementation of the plant, which would be characterized using different analyses and analytical techniques for consideration, mainly including qualitative screening, Fourier transform infrared (FTIR) and energy dispersive X-ray (EDX) spectroscopy, and gas chromatography–mass spectroscopy (GCMS) techniques with the assessment of biological potentialities.

The largest internal organ in humans is the liver, which contains intricate metabolic, biochemical, and physiological processes and exhibits detoxification action. In addition, the liver transports blood to the body by regulating blood filtration in the digestive tract [5,6]. In spite of this, the liver is electively damaged by hepatotoxic agents such as chloroquine, paracetamol, and isoniazid inducers, which alter the liver physiology and functional system and eventually result in liver necrosis [7]. *N*-acetyl-*p*-aminophenol (acetaminophen), commonly called paracetamol (PC), is prescribed as an analgesic and antipyretic drug. A high intake of PC could result in destructive hepatic rupture [8]. In the liver, the cytochrome P450 enzymatic system will transform PC into *N*-acetyl-*p*-benzoquinone (NAPQI). In the case of humans, NAPQI is excreted by the urinary tract upon its binding to liver glutathione (GSH). However, tumor necrosis factor-alpha (TNFα) synthesis is promoted when NAPQI accretion damages the liver GSH supply, which occurs during a PC overdose. In liver hepatocytes, this leads to molecule (lipids, proteins, RNA, and DNA) distortion due to the consequent formation of reactive oxygen species (ROS). This increase in pathological prooxidants causes cells to undergo inflammation and apoptosis, which triggers the death of liver cells [9–11]. Moreover, this undesired cellular injury is controlled by endogenous resistance mechanisms, but this resistance is not enough. To improve the protection, dietary antioxidants have been suggested as a means to scavenge ROS for the minimization of cellular damage [12]. Certainly, by looking into the concrete harmful effects of synthetic drugs and xenobiotics, it is clear that plant-based treatments and product consumption have to be normalized and substituted. The concept of implementation of herbal treatments is fascinating for researchers, who are investigating plants' phytochemical and biological profiles as an alternate source for disease control [11].

The plant species of the *Cleome* genus are valuable and are frequently investigated for their nutritional (used as a vegetable, flavoring compounds, seeds as alternate for mustard, and in making pickles, curries, and cakes), phytochemical (like essential oils, fatty acids, anthocyanins, glucosinolates, terpenes, flavonoids, polyphenols, and carboxylic acids), while certain compounds have been isolated and identified (namely, citronellic acid, undecane, β-pinene, α-terpeniol, α-amorphene, camphene, myrcene, limonene, α-pinene, allo-ocimene, cedrene, ethyl palmitate, dehydrolinalool, decan-2-ol, E-ocimene, deca-2,4-dien-1-al, p-cymene, 6-methylhept-5-en-2- one, limonene oxide, and heptan-4-

one) with pharmacological aspects (like antioxidant, antimicrobial, anticancer, analgesic, anti-inflammatory, anti-proliferative, hepatoprotective, antibacterial, insecticidal, etc.) [13]. While several Cleomaceae species were studied for in vivo hepatoprotective ability in experimental animals, for instance, the methanolic stem extracts of *Cleome chelidonii* and *Cleome viscosa* plants of Cleomaceae have been assessed for hepatoprotective ability against carbon tetrachloride (CCl_4)-intoxicated rats to monitor ALT and AST biochemical level [14]. Similarly, coumarinolignoids (cleomiscosins A, B, and C) isolated from *Cleome viscosa* seeds showed hepatoprotective protection for CCl_4-damaged livers in rats [15]. The *Cleome* genus belongs to the family Cleomaceae of flowering plants. *Cleome* is the largest genus of family which is distributed across tropical and subtropical regions, including herbaceous annual or perennial shrubs and plants [16]. As such, another species of the genus *Cleome*, *Cleome simplicifolia* (Cambess.) Hook. f. and Thomson, is one among them. *C. simplicifolia*, commonly known as Clammy "weed", while its Indian vernacular name is "Gawati tilwan", luxuriantly grows in moist, loamy black soil during the rainy season and has a short life span of about 3–4 months [17]. *C. simplicifolia* is native to Asia, and in India, it is distributed in Andhra Pradesh, Goa, Karnataka, Gujarat, and Chhattisgarh and is commonly found in parts of Maharashtra. Each part of *C. simplicifolia* is used in medicines and by tribal communities in the traditional medicine system [18]. Nevertheless, *C. simplicifolia* leaves and seeds have been part of several previous research studies that focused on seed ornamentation [19], seed oil fatty acid composition [20], protein composition [17], antioxidant activities [21], infrared spectroscopy analysis [22], growth and development [23], microscopic pollen analysis [24], and nanoparticle synthesis [18], but none of these studies has given complete medicinal information about the *C. simplicifolia*. Hence, this study was aimed to determine the medicinal properties of *C. simplicifolia* leaves by examining the phytochemical integrant and paracetamol-triggered in vivo hepatoprotective activity in Wister albino rats.

2. Materials and Methods

2.1. Collection, Authentication, Drying, and Extraction of Research Specimen

The collection place of the *Cleome simplicifolia* (Cambess.) Hook. f. and Thomson research plant specimen was Darikonur village—17.0268155 N, 75.3761952 E (Sangli, Maharashtra, India). Further, Dr. Manoj M. Lekhak, assistant professor at the Department of Botany, Shivaji University, Kolhapur, identified the collected plant specimens, and the herbarium (voucher specimen number HYS-01) that was maintained at the Shivaji University, Kolhapur.

The healthy leaves of *C. simplicifolia* were detached from the plant and made free of contaminants by washing them under running water, followed by removing excess water by pressing them between bundles of newspapers. Further, the leaves were shade-dried for up to 30–35 days; the dried material was blended in an electric blender to obtain a powder form. The leaf powder was stored at 4 °C in an airtight container and utilized during further research studies.

The leaf powder (30 g) was processed for extraction using a Soxhlet apparatus for 8–10 h using acetone, methanol, and distilled water as a solvent separately in the increasing polarity range. The extracts were filtered to remove powder particles and freed from the solvent by applying vacuum pressure. The solvent-free extracts were stored in airtight glass vials for utilization in subsequent evaluations.

2.2. The Extract Concentration of C. simplicifolia Leaf

An equation was used to get the extract percentage yield by subtracting the first-day extract concentration from the solvent-free extract concentration. The obtained mass, after subtracting, was then divided by the total powder concentration taken. Finally, the percentage yield was obtained by multiplying the results by 100. The extraction yield was

performed thrice. The percentage ratio of the extract was utilized to calculate the extract in terms of g/100 g. The equation to calculate extract percentage yield was as follows.

$$\text{Extract yield } (\%) = \frac{\text{weight of extract} - \text{weight of solvent free extract}}{\text{Total weight of powder}} \times 100$$

2.3. Qualitative Assessment of Phytochemicals in C. simplicifolia Leaf Extracts

2.3.1. Carbohydrates (Fehling's Test)

Fehling's solution A (copper sulfate solution) and Fehling's solution B (Rochelle salt solution) were combined in the same concentrations, to which Fehling's solution was mixed with the preheated solutions of the tested leaf extracts. Reducing sugar positivity was confirmed upon brick-red precipitation [25].

2.3.2. Proteins (Biuret Test)

In total, 1 mL of 10% NaOH was mixed with the tested leaf extracts and heated. Further, upon adding a few drops of 7% $CuSO_4$ solution to the preheated solution, a purplish-violet color appeared, that implies presence of protein [26].

2.3.3. Amino Acids (Ninhydrin Test)

Two to five drops of 2%-Ninhydrin solution were dropped into the tested leaf extracts; later, the solutions were kept in a boiling water bath (120 s). Purple color examination determines the amino acid occurrence [27].

2.3.4. Glycosides (Salkowski's Test)

To the tested extracts, 2 mL chloroform was added and then treated with H_2SO_4 and gently shaken for proper mixing of the solutions. This results in the development of a lower steroidal layer with a reddish-brown color at interference and confirms the presence of glycoside [25].

2.3.5. Cardiac Glycosides (Keller–Kiliani Test)

The mixture of distilled water (5 mL) and tested leaf extracts (2 mL each) was shaken vigorously, into which 2 mL of acetic acid (glacial) (having certain $FeCl_3$ drops) was mixed. Then, 1 mL of H_2SO_4 was poured through the side of the test tube walls. This allowed for a ring (brown) to appear at the interface, indicating cardiac glycoside positivity. In addition, the formation of a violet-colored ring can be seen beneath the ring (brown) [28].

2.3.6. Phenols (Ferric Chloride Test)

The tested leaf extracts were diluted (distilled water—3 mL), and this aqueous solution of 5% ferric chloride was combined. Phenols were determined by the observation of a deep blue or black color [29].

2.3.7. Flavonoids (Alkaline Reagent Test)

The tested leaf extracts were treated with 2 mL of 2% NaOH solution, which gave a deep yellow color. Upon dilute acid addition (a few drops), if the mixture turned colorless, flavonoid positivity was implied [29].

2.3.8. Saponins (Foam Test)

A 2 mL solution of each tested leaf extract was poured into 10 mL of water (distilled) and later agitated thoroughly. Saponins occurrence was shown by the stable fourth appearance [28].

2.3.9. Terpenoids (Salkowski's Test)

A homogenous mixture of the tested leaf extracts and chloroform was shaken thoroughly. Then, 2 mL of concentrated H_2SO_4 was poured through the sides of the test tube

wall. Further acid treatment resulted in the formation of a ring (reddish-brown) at interface, indicating terpenoids positivity [30].

2.3.10. Anthraquinone Glycosides (Borntrager's Test)

The benzene solution was mixed with tested leaf extracts and then shaken thoroughly, followed by separation of the benzene layer. Ammonia (10%) was added to the remaining solution (in 1/2 volume of the benzene layer taken out). The appearance of a specific color (pink, red, or violet) at the ammoniacal phase indicated anthraquinone glycosides positivity [26].

2.3.11. Tannins

The tested leaf extracts were mixed in 10 mL of distilled water and filtered. Then, drops of $FeCl_3$—5% were added. Appearance of black or blue-green color or precipitation indicated the occurrence of tannins [30].

2.3.12. Alkaloids

Each tested leaf extract—2 mL was reacted with 1.5% v/v HCl and then filtered. Mayer's reagent was added to these filtrates. The observation of yellow cream precipitation indicated alkaloids occurrence [29].

2.3.13. Betacyanins

The tested leaf extracts were treated with NaOH-2 N, followed by 5 min heating at 100 °C. Upon examination, a yellow color specified the presence of betacyanins [31].

2.3.14. Quinone's

The same concentration of the tested leaf extracts was mixed with concentrated H_2SO_4. The appearance of color (red) characterizes for quinine's positivity [32].

2.4. Functional Group Determination Using the FTIR Technique and Element Characterization Using the EDX Technique

The methanolic extract from the leaf of *C. simplicifolia* were estimated for functional group determination with an FTIR spectrophotometer (NICOLET 67000, Thermo Fisher Scientific, Waltham, MA, USA). The methanolic leaf extract was processed under vacuum pressure to retain the moisture-free extract. The thin discs of the extract were made by grinding the extract with potassium bromide crystals and suppressing them between the guides using a vacuum pump. The spectrum of the functional groups in the extract was drawn at the 400 cm^{-1} and 4000 cm^{-1} transmittance modes [33].

Energy-dispersive X-ray spectroscopy (EDX) (JSM-IT 500LA, Kyoto, Japan) was used to investigate the elemental constitution of the leaf powder. For the analysis, leaf powder was kept on a stub wrapped with carbon tape and covered. Later, the filled stub was kept under the chamber to conduct subsequent analysis. The analysis was carried out by applying the accelerating voltage of 20.00 kV at a magnification of ×250 with the high vacuum mode. Finally, the spectrum was recorded for micro and macro elements in mass % [34].

2.5. Phytochemical Characterization Using GCMS of the Methanolic Extract of C. simplicifolia Leaf

The volatile metabolites of the methanolic leaf extract were characterized using the GCMS technique (GC-Agilent 8890 coupled with MS-Agilent 5977B detector, Palo Alto, CA, USA). The fused silica capillary column (dimension 30 m × 250 µm × 0.25 µm) was prepared to segregate compounds. One µL sample was introduced at the injector (temperature—250 °C) in a split mode (split ratio 15:1), while carrier gas (helium) flowed in the concentration of 3 mL/min. Temperature monitoring was initially—75 °C retained for 0.5 min; then gradually, the temperature was raised at 5 °C/min up to 300 °C, retained for up to 20 min. Further, the mass spectrometer detector (MSD) transfer line temperature

was assigned to 280 °C. MSD was performed with the electron ionization mode (ionization energy—70 eV and ion source temperature—230 °C) for mass spectra determination at 50 m/z mass range by keeping the MS quadrupole temperature at 150 °C. A spectrum was recorded to determine the compounds in the methanolic leaf extract by name, molecular weight, and structure. The compound identification was sourced by comparison of the extract mass spectra to the automated library search of known compounds generated using the NIST MS Search program (version 2.3 accessed on 13 April 2023) [35].

2.6. Estimation of In Vitro Antiradical Capacity of C. simplicifolia Methanolic Leaf Extract

An antioxidant assay was performed using 2,2-diphenyl-1-picrylhydrazyl (DPPH) using the method described by Yadav et al. [36], including minor variations. In total, 3.0 mL of DPPH methanolic reagent (0.1 mM) was added to the methanolic leaf extract in different concentrations of 20, 40, 60, 80, and 100 µg/mL, and the final volume was brought to 4 mL. Then, the test tubes were incubated up to 1800 s, and readings were noted at 517 nm. Gallic acid was used as a standard. The DPPH inhibition activity of the tested extract was presented as a percentage of radical scavenging activity, which was measured using the following formula [37].

$$\% \text{ Radical scavenging activity} = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

2.7. Experiment Animals

Wister albino rats were procured from a central animal facility at H.S.K. College of Pharmacy and Research Centre, Bagalkot. Controlled environmental conditions were provided to the Wister albino rats, including a 22–28 °C temperature and a 65 ± 10% relative humidity with a 12 h light and dark cycle. Observations were made following Institutional Animal Ethics Committee (IAEC) guidelines, and the rats were given laboratory-grade food (Amruth, Sangli, Maharashtra, India) with steady water accession. Ethical approval, with reference number HSKCP/IAEC, Clear/1/2022-23/R&D/KUD 02, was sourced from the Institutional Animal Ethics Committee (IAEC) of Hangal Shri Kumareshwar College of Pharmacy, Bagalkot-587101, Karnataka, India.

2.8. Acute Toxicity of the Methanolic Extract of C. simplicifolia Leaves

Acute toxicity of the *C. simplicifolia* leaf extract (methanolic) was determined by considering the Organization for Economic Co-operation and Development (OECD) guidelines 425 [38]. A fixed single dose was used to assess toxicity via oral administration of the methanolic extract of *C. simplicifolia* leaves prepared by dissolving in distilled water, which was given to male Swiss albino mice. Fixed single doses of 72, 78, and 82 mg/kg b.wt. (36, 39, and 41 g of body weight) were given to each Swiss albino mouse. After the treatment, the mice were monitored for any clinical toxicological signs within 4 h and up to 24 h. After that, the observation was continued for toxic manifestations (salivation, draping, and modulations concerning skin, fur, eyes, and mucous membranes; special attention was given to tremors, convulsions, diarrhea, lethargy, sleep, coma, and mortality) for up to 14 days. The dose screening of methanolic leaf extract was used for dose determination in the hepatoprotective activity evaluation.

2.9. In Vivo Hepatoprotective Activity against Paracetamol-Intoxicated Wister Albino Rats

The experiment was designed to include five groups of Wister albino rats, 4 in each group. The group-1 (normal) rats received 10 mL/kg b.wt. normal saline solution (vehicle), and the group-2 (control) rats were administered *N*-acetyl-*p*-aminophenol (acetaminophen), generally called paracetamol (PC), while the group-3 (standard) rats were treated with silymarin 20 mg/kg b.wt. The group-4 (low dose) rats were given 50 mg/kg b.wt. methanolic leaf extract, and group-5 (high dose) rats were given 100 mg/kg b.wt. methanolic leaf extract. The group-4 and group-5 rats were treated with paracetamol for the first seven days along with the methanolic extract of *C. simplicifolia* leaves, and after seven days, the

rats in these groups were treated only with the methanolic extract of *C. simplicifolia* leaves. The dose administered to the rats in each group was given orally for 12 days. The rats in each group were humanly sacrificed after the last dose administration (after 24 h) for 12 days. The serum blood was collected, and the liver was excised from the rats' bodies, which was further used to determine serum blood enzymes and in vivo antioxidant activity in the liver.

2.9.1. Serum Blood Biochemical Markers Estimation

The serum blood was retrieved via tail vein from rats in each group after 1440 min of the final dosage. After that, the serum was segregated from the blood by centrifuging the samples for 600 s at 3000 rpm. The collected serum was estimated for the assessment of aspartate transaminase/serum glutamic oxaloacetic transaminase (AST/SGOT), alanine transaminase (ALT), alkaline phosphatase/serum glutamic pyruvic transaminase (ALP/SGPT), total bilirubin, and cholesterol concentrations using standard procedures [39] and standard Biochemical diagnostic kits BIO-LA-TEST (Erbamannheim, Transasia Bio-Medicals Ltd. Bagalkot, Karnataka, India).

Measurement of Aspartate Transaminase

AST concentration determination was performed using the procedure of Iqbal et al. [39] with minor modifications. The fresh working reagent was obtained with the combination of Reagent 1 and Reagent 2 (4:1). Distilled water was used as a blank to form a baseline in the spectrometric readings. In the analysis of the test serum sample, the serum (50 µL) was treated with a working reagent (500 µL) at a 37 °C temperature. The NADH oxidation rate was recorded at 340 nm using an auto-analyzer (CPC Stat Fax 3000 Plus, Shimadzu, Kyoto, Japan) by recording a decrease in absorbances. Finally, the level of AST was measured in terms of units per liter of blood serum (U/L).

Measurement of Alanine Transaminase

An ALT level was measured using the reported method of Iqbal et al. [39] with certain modifications. In the experiment, preparing the working reagent involved mixing Reagent 1 with Reagent 2. Distilled water served as the blank. Then, the test serum was analyzed by reacting 0.05 mL of the test serum with 0.5 mL of a working reagent at a 37 °C temperature. The decrease in absorbances was noted at 340 nm using an auto-analyzer (CPC Stat Fax 3000 Plus, Shimadzu, Kyoto, Japan) to measure the NADH oxidation rate. The level of ALT was measured in U/L.

Measurement of Alkaline Phosphatase

An ALP concentration assessment was performed using the Tietz et al. [40] protocol, including a few variations. During analysis, test serum (20 µL) was mixed with 1 mL of ALP reagent (Reagent 1). The yellow color that formed was kinetically measured, which caused changes in absorbance, recorded at 405 nm with an auto-analyzer (CPC Stat Fax 3000 Plus, Shimadzu, Kyoto, Japan). Changes in absorbances are proportional to the ALP level of the test serum. ALP amount was expressed in U/L.

Measurement of Total Bilirubin

The total bilirubin level estimation was performed following the Diazo method [41]. During the experiment, a fresh working reagent was made by mixing Reagent 1 (total bilirubin reagent) and Reagent 2 (sodium nitrite reagent). For the blank, distilled water (25 µL) was combined with a working reagent (500 µL). Similarly, the test serum (25 µL) was reacted with a working reagent (500 µL) in the test serum sample analysis. Then, the solutions were homogenized well, followed by 5 min of incubation (37 °C). An auto analyzer (CPC Stat Fax 3000 Plus, Shimadzu, Kyoto, Japan) was used to record the absorbances at 546 nm. Subsequently, the concentration of total bilirubin was expressed as a milligram per deciliter of blood serum (mg/dL).

Measurement of Cholesterol

Cholesterol estimation was carried out using the dictated experiment of Iqbal et al. [39] with minor variations. The polyethylene glycol-cholesterol oxidase-peroxidase-4-aminoantipyrine procedure and lipid clearing factor were implemented for end-point determination. The analysis included the preparation of a standard reagent (solution of 0.1 mL Reagent 1 and 0.01 mL Reagent 2), which contained cholesterol, preservatives, and stabilizers. The blank reference selected was distilled water. The serum sample (10 µL) of rats was reacted with the methanolic extract of *C. simplicifolia* leaf combined with an enzyme reagent (1000 µL) followed by a 10 min incubation at 37 °C. Absorbances of the sample were noted for 505 nm with an auto-analyzer (CPC Stat Fax 3000 Plus, Shimadzu, Kyoto, Japan). Cholesterol was measured as mg/dL.

2.9.2. Assessment of In Vivo Antiradical Potential of Liver Hepatic Tissues

Assessment of liver hepatic tissues for in vivo antioxidant level was performed by following Shaikh et al.'s [42] method. Before liver excision, the serum blood samples were collected via tail vein using sterile centrifuge tubes containing heparin from rats in the individual groups. Then, the rats in all groups were humanly sacrificed to excise the liver. The excised livers were made free of blood traces by rising with ice-cold normal saline. Later, the weight of the livers was noted, followed by homogenization of individual liver tissues in 0.1 M cold phosphate buffer (pH 7.4) to obtain liver hepatic tissue homogenate. Subsequently, liver tissue homogenates post-mitochondrial supernatant (PMS) were obtained with the application of centrifugal force at 4 °C maintained up to 10 min at 10,000 rpm. The tissue homogenate was re-forced with centrifugal force for an hour at 17,000 rpm. Finally, liver tissue homogenates were taken for assessment using the liver function test, which was achieved by determining the reduced glutathione (GSH), catalase (CAT), superoxide dismutase (SOD), antioxidant, and lipid peroxidation (LPO) levels.

Measurement of Glutathione

The reduced glutathione analysis was completed with the use of the Islam et al. [43] procedure, including minor changes. In this experiment, homogenized liver tissues (1 mL) were reacted with the 6 mL phosphate buffer solution—PBS (potassium hydrogen phosphate—9400 µL and potassium di-hydrogen phosphate—3000 µL were mixed) at a pH of 8 and 1000 µL of 0.6 mM 5,5′-dithiobis (2-nitrobenzoic acid)-DTNB (2.38 mg DTNB was dissolved in 10,000 µL methanol), followed by further incubation for 600 s at room temperature. The absorbances were taken at 412 nm. Glutathione content was measured by plotting the graph of standard, which was examined with the application of a similar experimental procedure and expressed as the number of moles per milligram of protein (nmol/mg of protein).

Measurement of Catalase

Catalase concentration estimation was obtained using the Claiborne method [44] with a few alterations. During the analysis, 50 units of homogenized liver tissue were treated with 1950 µL of phosphate-buffered saline (PBS—50 mM) at a pH of 7 and 1 mL of 0.7 mM hydrogen peroxide—H_2O_2 (0.160 mL of H_2O_2 added to 100 mL PBS). The record absorbances were noted at 240 nm for 0 min and 1 min, which were measured as nmol/mg of protein.

Measurement of Superoxide Dismutase

The superoxide dismutase level was assessed using the Natikar et al. [45] method, with some alterations. A solution of sodium carbonate buffer, hydrochloric acid (0.5), and epinephrine was prepared for the analysis. To the homogenized liver tissue (0.1 mL), the sodium carbonate buffer (0.8 g) with epinephrine solution was added and taken into the cuvette. The second cuvettes with sodium carbonate buffer (0.8 g) and homogenized liver tissue (0.1 mL) were combined and analyzed at 295 nm with an auto-analyzer (CPC Stat

Fax 3000 Plus, Shimadzu, Kyoto, Japan) for 0 min and 1 min and defined as nmol/mg of protein.

Measurement of Lipid Peroxidation

With some modifications, LPO activity was measured using the method of Shaikh et al. [42]. The homogenized liver tissue was assessed to identify the concentration of thiobarbituric acid. During the experiment, 10% tissue homogenate (0.5 mL) was treated with 15% trichloroacetic acid—TCA (0.3 mL) and 0.375% thiobarbituric acid—TBA (0.3 mL) along with 5 N HCl (0.03 mL) followed by a 15 min incubation at 95 °C in a hot water bath. Later, the solutions were brought to normal temperature and subjected to a centrifugal force of 2000 rpm. Thereafter, the resultant separated solution was taken for analysis, which was read for absorbance at 535 nm in contrast to the suitable blank. The lipid peroxidation reaction was calculated for nmol/mg of protein.

2.10. Statistical Analysis

Each statistically measured value is given as mean ± SE and SEM, determined with the application of One-Way Analysis of Variance (ANOVA) with Duncan's multiple range test (IBM SPSS Statistics software version 20) and multiple Dunnett's Test (GraphPad Prism 10). Significant values with $p < 0.05$ indicated differences in variance among the group of treated rats in comparison with the normal group rats.

3. Results

3.1. Extract Concentration of C. simplicifolia Leaves

This analysis was performed to assess the *C. simplicifolia* (Figure 1) leaf extraction yield of solvents, namely, acetone, methanol, and distilled water (Table 1). The percent quantities of each extract obtained from the leaf were acetone—3.49, methanol—14.76, and water—10.02 g/100 g, correspondingly.



Figure 1. *C. simplicifolia*: (**A**) habit of the plant and (**B**) flower of the plant.

Table 1. Extract yield from *C. simplicifolia* leaves.

Name of Solvent	Extract Yield g/100 g
	***C. simplicifolia* Leaves**
Acetone	3.49 ± 0.004^{c}
Methanol	14.76 ± 0.06^{a}
Water	10.02 ± 0.12^{b}

Values are mean ± standard error (n = 3); a significant difference ($p < 0.05$) is displayed using alphabetic letters in superscript.

3.2. Qualitative Assessment of Phytochemicals in C. simplicifolia Leaf Extracts

The *C. simplicifolia* acetone, methanol, and distilled water leaf extracts were qualitatively screened to determine the occurrence of active phytochemicals, like primary and secondary metabolites, by following standard protocols. The outcome of the present investigation of *C. simplicifolia* leaves depicted the important class of phyto-compounds, namely, carbohydrates, proteins, amino acids, glycosides, cardiac glycosides, phenolics, flavonoids, saponins, terpenoids, anthraquinones glycosides, betacyanins, and quinones, that is useful for solvent selection to conduct further research studies (Table 2). Meanwhile, the acetone extract showed positivity toward carbohydrates, proteins, flavonoids, anthraquinones, and glycosides. In comparison, the methanol extract exhibited the presence of carbohydrates, protein, amino acids, glycosides, cardiac glycosides, phenolics, flavonoids, saponins, terpenoids, anthraquinones glycosides, and quinones. Similarly, the distilled water extract revealed the appearance of protein, amino acids, cardiac glycoside, phenolic, flavonoids, saponins, anthraquinones glycosides, betacyanins, and quinines, respectively.

Table 2. Qualitative screening of phytochemical in extracts of *C. simplicifolia* leaves.

Molecule Name	Test Type	Leaves		
		Acetone	Methanol	Water
Phenolics	Ferric chloride test	−	+	+
Amino acids	Ninhydrin Test	−	+	+
Cardiac glycosides	Keller–Killiani test	+	+	+
Anthraquinones glycosides	Borntrager's Test	+	+	+
Proteins	Biuret Test	+	+	+
Glycosides	Salkowski's test	−	+	−
Flavonoids	Alkaline reagent test	+	+	+
Carbohydrates	Fehling's Test	+	+	−
Terpenoids	Salkowski's test	−	+	−
Alkaloids	Mayer's Test	−	−	+
	Wagner's Test	−	+	+
Quinones		−	+	+
Saponins	Froth Test	−	+	+
Betacyanins	Sodium hydroxide test	−	−	+
Tannins	Ferric chloride test	−	+	+

Table symbols indicate "+" positive for the test, and "−" negative for the test.

3.3. Functional Group Determination Using the FTIR Technique and Element Characterization Using the EDX Technique

The *C. simplicifolia* leaves (methanolic extract) were assessed using FTIR to determine the active functional groups (Figure 2 and Table 3) using a NICOLET 67000 FTIR spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The strong, broad, and medium absorption peaks were obtained between the 400 cm^{-1} and 4000 cm^{-1} IR region. The result of the current analysis displayed peak values at 3367.14, 2920.79, 2850.32, 1631.04, 1384.59, 1168.64, 1063.78, 824.78, and 615.25 cm^{-1}, respectively. O-H stretching determining alcohol and carboxylic acid groups of strong and broad peaks at 3367.14 cm^{-1} and 2920.79 cm^{-1} were recorded. The peak value at 2850.32 cm^{-1} (strong), indicating the occurrence of N-H stretching for amine salts, was observed, and strong and medium peaks at 1631.04 cm^{-1} and 1384.59 cm^{-1} displayed C=C and S=O stretching for conjugated alkenes and sulfate groups. Furthermore, the tertiary alcohols and amine groups stretched with C-O and C-N bands were affirmed at strong—1168.64 cm^{-1} and medium—1063.78 cm^{-1} peak sizes. In addition, the intense peak ranging from 824.78 cm^{-1} to 615.25 cm^{-1} confirmed halo compounds with C-Cl and C-Br bending.

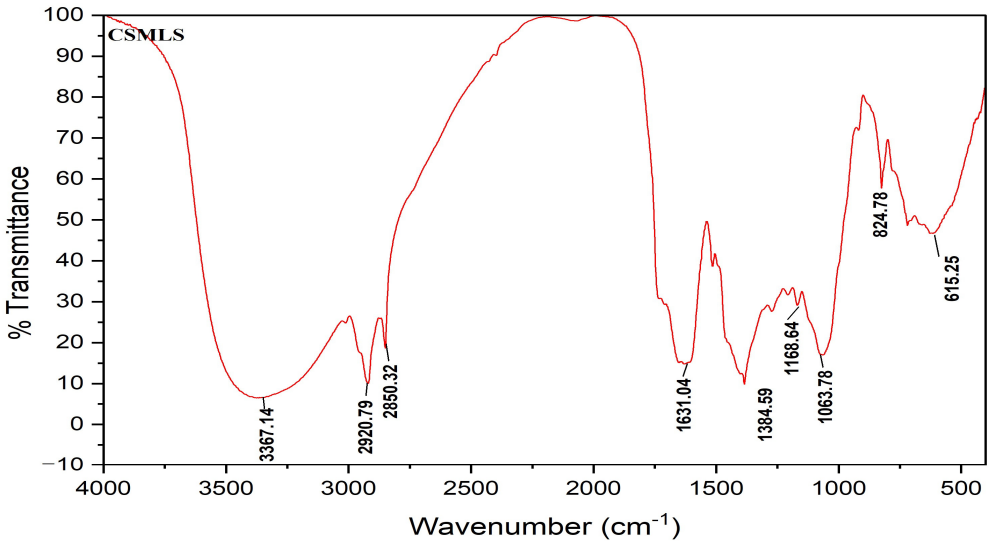

Figure 2. FTIR chromatogram showing wavenumber, indicating the occurrence of functional compound moiety in the methanol extract of *C. simplicifolia* leaves.

Table 3. Functional group characterization with FTIR and wavenumber interpretation of methanolic leaf extract of *C. simplicifolia*.

Wavenumber (cm^{-1})	Chemical Bond Determining Functional Group
3367.14	O-H stretching for alcohols
2920.79	O-H stretching for carboxylic acid
2850.32	N-H stretching for amine salts
1631.04	C=C stretching for conjugated alkene
1384.59	S=O stretching for sulfate
1168.64	C-O stretching for tertiary alcohol
1063.78	C-N stretching for amines
824.78	C-Cl stretching for halo compounds
615.25	C-Br stretching for halo compounds

EDX spectroscopy was used to characterize the elemental constituents of *C. simplicifolia* leaves. This study attempted to obtain the mass % composition that exhibited the peak intensities for major and minor elements (Figure 3 and Table 4). The various active elements with their mass percent were identified, and those were carbon—33.17 ± 0.11%, oxygen—42.57 ± 0.27%, magnesium—4.46 ± 0.05%, aluminum—0.21 ± 0.02%, silicon—0.82 ± 0.02%, phosphorus—1.03 ± 0.02%, sulfur—2.01 ± 0.03%, chlorine—1.12 ± 0.02%, potassium—2.59 ± 0.04%, and calcium—12.02 ± 0.08%.

Table 4. Element composition of *C. simplicifolia* leaves.

Sl.No	Elements	Mass %
1	C	33.17 ± 0.11
2	O	42.57 ± 0.27
3	Mg	4.46 ± 0.05
4	Al	0.21 ± 0.02
5	Si	0.82 ± 0.02
6	P	1.03 ± 0.02
7	S	2.01 ± 0.03
8	Cl	1.12 ± 0.02
9	K	2.59 ± 0.04
10	Ca	12.02 ± 0.08
Total		100.00

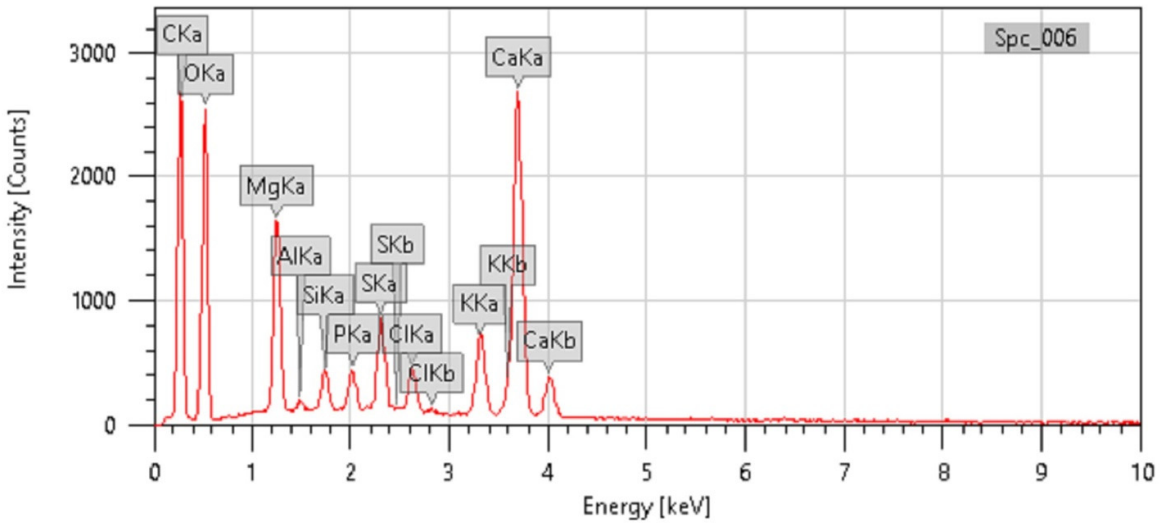

Figure 3. EDX chromatogram of *C. simplicifolia* leaves indicating elemental peaks.

3.4. Phytochemical Characterization Using GCMS of the Methanolic Leaf extract of C. simplicifolia

The present study reports on the volatile functional phytochemicals in the methanolic extract of *C. simplicifolia* leaves using the GCMS technique. The NIST library mass spectrum confirmed the compound identification. The analysis documented 10 different phytochemicals in the methanolic leaf extract for major and minor composition, which was characterized by its retention time, peak area, area %, metabolite name, and molecular formula and weight (Figure 4 and Table 5). The major phyto-compounds detected with the GCMS analysis were *n*-Heptyl acrylate—18.87%, undecane—17.49%, 2-Propenoic acid, 3-(3,4-dimethoxyphenyl)-, (E)—11.40%, Neophytadiene—11.02%, *n*-Hexadecanoic acid—10.78%, and Glafenin—10.09% respectively. Consequently, some are found in minor quantity, and they are Decane—7.45%, Phytol—6.0%, Benzene, (1-methyldodecyl)—3.48%, and 4-Cyclohexyl-1-butanol—3.41% respectively. The depicted data of the GCMS analysis implies the leaves' importance in terms of phytochemical aspects and as well as biological properties.

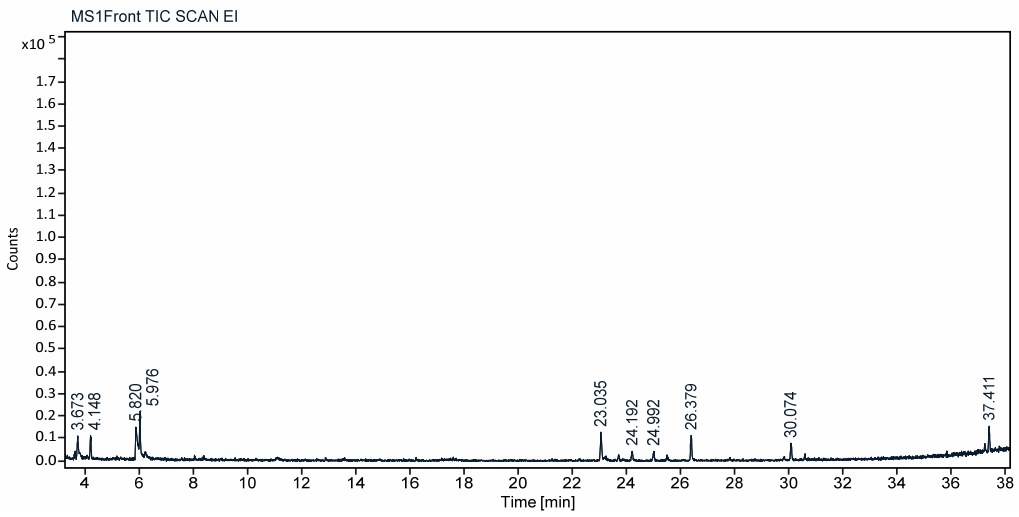

Figure 4. GCMS graph displaying peak values for volatile phyto-compounds of the methanolic leaf extract of *C. simplicifolia*.

Table 5. Phytochemical concentration of *C. simplicifolia* methanolic leaf extract screened with GCMS analysis.

Retention Time	Area	Compound Name	Area %	Molecular Formula	Molecular Weight
3.673	44,283.333	2-Propenoic acid, 3-(3,4-dimethoxyphenyl)-, (E)-	11.41	$C_{11}H_{12}O_4$	208.21 g/mol
4.148	28,934.119	Decane	7.45	$C_{10}H_{22}$	142.28 g/mol
5.820	73,312.043	*n*-Heptyl acrylate	18.87	$C_{10}H_{18}O_2$	170.25 g/mol
5.976	67,941.218	Undecane	17.49	$C_{11}H_{24}$	156.31 g/mol
23.035	42,791.077	Neophytadiene	11.02	$C_{20}H_{38}$	278.5 g/mol
24.192	13,260.050	4-Cyclohexyl-1-butanol	3.41	$C_{10}H_{20}O$	156.26 g/mol
24.992	13,523.040	Benzene, (1-methyldodecyl)-	3.48	$C_{19}H_{32}$	260.5 g/mol
26.379	41,868.951	*n*-Hexadecanoic acid	10.78	$C_{16}H_{32}O_2$	256.42 g/mol
30.074	23,301.159	Phytol	6.0	$C_{20}H_{40}O$	296.5 g/mol
37.411	39,202.810	Glafenin	10.09	$C_{19}H_{17}ClN_2O_4$	372.8 g/mol
Total %			100.00		

3.5. Estimation of In Vitro Antiradical Activity

The present work depicted the antiradical capacity, performed using 2,2-diphenyl-1-picrylhydrazyl (DPPH) of the *C. simplicifolia* methanolic leaf extract (Table 6). The DPPH is an unstable free radical in which hydrogen atoms donated by stable antioxidants can stabilize. The DPPH inhibition concentration of the methanolic leaf extract was calculated against gallic acid at 517 nm. The analysis found that the methanolic leaf extract showed strong DPPH inhibition in a concentration-dependent manner. Eventually, the methanolic leaf extract at concentrations of 20, 40, 60, 80, and 100 µg/mL displayed significant DPPH inhibition of 46.18%, 53.83%, 66.64%, 74.03%, and 85.05% respectively.

Table 6. In vitro antioxidant capacity of the methanolic leaf extract of *C. simplicifolia* against the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicle.

Concentration µg/mL	DPPH Inhibition Percent	
	Gallic Acid %	CSMLS %
20	60.02 ± 0.25^{g}	46.18 ± 0.61^{i}
40	70.91 ± 0.34^{e}	53.83 ± 0.31^{h}
60	75.71 ± 0.27^{c}	66.64 ± 0.30^{f}
80	81.89 ± 0.37^{b}	74.03 ± 0.53^{d}
100	86.06 ± 0.27^{a}	85.05 ± 0.48^{a}

Values are mean ± standard error (n = 3). Different superscript letters show significant differences ($p < 0.05$); the same superscript letters show no significant difference ($p > 0.05$) between each value.

3.6. Acute Toxicity of the Methanolic Leaf Extract of C. simplicifolia

The methanolic extract of *C. simplicifolia* leaves was assessed for acute toxicity via oral administration of the extract (dose of 72 mg, 78 mg, and 82 mg of the body weight) following guideline 425 of the Organization for Economic Co-operation and Development (OECD), in Swiss albino mice. The observation made for the toxicological effect of the extract showed no mortality rate in the treated mice. Furthermore, visible signs of toxic effects such as salivation, rising fur, writhing, aggression, skin and eye color change, constipation, urination, food and water intake, and other behavioral symptoms were not noticed for 14 days.

3.7. In Vivo Hepatoprotective Capacity of C. simplicifolia Methanolic Leaf Extract Antagonist toward Paracetamol-Intoxicated Wister Albino Rats

3.7.1. Serum Blood Biochemical Marker Estimation

In the current study, *C. simplicifolia* methanolic leaf extract in vivo hepatoprotective activity against paracetamol intoxication (experimental animals—Wister albino rats) was

determined. Serum blood biochemical marker estimation was performed to monitor early hepatic damage and liver dysfunction by examining the range of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), total bilirubin, and cholesterol (Table 7) performed 24 h after the last dose. This study showed that group-1 rats displayed a significant range of serum blood AST (116.7 ± 1.45 U/L), ALT (41.53 ± 6.26 U/L), ALP (195.0 ± 7.55 U/L), total bilirubin (0.15 ± 0.02 mg/dL), and cholesterol (94.00 ± 2.60 mg/dL) levels, respectively. Whereas, the PC-treated group-2 rats showed insignificant increased concentrations of blood plasma AST (134.5 ± 0.50 U/L), ALT (164.9 ± 23.13 U/L), ALP (434.7 ± 27.70 U/L), total bilirubin (1.36 ± 0.03 mg/dL), and cholesterol (257.4 ± 0.45 mg/dL) compared with the untreated group-1 rats serum biochemical levels. In the case of group 3, a drastic decline in the serum blood values of AST (126.5 ± 0.50 U/L), ALT (112.9 ± 16.50 U/L), ALP (350.0 ± 11.59 U/L), total bilirubin (0.35 ± 0.01 mg/dL), and cholesterol (23.00 ± 1.00 mg/dL) was observed, which was significant compared with the untreated (normal group) and PC-intoxicated (control group) rats. However, the methanolic leaf extract at the low dose of 50 mg/kg b.wt. (group-4 rats) showed a significant reduction in serum blood AST (129.9 ± 0.65 U/L), ALT (133.0 ± 4.12 U/L), ALP (398.4 ± 32.16 U/L), total bilirubin (0.33 ± 0.03 mg/dL), and cholesterol (57.87 ± 2.76 mg/dL) when compared with the normal group rats and control group rats. Similarly, the high dose of 100 mg/kg b.wt. methanolic leaf extract (group-5 rats) exhibited a reduction in the level of AST (130.5 ± 0.50 U/L), ALT (106 ± 7.67 U/L), ALP (334.3 ± 11.85 U/L), total bilirubin (0.36 ± 0.03 mg/dL), and cholesterol (46.25 ± 2.76 mg/dL) serum blood biochemical markers compared with the normal and control rats.

Table 7. *C. simplicifolia* leaf efficacy for serum blood biochemical markers.

Group	Administration	AST U/L	ALT U/L	ALP U/L	Bilirubin mg/dL	Cholesterol mg/dL
1	Normal—vehicle	116.7 ± 1.45	41.53 ± 6.26	195.0 ± 7.55	0.15 ± 0.02	94.00 ± 2.60
2	Control—paracetamol (PC)	134.5 ± 0.50 ****	164.9 ± 23.13 ****	434.7 ± 27.70 ****	1.36 ± 0.03 ****	257.4 ± 0.45 ****
3	Standard—silymarin	126.5 ± 0.50 **	112.9 ± 16.50 **	350.0 ± 11.59 **	0.35 ± 0.01 **	23.00 ± 1.00 ****
4	PC + CSMLS low dose (50 mg/kg p.o)	129.9 ± 0.65 ***	133.0 ± 4.12 **	398.4 ± 32.16 ***	0.33 ± 0.03 **	57.87 ± 2.76 ****
5	PC + CSMLS high dose (100 mg/kg p.o)	130.5 ± 0.50 ***	106 ± 7.67 *	334.3 ± 11.85 **	0.36 ± 0.03 ***	46.25 ± 2.76 ****

All table values are mean ± SEM; One-Way Analysis of Variance (ANOVA) and multiple Dunnett's *t*-tests; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$ compared with normal rats was followed. (CSMLS: *C. simplicifolia* methanolic leaf extract).

3.7.2. Assessment of In Vivo Antiradical Potential of Liver Hepatic Tissues

The efficacy of the methanolic extract of *C. simplicifolia* leaves toward in vivo hepatoprotective properties against paracetamol-damaged Wister albino rats was evaluated by measuring the concentrations of oxidative stress scavengers, namely, the glutathione (GSH), catalase (CAT), superoxide dismutase (SOD), and lipid peroxidation (LPO) levels in the liver hepatic homogenates of treated and untreated rats during the experiment at the end 24 h after the last dose (Table 8). The in vivo antioxidant potential of the hepatic enzyme and lipid peroxidation level was attributed to the scavenging free radical ability of the treated and untreated group rats. The outcome of the current assay explored the significant elevation of hepatic antioxidants and reduction in lipid peroxidation. Normal group-1 rats delivered relevant concentrations of GSH (33.72 ± 1.74 nmol/mg of protein), CAT (7.35 ± 0.54 nmol/mg of protein), SOD (204.0 ± 5.38 nmol/mg of protein), and lipid peroxidation (33.63 ± 8.63 nmol/mg of protein), respectively. On the contrary, the PC-treated rats showed an insignificant decrease in GSH (21.63 ± 3.15 nmol/mg of protein), CAT (13.90 ± 1.15 nmol/mg of protein), and SOD (140.1 ± 4.56 nmol/mg of protein) and a dramatic rise in the lipid peroxidation (272.7 ± 2.45 nmol/mg of protein) reaction compared with the normal group rats. Silymarine-treated rats, compared with the normal and control rats, displayed a high range of GSH (43.80 ± 1.39 nmol/mg of pro-

tein), CAT (16.04 ± 1.71 nmol/mg of protein), and SOD (246.6 ± 9.78 nmol/mg of protein) and a decrease in lipid peroxidation (69.33 ± 5.08 nmol/mg of protein). Subsequently, in comparison with the normal rats' antioxidant capacity, the methanolic leaf extract at a low dose expressed a significant increase in the level of GSH (24.78 ± 1.31 nmol/mg of protein), CAT (20.50 ± 1.13 nmol/mg of protein), and SOD (135.2 ± 10.65 nmol/mg of protein) with a reduction in lipid peroxidation (107.7 ± 4.85 nmol/mg of protein). Similarly, a high dose of methanolic leaf extract showed elevated concentrations of GSH (41.42 ± 0.58 nmol/mg of protein), CAT (26.87 ± 1.01 nmol/mg of protein), and SOD (254.8 ± 22.12 nmol/mg of protein) and a reduction in lipid peroxidation (71.82 ± 6.44 nmol/mg of protein) in contrast to normal rats.

Table 8. *C. simplicifolia* leaf efficacy for in vivo antioxidants and lipid peroxidation.

Group	Administration	GSH nmol/mg of Protein	CAT nmol/mg of Protein	SOD nmol/mg of Protein	LPO nmol/mg of Protein
1	Normal—vehicle	33.72 ± 1.74	7.35 ± 0.54	204.0 ± 5.38	33.63 ± 8.63
2	Control—paracetamol (PC)	21.63 ± 3.15 **	13.90 ± 1.15 **	140.1 ± 4.56 **	272.7 ± 2.45 ****
3	Standard—silymarin	43.80 ± 1.39 *	16.04 ± 1.71 ***	246.6 ± 9.78 *	69.33 ± 5.08 *
4	PC + CSMLS low dose (50 mg/kg p.o)	24.78 ± 1.31 *	20.50 ± 1.13 ****	135.2 ± 10.65 **	107.7 ± 4.85 ***
5	PC + CSMLS high dose (100 mg/kg p.o)	41.42 ± 0.58 *	26.87 ± 1.01 ****	254.8 ± 22.12 *	71.82 ± 6.44 *

All table values are mean ± SEM; One-Way Analysis of Variance (ANOVA) with multiple Dunnett's *t*-tests; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$ compared with normal rats was followed. (CSMLS: *C. simplicifolia* methanolic leaves extract).

4. Discussion

Humans and animals are lavished with nature's gift of massive therapeutic knowledge with a broad spectrum of medicinal plants [46]. Since the 1980s, the World Health Organization (WHO) has recognized the contribution of traditional medicines and practices in the management of various diseases [47]. It was reported that more than 5000 plant species out of 422,000 were utilized medicinally, as medicinal herbs have antioxidant properties that make them less toxic than pharmaceuticals. Antioxidants are secondary metabolites, which are phytochemicals that act as scavengers of free radicles and reactive oxygen species and help manage disease. Phytochemicals are commonly called chemicals of plants, which are not nutritive but are synthesized by plants. Upon consumption, extraction, and isolation, these phytochemicals become crucial and confer health benefits to well-being [48,49].

A plant's phytochemicals can be extracted using different solvents with various techniques, such as the Soxhlet apparatus, which is a temperature-dependent technique that allows for the extraction of crude compounds using organic solvents. In the current study, *C. simplicifolia* leaves were extracted with acetone, methanol, and distilled water using the Soxhlet apparatus, which showed that the methanol solvent had a higher extract yield of 14.76 ± 0.06 g/100 g than the water (10.02 ± 0.12 g/100 g) and acetone (3.49 ± 0.004 g/100 g) solvents. The study reported by Borges et al. [50] provided clear discrimination about the different techniques for extraction, such as solid–liquid, ultrasound, Soxhlet, and microwave techniques. They determined that the Soxhlet technique was the most reliable technique among the others mentioned for extraction of crude phytocompounds as it is simple and requires no filtration for the extracts. On the other hand, Rao et al. [51] specified the reason for obtaining high extract yield in methanol by justifying that the polar solvents enhance the cell wall permeability of chemical compounds, which allows for good contraction between the solvent and solute that triggers high extract yield. Like other polar solvents, methanol is also a highly polar solvent with less viscosity and surface tension.

The qualitative screening of phytochemicals was achieved in this research. The methanolic leaf extract of *C. simplicifolia* delivered the most phytochemicals such as carbohydrates, proteins, amino acids, glycosides, cardiac glycosides, phenolics, flavonoids, saponins, terpenoids, anthraquinones glycosides, and quinones. Meanwhile, distilled water and

acetone extract were found to exhibit considerable phytochemicals. The obtained outcome of the analysis implies the biological and medicinal importance of *C. simplicifolia*. Similarly, the published reports by Mohammadi et al. [30] on *Anabasis setifera* and *Salsola tomentosa* showed the occurrence of tannins, phenols, terpenoids, saponins, and flavonoids, which are more or less proven to have anti-inflammatory, anti-diabetic, anti-malarial, anti-cancer, radical scavenging, anti-allergenic, and anti-viral pharmacological applications. This can be more related to the recently conducted research work of Jabeen et al. [52], wherein the authors carried out a comparative analysis on *Cenchrus ciliaris*, Polypogon monspeliansis, and Dicanthium annulatum for secondary metabolite detection, which included alkaloid, flavonoid, phenol, coumarin, tannin, and saponin determination, and explained the specific function of each metabolite as an oxidative stress reducer, an inflammation reducer, or an antifungal fungal agent and their involvement in improving digestion and absorbing nutrients, contrary to enhancing feed efficiency.

The current FTIR spectroscopy assessment of the methanolic leaf extract of *C. simplicifolia* revealed paramount functional groups such as O-H, N-H, C=C-, S=O, C-O, C-N, C-Cl, and C-Br, which stretched at 3367.14, 2920.79, 2850.32, 1631.04, 1384.59, 1168.64, 1063.78, 824.78, and 615.25 cm^{-1} wavelengths, respectively. The FTIR results indicated the bonds' nature and molecular structure for the phyto-compounds present in the methanolic leaf extract; this information helps predict integrant molecules in the methanolic leaf extract of *C. simplicifolia*, which would imply the therapeutic values [53]. In contrast to the present study, the earlier data of Karpagasundari and Kulothungan [54] on the FTIR analysis of *Physalis minima* leaf extract depicted the presence of phenols, aldehydes, alkanes, aromatic amines, secondary alcohols, amino acids, and halogen compounds. Another report by Singh et al. [55] on the FTIR spectroscopy of *Schefflera vinosa* methanol and chloroform leaf extracts identified various functional groups, namely, phenols, amines, aldehyde carboxylic acids, aliphatic, aromatics, and carbonyl compounds determining the functional groups as N-H, O-H, C-O, C-N, C-H, C=O, C≡C, and C-O-H vibrating at specific frequencies.

The EDX screening of *C. simplicifolia* leaves displayed the macro- and micro-elemental composition of the mass % present in the leaves. The major detected elements were oxygen, carbon, and calcium. Meanwhile, elements like magnesium, potassium, sulfur, chlorine, phosphorus, silicon, and aluminum were detectable in the trace concentration. The elucidated mineral elements in the leaves indicate the proper maintenance of plants' physiological and metabolic processes. Moreover, these elements are key characteristics responsible for setting (of seeds and fruits), transportation (of carbohydrates, calcium, and hormones), and development (of tissues, roots, flowers, and cell walls), and other activities [56]. Despite playing a characteristic position in plant growth and development, the consumption of plants enriched with essential mineral elements by humans promotes metabolic processes, meets nutritive requirements, and contributes to mankind's medicinal properties [57].

The present GCMS analysis of *C. simplicifolia* methanolic leaf extract identified ten indispensable phyto-compounds contributing to the medicinal and pharmacognosy field. The imperative major and minor compounds detected in the methanolic leaf extracts were *n*-Heptyl acrylate, undecane, 2-Propenoic acid, 3-(3,4-dimethoxyphenyl)-, (E)-, Neophytadiene, *n*-Hexadecanoic acid, Glafenin, Decane, Phytol, Benzene, (1-methyldodecyl), and 4-Cyclohexyl-1-butanol, respectively. *n*-Heptyl acrylate, also called heptyl acrylate, is an acrylic acid ester formed by polymerization, giving a soapy and fruity odor. *n*-Heptyl acrylate is used in a broad range of applications, like being used in plastics and super absorbers (making diapers) and in adhesives and coatings [58]. Undecane is a natural plant compound detected in the seed oil of *Salvia verbenaca* [59]. Choi et al. [60] reported that undecane exhibited inhibition activity against degranulation, histamine secretion, and tumor necrosis factor-alpha (TNF-α) and also has applications in curing skin inflammatory disorders, including atopic dermatitis and various allergic diseases. 2-Propenoic acid, 3-(3,4-dimethoxyphenyl)-, (E)- is a hydroxycinnamic acid. Neophytadiene is a diterpene that was proven to exert anxiolytic and anticonvulsant properties [61]. In addition, Bhardwaj et al. [62] presented the potential anti-inflammatory activity

isolated from *Turbinaria ornate* and described the antioxidant nature of Neophytadiene. *n*-Hexadecanoic acid (terpenoid) was the most commonly detected compound reviewed with versatile properties like hepatoprotective, antioxidant, anti-inflammatory, anticancer, and hypocholesterolemic activities [63,64]. Glafenin (Glafenine) is a very reactive nonnarcotic analgesic agent [65]. Decane belongs to the class of hydrocarbons with antimicrobial properties [66]. Phytol (terpenoids) is a frequently detected compound in crude extracts of plants and was reported to show antidiabetic, immunostimulatory, anti-coronary, hypocholesterolemic, antinociceptive, diuretic, fragrance, anti-inflammatory, and some other activities [25,55,64,67]. Whereas, Benzene, (1-methyldodecyl)- is a hydrocarbon compound type [68], and 4-Cyclohexyl-1-butanol was determined as a phenyl alcohol [69]. In contrast to the present GCMS data, Khlifi et al. [70] earlier characterized the constituents of leaves and stem of *Cleome amblyocarpa* that revealed major compounds like β-Caryophyllene, eugenol, Ethyl 3-methylpentanoate, 1,8-Cineole, Ethyl hexanoate, β-Pinene, Ethyl octanoate, and many more which are involved in biological process like anti-inflammatory, antioxidant, antibacterial, etc.

Antioxidants are molecules involved in controlling and protecting the cellular damage caused by forming free radicals, which was exerted due to excess oxidative stress build-up, eventually leading to chronic and degenerative diseases. Natural antioxidants obtained or consumed in the diet are sourced from medicinal plants, as plant-based products are nontoxic and have no harmful reactions in the human body. Plant polyphenols are frequently assessed and determined to possess antioxidant activities [71,72]. The present study determined the antioxidant capacity (in vitro) antagonist to DPPH free radicals for different concentrations of methanolic leaf extract of *C. simplicifolia*. The results of the analysis depicted a significant elevation in antioxidant activity by inhibiting DPPH radicals in a concentration-dependent manner in comparison with gallic acid. The lowest DPPH inhibition percent was 46.18% at the 20 µg/mL concentration, while 85.05% was the maximum DPPH inhibition concentration observed at the 100 µg/mL concentration of the methanolic extract of *C. simplicifolia* leaves. The determination of antioxidant properties by measuring the DPPH radical concentration is the usual method used during most of the studies. For example, earlier, Al-Musawi et al. [73] reported that the antioxidant capacity of the ethanolic extract of *Cordia myxa* fruit by assessing the concentration of DPPH radicals, and the highest inhibition concentration of 86.45% at a 60 µg/mL concentration was obtained. Corresponding to this, another investigation of antioxidant activity by Abebe et al. [74] using aqueous leaf extract of *Combretum microphyllum* for DPPH and other radical scavenging activity showed 74.2% of DPPH inhibition at 175 mg/mL in contrast with vitamin C.

Medicinal plants, despite having therapeutic applications, may also contain toxic substances. Hence, it is mandatory to evaluate a plant's toxicity before it is considered by society. In this study, the *C. simplicifolia* methanolic leaf extract was analyzed for its toxicity with acute toxicity of single-dose administration. The results showed that the methanolic leaf extract was nontoxic as it did not exhibit any abnormal behavioral changes, and the mortality rate depicted was zero at the limit doses of 72 mg, 78 mg, and 82 mg/kg b.wt. The same efforts were made by Ng'uni et al. [75] to evaluate the toxicity of *Galenia africana* by performing oral acute toxicity at single-dose administration of 300 and 2000 mg/kg b.wt., which was determined as nontoxic and nonlethal at the mentioned doses. Eventually, the leaves of *Pericampylus glaucus* were also tested for toxicity and showed no behavioral changes, other symptoms, or mortality at 2000 and 4000 mg/kg b.wt. [76].

The *C. simplicifolia* methanolic leaf extract in vivo hepatoprotective ability triggered by paracetamol in Wister albino rats was evaluated in the present work. An exceeded dose of paracetamol can cause the activation of highly reactive NAPQI, which causes damage to macromolecules as a result of an increased ratio of lipid peroxidation that damages the liver, indicating cellular necrosis; this accelerates overproduction of blood biochemical markers like AST, ALT, and ALP [77]. In the current study, the level of biochemical indicators was found to be high in PC-treated rats compared with the normal group rats. While the

methanolic extract of *C. simplicifolia* leaves at low (50 mg/kg b.wt.) and high (100 mg/kg b.wt.) doses manifested a potential attenuation of serum blood biochemical markers such as AST, ALT, and ALP, in addition to this, a reduction in total bilirubin and cholesterol level was also observed in comparison with the normal group rats. Injury to cellular macromolecules in the liver promotes NAPQI to alkylate and oxidize intracellular GSH and thiol groups of protein, leading to a reduction in the GSH pool and rapid elevation of LPO and liver damage [78]. In vivo antioxidant ability of the liver was investigated, and the GSH level of PC-treated rats was found to be decreased, while low (50 mg/kg b.wt.) and high (100 mg/kg b.wt.) doses of the methanolic extract of *C. simplicifolia* leaves illustrated the significant acclivity of GSH, CAT, and SOD with an indicative dropdown in LPO activity. Precisely, this finding gives remarkable evidence for *C. simplicifolia* hepatoprotective activity that can be compared to the earlier investigation of artichoke leaf extract hepatoprotective activity induced by acetaminophen, which was reported to exhibit novel action on the reduction in ALT, AST, and ALP along with malondialdehyde (MDA) and glutathione reductase reduction [79]. A study conducted by Martić et al. [80] for paracetamol-induced in vivo hepatoprotective activity of *Ceratonia siliqua* pulp flour extract enumerated a notable decrease in the level of AST, ALT, and ALP followed by suppression of the MDA level. In addition, an increase in superoxide dismutase, catalase, glutathione peroxidase, and glutathione S-transferase enzyme levels was reported. Similarly, Zakaria et al. [81] and Menon et al. [82] investigated the hepatoprotective property against paracetamol of *Dicranopteris linearis* leaf extract, and *Annona muricata* fruit pulp lyophilized powder was noteworthy.

The current investigation identified the substantial medicinal potentiality of the immense integrant compounds of *C. simplicifolia* leaves. This study provides a scientific base for *C. simplicifolia* leaf deliberation of essential active phytochemicals and identifies the novel hepatoprotective property against paracetamol. The defined prevention of paracetamol hepatotoxicity with methanolic leaf extract could be indexed to the detected volatile compounds, namely, undecane, Neophytadiene, *n*-Hexadecanoic acid, and phytol, wherein these molecules were screened to show hepatoprotective, antidiabetic, immunostimulatory, anti-coronary, hypocholesterolemic, antinociceptive, diuretic, anti-inflammatory, and some other activities.

5. Conclusions

The outcomes drawn from the current investigation add up to significant remarks in the field of pharmacognosy, medicine, and traditional systems. This study provides a thorough phytochemical integrant evaluation of *C. simplicifolia* leaves, which were assessed using different analyses and techniques. The results exhibited the occurrence of carbohydrates, proteins, amino acids, glycosides, cardiac glycosides, phenolics, flavonoids, saponins, terpenoids, anthraquinones glycosides, betacyanins, and quinones in different extracts of *C. simplicifolia* leaves. Furthermore, significant functional groups such as O-H, N-H, C=C-, S=O, C-O, C-N, C-Cl, and C-Br were identified using FTIR, and imperative minerals like oxygen carbon, calcium, magnesium, potassium, sulfur, chlorine, phosphorus, silicon, and aluminum were identified using the EDX technique. In addition to this, the active pharmacological potent phyto-compounds were estimated with GCMS analysis. Which showed the presence of n-Heptyl acrylate, undecane, 2-Propenoic acid, 3-(3,4-dimethoxyphenyl)-, (E)-, Neophytadiene, *n*-Hexadecanoic acid, Glafenin, Decane, Phytol, Benzene, (1-methyldodecyl)-, and 4-Cyclohexyl-1-butanol. Despite the chemical evaluation, *C. simplicifolia* was also assessed for biological activities, namely, in vitro antioxidant activity, in vivo acute toxicity, and paracetamol-induced hepatoprotective properties. The in vitro antioxidant activity against DPPH radical ameliorated the cogent effect by methanolic leaf extract. After that, the methanolic leaf extract was found to be safe and exert no toxic signs at the mentioned dose, and investigations drew the patent hepatoprotective activity against the paracetamol. The abbreviated in vitro antioxidant and in vivo hepatoprotective properties would result from existing compounds including undecane, Neophytadiene, *n*-Hexadecanoic acid, and phytol. The conclusion of the current study is

that the leaves of *C. simplicifolia* are the key source for the extraction of pharmacologically active compounds like undecane, Neophytadiene, *n*-Hexadecanoic acid, and phytol exerting medicinal properties like antioxidant and hepatoprotective activity against paracetamol damage. Importantly, paracetamol is a widely suggested drug for the relief of pain and fever, and thus, the presented work would help cure liver injury as the extract was shown to balance hepatic redox and stabilize the liver architecture in the treated rats. Due to the detection of vast major chemical compositions, the plant could also be considered for use in industries like plastics and super absorbers (making diapers) as well as in adhesives and coatings and as a flavoring agent. Future research work should attempt to isolate and screen the specific compounds for the mentioned applications in support of the present research work.

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