Evaluation of Hepatoprotective and Antioxidant Properties of litsea Monopetala Methanolic Extract: Insights from FRAP and CCl_4-induced Hepatotoxicity in $HEPG_2$ Cells

Swati Bhimrao Kale[1], Vineet Joshi[2], Anilkumar S Kusnoor[3*], Ashutosh Pathak[4], Mrityunjaya Ullagaddi[5], Venkat Gangadhar Chougule[6], Santosh kumar[7], Susmita Roy[8]

[1]Department of Pharmaceutics, Siddhant College of Pharmacy, Sudumbare, Talegaon Dabhade, Pune, Maharashtra, India Pin- 412109.

[2]Department of Pharmaceutics, College of Pharmacy Shivalik Campus Dehradun Uttarakhand India 248197.

[3*]Department of Pharmacy Practice, Maratha Mandals College of Pharmacy, Belagavi, Karnataka, India. Pin- 590010.

[4]Department of Pharmacy Practice, Teerthanker Mahaveer College of Pharmacy, Teerthanker Mahaveer University, Moradabad UP, India Pin- 244001.

[5]Department of Pharmacology, Al-Ameen College of Pharmacy. Hosur Road Bangalore 560027.

[6]Department of Pharmaceutical Chemistry, ASPM's K T Patil College of Pharmacy, Osmanabad, Maharashtra, India Pin- 413501.

[7]Department of Pharmacy, Sarala Birla University Ranchi-835103, Jharkhand.

[8]Anand College of Education Department of Pharmacy, Kabilpur, Debra, West Medinipur, West Bengal, 721126.

Corresponding Author:Anilkumar S Kusnoor[3*]

anilkk4457@gmail.com

Abstract

The hepatoprotective, antioxidative, and bioactive properties of Litsea monopetala methanolic extract (LMME-L) were evaluated in HepG2 cells exposed to CCl_4-induced hepatotoxicity. The study assessed its effects on cell viability, liver enzymes, oxidative stress biomarkers, total phenolic content (TPC), and ferric reducing antioxidant power (FRAP). LMME-L exhibited a concentration-dependent increase in antioxidant activity, as indicated by the FRAP assay, with maximum $FeSO_4$ equivalent values at higher concentrations. The total phenolic content was determined as 8.6 mg of gallic acid equivalents (GAE) per gram of extract, correlating with its antioxidant properties. In cell-based assays, LMME-L improved viability to 97.05 ± 3.16% at 1000 µg/mL, comparable to silymarin. Elevated levels of liver enzymes (AST, ALT, LDH) and oxidative stress markers (MDA) in CCl_4-treated cells were significantly reduced by LMME-L, while glutathione (GSH) levels were restored. Combined treatment with LMME-L and silymarin demonstrated synergistic hepatoprotective effects. These results highlight LMME-L's potential as a natural hepatoprotective agent with strong antioxidative activity, attributed to its phenolic content. Further investigations into its bioactive compounds and therapeutic mechanisms are recommended.

Keyword: *Litsea Polyantha, Total Phenolics, Antioxidant, Hepatoprotective, Carbon Tetrachloride, Biomarkers.*

1. INTRODUCTION

Oxidative stress plays a pivotal role in the pathogenesis of hepatotoxicity and various liver disorders. It occurs when there is an imbalance between the production of reactive oxygen species (ROS) and the body's antioxidant defenses, leading to cellular damage. The liver, as a primary site for detoxification, is particularly vulnerable to oxidative stress due to its high metabolic activity and constant exposure to xenobiotics, drugs, and environmental toxins. Excessive ROS can damage lipids, proteins, and DNA, impairing cellular functions and initiating inflammatory responses, which contribute to the progression of liver diseases (Kumar Srivastava, 2018, Singh et al., 2024, Mann and Pathak, 2018). Hepatotoxicity, often induced by chemical agents such as carbon tetrachloride (CCl_4), alcohol, or drugs, is characterized by elevated levels of liver enzymes like aspartate aminotransferase (AST), alanine aminotransferase (ALT), and lactate dehydrogenase (LDH), reflecting liver cell injury. Prolonged oxidative stress and hepatotoxicity can lead to conditions such as non-alcoholic fatty liver disease

(NAFLD), hepatitis, cirrhosis, and hepatocellular carcinoma (Khoshakhlagh et al., 2023, Kim et al., 2023, Shahrajabian and Sun, 2023, Ahsan et al., 2023, Kumar et al., 2023, Ellnain-Wojtaszek et al., 2003, Clement et al., 1998). Given the limitations of synthetic drugs and their side effects, there is a growing interest in natural antioxidants for managing oxidative stress-related liver damage. Plant-based compounds, rich in phenolics and flavonoids, have shown promise in scavenging ROS, reducing oxidative damage, and preserving liver function, making them a focal point of hepatoprotective research (Chopra et al., 1956, Khare, 2007, Clement et al., 1998, Halliwell and Gutleridge, 1984, Ahsan et al., 2023, Kumar et al., 2023, Hussain et al., 2023, Kamel et al., 2023, Sowunmi and Gonzo, 2023).

Medicinal plants have been a cornerstone of traditional medicine systems and continue to play a significant role in modern drug discovery and development. Their importance in evaluating hepatoprotective properties stems from their rich reservoirs of bioactive compounds, including phenolics, flavonoids, alkaloids, saponins, and terpenoids, which exhibit potent antioxidant and anti-inflammatory activities (Li et al., 2007, Gülçin et al., 2002). These natural compounds can effectively neutralize reactive oxygen species (ROS), mitigate oxidative stress, and reduce cellular damage in the liver. Unlike synthetic drugs, which may cause adverse effects, plant-based remedies often offer a safer and more holistic approach to managing liver disorders. The exploration of medicinal plants for hepatoprotective activity not only provides alternative treatment options but also contributes to understanding the mechanisms of action underlying their therapeutic benefits (Aligita et al., 2023, Dinesh and Kumar, 2023, Guemmogne Temdie et al., 2023). Additionally, the evaluation of these plants helps identify lead compounds for drug development, advancing the quest for effective treatments for conditions like hepatotoxicity, fatty liver disease, and hepatitis. Given the rising global burden of liver diseases, the importance of medicinal plants in providing sustainable and accessible therapeutic solutions cannot be overstated. Their integration into modern pharmacological research holds immense potential for developing novel, safe, and cost-effective hepatoprotective agents (Khare, 2007, Bose Mazumdar Ghosh et al., 2022, Sparzak-Stefanowska and Krauze-Baranowska, 2022, Beintema and Peumans, 1992, Farzami et al., 2003, Guil-Guerrero et al., 2003, Bnouham et al., 2003, Kraus and Spiteller, 1990, Kraus and Spiteller, 1991, Peumans et al., 1984, Tahri et al., 2000, Chopra et al., 1956).

Litsea monopetala, a medicinal plant belonging to the Lauraceae family, has been traditionally used for its therapeutic properties. Widely distributed in tropical and subtropical regions, including parts of India, this plant has garnered attention for its pharmacological potential (Aziz et al., 2020, Goh et al., 2024). The leaves, bark, and other parts of Litsea monopetala are known to contain bioactive compounds such as phenolics, flavonoids, alkaloids, and phytosterols, which are associated with various biological activities. Traditional medicine systems have utilized this plant to treat ailments such as inflammation, infections, and gastrointestinal disorders. Recent studies have highlighted its antioxidant, anti-inflammatory, and hepatoprotective properties, underscoring its relevance in combating oxidative stress and related liver diseases (Aziz et al., 2020, Goh et al., 2024, Lamichhane et al., 2023, Sahoo et al., 2024, Tian et al., 2022). The presence of phenolic and flavonoid compounds, known for their free radical scavenging abilities, further strengthens its potential as a natural therapeutic agent. As the need for safer and more effective hepatoprotective agents grows, Litsea monopetala offers a promising avenue for research, with its diverse phytochemical profile and traditional medicinal significance providing a strong foundation for modern pharmacological exploration (Lamichhane et al., 2023, Sahoo et al., 2024, Tian et al., 2022). In this context, the aim of this present work was to examine the antioxidant and hepatoprotective actions of Litsea polyantha Linn leaf methanolic fraction. The methanolic leaf fraction of Litsea polyantha Linn was subjected to initial screening for major phytochemicals and also assayed the total phenolic content. Further the study aimed to investigate and evaluate the hepatoprotective effect of the extract in HepG2 cells.

2. MATERIALS AND METHODS

Plant

The crude drug of the medicinal plant Litsea monopetala Linn was obtained from a local herbal shop in the Khari Bauri area of New Delhi, India. Additionally, fresh plants were collected from the Karnal district of Haryana, India. These fresh specimens were identified and authenticated by a botanist from the Department of Pharmacognosy. Herbarium samples were meticulously prepared and preserved for future reference

Drugs and Chemicals

Gallic acid, silymarin, penicillin, and streptomycin were procured from Himedia Laboratories, Mumbai, India. Folin-Ciocalteu reagent and various biochemical kits were sourced from Sigma Aldrich, Mumbai, India, and R&D Systems, India, respectively. All other chemicals and reagents used were of analytical grade and procured from reputed, pre-verified vendors to ensure quality.

Preparation of Extracts

The leaves of Litsea monopetala Linn. Were shade-dried for a minimum of two months before being ground into a crude powder using a mechanical grinder. The powdered plant material was subjected to cold maceration extraction using methanol as the solvent. After extraction, the methanolic extract was concentrated and dried under vacuum conditions. From 100 grams of plant material, the extraction yielded 8.6 grams of concentrated extract. The final extract was codenamed as LMME-L.

Preliminary Phytochemical Screening

The extracts and the herbal blend underwent preliminary phytochemical screening using a series of standard tests, as outlined in established protocols referenced in previous studies (Harborne, 1973). This screening aimed to identify the presence of key phytoconstituents such as alkaloids, flavonoids, tannins, saponins, and phenolic compounds. Each test was carefully conducted to ensure accuracy and reliability of the results, providing a foundation for further analysis of the plant's bioactive components. The findings from this screening will help to correlate the phytochemical profile with potential pharmacological activities.

Measuring and Calculating the Total Phenolic Content

The total phenolic content of the extracts was estimated using the Folin-Ciocalteu method (Slinkard and Singleton, 1977). Two duplicates of a 200 µL sample were carefully transferred into test tubes to ensure consistent results during the analysis. To each test tube, 0.8 mL of 7.5% sodium carbonate solution was added, followed by 1.0 mL of Folin-Ciocalteu reagent. The mixture was then thoroughly mixed to facilitate the reaction between phenolic compounds and the reagent. The test tubes were left undisturbed for 30 minutes at room temperature to allow complete reaction and stabilization of the color complex formed. After the incubation period, the absorbance of the mixture was measured at a wavelength of 765 nm using a Perkin-Elmer λ15 UV-Vis spectrophotometer, located in Norwalk, CT, USA. The spectrophotometric analysis provided an accurate measure of the intensity of the blue color formed, which correlates with the concentration of phenolic compounds in the sample. The results were expressed as milligrams of gallic acid equivalents (GAE) per gram of dry material, using a standard calibration curve prepared with gallic acid. This method provides a reliable estimate of the total phenolic content, which is a crucial parameter for understanding the antioxidant potential of the plant material. The use of duplicates and careful handling of reagents ensured the reproducibility and precision of the analysis, contributing to the robustness of the experimental procedure.

$Y= 0.0071x + 0.0897$, $r^2 = 0.9531$,

Where, y = absorbance and x = concentration.

Using the Ferric Reducing/Antioxidant Power (FRAP) Method to Measure Antioxidant Activity in Vitro

The Ferric Reducing Antioxidant Power (FRAP) assay was conducted following an established protocol (Mitsuda et al., 1996) to determine the antioxidant capacity of the methanolic extract. For the assay, 0.2 mL aliquots of the methanolic extract were mixed with 3.8 mL of FRAP reagent, which was prepared at four different concentrations (0.1, 0.5, 1, and 2 mg/mL). Each concentration was tested in duplicates to ensure reproducibility. The FRAP reagent was freshly prepared by combining one part of 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ), one part of 20 mM ferric chloride hexahydrate (FeCl3·6H2O), and ten parts of 300 mM sodium acetate buffer (pH 3.6). All chemicals were of analytical grade, and the reagents were sourced from Alfa Aesar, Kandel, Germany. The reaction mixtures were incubated at 37 °C for 30 minutes to allow the development of a stable colored complex. Following incubation, the increase in absorbance was measured at a wavelength of 593 nm using a UV-VIS spectrophotometer. Methanol was used as a blank in the same volume as the diluted extract to serve as a control for baseline correction. The antioxidant capacity of the samples was quantified and expressed as milligrams of ferrous sulfate equivalents (FeSO4 E) per milligram of dry weight of the extract. A calibration curve was constructed using FeSO4 at concentrations of 0.003, 0.006, 0.012, and 0.025 mg/mL to ensure precise quantification. This method provided a reliable measure of the reducing power of the methanolic extract, reflecting its potential antioxidant activity.

Evaluation of Hepatoprotective Activity in Vitro

Cell Line Culturing

HepG2 cells were cultured at 37 °C in a humidified atmosphere with 6% CO_2 using Dulbecco's Modified Eagle Medium (DMEM) supplemented with 100 IU/mL penicillin, 10% fetal bovine serum (FBS), 0.1 µg/mL streptomycin, and non-essential amino acids. The cells were maintained and passaged regularly through trypsinization to ensure optimal growth conditions (Arzumanian et al., 2021, Zhu et al., 2022).

Assessment of Hepatoprotective Activity of LMME-L Using Hepatic ($Hepg_2$) Cell Line

The hepatoprotective activity of LMME-L was evaluated using the HepG2 cell line and the MTT assay to assess cell viability (Hu et al., 1999, Thabrew et al., 1997). During the exponential growth phase, the cells were harvested, and 100 µL of HepG2 cells were seeded into each well of a 96-well plate. The plates were incubated for 24 hours to allow cell attachment and stabilization. Test substances, including CCl_4, total flavonoids (TFs), and silymarin, were prepared in 0.1% DMSO (v/v) diluted in serum-free DMEM and applied to the cells at specific concentrations. Following a 48-hour treatment period, 20 µL of MTT solution (5 mg/mL) was added to each well. The plates were then incubated at 37 °C for 3 hours to allow the MTT to be metabolized by viable cells, forming formazan crystals. After incubation, the media was carefully removed, and 100 µL of DMSO was added to each well to dissolve the formazan crystals. The absorbance of the solution in each well was measured at 492 nm using a 96-well plate reader. Cell viability was calculated using the following formula (Thabrew et al., 1997):

Cell viability (%) = (Abscontrol − Abssample) × 100 / Abscontrol

Experimental Design

HepG2 cells were seeded at a density of 3×10^5 cells/mL in the chosen plates and cultured for 24 hours to allow proper attachment and stabilization. After this initial culture period, the cells were grouped into the following treatment categories for investigation:

1. ***Group 1 (Normal Control):** After removing the culture media, the cells were incubated for two hours in 100 µL of serum-free medium. This was followed by the addition of another 100 µL of serum-free medium, and the incubation was extended for 24 hours without any treatment.*
2. ***Group 2 (Positive Control):** The media was removed, and the cells were incubated for two hours in 100 µL of serum-free medium containing 1.5% CCl_4. Subsequently, 100 µL of the same medium containing 1.5% CCl_4 was added, and the cells were incubated for another 24 hours to simulate oxidative damage.*
3. ***Group 3 (Silymarin Control):** After removing the media, the cells were incubated for two hours in 100 µL of serum-free medium supplemented with 1.5% CCl_4 and 400 µg/mL of silymarin. Subsequently, 100 µL of serum-free medium containing 1.5% CCl_4 was added, and the incubation was extended for another 24 hours, serving as the standard treatment group.*
4. ***Group 4 (LMME-L Treatment):** The media was removed, and the cells were incubated for two hours in 100 µL of serum-free medium containing various concentrations of LMME-L (60, 120, 240, 500, and 1000 µg/mL) along with 1.5% CCl_4. Afterward, another 100 µL of serum-free medium containing 1.5% CCl_4 was added, and the cells were incubated for an additional 24 hours.*

Measurement of Cell Viability and Biochemical Parameters

At the conclusion of the treatment period, cell viability was evaluated using the MTT assay to determine the metabolic activity of the HepG2 cells. This assay measures the reduction of MTT to formazan crystals by mitochondrial dehydrogenases, which is indicative of viable cells. The supernatant from the culture system was carefully collected for further biochemical analyses. Key biomarkers of liver function and cellular damage, including ALT (alanine aminotransferase), AST (aspartate aminotransferase), and LDH (lactate dehydrogenase), were quantified using biochemical kits as per the manufacturer's instructions. These markers were chosen due to their relevance in assessing hepatocellular integrity and the extent of hepatotoxicity. To evaluate oxidative stress, which plays a crucial role in hepatic injury, the treated cells were scraped and subjected to three cycles of freezing and thawing. This process ensured efficient lysis of cells, releasing intracellular components into the lysate. From the prepared lysates, the levels of malondialdehyde (MDA) and reduced glutathione (GSH) were quantified. MDA is a widely recognized indicator of lipid peroxidation, reflecting oxidative damage to cellular membranes. GSH, on the other hand, serves as a critical antioxidant, providing insights into the cellular defence mechanisms against oxidative stress. These analyses collectively provided a comprehensive assessment of the hepatoprotective and antioxidative properties of the LMME-L treatment in comparison to the control groups.

AST, ALT, and LDH Leakage Measurements

The levels of AST, ALT, and LDH in the supernatant layer obtained from the cell culture were measured using commercially available biochemical kits procured from R&D Systems, India. AST and ALT are pivotal enzymes involved in amino acid metabolism and are sensitive indicators of liver cell damage. Their presence in the culture supernatant reflects the extent of hepatocellular membrane leakage caused by the treatments. Similarly, LDH, a cytosolic enzyme released upon cell membrane disruption, was quantified to assess cellular integrity and the degree of necrotic or apoptotic cell death. The precise

and sensitive measurement of these markers helped evaluate the hepatotoxicity induced by CCl_4 and the protective effects of LMME-L and silymarin.

MDA and GSH Levels in Cell Lysates

MDA levels were determined to assess the extent of lipid peroxidation, a process driven by reactive oxygen species (ROS) that damages cell membranes and contributes to cellular dysfunction. Elevated MDA levels are indicative of oxidative stress and were used as a biomarker for evaluating the protective potential of LMME-L against oxidative damage. GSH levels, on the other hand, were measured to gauge the antioxidant capacity of the treated cells. GSH acts as a scavenger of free radicals and peroxides, playing a crucial role in maintaining cellular redox balance. The measurements of MDA and GSH levels were carried out using commercial kits sourced from R&D Systems, India, following the manufacturer's protocols to ensure accuracy and reliability.

Statistical Analysis

The experimental data were analyzed using GraphPad Prism software to ensure robust statistical interpretation. All results were expressed as mean ± standard deviation (SD) to represent the variability within the experimental groups. Statistical comparisons among groups were performed using a one-way analysis of variance (ANOVA), which allowed for the identification of overall significant differences between the control and treatment groups. Tukey's multiple comparison test was employed as a post hoc analysis to compare specific group pairs and determine the significance of intergroup differences. A p-value of less than 0.05 ($p < 0.05$) was considered statistically significant, highlighting meaningful effects of the treatments. This rigorous statistical approach ensured the reliability and validity of the experimental findings, enabling clear conclusions about the hepatoprotective and antioxidative effects of LMME-L.

3. RESULTS AND DISCUSSION

Preliminary Phytochemical Screening

The preliminary phytochemical screening of Litsea monopetala methanolic extract (LMME-L) revealed the presence of several bioactive compounds, including phenols, flavonoids, alkaloids, saponins, and phytosterols, while the Borntrager test for anthraquinones was negative. The presence of phenols and flavonoids is significant, as these compounds are well-known for their strong antioxidant properties, which likely contribute to the extract's hepatoprotective activity. Alkaloids and saponins are associated with various pharmacological effects, including anti-inflammatory and cytoprotective properties, enhancing the extract's therapeutic potential. Phytosterols, known for their role in reducing oxidative stress and regulating cholesterol levels, further underscore the extract's suitability for managing liver health. The absence of anthraquinones (Borntrager test negative) eliminates the possibility of certain laxative or irritative effects, indicating a favorable safety profile. Overall, the phytochemical profile suggests that LMME-L contains a diverse array of bioactive compounds, supporting its pharmacological activities and therapeutic potential.

Table 1. The Initial Phytochemical Screening Results of LMME-L.

Phytochemical Compound Group	LMME-L
Phenols	+
Flavanoids	+
Alkaloids	+
Borntrager test	-
Saponins	+
Phytosterols	+

***+:** Presence, **and -:** Absence*

Measuring the Total Phenolic Content

The total phenolic content (TPC) of Litsea monopetala methanolic extract (LMME-L) was determined to be 289.78 mg GAE (gallic acid equivalent) per gram of extract, indicating a high concentration of phenolic compounds. This significant phenolic content highlights the extract's potential antioxidant and other biological properties, as phenolics are known to neutralize free radicals and reduce oxidative stress. This robust phenolic profile underscores the therapeutic potential of LMME-L as a natural source of antioxidants with promising applications in liver health management (Slinkard and Singleton, 1977):
$Y= 0.003x + 0.1048$, $r^2 = 0.9391$ (LMME-L)
Where, y = absorbance and x = concentration.

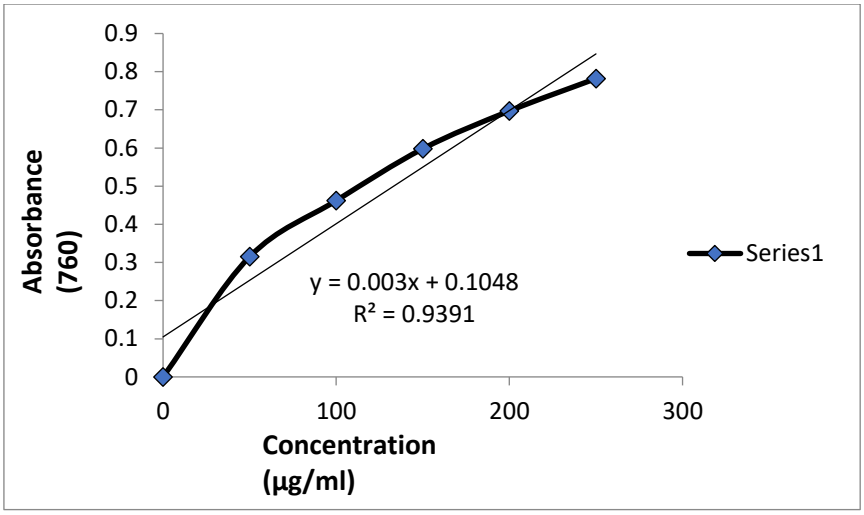

Figure 1. The Estimation of Total Phenolic Content in LMME-L.

In Vitro Antioxidant Activity: Ferric Reducing System Using FRAP Technique

The data demonstrates the ferric reducing antioxidant power (FRAP) of the samples at varying concentrations, expressed as ferrous sulfate ($FeSO_4$) equivalents. The antioxidant activity shows a clear concentration-dependent increase, with higher concentrations yielding greater $FeSO_4$ equivalent values. For instance, at 0.1 mg/mL, the $FeSO_4$ equivalent is 0.005, which rises significantly to 0.052 at 0.5 mg/mL. However, the rate of increase diminishes at higher concentrations, indicating a possible plateau effect. This is evident as the $FeSO_4$ equivalent increases modestly from 0.071 at 1 mg/mL to 0.082 at 2 mg/mL. The data suggests that the sample reaches its maximum ferric reducing capacity at 2 mg/mL, efficiently reducing Fe^{3+} to Fe^{2+} at this concentration. These results highlight the sample's strong antioxidant potential, which increases with concentration but may approach a saturation point at higher levels.

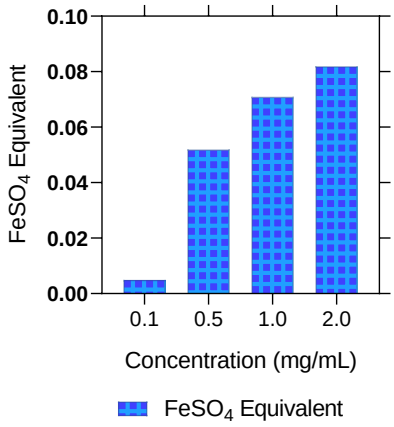

Figure 2. The Results of Antioxidant Activity Using the Ferric Reducing/Antioxidant Power (FRAP) Of LMME-L.

In Vitro Hepatoprotective Activity: Assessment Of LMME-L Using Hepg2 Cell Line

The data demonstrates the hepatoprotective effects of Litsea monopetala methanolic leaf extract (LMME-L) against CCl_4-induced cytotoxicity in HepG2 cells, as assessed by percentage cell viability. The untreated normal control group showed 100% viability, serving as a baseline for healthy cells, while the CCl_4-induced positive control group exhibited a dramatic reduction in viability to 14.71 ± 2.07%, confirming the severe cytotoxic effects of CCl_4 ($p < 0.001$ compared to the normal group). Silymarin, used as a standard hepatoprotective agent, significantly restored cell viability to 96.76 ± 3.01% at a concentration of 400 µg/mL ($p < 0.01$ compared to the positive control), highlighting its effectiveness in counteracting CCl_4-induced damage. The test groups treated with LMME-L showed a concentration-dependent protective effect. At the highest concentration of 1000 µg/mL, LMME-L restored cell viability to 97.05 ± 3.16%, closely comparable to the silymarin-treated group. Moderate concentrations of 500 µg/mL and 240 µg/mL also demonstrated high levels of protection, with cell viability at 94.64 ± 3.02%

and 92.85 ± 3.75%, respectively. Lower concentrations of 120 µg/mL and 60 µg/mL exhibited reduced, but still significant, protection, with viability levels of 85.87 ± 3.16% and 79.78 ± 3.00%, respectively, compared to the positive control group ($p < 0.01$). These findings indicate that LMME-L exhibits strong hepatoprotective activity, particularly at higher concentrations, where its efficacy approaches that of silymarin. The results underline the potential of LMME-L as a natural hepatoprotective agent, demonstrating significant improvements in cell viability across all tested concentrations. The dose-dependent increase in viability suggests that the extract counteracts CCl_4-induced oxidative damage, likely through antioxidant mechanisms. The effectiveness of LMME-L at 1000 µg/mL, comparable to silymarin, highlights its therapeutic promise. Future studies should focus on isolating and characterizing the bioactive compounds within LMME-L and investigating their mechanisms of action to better understand and optimize its hepatoprotective potential.

Table 2. Hepatoprotective Efficacy of LMME-L in Hepg2 Cells Damaged By Ccl4.

Sample (Treatment Groups)	Concentration (µg/ml)	% Cell Viability
Control (Normal Group)	-	100
Control (Positive Group, CCl_4 induced)	-	$14.71 \pm 2.07^{*}$
Silymarin Treated + CCl_4 (1.5 %) induced (Standard Group)	400	$96.76 \pm 3.01^{\#}$
LMME-L treated + CCl_4 (1.5 %) (Test Group)	1000	$97.05 \pm 3.16^{\#}$
	500	$94.64 \pm 3.02^{\#}$
	240	$92.85 \pm 3.75^{\#}$
	120	$85.87 \pm 3.16^{\#}$
	60	$79.78 \pm 3.00^{\#}$
Data were represented as Mean ± SD for 3 replicate determinations as an average. * indicated significant differences compared to normal group ($p< 0.001$); and # indicated significant as compared to Toxicant group (Positive control) ($p< 0.01$).		

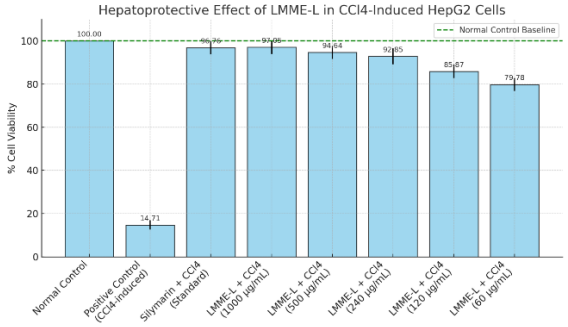

Figure 3. Hepatoprotective Effect of LMME-L in Ccl4 Induced Injury in Hepg2 Cells.

Effect of LMME-L on Biomarkers of Hepatotoxicity

The data in Table 3 highlights the effect of Litsea monopetala methanolic extract (LMME-L) on key liver enzymes—AST, ALT, and LDH—which serve as biological markers of hepatotoxicity in HepG2 cells. The normal control group exhibited baseline enzyme levels of 3.78 ± 0.36 U/L for AST, 4.02 ± 0.21 U/L for ALT, and 180.75 ± 5.57 U/L for LDH, indicative of healthy liver function. In contrast, the positive control group (CCl_4-intoxicated) demonstrated significant increases in all enzyme levels: AST (9.78 ± 0.65 U/L), ALT (6.66 ± 0.50 U/L), and LDH (400.48 ± 15.10 U/L), reflecting substantial hepatocellular damage induced by CCl_4 ($p < 0.01$ for AST and ALT, $p < 0.001$ for LDH). Treatment with silymarin as the standard significantly reduced these elevated enzyme levels, with AST, ALT, and LDH decreasing to 4.45 ± 0.25 U/L, 4.75 ± 0.34 U/L, and 222.89 ± 9.78 U/L, respectively ($p < 0.01$–0.001 compared to the positive control). This reduction confirms silymarin's established hepatoprotective efficacy. Treatment with a combination of LMME-L and silymarin also resulted in marked improvement, with enzyme levels comparable to those of the silymarin-treated group: AST (4.80 ± 0.33 U/L), ALT (4.56 ± 0.66 U/L), and LDH (325.37 ± 11.27 U/L), although LDH reduction was less pronounced. The hepatoprotective effects of LMME-L alone were evident at different concentrations, showing a dose-dependent reduction in enzyme levels. However, at lower concentrations (e.g., 6.54 ± 0.23 U/L for AST and 5.48 ± 0.34 U/L for ALT), the protection was less robust, and LDH levels (381.96 ± 7.36 U/L) remained relatively higher. These findings suggest that LMME-L effectively mitigates CCl_4-induced

hepatotoxicity, particularly when combined with silymarin, but its standalone efficacy may vary depending on the concentration used. In conclusion, the results demonstrate that LMME-L exerts a protective effect against CCl_4-induced hepatotoxicity by reducing AST, ALT, and LDH levels, with the most significant improvements observed at higher concentrations and in combination with silymarin. These findings suggest that LMME-L has promising hepatoprotective potential, though its effectiveness as a monotherapy may require optimization. Further investigations into the extract's bioactive constituents and mechanisms of action are warranted to enhance its therapeutic utility.

Table 3. Effect Of LMME-L on the Hallmark Liver Enzymes as Biological Markers of Hepatotoxicity in $Hepg_2$ Cells.

Experimental Groups	AST (Unit/L)	ALT (Unit/L)	LDH (Unit/L)
Normal Control	3.78±0.36	4.02±0.21	180.75±5.57
Positive Control (CCl4 intoxicated)	9.78±0.65**	6.66±0.50**	400.48±15.10***
CCl4 (1%) intoxicated + Treated with Standard Silymarin	4.45±0.25##	4.75±0.34##	222.89±9.78###
CCl4 (1%) intoxicated + Treated with [LMME-L + Silymarin]	4.80±0.33#	4.56±0.66##	325.37±11.27##
	6.01±0.41#	5.05±0.21#	346.84±10.47#
	6.54±0.23#	5.48±0.34	381.96±7.36

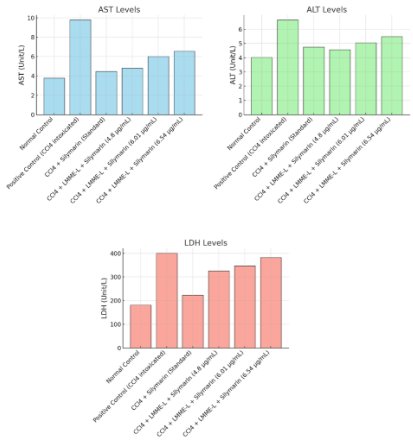

Figure 4. Hepatoprotective Effect in Terms of AST, ALT and LDH Levels of LMME-L in Ccl4 Induced Injury in Hepg2 Cells.

Effect of LMME-L on Oxidative Stress Biomarkers

The data in Table 4 highlights the effect of Litsea monopetala methanolic extract (LMME-L) on oxidative stress biomarkers—malondialdehyde (MDA) and reduced glutathione (GSH)—in HepG2 cells, which serve as indicators of oxidative damage and antioxidant defense, respectively. The normal control group exhibited baseline levels of MDA (5.02 ± 0.43 nM/mg protein) and GSH (97.19 ± 1.77 nM/mg protein), reflecting a state of minimal oxidative stress and robust antioxidant capacity. In contrast, the positive control group (CCl_4-intoxicated) showed a significant increase in MDA levels (6.71 ± 0.29 nM/mg protein, $p < 0.01$), indicating enhanced lipid peroxidation and oxidative damage. Simultaneously, GSH levels were drastically reduced (30.39 ± 2.65 nM/mg protein, $p < 0.01$), signifying depleted antioxidant reserves due to oxidative stress caused by CCl_4. Treatment with silymarin as the standard reference resulted in partial restoration of both biomarkers. MDA levels were slightly reduced to 6.24 ± 0.28 nM/mg protein, while GSH levels improved significantly to 54.38 ± 2.75 nM/mg protein ($p < 0.01$ compared to the positive control), highlighting silymarin's antioxidative efficacy. Treatment with LMME-L in combination with silymarin also led to improvements in oxidative stress markers. MDA levels were reduced in a dose-dependent manner, with the lowest levels observed at higher concentrations of LMME-L (e.g., 5.73 ± 0.31 nM/mg protein). Similarly, GSH levels were significantly restored, reaching 47.86 ± 1.57 nM/mg protein at the most effective dose. Lower doses of LMME-L (e.g., 6.30 ± 0.36 nM/mg protein for MDA and 40.71 ± 1.21 nM/mg protein for GSH) exhibited lesser efficacy but still provided substantial protection compared to the CCl_4-intoxicated group. These results suggest that

LMME-L effectively mitigates oxidative stress in CCl_4-induced hepatotoxicity, as evidenced by reduced MDA levels and restored GSH levels. The combination of LMME-L with silymarin produced synergistic effects, improving the oxidative stress markers more significantly than LMME-L alone at lower doses. However, the data also indicate that LMME-L's efficacy is concentration-dependent, with higher doses showing more pronounced benefits. In conclusion, the findings demonstrate that LMME-L possesses potent antioxidative properties, reducing lipid peroxidation and enhancing antioxidant defenses in HepG2 cells subjected to CCl_4-induced oxidative stress. Its therapeutic potential is further enhanced when used in combination with silymarin, making it a promising candidate for hepatoprotective interventions. Further research should focus on elucidating the mechanisms underlying its antioxidative effects and exploring its bioactive components.

Table 4. Effect of LMME-L on the Hallmark Oxidative Bio-Markers of Hepatotoxicity in $Hepg_2$ Cells

Experimental Groups	MDA (nM/mg protein)	GSH (nM/mg protein)
Normal Control	5.02±0.43	97.19±1.77
Positive Control (CCl4 intoxicated)	6.71±0.29**	30.39±2.65**
CCl4 (1%) intoxicated + Treated with Standard Silymarin	6.24±0.28#	54.38±2.75##
CCl4 (1%) intoxicated + Treated with [LMME-L + Silymarin]	5.73±0.31#	47.86±1.57##
	6.07±0.27#	45.23±1.36#
	6.30±0.36#	40.71±1.21#

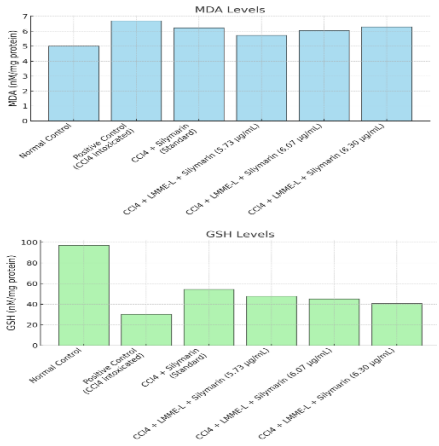

Figure 5. Hepatoprotective Effect in Terms of GSH and MDA Levels of LMME-L in Ccl4 Induced Injury in Hepg2 Cells

4. CONCLUSIONS

This study highlights the hepatoprotective, antioxidative, and bioactive potential of Litsea monopetala methanolic extract (LMME-L). The FRAP assay confirmed its strong antioxidant activity, which increased with concentration, while the total phenolic content (8.6 mg GAE/g) correlated with its reducing power. LMME-L effectively countered CCl_4-induced hepatotoxicity, as evidenced by improved cell viability and significant reductions in AST, ALT, LDH, and MDA levels, alongside restored GSH levels. The combination of LMME-L with silymarin enhanced these protective effects, indicating a synergistic mechanism. These findings establish LMME-L as a promising natural therapeutic agent for liver protection, combining strong antioxidative properties with effective hepatocellular protection. The concentration-dependent effects of LMME-L underline its potential for dose optimization. Future research should focus on the isolation of active compounds, elucidation of molecular mechanisms, and evaluation of its clinical applicability in managing oxidative stress-related liver damage. The study provides a robust foundation for further exploration of LMME-L's therapeutic potential in hepatoprotection.

5. REFERENCES

[1] AHSAN, R., MISHRA, A., BADAR, B., OWAIS, M. & MISHRA, V. 2023. Therapeutic Application, Phytoactives and Pharmacology of Tinospora cordifolia: An Evocative Review. Chin J Integr Med, 29**,** 549-555.

[2] ALIGITA, W., SINGGIH, M., SUTRISNO, E. & ADNYANA, I. K. 2023. Hepatoprotective Properties of Water Kefir: A Traditional Fermented Drink and Its Potential Role. Int J Prev Med, 14**,** 93.

[3] ARZUMANIAN, V. A., KISELEVA, O. I. & POVERENNAYA, E. V. 2021. The Curious Case of the HepG2 Cell Line: 40 Years of Expertise. International journal of molecular sciences, 22**,** 13135.

[4] AZIZ, M. A., KHAN, A. H. & PIERONI, A. 2020. Ethnoveterinary plants of Pakistan: a review. J Ethnobiol Ethnomed, 16**,** 25.

[5] BEINTEMA, J. J. & PEUMANS, W. J. 1992. The primary structure of stinging nettle (Urtica dioica) agglutinin A two-domain member of the hevein family. FEBS Letters, 299**,** 131-134.

[6] BNOUHAM, M., MERHFOUR, F.-Z., ZIYYAT, A., MEKHFI, H., AZIZ, M. & LEGSSYER, A. 2003. Antihyperglycemic activity of the aqueous extract of Urtica dioica. Fitoterapia, 74**,** 677-681.

[7] BOSE MAZUMDAR GHOSH, A., BANERJEE, A. & CHATTOPADHYAY, S. 2022. An insight into the potent medicinal plant Phyllanthus amarus Schum. and Thonn. Nucleus (Calcutta), 65**,** 437-472.

[8] CHOPRA, R. N., NAYAR, S. L. & CHOPRA, I. C. 1956. Supplement to Glossary of Indian Medicinal Plants, New Delhi, Council of Scientific & Industrial Research.

[9] CLEMENT, M. V., HIRPARA, J. L., CHAMDHURY, S. H. & PERVAIZ, S. 1998. Chemopreventive agent resveratrol, a natural product derived from graps, triggers CD 95 Signaling- dependent apoptosis in human tumor cells. Blood 1998, 92**,** 996-1002.

[10] DINESH, A. & KUMAR, A. 2023. A review on bioactive compounds, ethnomedicinal importance and pharmacological activities of Talinum triangulare (Jacq.) Willd. Chem Biodivers**,** e202301079.

[11] ELLNAIN-WOJTASZEK, M., KRUCZYNSKI, Z. & KASPRZAK, J. 2003. Investigation of the free radical scavenging activity of Ginkgo biloba L. leaves. Fitoterapia, 74**,** 1-6.

[12] FARZAMI, B., AHMADVAND, D., VARDASBI, S., MAJIN, F. J. & KHAGHANI, S. 2003. Induction of insulin secretion by a component of Urtica dioica leave extract in perifused Islets of Langerhans and its in vivo effects in normal and streptozotocin diabetic rats. Journal of Ethnopharmacology, 89**,** 47-53.

[13] GOH, M. P. Y., SAMSUL, R. N., MOHAIMIN, A. W., GOH, H. P., ZAINI, N. H., KIFLI, N. & AHMAD, N. 2024. The Analgesic Potential of Litsea Species: A Systematic Review. Molecules, 29.

[14] GUEMMOGNE TEMDIE, R. J., YMELE CHIOGO VOUFFO, E., TIETCHEU GALANI, B. R., DABOLE LADANE, B., BIANZOUMBE, J., KUUM MINOUE, M. G., NTCHAPDA, F. & DIMO, T. 2023. Potential Curative Effects of Aqueous Extracts of Cissus quadrangularis (Vitaceae) and Jatropha gossypiifolia (Euphorbiaceae) on Acetaminophen-Induced Liver Injury in Mice. Curr Ther Res Clin Exp, 99**,** 100716.

[15] GUIL-GUERRERO, J. L., REBOLLOSO-FUENTES, M. M. & ISASA, M. E. T. 2003. Fatty acids and carotenoids from Stinging Nettle (Urtica dioica L.). Journal of Food Composition and Analysis, 16**,** 111-119.

[16] GÜLÇIN, I., OKTAY, M., KÜFREVIOGLU, Ö. I. & ASLAN, A. 2002. Determination of antioxidant activity of lichen Cetraria islandica (L) Ach. Journal of Ethnopharmacology, 79**,** 325-329.

[17] HALLIWELL, B. & GUTLERIDGE, M. 1984. Review article. Oxygen toxicity, oxygen radicals, transition metals and disease Biochemical Journal, 219**,** 1-4.

[18] HARBORNE, J. B. 1973. Phytochemical methods: A guide to Modern Techniques of Analysis, London, Chapman and Hall Publishers.

[19] HU, K., KOBAYASHI, H., DONG, A., JING, Y., IWASAKI, S. & YAO, X. 1999. Antineoplastic agents. III: Steroidal glycosides from Solanum nigrum. Planta Med, 65**,** 35-8.

[20] HUSSAIN, S., JALI, A. M., ALSHAHRANI, S., KHAIRAT, K. H. M., SIDDIQUI, R., ALAM, M. I., ALI, R., MOHAMMED, M., KHAN, A., AL SHAHI, H., HANBASHI, A., QADRI, M. & ASHAFAQ, M. 2023. Hepatoprotective and Antioxidant Effects of Nanopiperine against Cypermethrin via Mitigation of Oxidative Stress, Inflammations and Gene Expression Using qRT-PCR. Int J Mol Sci, 24.

[21] KAMEL, F. O., KARIM, S., BAFAIL, D. A. O., ALDAWSARI, H. M., KOTTA, S. & ILYAS, U. K. 2023. Hepatoprotective effects of bioactive compounds from traditional herb Tulsi (Ocimum sanctum Linn) against galactosamine-induced hepatotoxicity in rats. Front Pharmacol, 14**,** 1213052.

[22] KHARE, C. P. 2007. Indian Medicinal Plants: An Illustrated Dictionary, New York, Springer Science+BusinessMedia, LLC.

[23] KHOSHAKHLAGH, M., BUTLER, A. E., JAMIALAHMADI, T. & SAHEBKAR, A. 2023. Protective Effects of Curcumin Against Alcoholic Fatty Liver. Curr Med Chem.

[24] KIM, H., HONG, J., LEE, J., JEON, W., YEO, C., LEE, Y., BAEK, S. & HA, I. 2023. Curcuma aromatica Salisb. Protects from Acetaminophen-Induced Hepatotoxicity by Regulating the Sirt1/HO-1 Signaling Pathway. Nutrients, 15.

[25] KRAUS, R. & SPITELLER, G. 1990. Phenolic compounds from roots of Urtica dioica. Phytochemistry, 29**,** 1653-1659.

[26] KRAUS, R. & SPITELLER, G. 1991. Terpene diols and terpene diol glucosides from roots of Urtica dioica. Phytochemistry, 30**,** 1203-1206.

[27] KUMAR, M., HASAN, M., SHARMA, A., SUHAG, R., MAHESHWARI, C., RADHA, CHANDRAN, D., SHARMA, K., DHUMAL, S., SENAPATHY, M., NATARAJAN, K., PUNNIYAMOORTHY, S., MOHANKUMAR, P., DEY, A., DESHMUKH, V., ANITHA, T., BALAMURUGAN, V., PANDISELVAM, R., LORENZO, J. M. & KENNEDY, J. F. 2023. Tinospora cordifolia (Willd.) Hook.f. & Thomson polysaccharides: A review on extraction, characterization, and bioactivities. Int J Biol Macromol, 229**,** 463-475.

[28] KUMAR SRIVASTAVA, A. 2018. Chapter 1 - Significance of medicinal plants in human life. In: TEWARI, A. & TIWARI, S. (eds.) Synthesis of Medicinal Agents from Plants. Elsevier.
[29] LAMICHHANE, G., SHARMA, G., SAPKOTA, B., ADHIKARI, M., GHIMIRE, S., POUDEL, P. & JUNG, H. J. 2023. Screening of Antioxidant, Antibacterial, Anti-Adipogenic, and Anti-Inflammatory Activities of Five Selected Medicinal Plants of Nepal. J Exp Pharmacol, 15**,** 93-106.
[30] LI, X. M., LI, X. L. & ZHOU, A. G. 2007. Evaluation of antioxidant activity of the polysaccharides extracted from Lycium barbarum fruits in vitro. European Polymer Journal, 43**,** 488-497.
[31] MANN, M. & PATHAK, S. R. 2018. Chapter 12 - Ayurveda: A new dimension in the era of modern medicine. In: TEWARI, A. & TIWARI, S. (eds.) Synthesis of Medicinal Agents from Plants. Elsevier.
[32] MITSUDA, H., YUASUMOTO, K. & IWAMI, K. 1996. Antioxidation action of indole compounds during the autoxidation of linoleic acid. Eiyo to Shokuryo, 19**,** 210-214.
[33] PEUMANS, W. J., DE LEY, M. & BROEKAERT, W. F. 1984. An unusual lectin from stinging nettle (Urtica dioica) rhizomes. FEBS Letters, 177**,** 99-103.
[34] SAHOO, S., KHAN, H. A., KAR, D. M., PATTANAIK, S. & RATH, D. 2024. LC-QTOF-MS/MS metabolic profiling and hepatoprotective effects of Litsea monopetala bark methanol extract against liver injury in rats and HepG2 cells. Cell Mol Biol (Noisy-le-grand), 70**,** 156-169.
[35] SHAHRAJABIAN, M. H. & SUN, W. 2023. The Golden Spice for Life: Turmeric with the Pharmacological Benefits of Curcuminoids Components, including Curcumin, Bisdemethoxycurcumin, and Demethoxycurcumin. Curr Org Synth.
[36] SINGH, V., AKANSHA, ISLAM, Z. & SHAIDA, B. 2024. Medicinal Plants: Sustainable Scope to Nutraceuticals. In: THAKUR, M. (ed.) Sustainable Food Systems (Volume II): SFS: Novel Sustainable Green Technologies, Circular Strategies, Food Safety & Diversity. Cham: Springer Nature Switzerland.
[37] SLINKARD, K. & SINGLETON, V. L. 1977. Total Phenol analysis: Automation and comparison with manual methods. American Journal of Enology and Viticulture, 28**,** 49-55.
[38] SOWUNMI, B. O. & GONZO, M. 2023. The effect of Moringa oleifera crude extract on liver cell line, HepG2. BMC Complement Med Ther, 23**,** 380.
[39] SPARZAK-STEFANOWSKA, B. & KRAUZE-BARANOWSKA, M. 2022. Phyllanthus amarus shoot cultures as a source of biologically active lignans: the influence of selected plant growth regulators. Sci Rep, 12**,** 11505.
[40] TAHRI, A., YAMANI, S., LEGSSYER, A., AZIZ, M., MEKHFI, H., BNOUHAM, M. & ZIYYAT, A. 2000. Acute diuretic, natriuretic and hypotensive effects of a continuous perfusion of aqueous extract of Urtica dioica in the rat. Journal of Ethnopharmacology, 73**,** 95-100.
[41] THABREW, M. I., HUGHES, R. D. & MCFARLANE, I. G. 1997. Screening of hepatoprotective plant components using a HepG2 cell cytotoxicity assay. J Pharm Pharmacol, 49**,** 1132-5.
[42] TIAN, S., LI, Y., XIAO, L. & RAN, T. 2022. The complete chloroplast genome sequence of Litsea coreana var. lanuginosa (Lauraceae): genome structure and phylogenetic analysis. Mitochondrial DNA B Resour, 7**,** 1617-1618.
[43] ZHU, L., ZHANG, Y., LI, Y., WANG, H., SHEN, G. & WANG, Z. 2022. Inhibitory effect of lingonberry extract on HepG2 cell proliferation, apoptosis, migration, and invasion. PloS one, 17**,** e0270677-e0270677.