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Original Article

LC-QTOF-MS/MS metabolic profiling and hepatoprotective effects of *Litsea monopetala* bark methanol extract against liver injury in rats and HepG2 cells

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Abstract

Fresh stem bark decoction of *Litsea monopetala* has been practiced for the treatment of jaundice and other liver disorders by the tribal communities of Thakht-e-Sulaiman hills from West Pakistan. As per the folkloric claim, this study aims to identify the phytoconstituents and evaluate the hepatoprotective action of stem bark methanol extract of *L. monopetala* (LMME). The *in-vitro* hepatoprotective effect of *L. monopetala* was performed by H_2O_2-induced toxicity in the HepG2 cell line and *in-vivo* by ccl_4-induced hepatotoxicity in Wistar albino rats taking Silymarin as standard drug. Phytoconstituents were identified using LC-QTOF-MS analysis followed by *in-silico* docking and validation. Molecular docking interactions between identified compounds of *L. monopetala* and two target proteins, namely 1VJY and 5HYK were presented. In this study, treatment with LMME at 100 µg/mL showed 67.73 % cell viability as compared to H_2O_2 (100 µM) treated alone i.e., 18.55 % in the HepG2 cell line. *In-vivo* treatment of LMME reversed the altered serum biochemical parameters and reduced the inflammatory response similar to that of the Silymarin-treated group supported by histopathological investigation. This research reveals that *L. monopetala* is a rich source of flavonoids and phenols which supports its hepatoprotective effects and is proposed for its usage as a promising hepatoprotective agent after controlled trials.

Keywords: Hepatoprotective, *Litsea monopetala*, toxicity, *in-silico*, *in-vivo*, *in-vitro*

1. Introduction

The liver is the most vital organ in our body which not only completes the metabolism of carbohydrates, protein, and fat but also acts as a detoxification centre for various Xenobiotics and drugs. Hepatic damage can lead to various fatal health conditions such as renal infection, renal failure, and bleeding disorders which may be due to the formation of reactive oxygen species and inflammations by the Xenobiotics [1]. Due to the alteration in permeability of hepatocyte membranes, there is a rise in the level of Aspartate aminotransferase (AST), Alanine transaminase (ALT), and Alkaline phosphatase (ALP) as a result of the destruction of the hepatocytes. Carbon tetrahydrochloride (CCl_4) is a chemical agent that is used for inducing liver toxicity by producing a large number of free radicals in the case of animal studies. Silymarin is used for hepatoprotection but it has some drawbacks like poor oral-bioavailability, low stability, and poorly soluble in gastric juice as well [2]. Even if the Silymarin drug is well tolerated in humans, it has been noted some common side effects like itching and headache after being used at a high dosage for a prolonged period [3].

According to the World Health Organization (WHO), a significant majority of the world population, over 80 %, mostly depends on traditional therapies that use plant extracts or their active constituents [4]. Historically, individuals residing in rural areas, particularly those affiliated with Indian ethnic communities, have traditionally depended on botanical resources for the management of liver damage, mostly owing to their protective characteristics. Despite the acknowledgment of several botanicals used in the Indian system of medicine, there remain a considerable number of plants or plant usages, particularly those exclusive to rural populations, that have yet to get acknowledgment.

Litsea monopetala (Roxb.) Pers. from the Lauraceae family (popularly known as Meda, Menda, Bara, Lalkhori, Boi-bet) is widely distributed in India, Bangladesh, China, Burma, and Nepal worldwide [5]. It is an evergreen plant commonly known as *Tetranthera monopetala;* its height is 18 meters and its leaves are elliptic-oblong and usually rounded at both ends. Methanol extract of this

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plant showed antioxidant, cytotoxicity, anthelmintic, and thrombolytic effects [6]. Fresh stem bark decoction of *L. monopetala* has been practiced for the treatment of jaundice and other liver disorders in the case of humans and animals by the tribal communities of Thakht-e-Sulaiman hills from West Pakistan [7].

Crushed leaf extract of this plant (vernacular name as Kala deungra) was used for the treatment of dysentery; crushed bark extract was used for the semen loss and decoction of seed as diabetes by North Tripura district of Tripura state [8]. In Nepal, scientists have investigated 5 medicinal plants that have potent anti-adipogenic, antioxidant, and anti-inflammatory effects and the *L. monopetala* plant is one of them [9]. Antioxidant effects of *L. monopetala* were studied in the bark extracts *in-vitro* using Sephadex LH-20 column chromatography [9]. Another species of Litsea plant i.e., *L. floribunda* showed hepatoprotective action against paracetamol-induced toxicity and antioxidant effects in rats [10]. The bark of this plant is used as a stimulant, astringent, stomachic, spasmolytic, antidiarrheal, and jaundice [11]. Bioactive constituents like Humulene oxide and Caryophyllene oxide were found to be reported from *L. monopetala* plant [12].

Despite the existence of sophisticated medications, there are currently no pharmaceuticals available that can effectively repair hepatic damage, regenerate hepatic cells, or prevent the demise of liver cells. Therefore, it is crucial to discover an alternative medication that may efficiently treat liver illnesses with less toxicity [13]. Due to its traditional claim and antioxidant capabilities, the plant may have hepatoprotective benefits, as well as anti-inflammatory characteristics. The current research work was designed to evaluate the hepatoprotective effects of stem bark methanol extracts of *L. monopetala* (Roxb.) Pers. in H_2O_2 treated HepG2 cells (*In-vitro*) and CCl_4 treated Wistar albino rats (*In-vivo*).

2. Materials and Methods

2.1. Authentication and extraction of plant materials

The stem bark of *L. monopetala* was collected from Rairangpur, Mayurbhanj, Odisha in January and authenticated by taxonomist Dr. Pratap Chandra Panda at CBT, Siksha 'O' Anusandhan (Deemed to be University), Bhubaneswar with a field no. 2412/CBT Dt. 08.11.2022 and the specimen was stored at the herbarium for future reference. After washing *L. monopetala* stem bark, it was chopped into tiny pieces. The fragments were then dried in a shady area and ground into a coarse powder using a mechanical grinder. *L. monopetala* (LMME) defatted dry stem bark components were extracted using methanol as the solvent in Soxhlet apparatus. The liquid extract was dried at 45 ˚C by vacuum concentration in a Rotary evaporator. The percentage yield of the extract (LMME) was 14.23 % w/w. The dried extract was then stored and used later on in a vacuum desiccator [14].

2.2. *In-vitro* MTT assay

LMME was evaluated *in vitro* using the MTT test, a widely recognized colorimetric assay, to determine its effects on cell proliferation and cytotoxicity. This assay provides a precise method for assessing cell viability, as it measures the conversion of the water-soluble tetrazolium dye MTT from yellow to formazan crystals. Living cells produce an enzyme called mitochondrial lactate dehydrogenase. This enzyme has the amazing ability to convert MTT into insoluble formazan crystals. When these crystals are dissolved in the right solvent, they exhibit purple colour. By analyzing the colour intensity at a wavelength of 570 nm, we can accurately measure the number of viable cells. [15]. The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) with low glucose, supplemented with 10 % FBS and a 1 % antibiotic-antimycotic solution. The culture was maintained at a temperature of 37 °C in a carbon dioxide (CO_2) incubator with a 5 % CO_2 and 18 - 20 % oxygen (O_2) atmosphere. Subculturing of the cells was performed every two days using cell lines obtained from the National Centre for Cell Science (NCCS) in Pune, India [15,16].

Before applying the test agent, a 96-well plate with 200 µl of cell suspension at the required cell density (2 x 10^5) was well-seeded. The cells were allowed around 24 hours to mature. Two groups of treatment circumstances were developed: Batch 1, the right quantities of the test agent, the toxic control, and Silymarin were all applied. Batch 2: After cells were subjected to the toxicity induced by 100 µM of H_2O_2 for approximately two hours, the standard and the test drug (LMME) were subjected for evaluation of their protective effects. The plate was incubated for 24 hours at 37 °C in a 5 % CO_2 atmosphere. Following incubation, 10 µl of MTT reagent at a concentration of 0.5 mg/mL was added to each well. Aluminium foil was used to shield the plate from light exposure. The plates were placed back in the incubator and incubated for more three hours. *(Note: Incubation time might differ across cell lines. It's important to keep the incubation time the same from one experiment to the next so that results may be compared.)* 100 µl of solubilization solution (DMSO) was then added after the MTT reagent was discarded. Then, the dissolution was helped by mild agitation in a gyratory shaker. Pipetting up and down was sometimes required to completely dissolve the MTT formazan crystals when they were very thick. The absorbance was measured using an ELISA reader with a 570 nm wavelength. To get the percentage of cell viability, the formula applied is mentioned below:

% of cell viability = [Mean absorbance of treated cells / Mean absorbance of untreated cells] x 100

For the MTT test, the HepG2 cell line was used, and the experimental groups (culture media) were split into six categories: i.e.,

Group 1: Untreated group (without any treatment);
Group 2: H_2O_2 treated alone group (100 µM/ml);
Group 3: Silymarin alone (100 µg/ml),
Group 4: Methanol extract of *L. Monopetala* (LMME) (100 µg/ml), i.e., without induction of H_2O_2;
Group 5: H_2O_2+Silymarin (100 µM+100 µg/ml);
Group 6: H_2O_2+LMME (100 µM+100 µg/ml).

2.3. Ethical approval from IAEC

The following research work was conducted in accordance to OECD and ARRIVE 2.0 guidelines followed by the approval of the Institutional Animal Ethical Committee (IAEC) before conducting an *In-vivo* animal study and the proposal no is IAEC/SPS/SOA/108/2022 dated 26.04.2022.

2.4. Acute toxicity study

The test samples were subjected to an acute toxicity evaluation in accordance with OECD standards 423 gui-

delines. Wistar albino rats (200 - 250 g) were divided into test groups, which received extract doses ranging from minimal dose to a maximum of 4000 mg/kg body weight, and control groups, which got distilled water and Tween 80 by mouth. All animals were closely monitored for the first 4 hours, then again for the next 72 hours, looking for signs of acute toxicity. The effects of extracts on animal mortality were monitored for up to 14 days [17].

2.5. Animal and experimental groups for *in-vivo* hepatoprotective study

Wistar albino rats were procured from the animal house at the School of Pharmaceutical Sciences, Siksha 'O' Anusandhan (Deemed to be University), Odisha, India, weighing between 200 ± 20 g for use in the experiments and they were fed with standard diet and water ad libitum freely. They were housed in polycarbonate cages maintained at room temperature 25 ± 2 ℃ with a relative humidity of 50 - 60 % and 12 h light and dark cycle. Experiments were conducted following the rules of OECD 423. Five groups of six animals each (n=6) were formed at random. Group A was considered as without any treatment (Control). The vehicle (1 % Tween-80 in distilled water) was administered orally once a day for 8 days to Group B at a dosage of 10 mL/kg body weight. The standard group (Group C) received 100 mL/kg body weight of standard Silymarin orally once daily for 8 consecutive days. Group D and E underwent 8 days of testing during which the protective effects of LMME at 200 and 400 mg/kg body weight were evaluated.

On day 8, rats in groups B-E received intraperitoneally at a dose of 1 mL/ kg body weight of CCl_4 diluted in olive oil at a 1:1 ratio, whereas rats in Group A received olive oil alone. On the 9th day, blood samples were collected from the 12 h fasted rats by the heart puncture method under anaesthesia (Ketamine-Xylazine at dose 100 mg/kg + 10 mg/kg body weight) for the evaluation of serum biochemical parameters. At last, all the animals were sacrificed by the high dose of Ketamine, and liver organs were extracted for histopathological investigation [18]. The above-mentioned *in-vivo* analysis was performed by strictly adhering to the OECD 423 and IAEC (IAEC/SPS/SOA/108/2022) guidelines.

2.6. Determination of serum marker enzyme

Blood samples were collected on the ninth day and thereafter transferred into test containers in preparation for centrifugation at a rotational speed of 2000 RPM. Following the guidelines provided by the manufacturer for the Ecoline diagnostic kits, serum samples were obtained and utilized to measure different biochemical parameters such as aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), total protein (TP), and albumin [19].

2.7. Histopathological analysis

The liver tissue was obtained and stored in a solution of formalin with a concentration of 10 %. The specimen was then dehydrated using a sequence of ethanol solutions with concentrations ranging from 50 % to 100 %. Afterwards, the tissue was purified using xylene and ultimately encased in paraffin. The liver slices were obtained by slicing them into 5 µm thickness using a microtome. Subsequently, these sections were treated with haematoxylin and eosin dye to aid in the microscopic investigation and study of histological changes. The liver slices were evaluated for the degree of hepatic injury using a microscope produced by Nikon, a Japanese manufacturer, and visual documentation was performed [20].

2.8. LC-QTOF-MS conditions

The Waters Acquity UPLC system was used for the LC-QTOF-MS analysis with an ACQUITY UPLC BEH C18 Column (1.7 µm, 2.1 mm X 50 mm). Solvent A was water with 0.1 % formic acid, and Solvent B was Acetonitrile; these two solvents comprised the mobile phase and 0.2 ml/m flow rate was observed with the following gradient pattern: 0-1 min, 2 B, 1-7 min, 2-50 % B, 7-16 min, 50-95 % B, 16-20 min, 95-2 % B. The characterization was carried out in a positive mode of ionization and a 5 µl injection volume was used. The mass spectrometer used in this investigation was the Waters Xevo G2-XS QTOF, produced by Waters Corporation in Milford, MA, USA. The device was outfitted with an electrospray ionization (ESI) source, which allowed for the identification of mass spectrometric signals. This apparatus enabled the concurrent acquisition of precursor and product ion measurements by a single injection. The testing settings included a source temperature of 150 ˚C and a desolvation gas temperature of 450 ˚C. Moreover, the flow rates of the cone gas and desolvation gas were continually maintained at 50 and 800 L/h, respectively, while maintaining a capillary voltage of 3.0 kV and a collision energy of 20 volts and mass range of 500 to 2000 amu. The data were collected for each test sample from 200 to 1,500 Da with 0.25 s scan time [21, 22]. Masslynx v4.1 was utilized for data acquisition and compilation. The observed fragments were then compared with entries in the MassBank Europe Mass Spectral Database, the Human Metabolome Database, and relevant literature sources [23].

2.9. *In-silico* docking study

2.9.1. Selection of receptors

The receptor selection procedure was carried out in accordance with the literature, using the three-dimensional protein structure obtained from the Protein Data Bank (PDB). This structure includes the amino acid sequence, along with some water molecules and other elements. This structure is enhanced using the Swiss-PDB viewer (SPDBV software). The National Center for Biotechnology Information (NCBI) has made available the Peroxisome Proliferator-Activated Receptor Alpha receptor (PPAR-α) protein data bank (PDB ID: 5HYK) in FASTA format. The protein showed a query coverage of 57 % for Chain A and an impressive identification rate of 99.63%. Similarly, the Transforming growth factor-beta 1 protein (PDB ID: 1VJY) was obtained from the NCBI, showing a query coverage of 60 % for Chain A and a 100% identification rate. Using the X-ray diffraction technique, the protein in question was successfully resolved at a resolution of 2.0 Å. The R-Value Free and work values for 1VJY were 0.275 and 0.223, respectively. Similarly, the observations recorded in the Protein Data Bank revealed that it was resolved at a resolution of 1.83 Å with R-Value Free and work values of 0.262 and 0.214 for 5HYK.

The Autodock tool (ADT) program was used to eliminate water molecules and heteroatoms that were coexisting with the obtained protein. This was done to prevent any

interference during drug docking, and the resulting structure was stored in PDB format. The 3-dimensional protein structure, amino acid sequence, and other data were obtained from the Protein Data Bank (PDB) and binding tests were conducted using the Biovia Discovery Studio visualizer (DSV) server.

2.9.2. Selection of ligand

From the PubChem database, all the 3D structures of the selected isolated components obtained from LC-QTOF-MS analysis of *L. monopetala* were retrieved in structural data format (SDF).

2.9.3. Docking of receptor-ligand

The docking study under investigation by using autodock vina application was set to be carried out with grid box dimensions (Angstroms) X = 15.29, Y = 17.27, Z = 15.94 for 1VJY receptor and X = 22.23, Y = 23.14, Z = 26.62 for 5HYK receptor separately. The grid box dimension for each receptor ensures covering all the active residues. The result of each docking interaction was studied by using three different software PYMOL, Biovia Discovery Studio, and ADT sphere view tools for visualization purposes.

2.10. Statistical analysis

The experimental results were shown as mean ± SEM. Statistical analysis was conducted using ANOVA, followed by Tukey's multiple range test, using GraphPad Prism software (version 10.0; GraphPad, Sandiego, CA, USA). A statistically significant difference was observed between the groups at a significance level of $p < 0.0001$.

3. Results

3.1. Effects of *L. monopetala* on H_2O_2-induced hepatotoxicity in HepG2 cells

MTT assay of LMME was performed at a concentration of 100 µg/mL and showed cell viability of 98.69 %; Silymarin at a concentration of 100 µg/mL showed 100.46 % viability which indicates the non-toxicity nature of the compound. Further, in combination with H_2O_2, LMME, and Silymarin revealed 67.73 and 86.97 % of cell viability respectively showing hepatoprotective effects after recovery from H_2O_2 at 100 µM concentration. Only treatment with H_2O_2 at 100 µM exhibited cell viability of 18. 55 % showing its hepatotoxicity effect (Table 1) (Figure 1).

The results of the hepatoprotective study conducted using the MTT assay indicated that the test compound, LMME at a concentration of 100 µg/mL, did not exhibit cytotoxic effects on Human liver (HepG2) cells, as evidenced by a cell viability of 98.69 %. Silymarin used as a standard control in the study at a concentration of 100 µg/mL, demonstrated a cell viability of 100.46 %. In contrast, H_2O_2 alone at a concentration of 100 µM exhibited cytotoxicity, resulting in a cell viability of 18.55 %.

In the H_2O_2-induced model targeting HepG2 cells, the administration of LMME at a concentration of 100 µg/ml and Silymarin at a concentration of 100 µg/ml had a protective effect, resulting in cell survival values of 67.73 and 86.97 %, respectively (Table 1).

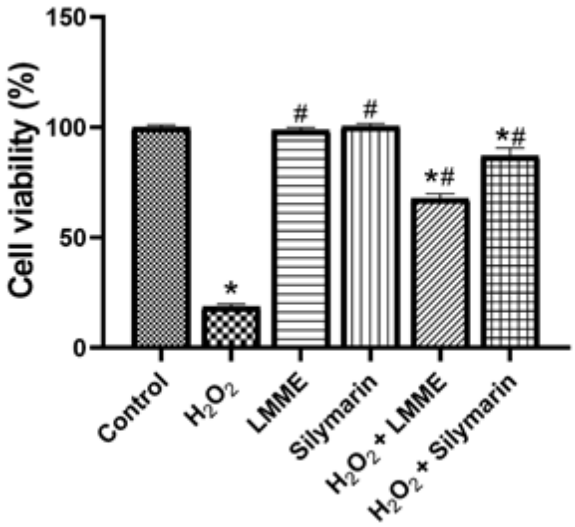


Fig. 1. This graph represents the impact of *L. monopetala* extract (LMME) on the viability of HepG2 cells after 24 h incubation. The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) with low glucose, supplemented with 10 % fetal bovine serum (FBS) and a 1 % antibiotic-antimycotic solution. The culture was maintained at a temperature of 37 °C in a carbon dioxide (CO_2) incubator. The cell viability percentage obtained from the MTT experiment is shown as the mean ± standard error of the mean (SEM), with a sample size (N) of 3. Asterisks are used in statistical analysis to denote statistically significant disparities (*p<0.0001 compared to the control group, #p<0.0001 compared to the H_2O_2 treatment group) as established by the utilization of ANOVA and Tukey's multiple range test. In this study, we used *L. monopetala* methanol extract (LMME), hydrogen peroxide (H_2O_2), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT).

Table 1. Cell viability values of different culture conditions against HepG2 cells after the treatment period of 24 hrs. The presented values were the mean of 3 independent individual experiments. (N=3). Asterisks are used in statistical analysis to denote statistically significant disparities (*p<0.0001 in relation to the control group, #p<0.0001 in relation to the H_2O_2 treatment group) as ascertained by the implementation of ANOVA and Tukey's multiple range test. In this study, we used the *L. monopetala* methanol extract, denoted as LMME, and hydrogen peroxide, referred to as H_2O_2.

MTT Assay	
Culture condition	**Cell viability ± SEM (%)**
Untreated	100
H_2O_2-100 µM	$18.55 \pm 1.36^{*}$
LMME-100 µg/ml	$98.69 \pm 1.09^{\#}$
Silymarin-100 µg/ml	$100.46 \pm 1.24^{\#}$
H_2O_2+LMME-100 µg/ml	$67.73 \pm 2.08^{*\#}$
H_2O_2+Silymarin-100 µg/ml	$86.97 \pm 3.66^{*\#}$

3.2. Acute toxicity study

The methanol extract of *L. monopetala* stem bark did not exhibit any mortality or acute symptoms of toxicity, including convulsions, alterations in skin or fur, lethargy, salivation, diarrhoea, sleep disturbances, or other indicators of toxicity, even when the concentration of the extract was raised to 4,000 mg/kg body weight. This lack of observed toxicity was seen for a duration of 72 hours. The assessment of acute toxicity revealed that the animals subjected to treatment exhibited no discernible toxic effects for 7 days of observation. We have fixed our doses at 200 and 400 mg/kg body weight for further investigation *in vivo*.

3.3. Evaluation of serum biochemical parameters in Wistar albino rats

The impact of methanol extracts derived from the stem bark of *L. monopetala* on the toxicity produced by ccl_4 in rats was evaluated using biochemical parameters like aspartate aminotransferase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), total protein, and albumin at the end of the *in-vivo* hepatoprotective study i.e., on the 9th day of the study (Fig. 2A-E). The animals treated with ccl_4 alone intraperitoneally showed elevated levels of serum biochemical parameters like AST, ALT, and ALP due to the severe toxicity of hepatic cells and reduction in total protein and albumin. Whereas, pre-treatment with LMME (both the dose levels) and Silymarin-treated groups significantly decreased the levels of AST, ALT, and ALP values and normalized the total protein and albumin levels. However, LMME at 400 mg/kg body weight showed better potency as compared with LMME at 200 mg/kg body weight in a dose-dependent manner.

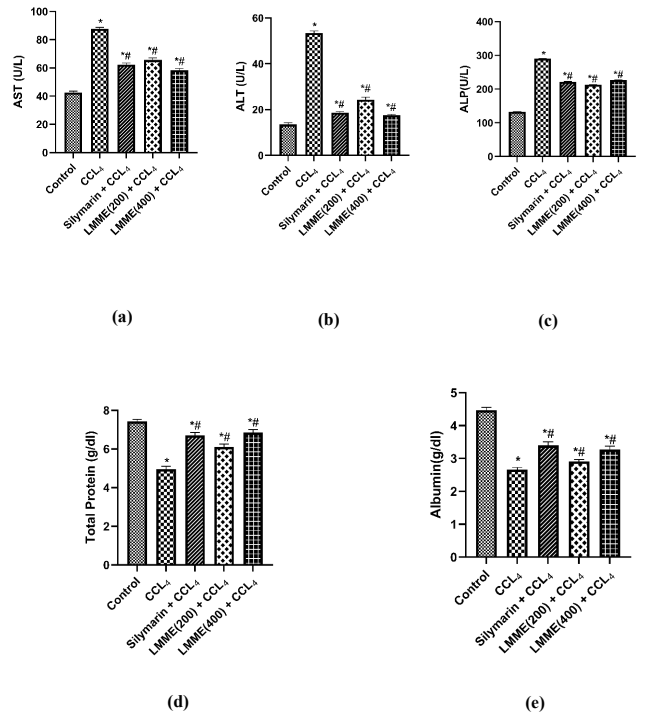


Fig. 2. The above graphs demonstrate the impact of stem bark methanol extracts of *L. monopetala* on various biochemical parameters such as (a) AST (b) ALT (c) ALP (d) Total Protein (e) Albumin after ccl_4-induced toxicity in Wistar rats. Group A: Control; Group B: ccl_4; Group C: Silymarin + ccl_4; Group D: LMME 200 mg/kg body weight+ ccl_4 and Group E: LMME 400 mg/kg body weight + ccl_4. The values are represented in mean ± SEM (n=6). Asterisks indicate differences that were found to be statistically significant (*p <0.0001 as compared with the control group, #p <0.0001 as compared with the CCl_4 treated group).

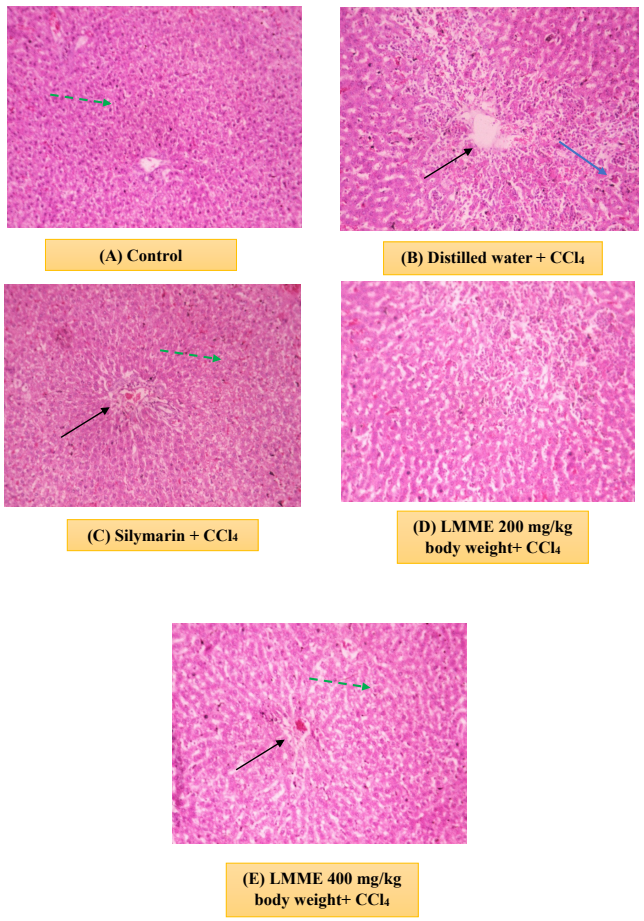


Fig. 3. Histopathology changes of liver tissues in CCl_4 intoxicated rats and hepatoprotective effects of extract LMME at 200 and 400 mg/kg body weight from *L. monopetala* (Staining with haematoxylin and eosin) at 10x magnification; (A) Control; (B) Distilled water + CCl_4, (C) Silymarin + CCl_4 (D) LMME at 200 mg/kg body weight + CCl_4, (E) LMME at 400 mg/kg body weight + CCl_4. The black arrow indicates the central vein where hepatic cells are radiating, the blue arrow indicates hepatic fibrosis surrounding connective tissue, and the green dotted arrow indicates hepatocytes having a nucleus.

3.4. Histopathology of liver tissue after CCl_4-induced toxicity

The histopathological analysis of liver tissue samples stained with haematoxylin and eosin the pictures were captured using 10x magnification with the help of Nikon D5600. The control group had typical hepatic cells and clearly discernible central veins (Fig. 3A). However, the liver tissues obtained from the CCl_4-induced group exhibited significant liver damage. This damage was characterized by widespread multifocal necrosis, disordered hepatic plate structure, degraded nuclei, severe lymphocytic infiltrates, considerable cellular edema, and fatty alteration (Fig. 3B). Liver tissue pretreated with LMME at 200 mg/kg body weight revealed mild inflammation with partially recovered liver cells after CCl_4-induced toxicity (Fig. 3D). The hepatic damage that was generated by $CC1_4$ was significantly mitigated in the liver tissue specimens obtained from rats that were pre-treated with a dose of 400 mg/kg of LMME and 100 mg/kg of standard drug Silymarin

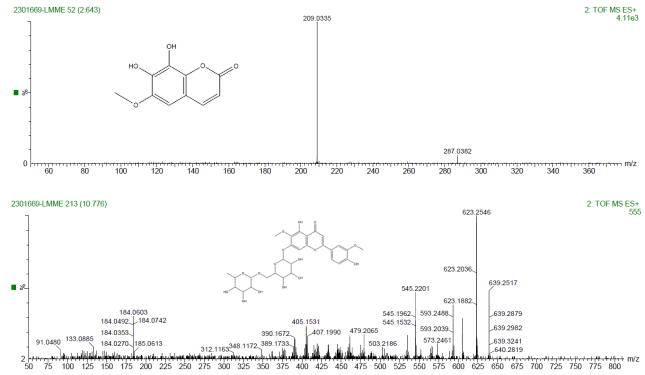


Fig. 4. Mass Spectra of the most promising compounds identified tentatively by LC-QTOF-MS/MS of *L. monopetala* extract.

which provides evidence of hepatoprotective activity of *L. monopetala* (Fig. 3C, E).

3.5. Characterization of *L. monopetala* extract by LC-QTOF-MS analysis.

The identification of phytochemicals derived from methanol extracts of *L. monopetala* (LMME) was carried out using LC-QTOF-MS analysis. Ten major compounds were identified by matching mass spectra with the reference Mass Bank Europe Mass Spectral Database, Human Metabolome Database, and relevant literature. The results of the LMME analysis revealed the existence of a variety of secondary metabolites, including coumarins, flavonoids, pyranocoumarins, lipids, etc. Figure 4 and Table 2 demonstrate the mass spectra of the promising compounds and MS data of the compounds identified tentatively by LC-QTOF-MS analysis respectively. Furthermore, the chemical compounds, along with their corresponding structures, were verified using a comprehensive analysis that included comparing the precise masses, mass spectrometry (MS) data, and pertinent literature sources.

3.6. *In-silico* study of identified compounds from the stem bark of methanol extract of *L. monopetala*

In accordance with the literature review, a computational molecular docking analysis was conducted to investigate the potential hepatoprotective effects of target selection. TGF-β1 primarily functions as a regulator of antioxidant mechanisms and liver fibrosis. Conversely, PPAR-α, a member of the nuclear receptor family, plays a significant role in maintaining lipid and glucose homeostasis through the regulation of gene expression in hepatocytes [30]. According to reports, the potential mechanism for hepatoprotection may include the suppression of TGF-β1 and PPAR-α proteins (PDB ID: 1VJY, 5HYK, respectively) [31].

4. Discussion

The present study was undertaken to validate scientifically the traditional claim of hepatoprotective effects of stem bark methanol extract of *Litsea monopetala* (LMME) in H_2O_2-treated HepG2 cells and CCl_4-treated Wistar albino rats followed by LC-QTOF-MS analysis and molecular docking. Moreover, this study would give a juxtaposition between the *in-vivo* and *in-vitro* hepatoprotective study with a molecular approach to be used for the benefit of mankind. Human hepato-carcinoma cell lines or HepG2 cell lines are popularly used due to the presence of numerous enzymes that are responsible for the stimulation of different xenobiotics, easy to handle and similar to the human hepatocytes [32]. ccl_4-induced hepatotoxicity is one of the common and effective methods to study the *in-vivo* hepatoprotective effect or acute liver injury in animals as it has a resemblance to human liver disorders. After induction, CCl_4 is metabolized to trichloromethyl radical (CCl_3^*) in the presence of the enzyme Cytochrome P450. Again, the CCl_3^* produces trichloromethyl peroxyl radical ($OOCCl_3$) with the influence of reactive oxygen species (ROS) thereby increasing oxidative stress and causing alteration in lipid peroxidation or glutathione pathway or formation of TNF-α proinflammatory cytokines that lead to the destruc-

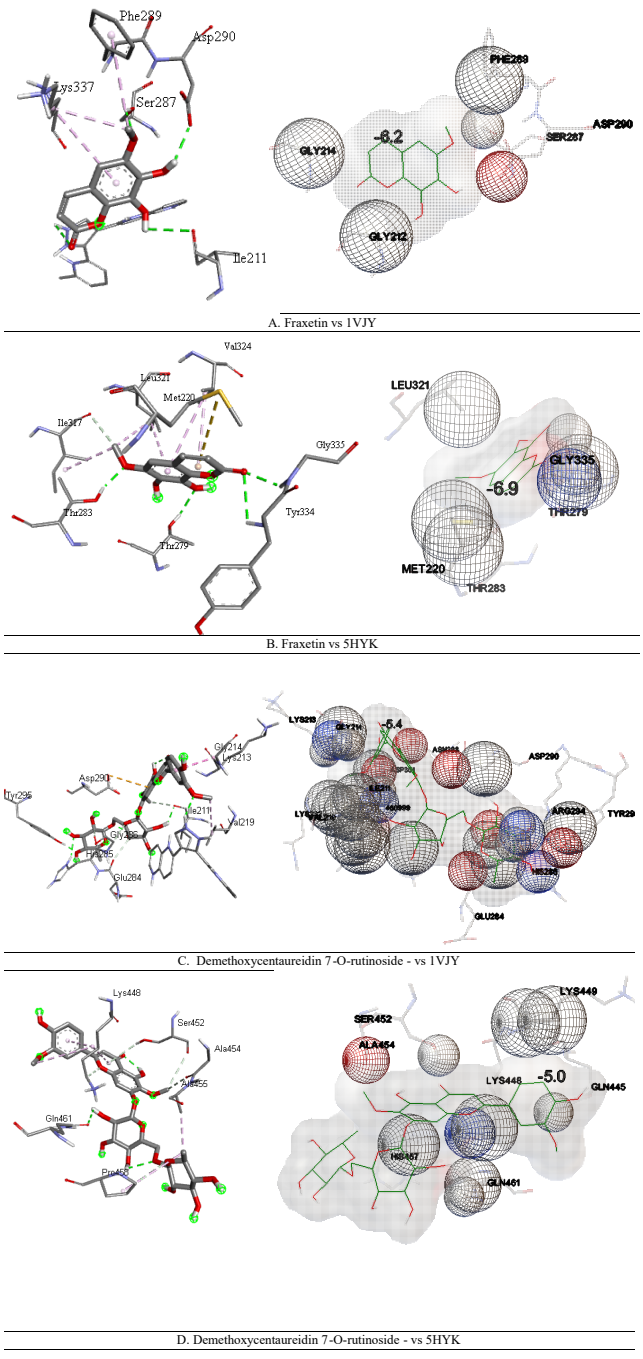


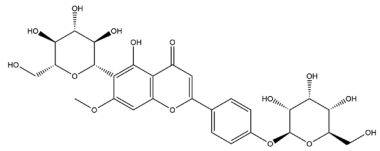
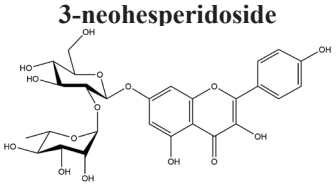
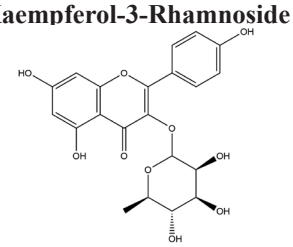
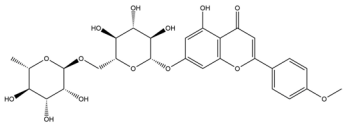
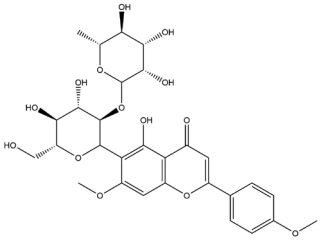
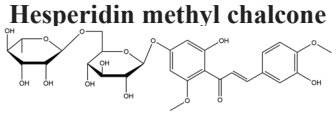
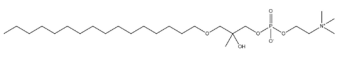
Fig. 5. The interactions of the most effective discovered compounds were shown using Discovery Studio Visualizer 3.5 and Auto Dock Tool, which provided a graphical representation of docked complexes in three dimensions.

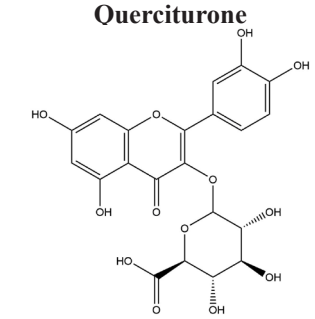
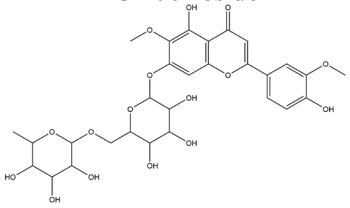
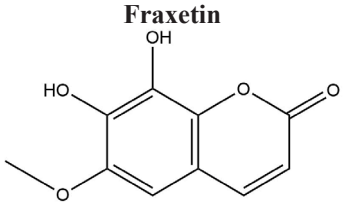
Table 2. List of bioactive constituents tentatively identified in the *L. monopetala* extract by LC-QTOF-MS method.

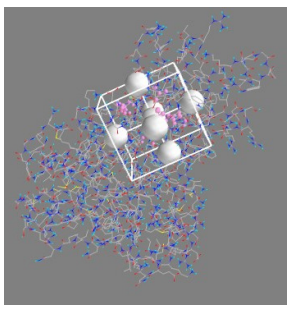
Sl No	RT (min)	Adduct	Mass (m/z)	Main fragment ions	Tentative identification	Molecular Formula	Exact mass	Reference
1	2.64	[M+H] +	209.03	209.0335, 287.0382	Fraxetin	$C_{10}H_8O_5$	208.03	[24]
2	3.60	[M+H] +	479.05	287.0347, 303.0284, 479.0542	Querciturone	$C_{21}H_{18}O_{13}$	478.07	**HMDB0029212**
3	4.00	[M+H] +	433.08	287.0347, 433.0838	Kaempferol-3-O-alpha-L-rhamnoside	$C_{21}H_{20}O_{10}$	432.10	[25]
4	4.51	[M+H] +	595.08	119.0441, 147.0359, 287.0382, 595.1097	Kaempferol-3- neohesperidoside	$C_{27}H_{30}O_{15}$	594.15	HMDB0302645
5	8.55	[M+H] +	496.31	98.9784, 104.0989, 124.9895, 184.0603, 496.3144	1-O-hexadecyl-2-C-methyl-3-phosphatidylcholine	$C_{25}H_{54}NO_6P$	495.36	[26]
6	9.91	[M+H] +	625.25	184.0714, 389.1410, 405.1696, 479.0900, 479.2110, 480.2151, 552.1860, 552.2341, 609.2272, 625.1978, 625.2592, 626.2575	Hesperidin methyl chalcone	$C_{29}H_{36}O_{15}$	624.20	**HMDB0253112**
7	10.11	[M+H] +	609.22	184.0603, 377.1471, 391.1461, 461.1804, 476.1736, 550.2351, 591.2217, 609.1514, 609.1716, 609.1968, 609.2423, 610.2278	Flavocommelin	$C_{28}H_{32}O_{15}$	608.17	[27]

8	10.42	[M+H] +	593.23	431.1608, 445.1694, 460.1964, 533.2153, 534.2224, 593.1541, 593.2338, 594.2512	Acaciin	$C_{28}H_{32}O_{14}$	592.17	[28]
9	10.77	[M+H] +	639.25	91.0480, 133.0885, 184.0270, 184.0353, 184.0492, 184.0603, 184.0742, 185.0613, 312.1163, 384.1172, 389.1733, 390.1672, 405.1531, 407.1990, 479.2065, 503.2186, 545.1532, 545.1962, 545.2201, 573.2461, 623.1882, 623.2036, 623.2546, 639.2517, 639.2879, 639.2982, 639.3241, 640.2819	Demethoxycentaureidin 7-O-rutinoside	$C_{29}H_{34}O_{16}$	638.18	HMDB0037467

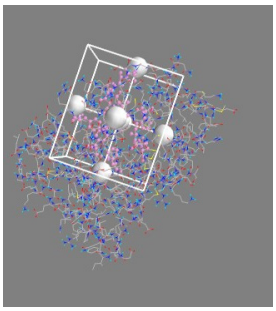
Table. 3. Molecular docking interactions between identified compounds of *L. monopetala* by LC-QTOF-MS with two target proteins, namely 1VJY and 5HYK.

SL No.	Compounds Name	AUTO DOCK MBE (kcal/mol)		Interacting residues		Hydrogen-bond interacting active binding site residues	
		1VJY	5HYK	1VJY	5HYK	1VJY	5HYK
1	**Flavocommelin** 	2.0	-5.5	ILE 211	ALA 455, PRO 458	LYS 337	ALA 454, LYS 448, LEU 456, GLN 461
2	**Kaempferol 3-neohesperidoside** 	-4.2	-5.8	VAL 219, ASP 290	PRO 458, LEU 456	GLU 209, GLU 284	HIS 274, HIS 457, ALA 455
3	**Kaempferol-3-Rhamnoside** 	-4.3	-4.5	ASP 290, GLY 212	PRO 458	SER 287, ASP 351, LYS 213	CYS 278, LEU 456, HIS 274
4	**Acaciin** 	-4.7	-3.6	GLY 212, LYS 213	LEU 456, LYS 448, HIS 457, PRO 458	GLU 284, SER 287, LYS 337	SER 452
5	**Embinin** 	-4.7	-3.9	ASN 338, GLY 214, LYS 337, SER 287	HIS 457, PRO 458, ALA 455	ASP 290	LYS 257
6	**Hesperidin methyl chalcone** 	-4.7	-5.3	ILE 211, ARG 294, TYR 291	PRO 458, CYS 278, HIS 457, HIS 274	ASP 290, TYR 295	SER 452, LEU 456, GLN 461
7	**1-O-hexadecyl-2-C-methyl-3-phosphatidylcholine** 	-4.9	-3.7	VAL 219, ILE 211, ARG 294, TYR 295	PRO 458, HIS 457, HIS 274, LYS 448, ALA 455	LYS 213, ASN 338, SER 287	LEU 456, SER 452

8	**Quercituron** 	-4.9	-4.4	VAL 219, ASP 290, ILE 211	ALA 455, PRO 458, HIS 457	SER 287, LYS 337	HIS 274
9	**Demethoxycentaureidin 7-O-rutinoside** 	-5.4	-5.0	ASP 290, LYS 213, VAL 219, GLU 284, GLY 286	ALA 454, SER 452, LYS 448, PRO 458, ALA 455	ILE 211, HIS 285, TYR 295	GLN 461
10	**Fraxetin** 	-6.2	-6.9	PHE 289, LYS 337	VAL 324, LEU 321	ILE 211, ASP 290, SER 287	TYR 234, GLY 335, THR 279, THR 283



A. 1VJY Grid box



B. 5HYK Grid box

Fig. 6. 1VJY (A) and 5HYK. (B) Grid boxes.

tion of hepatocytes as well as liver necrosis [33].

The result of the MTT assay of all tested compounds showed non-toxic and high survival rate in HepG2 cells as compared with H_2O_2-treated cells. Also, the morphological pictorial presentation of HepG2 cells corresponds with the results.

In order to evaluate cell viability, an MTT assay was conducted using HepG2 cells for the assessment of the drug's toxicity. The survival rate of cells treated with H_2O_2 was lower (18.55 %) than that of the untreated group. Methanol extract of *L. monopetala* was treated at a concentration of 100 µg/mL and showed cell viability of 98.69 %; Silymarin at a concentration of 100 µg/mL showed 100.46 % which indicates the non-toxic nature of the test compounds. The result of the MTT assay suggested, in combination with H_2O_2, LMME and Silymarin revealed 67.73 and 86.97 % of cell viability respectively showing hepatoprotective effects after recovery from H_2O_2 at 100 µM concentration (Table 1). Here, the LMME at a concentration of 100 µg/ml showed significant effects on hepatoprotection against H_2O_2 [34]. The cells treated with LMME and Silymarin alone at a concentration of 100 µg/mL exhibited no inflammation as well as toxicity after 24 h of incubation (Fig.1). HepG2 cells on treatment with LMME and Silymarin in addition to H_2O_2 showed more protection as compared to H_2O_2 treated group [18]. It shows that treatment with LMME has reversed the alterations in hepatic cells due to the presence of high content of flavonoid and phenolic bioactive compounds (Fraxetin, Kaempferol-3-Rhamnoside and Demethoxycentaureidin 7-O-rutinoside) which are well-known for hepatoprotective action in nature [35].

In-vivo hepatoprotective effects of test compounds at two dosage levels showed protection towards serum marker enzymes in a dose-dependent manner against the ccl_4 treated animals keeping Silymarin as standard and histopathology reports are in the same line with results.

In-vivo hepatoprotective study was carried out to strengthen the hepatoprotective nature of *L. monopetala* at lower and higher dose levels i.e., 200 and 400 mg/kg body weight. Silymarin is popularly taken as a standard drug that has been used as a hepatoprotective agent and is well tolerated by patients. It has anti-inflammatory and anti-fibrotic effects i.e., associated with the hepatic stellate cells (HSC) activation through the TGF-β1 expression and mast cell stabilization [36]. Serum marker enzymes like aspartate aminotransferase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), total protein, and

albumin were evaluated at the end of the study because these marker enzymes exist in the liver cells and variation of these parameters are the indication of hepatic damage [37]. In this present study, the animals treated with CCL_4 alone intraperitoneally showed elevated levels of serum biochemical parameters like AST, ALT, and ALP due to the severe toxicity of hepatic cells and reduction in total protein levels indicating difficulty in the synthesis of protein. Whereas, pre-treatment with LMME (both the dose levels) and Silymarin-treated groups decreased the levels of AST, ALT, and ALP values and normalized the total protein and albumin levels (Fig. 2; A-E) [18, 38]. CCl_4-induced hepatic damage is mainly associated with oxidative stress which is prevented by the antioxidative enzymes. LMME has protective effects on the serum marker enzymes as that of Silymarin due to the presence of antioxidant potential [37]. However, LMME at 400 mg/kg body weight showed more protection towards serum marker enzymes than that of LMME at 200 mg/kg body weight in a dose-dependent manner. Oxidative stress is closely associated with inflammatory response and is followed by cell death activation in hepatocytes [20]. PPAR-α belongs to the family of nuclear receptors which is highly active in hepatic cells, renal tubule, and cardiomyocytes [39]. As per the previous report, TGF-β1 is majorly associated with liver fibrosis and chronic liver diseases. The effects of *L. monopetala* (200 and 400 mg/kg body weight) and Silymarin (100 mL/kg body weight) on the histopathology of CCl_4 intoxicated hepatic cells are depicted in Fig. 3. Histopathology of liver cells of the control group showed regular architecture of distinguished hepatic cells and sinusoidal space (Fig. 3A). In contrast, the CCl_4 intoxicated group exhibited severe destruction of hepatocytes with lymphocyte infiltration, irregular cellular boundaries, and necrosis (Fig. 3B). Pretreated with LMME (200 and 400 mg/kg. body weight) and Silymarin with CCl_4 showed improved hepatic architecture with less cytotoxic and more protection as compared to the CCl_4 treated alone (Fig. 3C - E).

The LC-QTOF-MS was assessed in positive ionisation mode and the MS data showed tentative identification of 10 compounds which are mostly flavonoid and phenolic in nature. The water-acetonitrile system had better efficiency in the separation technique with low noise and the retention time (RT) of the compounds was more stable than the water-methanol system.

The results of the Liquid Chromatography Quadrupole Time of Flight Mass Spectrometry (LC-QTOF-MS) study of *L. monopetala* tentatively reported 10 major compounds like 3,15-Diacetyldeoxynivalenol; Fraxetin; Querciturone; Kaempferol-3-Rhamnoside; Kaempferol 3-neohesperidoside; 1-O-hexadecyl-2-C-methyl-3-phosphatidylcholine; Hesperidin methyl chalcone; Flavocommelin; Acaciin; Demethoxycentaureidin 7-O-rutinoside; Embinin and Triacylglycerol etc (Table 2.). They are mostly flavonoid, phenolic, fatty acids, and coumarin nature of compounds. Flavonoid compounds are mostly used to treat vascular, gastrointestinal, and respiratory disorders which are utilized in folk medicines [40]. The LC-QTOF-MS data of LMME has been represented in Table 2 with the retention time, major fragments and exact mass of the compounds. The structure of the identified compounds has been presented in Table 3 using ChemDraw Professional 16.0 software.

LC-QTOF-MS profiling of *L. monopetala* stem bark extract exhibited a total of 10 main peaks which were identified by comparing their MS data (in the positive ionization mode) as available in the literature. The compound Fraxetin was identified by comparing the adducts and a major fragment ion (209.03 m/z) with the exact mass i.e., 208.03 m/z at RT 2.64 min. Querciturone (3.61 min) and Kaempferol-3-Rhamnoside (4.00 min) were detected with a similar comparison of main fragment ions with the exact mass of the compound in relevant literature i.e., (479.05 m/z, 478.07 m/z) and (433.08 m/z, 432.10 m/z) respectively. Similarly, the compound Kaempferol 3-neohesperidoside (4.51 min) and 1-O-hexadecyl-2-C-methyl-3-phosphatidylcholine (8.55 min) were identified with a fragmentation of 595.08 and 496.31 m/z respectively. Hesperidin methyl chalcone; Flavocommelin; Acaciin and Demethoxycentaureidin 7-O-rutinoside were eluted with a fragmentation peak of 625.25 m/z, 609.22 m/z, 593.23 m/z, 639.25 m/z, and 607.24 m/z respectively (Table 3) which was confirmed by comparing the peaks with the MassData Bank, HMDB, PubChem database and relevant literature. The promising compound Fraxetin has a fragmentation of 209. 03 m/z which corresponds to the precursor adduct peak [M+H] + ions whereas the exact mass is 208.03 m/z. The other promising compound Demethoxycentaureidin 7-O-rutinoside showed fragmentation of 639. 25 m/z due to the presence of precursor adduct [M+H] + ions, whereas, the exact mass is 638.18 m/z as per the relevant databases and literature. The observed fragmentation and adducts recorded in MS are correlated with the reported spectra and the promising compounds i.e., Fraxetin, Demethoxycentaureidin 7-O-rutinoside and Kaempferol-3-Rhamnoside were tentatively identified [24].

It has been reported that Fraxetin or 7,8-dihydroxy-6-methoxy coumarin, a phenolic coumarin exhibited hepato-protective activities on ccl_4-induced hepatic fibrosis in rats because of its strong antioxidative effects [41]. As stated by the Pass online, Fraxetin is antioxidant, hepatoprotectant, anti-inflammatory, lipid peroxidase inhibitor, kidney function stimulant, chemo-preventive, etc. The test extract has identified the presence of the Fraxetin compound by LC-QTOF-MS analysis. It has similar interacting amino acids to that of the standard drug Silymarin (Table 3). It shows good binding energy along with hydrophobic interactions with the amino acids of the receptors. The identified compound Demethoxycentaureidin 7-O-rutinoside has free radical scavenger, vasoprotective, chemopreventive, hepatoprotective, and lipid peroxidase inhibitor as cited by Pass online.

The Fraxetin and Demethoxycentaureidin 7-O-rutinoside are subjected to *in-silico* study using the proteins 1VJY and 5HYK targeting the TGF-β1 and PPAR-α receptors responsible for hepatoprotective activity and found to possess a good binding affinity.

The ten identified major compounds of *L. monopetala* showed a docked score ranging from 2.0 to -6.9 in which 'Fraxetin' exhibited the highest score of docking -6.2 and -6.9 binding mode with the residues of receptor Transforming growth factor (TGF-β1) and Peroxisome Proliferator-Activated Receptor α (PPAR-α) respectively (Fig. 5). The compound 'Demethoxycentaureidin 7-O-rutinoside' showed the docking score of -5.4 and -5.0 binding mode with the residues of receptor TGF-β1 and PPAR-α respectively (Fig. 5). The compound 'Kaempferol-3-Rhamnoside' showed the docking score of -4.3 and -4.5 binding

mode with the residues of receptor TGF-β1 and PPAR-α respectively [15].

The crystal structure of targets (TGF-β1 and PPAR-α) was found in the Protein Data Bank bearing PDB ID: 1VJY and 5HYK respectively [30]. Further, the ADME and toxicity analysis of the top docked compounds Fraxetin, Kaempferol-3-Rhamnoside and Demethoxycentaureidin 7-O-rutinoside was carried out with the help of the software SwissADME developed by the Swiss Institute of Bioinformatics and ProTox v3.0 suggested that the compounds adhere to the Lipinski's rule of 5, Abbott oral bioavailability score > 0.55 and had good physicochemical and intestinal absorption properties that are requisite for an oral drug. TGF-β1 signalling induces hepatic damage by inhibition of NF-κB which therefore contributes to the accumulation of lipids by disturbed lipid metabolism as well as enhancement of hepatic cell death and inflammation [42]. TGF- β1 is a prime profibrogenic cytokine and is used as a target for the treatment of liver fibrosis [43]. PPAR-α is one of the master regulators in the case of lipid metabolism in the fasting situation and it directly or indirectly impacts lipogenesis. It is also involved with fatty acid synthesis in different conditions. Certainly, PPAR-α ligands act by enhancing the lipoprotein lipase activity (LPL), thereby hydrolysis of triglycerides in the lipoprotein of rat liver [44].

The novelty of our present study was the scientific exploration of hepatoprotective activity based on the traditional claim of the plant. The hepatoprotective potential of stem bark methanol extract of *L. monopetala* may be relevant to the synergistic effects of the compounds Kaempferol-3-Rhamnoside, Demethoxycentaureidin 7-O-rutinoside and Fraxetin which does not only resist inflammation but also reduce oxidative stress and mitigate cell necrosis. Test extract at 400 mg/kg body weight revealed more potency than that of the low dose at 200 mg/kg body weight of *L. monopetala*. The above results illustrated the scientific basis for the potential use of *L. monopetala* stem bark as a hepatoprotective drug and for the treatment of liver fibrosis which justified the traditional claim.

The strength of the study is that *in-vivo*, *in-vitro*, and *in-silico* results are unidirectional in support of the folkloric claim. The exact compound responsible for the said activity needs further investigation relating to the isolation and characterization of the compounds along with *in-vivo* and *in-vitro* assays.

5. Conclusion

The present study demonstrated the promising hepatoprotective effects of stem bark methanol extract of *L. monopetala* (LMME) using *in-vivo* and *in-vitro* methods following the identification of compounds by LC-QTOF-MS. Here, we have compared the test extracts with the H_2O_2 control group keeping normal control as a reference in the *in-vitro* study, similarly CCl_4 treated group was kept as a control in the *in-vivo* study. In the end, histopathological data, biochemical parameters, and *in-silico* molecular docking validation support our findings and justify the folkloric claim that has been previously reported. Here the interaction between the identified compounds Fraxetin, Kaempferol-3-Rhamnoside and Demethoxycentaureidin 7-O-rutinoside from *L. monopetala* could be responsible for its hepatoprotective effects on CCl_4 and H_2O_2-induced hepatotoxicity through target protein activation of PPAR-α and TGF-β1.

Further, detailed studies in Fraxetin, Kaempferol-3-Rhamnoside Demethoxycentaureidin 7-O-rutinoside compounds are required to know the exact underlying mechanism of hepatoprotection which could be taken as lead compound or safe hepatoprotective drugs for clinical application.

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Credit authorship contribution statement

Subhasish Sahoo: Experimentation, manuscript writing, Interpretation of results, **Haseeb A. Khan:** Supervision, Review and Editing**, Durga Madhab Kar**: Manuscript review and editing, **Sovan Pattanaik**: Data analysis, Software, **Diptirani Rath**: Conceptualisation, Manuscript writing, interpretation of results.

Conflict of Interest

The authors declare that there is no conflict of interest.

Data availability

Data will be made available on request.

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Appendix

List of Abbreviations

ANOVA: Analysis of Variance; AST: Aspartate aminotransferase, ALT: Alanine transaminase; ALP: Alkaline phosphatase, ADT: Autodock Tool, CCl_3: Trichloromethyl radical, CCL_4: Carbon tetrachloride, CBT: Centre of Biotechnology, CO_2: Carbon dioxide, DSV: Discovery Studio Visualizer, DMSO: Dimethyl Sulfoxide, DMEM: Dulbecco's Modified Eagle Medium, ELISA: Enzyme-linked immunosorbent assay, FBS: Fetal bovine serum, H_2O_2: Hydrogen peroxide, HepG2: Human Liver Hepatocellular Carcinoma Cell line, IAEC: Institutional Animal Ethical Committee, LC-QTOF-MS: Liquid chromatography Quadrupole Time of Flight mass spectrometry, LMME: *Litsea monopetala* methanol extract, min: Minute; mL: Millilitre, m/z: mass to charge ratio, µL: Microlitre, MTT: 3-[4,5-dimethyµthiazol-2-yl]-2,5 diphenyl tetrazolium bromide, NCCS: National Centre for Cell Science, $OOCCl_3$: Trichloromethyl peroxyl radical, OECD: The Organization for Economic Cooperation and Development, PDB: Protein Data Bank, PPAR-α: Peroxisome Proliferator-Activated Receptor-α ROS: Reactive Oxygen Species, RPM: Rotation per minute, SOA: Siksha 'O' Anusandhan, SOADU: Siksha 'O' Anusandhan (Deemed to be University), SPS: School of Pharmaceutical Sciences, SEM: Standard error of mean, SPDBV: Swiss-PDB viewer, SDF: Structural Data Format, TGF-β1: Transforming Growth Factor-β1, TNF-α: Tumour Necrotic Factor-α, TP: Total protein, UPLC: Ultra Performance Liquid Chromatogra-

phy.

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