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Protective Effects of *Lemna minor Linn*. On Hepatic and Cognitive Impairments in Acetaminophen Induced Hepatic Encephalopathy in Rats

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Aim: *Lemna minor* Linn., an aquatic plant, is a promising novel therapeutic agent that has been traditionally used in ethnobotanical practices as an ecofriendly supplement for the management of various ailments. This study involves the evaluation of ethanolic extract of *Lemna minor* Linn. against Paracetamol-induced Hepatic Encephalopathy using in vitro and in vivo Models.

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Methods: The acute oral toxicity study of the ethanolic extract of *Lemna minor* (EELM) was conducted following the OECD-425 guidelines over a 14-day period. A total of nine animals were used for this toxicity assessment. The EELM was tested in Paracetamol(PCM)-induced bioactivation animal model at two different dosages 200 and $400\mathrm{mg/kg}$ in comparison with silymarin as a standard compound. The In vivo experimental study was conducted using Sprague Dawley rats which was divided into 5 groups each group containing 6 animals so the total no of animals used in the study was 30 animals. The treatment groups included: normal control ($0.5\%\mathrm{Na}-\mathrm{CMC}$), 100mg/kg silymarin (standard) and the EELM of two different doses of 200 $\mathrm{mg/kg}$ and $400\mathrm{mg/kg}$, p.o were administered to rats 10 hr before paracetamol ($100\mathrm{mg/kg}$) treatment. Rats were orally administered their respective doses every day for total 30days. Paracetamol-induced oxidative liver damage disrupted normal levels of liver enzymes, total protein, and bilirubin, while also depleting antioxidant reserves. This oxidative stress was strongly associated with paracetamol toxicity, leading to a marked depletion of glutathione (GSH) and impairing both memory and cognitive function in the animals. Behavioral parameters are performed to evaluate the effect of drugs on cognitive behaviour of animals. Morris water maze test was performed to study how animals learn and remembers the spatial information relying on distal cues to locate the hidden platform in an opaque water.
Additionally, elevated plus maze was performed to measures the anxious behaviour of rats, the criterion was tested based on the conflict between rats innate instincts to explore new environment and avoid open, well-lit areas. The potential protective effects of EELM was evaluated by measuring serum enzyme levels and antioxidants status in the liver and brain, further histopathological analysis was performed respectively.
Results: The acute toxicity study did not report any mortality or toxicity signs in animals. PCM toxicity led to a statistically increase in the liver and body weight, along with brain water content. The PCM-intoxicated group exhibited a marked reduction in the level of superoxide dismutase (SOD) and glutathione (GSH) in the brain and liver, as well as an increase in lipid peroxidation and serum biomarkers (AST, ALT, ALP, and total bilirubin). EELM significantly ($\mathrm{P}<0.01$) reduced liver injury by inhibiting ALT, AST and ALP levels in serum. SOD, GSH and MDA liver content were significantly ($\mathrm{P}<0.001$) elevated by EELM, compared to PCM treated rats. Comparing the treatment and induced group, the treated group successfully recovered the activity of antioxidant levels and also been acknowledged for restored liver functioning by alleviating oxidative stress and also GSH level in brain was significantly $(\mathrm{p}<0.01)$ increased by EELM and preserved the histology of brain, which was chronically produced over a period of 30 days.
Conclusion: The findings of this investigation indicate the traditional use of *Lemna minor* in hepatoprotection and neuroprotection by regulating oxidative stress and mitigating reactive oxygen species (ROS). The insights gained from this research contribute to the development of novel therapeutic agents and paves the way for further studies on *Lemna minor* to enhance health outcomes, particularly in the management of neurodegenerative diseases.

Keywords: Lemna minor Linn; hepatic encephalopathy; paracetamol; acute toxicity; morris water maze; elevated plus maze, oxidative stress, reactive oxygen species.

1. INTRODUCTION

Chronic liver injury is a condition that gradually damages the liver, leading to its progressive destruction. If left untreated, it can impact other vital organs and result in various liver-related disorders (Wang et al. 2021). In contrast, acetaminophen overdose can induce severe acute liver injury and hepatic failure, which can lead to death or an emergency liver transplantation. Severe chronic liver damage and hepatic failure, caused by an overdose of paracetamol, could necessitate an immediate liver transplant.

Acute or chronic liver injury, if untreated, leads to hepatic encephalopathy (HE), a condition where toxin levels build up in the blood, impairing the liver's filtering capacity and allowing toxins to reach the brain. This results in metabolic disturbances and dysfunction in the central nervous system, which manifests as various behavioral and clinical symptoms (Badal and Bajaj 2023).

Precipitants of HE include electrolyte disturbances, renal insufficiency, constipation, infections, gastrointestinal bleeding, and use of

selected medications for prolonged period of time.

Currently, in the United States, Europe, and Australia, acetaminophen(N-acetyl-para-aminophenol-APAP) is the main factor contributing to acute liver failure (Andrade et al. 2019). Approximately 79% of the general population in the United States frequently consumes APAP (Atkinson and Fudin 2020). Since phenacetin was withdrawn from the market due to its nephrotoxic risks, APAP has been available in the United States. After being generally acknowledged as a safer substitute, APAP rose to prominence as the most often prescribed prescription for children and adults worldwide and became one of the most widely used over-the-counter analgesic-antipyretic combinations (Yang et al. 2022). It has been reported from previous studies that paracetamol overdose as low as 10 g (or $200\mathrm{mg/kg}$ for individuals weighing less than 50 kg) can lead to severe liver toxicity. Toxic effects may also occur from repeated consumption of doses only slightly exceeding the recommended daily therapeutic limit. Even medications which are considered as the safest for therapeutical use can cause toxicity if used predominantly over an extended period of time (Yang et al. 2022).

It has also been noted that paracetamol caused behavioral changes in chicks and pathological alterations in rats compared to the control group for chronic use (Buhrer et al. 2021). APAP is found in a lot of prescription and over-the-counter formulations, either by itself or in conjunction with other medications. The treatment of choice for paracetamol poisoning is N-acetylcysteine whereas for HE treatment regimen involves disaccharides like lactulose and lactitol, Branched-chain amino acids (BCAAs), rifaximin, L-Ornithine L-Aspartate, Zinc, etc. However there are very few FDA approved drugs for treating HE so this research focuses on developing potential outcome of therapeutic products for treating HE. Overdosing on self-medication is a prevalent habit that frequently results in APAP intoxication. Even at dosages below the threshold for hepatic toxicity, the metabolite N -acetyl-p-benzoquinone imine (NAPQI), which plays a key role in liver damage when repeatedly consumed, causes oxidative stress and depletes glutathione in the brain. NAPQI combines with mitochondrial proteins to produce adducts, which causes oxidative stress, nuclear DNA fragmentation, and ultimately necrosis of the cell. After paracetamol is de- acetylated to p-aminophenol, the hepatic microsomal cytochrome P450 enzyme system metabolizes it to produce the poisonous chemical p-benzoquinone (p-BQI). NAPQI and p-BQI are detoxified under normal physiological conditions by binding to glutathione (GSH) and gets eliminated by kidneys (Philippot et al. 2022).

Lemna minor Linn. (whole plant), is an aquatic plant commonly known as duckweed and is a well established herbal medicine recognized for its therapeutic safety in humans and animals (Sosa et al. 2024). The well-developed and diverse pharmacological activities of *Lemna minor* were evaluated through in vitro studies (Al-Snafi et al. 2019). The ethanolic extract of *Lemna minor* showcased a noteworthy reducing property, primarily attributed to its high content of flavonoids, carotenoids and amino acids. These compounds are postulated to contribute to the antioxidant activity, given that phenolic compounds are known for their direct antioxidant and anti-inflammatory properties. This study scrutinizes the potential of natural antioxidants to ameliorate oxidative modifications. Herbal agents, exemplified by flavonoid extracts, are deliberated upon for their antioxidative attributes, characterized by a diminished side-effect profile relative to synthetic analogs Silymarin, a well known ancient medicine, is a polyphenolic flavonoid extract commonly known as milk thistle. This chemical is an bioactive component used to standardize pharmaceutical substances and has been studied for its potential protective effect on liver and brain function (Elsawy et al. 2021). Therefore, the study was conducted to investigate the long-term chronic effect of paracetamol $(100\mathrm{mg/kg})$ on liver and brain without inducing acute high dose liver injury. The research is mainly targeted on long-term consumption of paracetamol and its impact on oxidative stress and cognitive behaviour in rats. Additionally, the study aims to evaluate the ameliorative properties of *Lemna minor* extract on hepatoprotection and neuroprotection through *in vitro* and *in vivo* (biochemical) and histological analyses.

2. MATERIALS AND METHODS

2.1 Collection of Plant Material

The *Lemna minor* Linn. Whole plant, which belongs to Araceae family, was collected from Chikkabemmathi, Hassan, India, during March and April of 2024. The plant's taxonomy was verified by botanists at the Central Ayurveda Research Institute, Bangalore and it was

identified and authenticated as *Lemna minor* Linn. belonging to the Araceae family (RRCBIMUS428). This identification was corroborated with the issuance of the official authentication number: SMPU/CARI/BNG/2023-24/2635(A).

The plant was shade-dried and pulverized into a fine powder using a mortar and pestle. A 500 g sample of the dried plant material was subjected to extraction via a Soxhlet apparatus, employing $1{,}500\ \mathrm{mL}$ of 70% ethanol as the solvent. The total yield obtained was 20 g of extract.

Chemicals: Alkaline phosphatase, Alanine aminotransferase, Total protein, Aspartate aminotransferase and Total bilirubin kits (Transasia Bio-medicals Ltd), Silymarin tablets. The remaining reagents utilised in the tests were all highly pure and analytical grade.

2.2 Experimental Animals

The animals used in the study was female Wistar rats, weighing 180-200 grams. They were procured from the Central Animal House at Krupanidhi College of Pharmacy. Three rats were housed in each polypropylene cage within dedicated enclosures. The bedding material was replaced every 24 hours to maintain hygiene. The animals were kept under controlled environmental conditions, including a regulated circadian rhythm, 50% humidity and a temperature range of $23-24^{\circ}\mathrm{C}$. The study protocol number to perform animal study is KCP/IAEC/PCOL/125/AUG-2023, which was approved by Institutional Animal Ethics Committee.

2.2.1 Experimental model

Prior to animal dosing, animals are weighed individually and are grouped into 5 of 6 rats in each and Complete dosing period is 30days.

Group-I: Negative control ($0.5\%\mathrm{Na}-\mathrm{CMC}$).
Group-II: Positive control (Paracetamol, 100mg/kg *p.o*), for 30 consecutive days (*p.o*).
Group-III: Low dose of *Lemna minor* Linn (200mg/kg p.o) and paracetamol (100mg/kg) for 30 consecutive days (*p.o*).
Group-IV: High dose of *Lemna minor* Linn (400mg/kg *p.o*) and paracetamol (100mg/kg) for 30 consecutive days (*p.o*).
Group-V: Standard drug (Silymarin 100mg/kg *p.o*) and paracetamol (100mg/kg) for 30 consecutive days (*p.o*).

2.3 Qualitative Phytochemical Analysis

2.3.1 Preliminary phytochemical screening

Using Kokate standard processes, qualitative chemical techniques was used to carry out the preliminary phytochemical screening. Proteins, amino acids, phenols, alkaloids, flavonoids, tannins and triterpenoids were all examined in *Lemna minor*'s ethanolic extract.

2.3.2 Antioxidant reducing power assay

One commonly observed phenomenon that indicates electron-donating activity-a critical mechanism of phenolic antioxidant activity is Fe (III) reduction. In this assay, antioxidants present in the samples facilitate the reduction of Fe^{3+} to Fe^{2+} by donating an electron. By measuring the intensity of Perl's Prussian blue colour, one can determine the extent of antioxidant property of the plant material at 700 nm. It was demonstrated that as the concentration of the extract increased, its reducing power correspondingly enhanced. The extract exhibited remarkable activity, indicating that *Lemna minor* possesses the ability to exhibit reducing activity and donate electron moieties.

2.4 Determination of Acute Toxicity Study

To conduct the acute toxicity study, healthy albino Wistar rats weighing 180-200 g was selected and maintained under standard laboratory condition, in complaince with OECD-425 criteria. Following the administration of ethanolic extract of *Lemna minor* upto 2000mg/kg, lethal signs are meticulously monitored for 4 hr and periodically during first 24 hr treatment period, during which the rats were deprived of food but had ad libitum access to water for the first 3-4 hours post-administration. Any signs of toxicity that was observed during the study was including grooming, sedation, sleep changes, tremors, diarrhoea and mortality for a total of 14days.

2.5 Tissue Preparation

The brain and liver were removed, weighed, and thoroughly cleaned with saline solution. Tissues were sliced individually and homogenized ($10\%\mathrm{w/v}$) in ice-cold sodium potassium phosphate buffer ($0.01\mathrm{M},\mathrm{pH}7.4$) with $1.15\%\mathrm{KCl}$ using a Remi electrotechnik homogenizer. The homogenate was then centrifuged at $10\times$ g for 20 minutes.". Antioxidant parameters of liver and

brain were performed by utilizing the obtained supernatant layer.

2.5.1 Biochemical analysis

After 30 days of consecutive dosing period, biochemical markers were evaluated by collecting 2 ml of blood from the retro orbital plexus whilst under a light ether anaesthesia. Collected blood is subjected to centrifugation for 10 minutes at 3000 rpm and $4^{\circ}C$ to obtain serum. Finally from the obtained serum biochemical analysis is performed.

2.5.2 Effect of EELM on serum biomarkers

The Effect of EELM on biochemical parameters i.e, ALT, AST, ALP, Total bilirubin and Total protein levels are measured by automated analyzer. All the mentioned biochemical serum markers were analyzed using Erba kits (Transasia biochemistry testing kits).

2.6 Behavioral Assessment

2.6.1 Morris water maze

The Water Maze consists of a circular pool filled with water, where rats are trained to reach an escape platform that is hidden below the surface of the water. The experimental setup included a circular pool measuring 200 cm in diameter and 60 cm in depth, filled with water at a temperature of $24-25^{\circ}C$, reaching 30 cm above the pool's base. Various visual cues were placed for animals. A hidden platform, 20 cm in diameter, was submerged below the water's surface i.e, 2 cm and remained fixed in one of the pool's quadrants. During the training phase, the rats were trained over six consecutive days, with three trials per day, to locate the hidden platform. The rats are permitted to stay for 15 sec, if it fails to find platform within 90 sec. The average time taken to find the platform during these training trials, known as the escape latency, was used to evaluate the rats spatial learning behaviour.

2.6.2 Elevated plus maze

In the elevated plus maze test, each rat from the experimental groups was placed on the central platform, initially facing the open arm, and given five minutes to explore the maze freely during training period. behavioural data were recorded using a digital camera for duration of 3min and observed for duration of time spent in open and closed arms.

2.7 Measurement of Brain Edema

The brain water content is determined by the wet/dry method. The animals are euthanized by A sample of cerebral cortex tissue, was extracted and weighed both before and after the collection of sample, it was incubated for 48 hours at $120^{\circ}C$ in an oven. The formula used to determine the water content of the brain samples was,

Water (%) = [(wet weight - dry weight) / wet weight] × 100.

3. HISTOPATHOLOGICAL EXAMINATION

Cervical dislocation is the euthanasia method followed, then extraction of brain and liver samples from each group which is fixed in saline with 10% formalin. Collected samples of liver and brain is weighed and further evaluation for structural alteration is processed.

4. STATISTICAL ANALYSIS

Graph pad prism was the platform used to conduct data analysis and collated data were analyzed using one-way analysis of variance (ANOVA) to assess the level of significance, followed by Dunnett's test to determine the level of significance followed by Dunnett's test. The results are expressed as Mean $\pm$ SEM ($n = 6$), with a P value of < 0.05 considered statistically significant.

5. RESULTS

5.1 Acute Toxicity Studies

During 14 days of toxicity study period no reported side effects or animal deaths was observed even at a single dose of 2000mg/kg of EELM has no toxicity signs. The dosage of extract to test on paracetamol induced-bioactivation model was selected at two different safe dosages i.e, 200 and $400 mg/kg$.

5.2 Preliminary Phytochemical Screening

EELM showed positive results for the presence of flavonoids, alkaloids, proteins, amino acids phenolic compounds, tannins, steroids, glycosides.

5.3 Antioxidant Reducing Power Assay

One of the most prominent indicators of electron-donating activity in phenolic antioxidants is Fe

(III) reduction. Fe^{3+} would be reduced to Fe^{2+} by giving up an electron due to the antioxidants present in the samples. The amount of antioxidant potential in the sample can be determined at 700 nm by measuring the intensity of Perl's Prussian blue color. It was observed that as the concentration of the extract increased, its reducing power also intensified. The ethanolic extract of *Lemna minor* showcased a noteworthy reducing power activity. It revealed that *Lemna minor* could have the capability to donate electron group and show reducing power activity. A lower IC_{50} value indicates the strongest reducing power activity. Although the extract's IC_{50} value of 94.07% was slightly higher than that of ascorbic acid (54.72%), this result highlights the extract's potential as a complementary agent to ascorbic acid.

5.4 Evaluation Parameters of Paracetamol Induced HE Model

5.4.1 The modulatory effects of EELM on Liver weight (LW), Body weight(BW) and Brain water content(BWC) in the PCM-intoxicated HE model

The PCM intoxicated group showed a significant ($p < 0.0001$) increase in the LW when compared with the normal control group (as indicated in Table 1). In terms of liver weight, treatment with EELM at both low and high doses (as indicated in Table 1) appeared to be lower liver weights, as a result they were able to reduce PCM-induced liver damage. There has not been notable distinctive changes observed in body weight of treated and untreated groups. The control group had less brain water content in contrast paracetamol treated group showed significantly higher brain water content $p < 0001$ indicating possible drug-induced edema or damage to brain tissue. Rats treated with EELM notably reduced the brain water content.

5.4.2 Effect of EELM on serum biomarkers in PCM-intoxicated HE model

The EELM exhibited substantial decline in the levels of biochemical parameters of AST, ALT, TB, and ALP significantly and were comparable to the control as well as the induced group and significant increase in total protein content. Effects of PCM and EELM on serum hepatic indices in rats with HE (Table 2).

5.4.3 Effects EELM on antioxidant parameters in PCM-intoxicated HE model

Paracetamol induced groups showed an significant reduction in the antioxidant levels SOD, GSH, MDA. In contrast treatment with EELM and silymarin groups resulted in notable increase in antioxidant levels (Table 3).

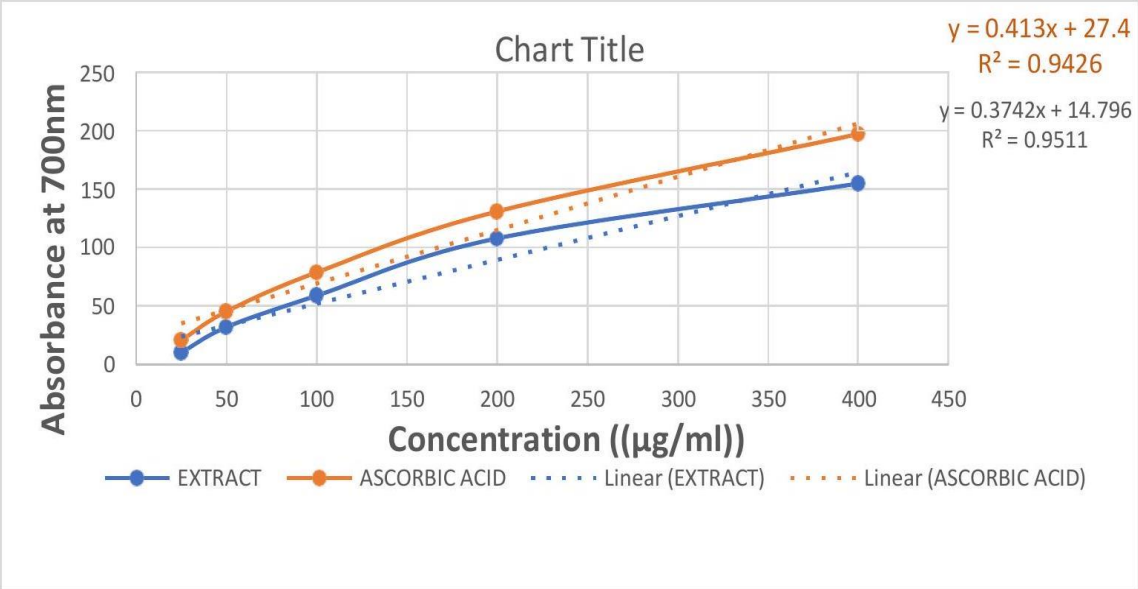


Fig. 1. The figure indicates the results of antioxidant power of ethanolic extract of *Lemna minor* extract

Table 1. The modulatory effects of EELM on Liver weight (LW), Body weight(BW) and Brain water content(BWC) in the PCM-intoxicated HE model

Sl.No	Groups	Liver(g)	Body weight(g)	Brain water content (%)
1.	Control	5.80 ± 0.10	206.5±1.13	64.78 ± 0.6
2.	Positive control group (Paracetamol)	$12.70 \pm 1.60^{****}$	$212.5 \pm 1.06^{***}$	82.76 1.98^{****}
3.	Paracetamol + Low Dose of EELM - 200mg/kg)	$8.10 \pm 0.80^{**}$	216.6±0.98*	78.89±0.8**
4.	Paracetamol + High Dose of EELM - 400mg/kg)	$6.88 \pm 0.08^{***}$	217.8 ± 0.56	71.7±0.18**
5.	Standard drug (Silymarin-100 mg/kg)	$7.45 \pm 0.10^{***}$	$209.5 \pm 0.21^{***}$	69.9 ± 0.87***

The Analysis is presented as Mean ± SEM of six rats, multiple ANOVA analogies were carried out followed by dunnetts test; groups I and II were compared, $^{****}p < 0.0001$ *and, when comparing all treated groups v/s positive control group significance values are denoted as follows respectively* $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ *on comparable days*

Table 2. Effects of PCM and EELM on serum cellular toxicity markers

Sl.No	Groups	ALT(U/L)	AST(U/L)	ALP(U/L)	Serum Bilirubin (g/dL)	Serum Protein (g/dL)
1.	Control	22.47 ± 0.3	114.5 ± 0.8	702.4 ± 0.6	0.35 ± 0.04	7.576
2.	Positive control group (Paracetamol100mg/kg)	$117.2 \pm 0.6^{****}$	$363.36 \pm 1.04^{***}$	$1073.93 \pm 1.5^{****}$	$1.56 \pm 0.08^{***}$	$4.313 \pm 0.48^{****}$
3.	Paracetamol + Low Dose of EELM (200mg/kg)	$75.9 \pm 0.20^{**}$	$203.03 \pm 1.18^{**}$	$862.03 \pm 0.7^{*}$	$0.69 \pm 0.01^{**}$	3.986 ± 0.45^{ns}
4.	Paracetamol + High Dose of EELM (400mg/kg)	$66.2 \pm 0.5^{***}$	$202.4 \pm 0.3^{**}$	$803.63 \pm 0.4^{**}$	$1.04 \pm 0.04^{**}$	5.27 ± 0.36**
5.	Standard drug (Silymarin-100mg/kg)	$58.5 \pm 0.2^{***}$	$147.66 \pm 0.2^{***}$	$772.4 \pm 0.2^{**}$	$0.69 \pm 0.07^{***}$	$5.45 \pm 0.61^{***}$

The Analysis is presented as Mean ± SEM of six rats, multiple ANOVA analogies were carried out followed by dunnets test; groups I and II were compared, $^{****}p < 0.0001$ *and, when comparing all treated groups v/s positive control group significance values are denoted as follows respectively* $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ *on comparable days*

Table 3. Effects of PCM and EELM on antioxidant parameters in rats with HE

Sl no	Groups	SOD(Units/ml)	GSH(units/ml)	MDA(enzyme level nmol/ml)
1.	Control	47.4 ± 1.43	4.47 ± 0.07	5.72 ± 0.11
2.	Positive control group (Paracetamol 100mg/kg)	$29.80 \pm 2.14^{***}$	$1.40 \pm 0.10^{****}$	$10.23 \pm 0.31^{****}$
3.	Paracetamol + Low Dose of EELM -200mg/kg)	$34.2 \pm 1.96^{**}$	$2.32 \pm 0.06^{*}$	$6.43 \pm 0.29^{**}$
4.	Paracetamol + High Dose of EELM -$400\mathrm{mg/kg}$)	$39.74 \pm 2.03^{***}$	$2.68 \pm 0.16^{**}$	$7.7 \pm 0.11^{***}$
5.	Standard drug (Silymarin-100 mg/kg)	$41.1 \pm 2.01^{**}$	$3.45 \pm 0.16^{***}$	$7.41 \pm 0.19^{****}$

The analysis is presented as the Mean $\pm$ SEM of six rats, and multiple ANOVA analogies were carried out; groups I and II were compared by assembling $^{****}p < 0.0001$, respectively, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$

Table 4. Effect of *Lemna minor* and Silymarin on GSH level in paracetamol induced oxidative stress in Brain

Sl.No	Groups	GSH(units/ml)
1.	Control	4.47 ± 0.07
2.	Positive control group (Paracetamol)	$1.40 \pm 0.10^{****}$
3.	Paracetamol + Low Dose of EELM- 200mg/kg)	$1.74 \pm 0.06^{**}$
4.	Paracetamol + High Dose of EELM - 400 mg/kg)	$1.98 \pm 0.16^{**}$
5.	Standard drug (Silymarin-100 mg/kg)	$2.39 \pm 0.16^{***}$

The analysis is presented as the Mean $\pm$ SEM of six rats, and multiple ANOVA analogies were carried out; groups I and II were compared by assembling $^{****}p < 0.0001$, respectively, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$

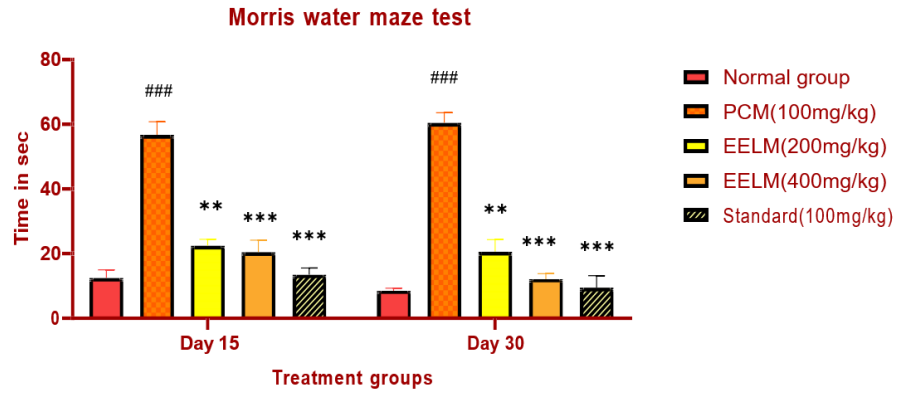


Fig. 2. Escape latency of rats in Morri's water maze test on Day15 and Day 30

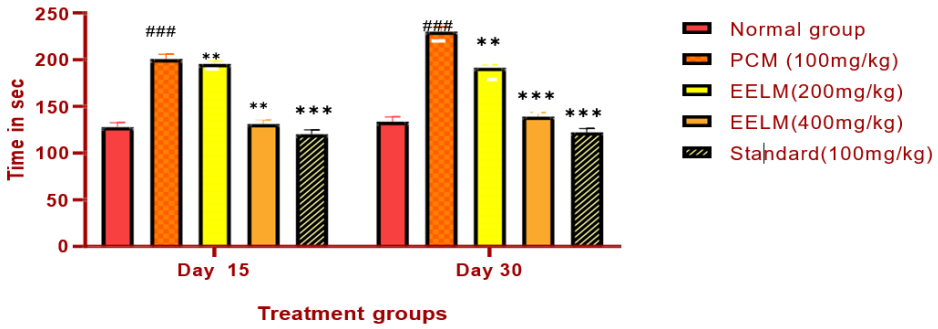


Fig. 3. Time spent in closed chamber on Day15 and Day 30

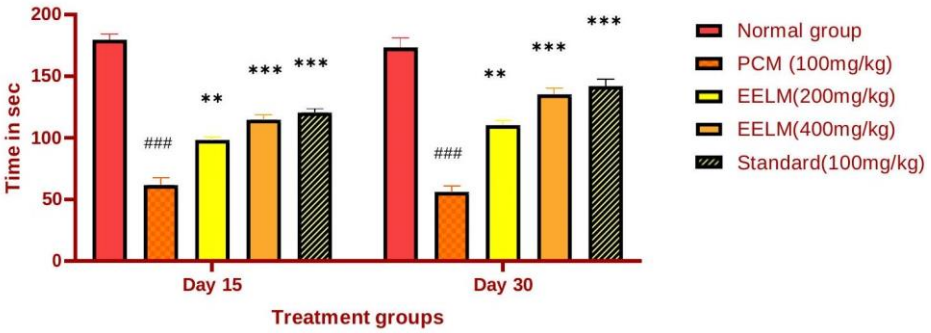


Fig. 4. Time spent in open chamber on Day15 and Day 30

5.4.4 EELM impact on GSH level in paracetamol induced oxidative stress in Brain

The results from Table-4 indicate that paracetamol significantly reduces GSH levels in the brain, implicating oxidative stress. Treatment with (EELM) at both low (200 mg/kg) and high (400 $\mathrm{mg/kg}$) doses increases GSH levels compared to the paracetamol-only group, with the high dose showing a more substantial effect of P value < 0.01. However, Silymarin ($100\mathrm{mg/kg}$) demonstrates the potential role, nearly restoring GSH levels to those of the control group this indicates the effective role brain normal physiological function.

5.5 Behavioral Assessment

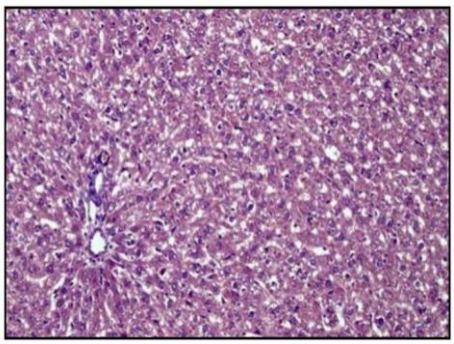
5.5.1 Morris water maze (MWM)

The test results in Fig. 2 includes, the paracetamol group treated exhibited a significant decrease in escape latency compared to the control group. The treatment with EELM at doses of 200 and $400\mathrm{mg/kg}$ effectively prevented the PCM induced increase in escape latency when compared to the PCM-induced group. The EELM treatment demonstrated the results comparable to those of the PCM treated group with the significance of P value < 0.001 of EELM($400\mathrm{mg/kg}$) and P value of < 0.01 of EELM($200\mathrm{mg/kg}$) is exhibiting the enhanced retention performance in the spatial navigation task.

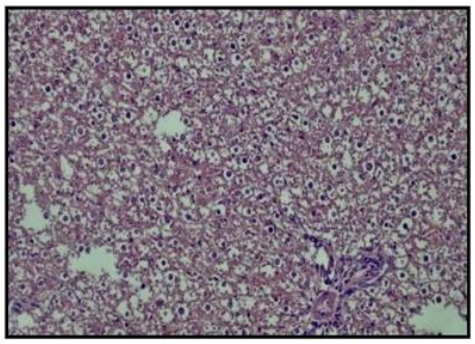
5.5.2 Elevated plus maze (EPM)

The obtained results showed that EELM treated animals substantially increased the open arm time and number of entries, while reduced the closed arm time and number of entries in EPZ (as indicated in Fig. 3 and Fig. 4).

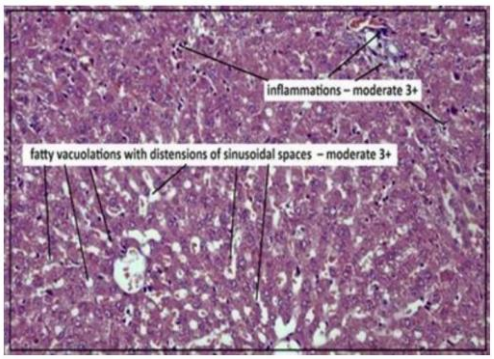
5.6 Histopathology Study of Liver tissue.



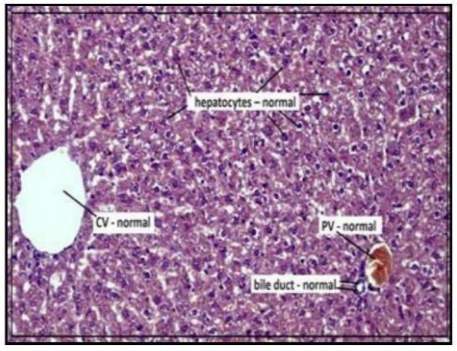
(a) Normal group histologic profile showing the hepatic lobule with no histopathological changes.



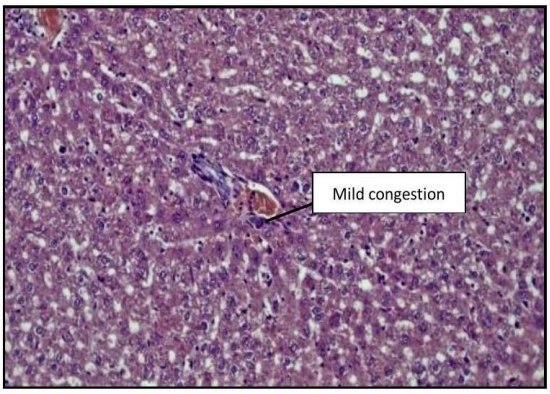
(b). Induced group (Paracetamol) showing hepatic necrosis distension of sinusoidal fatty vacuolations with distensions of sinusoidal spaces & inflammation moderate 3



(c) Induced & treated with ethanolic extract of *Lemna minor* low Dose ($200\mathrm{mg/kg}$), mild fatty changes and ballooning of hepatocytes- mild 2+, Bile ducts - normal & Portal Vein (PV) - normal - NAD+



(d) Induced & treated with ethanolic extract of *Lemna minor* high Dose ($400\mathrm{mg/kg}$) hepatic morphology - normal, Central Vein & Portal Vein - normal - NAD+, hepatocytes - normal, Central Vein, & Portal Vein with Bile ducts – normal- NAD+



(e) Induced and Silymarin treated group mononuclear aggregations in portal tract area- mild congestion (NAD+ indicates - No Abnormalities Detected)

Fig. 5. Liver tissue histologic profile

Table 5. Scoring of the liver tissue histopathological results

Groups	Inflammation	Necrosis	Steatosis	Hemorrhage
Normal group	-	-	-	-
Positive control group (Paracetamol)	+++	++	+++	+++
Paracetamol + Low Dose of EELM - 100mg/kg)	+	++	-	+
Paracetamol + High Dose of EELM- 200mg/kg)	-	+	+	-
Standard drug (Silymarin-100 mg/kg)	-	+	-	-

The scoring scheme is evaluated based on severity of hepatic injury when compared with control group: normal (-), mild effect (+), moderate effect (++), severe effect (+++)

5.7 Histapathological Study of Brain Tissue

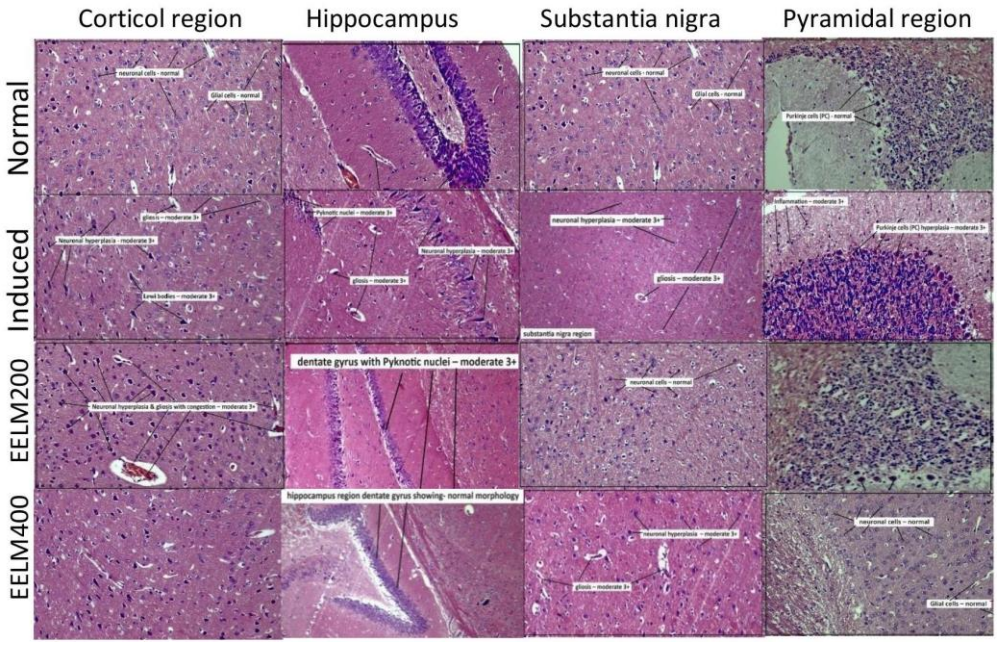


Fig. 6. The histopathological findings indicate that the effects of 30days of treatment of EELM and paracetamol therapy on brain tissue

(a) Histologic evaluation of showing control group with normal architecture of neuronal cells without any degeneration of brain tissue.
(b) Positive control group: The impact of 30days paracetamol treatment resulted in inflammation, neuronal and purkinje hyperplasia, and gliosis.
(c) Induced & treated group with ethanolic extract of *Lemna minor* of Low Dose (200 $\mathrm{mg/kg}$): Cortical and hippocampus region showing dentate gyrus, pyknotic nuclei (mild $2+$) and few degenerated neurons in pyramidal hippocampal region , substantia nigra region showed neuronal cells - normal (NAD+ - No Abnormalities Detected)
(d) Induced & treated with ethanolic extract of *Lemna minor* high Dose (400mg /kg): Cortical region showed Glial cells & neuronal cells normal morphology hippocampus region dentate gyrus showed normal morphology substantia nigra, pyramidal region showed neuronal cells & Purkinje cells (PC) normal morphology (Test Drug induced beneficial response).
(e) Induced and Silymarin treated group: showed an normal nerve cells with greater extent of reduction in necrosis of cells.

6. DISCUSSION

The uncommon but potentially life-threatening adverse drug reaction, known as drug-induced liver injury (DILI), is characterized by notable heterogeneity in both the onset and type of liver damage (Hayashi and Bjornsson 2018). Chronic liver injury can develop following prolonged exposure to the offending agent. Extended consumption of such substances, particularly those causing cholestatic damage, appears to exacerbate the risk of the condition progressing to chronicity (Hosack et al. 2019). The current study explores every avenue for understanding the potential hepatoprotective and neuroprotective benefits of EELM in rats suffering from hepatic encephalopathy due to the advanced stages of chronic liver condition (Ochoa-Sanchez and Rose 2018) induced by paracetamol (Teschke 2020). PCM intoxication model is an well-established preclinical paradigm model for induction of Hepatic encephalopathy (HE) because as it closely resembles the pathological symptoms and well-defined hepatic and cerebral changes observed in humans (Schiodt et al. 1999). The bioactive compounds derived from medicinal herbs have garnered significant interest for their potential in treating various disease conditions. In contrast, synthetic drugs have been associated with adverse clinical reactions, often outweighing their therapeutic benefits. One such medicinal plant is *Lemna minor* Linn (common duckweed) an aquatic plant possessing allelopathic potential (Gostynska et al. 2022), traditional and pharmacological investigations have demonstrated its antibacterial, antioxidant, antipruritic, antiscorbutic, cytotoxic, and immunomodulatory properties (Al-Snafi 2019). In general evaluation of ethanolic extracts of five Lemna species were identified and quantified with three chlorogenic acid derivatives and eleven apigenin and luteolin derivatives by HPLC-DAD/MS analysis and also containing rich source of amino acids (Chakrabarti 2018) from these studies it confers that the potential bioactive components presence can contribute to the treatment of various disease ailments.

The rich reservoir of bioactive components, notably twelve components with antioxidant property were identified in *Lemna minor* especially the flavone components i.e, apigenin(isovixetin) and luteolin(isoorientin) (Olasehinde and Olaokun 2024). Studies have revealed that apigenin possesses anxiolytic effects, which attenuate depressive-like behavior and improve sensorimotor and motor coordination in animals with cognitive impairment and neurobehavioral deficits (Cichon et al. 2021) by reducing the inflammatory markers (TNF-α, IL-6, IL-1β) (Olasehinde et al. 2024) and helpful in attenuating the liver inflammatory condition as it is confirmed by histopathological study (Fig. 5). Whereas, luteolin has potential to cross BBB and helps to suppress the oxidative stress, apoptosis and enhanced neuronal growth (Jayawickreme et al. 2024). Amoung amino acids, leucine and isoleucine was 48.67% of total essential amino acids (39.20%), whereas, Glutamic acid was 25.87% of total non-essential amino acids (53.64%). In case of hepatic encephalopathy glutaminergic transmission is affected leading to disturbance in the mental health. Glutamate, an essential amino acid and excitatory neurotransmitter, disruptions in its uptake can impair reward sensitivity, a key feature commonly associated with depression (Limon et al. 2021).

In the present study, Morris water maze method was employed to evaluate the cognitive functions related to special learning and memory deficits by measuring escape latency. Systemic inflammation plays a pivotal role in liver injury, and in cirrhotic patients with hepatic encephalopathy (HE), elevated proinflammatory cytokines are associated with cognitive decline compared to non-HE patients (Lalert et al. 2023).

Paracetamol (PCM)-intoxicated rats exhibited a significant increase in the escape latency (Fig. 2) indicating impairment in both acquisition phase and retention phase and this can be strongly correlate to the histopathological findings (Fig. 5), Paracetamol (PCM)-intoxicated rats exhibited a marked increase in escape latency (Fig. 2), indicating impairments in both the acquisition and retention phases of learning. These cognitive deficits strongly correlate with the histopathological findings (Fig. 5), which revealed degeneration of hippocampal neurons.
Conversely, treatment with EELM at doses of $200\mathrm{mg/kg}$ and $400\mathrm{mg/kg}$ significantly enhanced cognitive performance, as demonstrated by reductions in both escape latency and path length. These findings align with those of prior studies (Nantachai et al. 2022).

In the elevated plus maze(EPM) test, PCM induced anxiety-like behaviors which reduced the open arm time and number of entries, while increased closed arm time and number of entries in EPM(Fig. 3 and Fig. 4). The well-established correlation between oxidative stress, inflammation, and cognitive impairment (Rotundo and Pyrsopoulos 2020) provides compelling evidence of how PCM-induced toxicity adversely affects both cognitive and exploratory behavior, along with disrupting antioxidant homeostasis. This revealed anxiety-like behaviors and depression. In contrary EELM treated groups showed protective role by inhibiting further neuronal degeneration (Fig. 3c and Fig. 3d) and also maintaining the GSH level (Table 4) and also these observed results are in parallel with the previous findings. The correlation between oxidant/antioxidant imbalance, inflammation, and cognitive impairment is widely established (Rotundo and Pyrsopoulos 2020). this gives a strong evidence of how paracetamol induced toxicity can impact on cognitive and exploratory behaviour of rats alongside disturbedantioxidant status.

In our study, PCM induced hepatotoxicity successfully demonstrated its deleterious impact on liver enzymes, evidenced by elevating serum levels of ALT, AST and ALP along with disruptions in total bilirubin and protein levels indicating oxidative stress and inflammation induced by paracetamol for a long-term (Ahmed et al. 2023). These studies have demonstrated the loss of hepatocyte membrane integrity, which leads to the leakage of liver enzymes and disturbances in protein synthesis.

Oxidative stress, primarily driven by the accumulation of reactive oxygen species. The study assessed the condition of oxidative stress generated by paracetamol by measuring the levels of ROS buildup, antioxidant factors, and oxidative stress factors. The findings highlighted a significant decrease in antioxidant enzymes within the paracetamol group [Table 3].

The effects of PCM on the antioxidant profile include the formation of ROS in the brain and hepatic tissues of the rats, as evidenced by elevated MDA levels, the byproduct of lipid peroxidation, and the depletion of SOD and GSH reserves. Based on obtained results the depletion of antioxidants was markedly modulated by administration of ethanolic extract of *Lemna minor* along with improvement in the serum levels of protein and depletion of bilirubin level when compared to PCM intoxicated group. From Table 3 and Table 4 it can be concluded that EELM significantly antagonized the increase in bilirubin and decrease in SOD, MDA and GSH levels with significant level of $\mathrm{p} < 0.001$ (Salman et al. 2020). This coincides with the documented improvement in neurological problems and histological abnormalities of the liver linked to PMC-induced HE.

In our investigation, oxidative stress the increased lipid peroxidation is related to depleted levels of GSH in PCM poisoning is the compromised antioxidant defense mechanism in brain tissue. These results are strongly supported by the outcomes of histopathology indicating the possible protective role accompanied by EELM on brain by preserving the tissue integrity and architecture (Fig. 5) and also this supports the previous studies (Lu et al. 2020).

Histopathological findings, which revealed significant impact on tissue architecture and were consistent with previous research, provided additional evidence of PCM-induced hepatotoxicity. Hepatocellular necrosis, inflammation, and neuronal hyperplasia were all exhibited in the sections of the liver from PCM-intoxicated patients [Table 5]. Rats intoxicated with PCM were pretreated with EELM, stabilized the hepatic enzymes and changed the permeability of the membrane, which was caused by a harmful effect of PCM on the integrity of cell membrane [Fig. 4].

The chronic oxidative stress induced by PCM was also investigated through histological

evaluations on the 15th and 30th days findings Liver damage is closely associated with peripheral inflammation and cytokine storms, which can cross the BBB and contribute to neuroinflammatory disorders (Lu et al. 2020). To evaluate the neuroprotective effects of EELM against HE, histopathological analysis was conducted. The results on 15^{th} day showed that normal morphology of cortical and pyramidal region but negative impact on gliosis, neuronal hyperplasia and dentate gyrus with pyknotic nuclei in hippocampal and substantia nigra. Whereas 30days of consecutive paracetamol intoxication resulted in inflammation, neuronal and purkinje hyperplasia, and gliosis.

These results corroborates the prior study (Lalert et al. 2023). The impact of high dose of EELM restored the brain morphology by attenuating the degenerative changes with a moderate effect has been noted and low dose of EELM has the moderate effect with significant value of $p < 0.01$. Therefore we can conclude that in the HE rat model, the hepatoprotective and neuroprotective properties of EELM were comparable to those of silymarin (Teksoy et al. 2020). Treatment with EELM in PCM-intoxicated rats effectively mitigated the biochemical alterations associated with HE and alleviated anxiety and depression like behaviors. Therefore, by restoring oxidative and inflammatory balance through its antioxidant and neuromodulatory properties, EELM demonstrated the effects analogous to those of silymarin, suggesting its potential as an effective hepato- and neuroprotective agent. This highlights its ability to prevent hepatic injury and reduce the risk of developing HE.

7. CONCLUSION

In summary, the study demonstrated that *Lemna minor* extract possesses significant antioxidant and hepatoprotective properties. The preliminary phytochemical analysis suggests that the bioactive compounds, particularly aminoacids (leucine, glutamic acid), flavones (agigenin, luteolin) contributed to the neuroprotection through reduction of oxidative stress. The safety profile of the extract (acute toxicity study), along with antioxidant reducing power assay is coupled with its ability to reduce oxidative stress by controlling antioxidant profile and serum hepatotoxicity indices which paves the path for protective nature against liver and cerebral damage, leading it to a promising candidate for treating the hepatic encephalopathy. Additionally, its positive impact on cognitive functional behaviour and neuroprotection opens an avenue for further exploration in various neurodegenerative disease models.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

1. Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of this manuscript.

ETHICAL APPROVAL

The study protocol number to perform animal study is KCP/IAEC/PCOL/125/AUG-2023, which was approved by Institutional Animal Ethics Committee.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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