# Hepatoprotective Effects of *Rotula Aquatica* Lour Against Acetaminophen-Induced Liver Injury: Phytochemical and Mechanistic Insights

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**Abstract**
**Background**: Drug-induced liver injury is one of the major clinical concerns and the reason for the search for effective natural hepatoprotective agents.
**Objective:** This study is done to assess the ameliorative effect of ethanolic extract of *Rotula aquatica Lour* (EERA) in paracetamol induced liver damage in Wistar albino rats.
**Methods:** This experimental animal study was conducted using Wistar albino rats to evaluate the hepatoprotective potential of ethanolic extract of EERA (EERA) against paracetamol-induced liver injury. Preliminary phytochemical screening of the ethanolic extract was performed to identify its bioactive constituents. The antioxidant potential was assessed using standard free radical scavenging assays. The cytotoxic safety profile of the extract was evaluated using the MTT (methylthiazol tetrazolium) assay in Chang human liver cell lines. Hepatotoxicity was induced in Wistar albino rats through administration of paracetamol. In the curative model, animals received EERA at doses of 200 mg/kg and 400 mg/kg (p.o.), or silymarin at 100 mg/kg (p.o.), for seven consecutive days following induction of liver injury. In the preventive model, EERA or silymarin was co-administered with paracetamol for three days. Each experimental group consisted of six animals (n = 6 per group). Silymarin (100 mg/kg) was used as the standard reference drug. Biochemical evaluation included analysis of serum liver function markers, assessment of endogenous antioxidant enzymes such as superoxide dismutase and catalase, measurement of lipid peroxidation levels, and detailed histopathological examination of liver tissues.
**Results:** Analysis of phytochemical constituents revealed the presence of flavonoids, triterpenoids, alkaloids and steroids. EERA exhibited marked antioxidant activity ($p < 0.05$). In the *in vivo* study model, EERA significantly improved serum biochemical parameters, enhanced antioxidant enzyme levels and reduced lipid peroxidation compared to the toxic control group ($p < 0.05$). In the curative model, treatment with EERA at 400 mg/kg markedly restored altered liver enzyme levels, with SGOT decreasing from $3.3 \pm 0.139$ in the toxic control group to $2.25 \pm 0.07$ and SGPT improving toward near-normal values compared with the paracetamol-treated group. Histopathological findings confirmed its hepatoprotective effects. Treatment with 400 mg/kg EERA produced effects similar to Silymarin at 100 mg/kg ($p < 0.05$). This shows that EERA protects the liver from damage, which is further confirmed by histopathological analysis.
**Conclusion**: The findings suggest that EERA exhibits promising hepatoprotective and antioxidant activity against paracetamol-induced liver injury in rats, warranting further molecular and clinical investigation.

**Keywords:** Antioxidant potential, Hepatoprotection, Natural therapeutics, Oxidative stress; Paracetamol-induced hepatotoxicity, *Rotula aquatica Lour*.

## 1. Introduction

The liver is a fundamental organ that is instrumental in maintaining overall physiological health by regulating metabolism, storing nutrients and detoxification [1]. Liver disorders are significant global health concern because of its high mortality and scarcity of effective therapeutic options. The etiologies of liver disorders include viral infections, metabolic dysfunctions, autoimmune responses and notably, drug induced liver injury (DILI) remains a leading cause of acute liver failure [2]. Overdose of acetaminophen can cause serious liver toxicity. Although treatments such as antiviral medications, corticosteroids and vaccines are commonly prescribed, their utility is often limited by side effects and inefficacy, emphasizing the urgent need for safer and more efficient therapies [3].
Nowadays alternative or herbal medicines have been widely acknowledged for hepatic disorders. This is backed by the world health organization endorsement of their integration into global healthcare practices [4]. Many herbs have been traditionally used for their hepatoprotective effects like *Silybum marianum, Andrographis*

*paniculata* (kalmegh) etc. Out of these, major studies are based on the plant species *Silybum marianum* (milk thistle), whose main activeingredient,silymarin, possesses antioxidant and hepatoprotective properties [5]. Its clinical use however, is challenged by poor water solubility, prompting the development of improved formulations to enhance bioavailability [6].
Beyond these herbal medicines, *Rotula aquatica Lour.* has multiple pharmacological activities as it has significant anti-urolithiasis, hepatoprotective antioxidative effects and anti-inflammatory benefits [7]. Various studies reported that *Rotula aquatic* extracts exhibit strong antioxidant activity and effectively reduce oxidative stress [8]. Safety evaluations confirmed that the plant has low toxicity, while *in vivo* studies highlight it reduces pro-inflammatory mediators, thereby protecting hepatic tissue. These effects are attributed to the presence of various bioactive compounds, including flavonoids, phenolics and tannins known to support liver function [9].
Considering the pathophysiology of liver diseases and the drawbacks of available marketed treatments, the exploration of *Rotula aquatica Lour.* as a natural hepatoprotective agent holds considerable promise [10]. This study therefore aims to evaluate liver-protective capabilities of *Rotula aquatica Lour.*

## 2. Materials and Methods

This experimental animal study was carried out on Wistar albino rats to assess the hepatoprotective effect of EERA against paracetamol-induced liver injury.

**2.1. Extraction procedure**

*Rotula aquatica Lour* plant was collected from the Kaliyar River area in Thodupuzha, Kerala, and authenticated by an expert botanist (Voucher No. 2261). The plant material was dried in shade at room temperature and made into a coarse powder. A quantity of 250 g of this powder was extracted by refluxing at 60–80°C with 800 ml ethanol for 3 hours. The method was repeated twice with fresh solvent under identical conditions [11]. The extracts were combined and concentrated under reduced pressure to produce thick syrup like liquid. This was further heated in a water bath to obtain the crude ethanolic extract [12]. After concentration, the crude ethanolic extract was collected and preserved in an airtight amber-coloured container under refrigerated conditions at 4°C until further use. The stored extract was then subjected to preliminary qualitative phytochemical screening to identify the presence of various constituents, including alkaloids, glycosides, carbohydrates, proteins, amino acids, terpenoids and saponins. [7].

**2.2. *In vitro* antioxidant assays**

**2.2.1. 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay**

A 0.1 mM solution of DPPH was prepared. Then 2.96 ml of this solution was mixed with the test sample or standard antioxidant. Intubation of the mixture was done in the dark at room temperature for 20 minutes to allow the reaction to proceed. After incubation, the absorbance was recorded at 517 nm using a UV-visible spectrophotometer [13]. The baseline absorbance of control solution containing only DPPH were measured [14].

**2.2.2.Hydrogen Peroxide Scavenging Assay**

To assess the hydrogen peroxide scavenging effect, 1 ml of the test sample or standard was mixed with 0.6 ml of hydrogen peroxide solution [15]. The volume was then brought up to 5 ml using phosphate buffer (pH 7.4). The mixture was kept at room temperature for 10 minutes, after which the absorbance was measured at 230 nm using UV spectroscopy. A blank solution containing only phosphate buffer served as the control [16].

**2.2.3.Nitric Oxide Scavenging Assay**

Nitric oxide scavenging activity was evaluated by mixing the test sample or standard with sodium nitroprusside and incubating the mixture at a temperature of 25°C for 30 minutes [17]. After 30 minutes of incubation, 1.5 ml of this mixture was reacted with an equal volume of of 1% sulphanilamide, 2% phosphoric acid, and 0.1% N-1-naphthyl ethylenediamine dihydrochloride (griess reagent). Subsequently, the absorbance was measured using UV spectroscopy at 546 nm [18].

**2.2.4. *In vitro* evaluation of EERA biosafety**

Dulbecco's modified eagle medium (DMEM) were used to culture *Chang* liver cells at a temperature of 37°C with 5% $CO_2$ until confluent. Cells were first treated with trypsin (trypsinized) and seeded into T-flasks for growth. *Invitro* cytotoxicity was induced by treating *Chang* liver cells with 0.1% carbon tetrachloride ($CCl_4$) [19]. $CCl_4$ was used in the *in vitro* model because it can quickly produce oxidative stress and lipid peroxidation in cultured liver cells, making it useful for studying the antioxidant and cytoprotective effects of EERA under controlled laboratory conditions. In contrast, paracetamol was selected for the *in vivo* study since paracetamol-induced liver injury closely resembles clinically observed drug-induced hepatotoxicity and is a widely accepted experimental model for evaluating hepatoprotective activity in animals.
The cells were then exposed to different concentrations of EERA at 100, 500, and 1000 µg/ml, prepared from a 100 mg/ml stock. In this study, silymarin served as the positive control[20]. After incubation (24hrs), cells were washed with phosphate buffered saline (PBS) and again incubated for 3 hours with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution. After the incubation Formazan crystals were formed. They were dissolved by adding dimethyl sulfoxide (DMSO), and absorbance was measured by UV spectroscopy at 540 nm using DMSO as a blank, to assess cell viability [21].

**2.3. *In vivo* assessment of hepatoprotective activity**

**2.3.1 Experimental animals**

Male albino wistar rats weighing 180–220 g were used in the study. The rats were kept in polypropylene cages under standard laboratory conditions with a 12-hour light/dark cycle, adequate ventilation and provided them

with food and water. A one-week acclimatization period was provided prior to the experiment [22]. All procedures were performed according to CPCSEA guidelines and approved by the Institutional animal ethics committee (IAEC), India (Approval No. 005/MPH/UCP/CVR/13).

**2.3.2 Experimental Design**

Investigation of the hepatoprotective effect was done using both curative and preventive models of paracetamol-induced liver damage. In the curative protocol, thirty rats were divided into five groups (n = 6). The normal control received 1% carboxymethyl cellulose (CMC) orally for 10 days [23]. The toxic control group was administered paracetamol (2 g/kg, p.o.) for three days followed by CMC. Two treatment groups were given paracetamol for three days and subsequently administered EERA at doses of 200 mg/kg or 400 mg/kg from day 4 to 10. The standard group received paracetamol for three days followed by silymarin (100 mg/kg, p.o.) during the same period [24].

In the preventive protocol, thirty-six rats were allocated into six groups (n = 6). The normal control received CMC for three days, while the toxic control was administered paracetamol (2 g/kg, p.o.). The treatment groups were co-administered paracetamol with EERA at 200 mg/kg or 400 mg/kg, p.o., for three days [25]. The standard group received paracetamol along with silymarin (100 mg/kg, p.o.) for three days. At the end of treatment, blood samples were collected from the retro-orbital plexus for biochemical evaluation, and livers were excised, rinsed with saline, and fixed in 10% buffered formalin for histopathology [26].

**2.3.3 Biochemical analysis**

Serum samples were analyzed for alkaline phosphatase (ALP) serum glutamic-oxaloacetic transaminase (SGOT/AST), total bilirubin and total protein using standard protocols [27,28]. ALP was estimated by the PNPP kinetic method, SGOT by the modified IFCC procedure, bilirubin by the Jendrassik and Grof method, and protein by the Biuret assay [29].

**2.3.4 Histopathology**

Preserved liver tissues were processed by paraffin embedding, sectioned at 4–6 µm, and stained with hematoxylin and eosin. Hepatic architecture and pathological changes were assessed by examining the histopathology slides under light microscope [30]. Histopathological changes were examined under a light microscope at 40× and 100× magnifications. Liver damage was assessed using a semi-quantitative grading method based on features such as hepatocellular necrosis, inflammatory cell infiltration, hemorrhage, and disruption of normal hepatic architecture. Arrow marks were added in the representative photomicrographs to indicate the major histopathological changes.

**2.3.5. *Ex vivo* antioxidant studies**

Antioxidant activity was assessed by evaluating lipid peroxidation, measuring catalase and superoxide dismutase (SOD) levels in liver homogenates [31]. Thiobarbituric acid reactive substances (TBARS) method was used to estimate lipid peroxidation. This procedure was performed by heating the liver homogenates with thiobarbituric acid and ethanol, cooled, treated with acetone and centrifuged at 7000 rpm. The absorbance of the supernatant was measured using UV spectroscopy at absorbance of 535 nm to determine malondialdehyde equivalents formed [32]. Catalase activity was assessed by incubating tissue extracts with hydrogen peroxide prepared in phosphate buffer, and the decrease in absorbance at 260 nm was monitored at 15 seconds intervals for 1 -2 minutes. Buffer without hydrogen peroxide was used as blank to calculate the enzyme activity in units per milligram protein [33]. SOD activity was measured using a riboflavin nitroblue tetrazolium (NBT) assay system where homogenates were mixed with phosphate buffer (pH 7.8), methionine, and riboflavin, NBT and potassium ferricyanide [34]. The reduction of NBT was quantified at 600 nm using a spectrophotometer. The activity was expressed as percentage inhibition of NBT reduction. This shows at what extent the enzyme can remove the superoxide radicals [35].

**2.4. Statistical analysis**

GraphPad Prism 5.0 were used to analyze the data and it was expressed as mean SEM. Group differences were evaluated by one-way ANOVA followed by Tukey's post-hoc test, $p < 0.05$ considered statistically significant.

## 3. Results

**3.1. Extraction yield and phytochemical profile**

The extraction of the plant yielded 5.44% (w/w) and preliminary phytochemical screening revealed a wide range of chemical constituents, including flavonoids, tannins, phenolic substances, steroids, triterpenoids, alkaloids and glycosides.

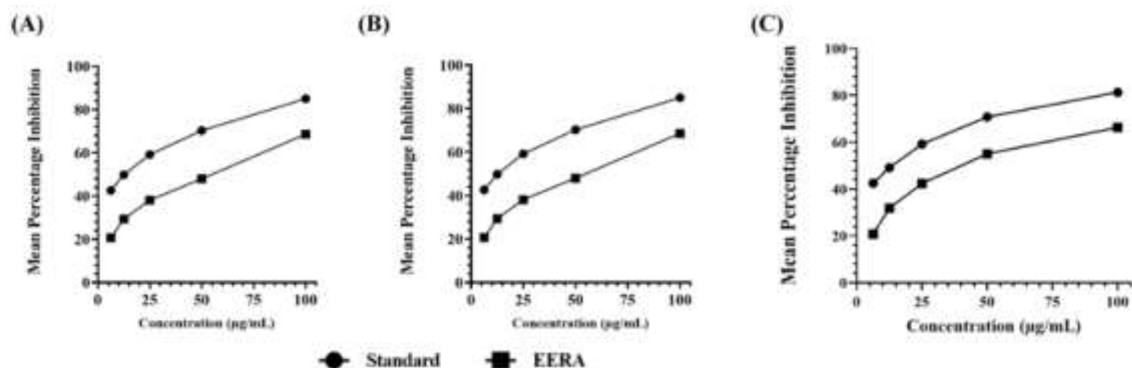
**3.2. Evaluation of *in vitro* antioxidant activity of EERA**

The antioxidant efficacy of EERA was assessed in several *in vitro* free radical scavenging assays. Figure 1 shows the concentration-dependent antioxidant activity of EERA in comparison with standard compounds. In the DPPH assay, EERA showed 82.18% inhibition at 100 µg/ml, and its $IC_{50}$ value was 29 µg/ml, while the antioxidant activity of EERA with standard Gallic acid showed a stronger effect with an $IC_{50}$ of 14 µg/ml.

In the Hydrogen peroxide scavenging test, EERA had an $IC_{50}$ of 32 µg/ml and showed 87% inhibition at 200 µg/ml, while standard Ascorbic acid showed better activity with an $IC_{50}$ of 13 µg/ml and 98.6% inhibition at the same concentration.

In the nitric oxide radical test, EERA showed an $IC_{50}$ of 54 µg/ml and 83.4% inhibition at 200 µg/ml, while gallic acid showed stronger activity with an $IC_{50}$ of 14 µg/ml and 97.3% inhibition.

**Figure 1**: **Concentration-dependent radical scavenging activity of EERA and standard compounds in (A) DPPH, (B) Nitric oxide, and (C) Hydrogen peroxide assays.**

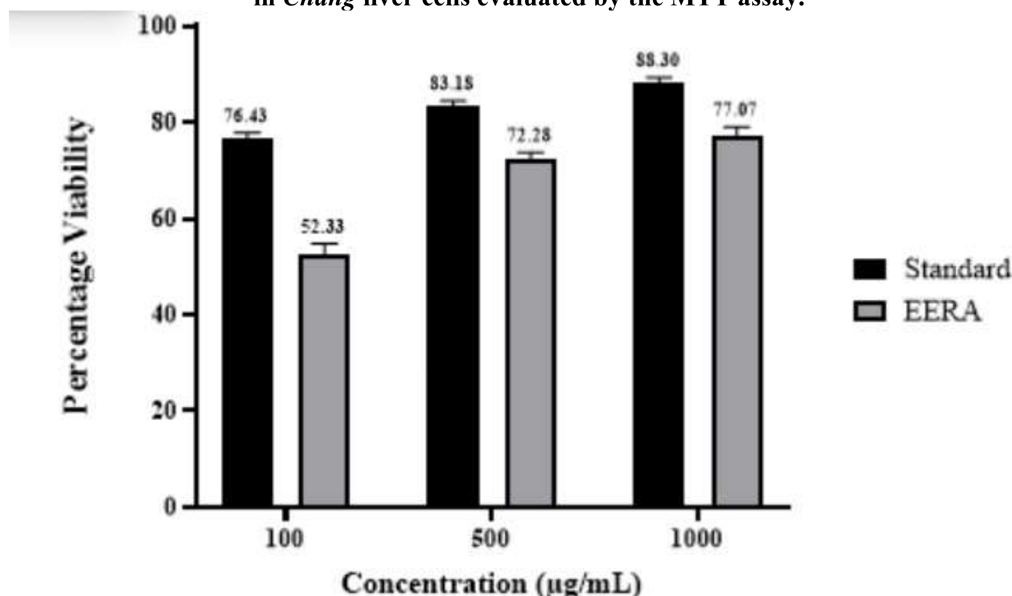



Values are expressed as mean ± SEM (n = 3). ***p*** < 0.05 considered statistically significant. $IC_{50}$: half maximal inhibitory concentration.

**3.3. Evaluation of EERA safety in Chang liver cells by MTT assay**

When Chang liver cells were exposed to Carbon tetrachloride, their metabolic activity decreased. This was measured using the MTT assay. However, when compared to the group treated only with $CCl_4$, the cells treated with the plant extract, shows significant increase in their survival (viability). Higher concentrations of the extract showed protective effect, meaning larger doses helped the cells survive better and the cell viability became close to that of normal untreated cells. Figure 2 shows the different concentrations of EERA and the percentage of cell survival, clearly indicating that the plant extract protects liver cells in a dose-dependent manner. Treatment with EERA increased the viability of Chang liver cells in a concentration-dependent manner. Compared with the $CCl_4$-treated toxic control group, cells treated with EERA at 100, 500, and 1000 µg/ml showed a gradual improvement in cell survival, and the difference was statistically significant ($p < 0.05$).

**Figure 2: Concentration dependent cytoprotective effect of EERA on $CCl_4$-induced damage in *Chang* liver cells evaluated by the MTT assay.**




Values are expressed as mean ± SEM (n = 3). Statistical analysis was performed using one-way ANOVA followed by Tukey's post-hoc test. $p < 0.05$ was considered statistically significant.

**3.4. *In vivo* studies**

**3.4.1. Determination of wet liver weight and wet liver volume**

In the curative groups, paracetamol administration caused a significant increase in wet liver weight and volume. The positive control animals showed the highest mean liver weight (6.458 ± 0.05 g/100 g) and volume (6.283 ± 0.03 ml/100 g). Treatment with the standard drug and EERA reduced both wet liver weight and volume. Table 1 shows the changes in liver weight and volume across different experimental groups. In the preventive model, EERA at 400 mg/kg reduced the liver weight significantly, but it did not clearly reduce the liver size.

**Table 1: Assessment of hepatoprotective Influence of EERA on liver weight and volume in curative and preventive models.**

Values expressed as Mean ± SEM,n=6 in each group. One way ANOVA followed by Tukey's multiple comparison test

| Model | Group | Treatment | Liver Weight (g/100 g) | Liver Volume (ml/100 g) |
|---|---|---|---|---|
| **Curative Study** | Normal | Vehicle (Distilled Water, P.O) | 4.218 ± 0.0591 | 4.233 ± 0.084 |
| | Positive Control | Paracetamol (2 g/kg, P.O) | 6.458 ± 0.0512 | 6.283 ± 0.03 |
| | Standard | Silymarin (100 mg/kg, P.O) | 5.025 ± 0.028*** | 4.617 ± 0.062*** |
| | EERALow Dose | 200 mg/kg, P.O | 5.707 ± 0.026***$^{x}$ | 5.733 ± 0.055***$^{x}$ |
| | EERA High Dose | 400 mg/kg, P.O | 5.368 ± 0.034***$^{x}$ | 5.200 ± 0.057***$^{x}$ |
| | | | | |
| **Preventive Study** | Normal | Vehicle (Distilled Water, P.O) | 4.215 ± 0.226 | 4.733 ± 0.298 |
| | Positive Control | Paracetamol (2 g/kg, P.O) | 7.140 ± 0.142 | 6.883 ± 0.208 |
| | Standard | Silymarin (100 mg/kg, P.O) | 4.435 ± 0.091*** | 5.633 ± 0.189** |
| | EERA Low Dose | 200 mg/kg, P.O | 6.188 ± 0.179**$^{x}$ | 5.833 ± 0.204* |
| | EERA High Dose | 400 mg/kg, P.O | 4.878 ± 0.146*** | 5.750 ± 0.168** |

*p<0.05 , **p<0.01, ***p<0.001 as compared to positive control $^{z}$ p< 0.05, $^{y}$ p<0.01, $^{x}$ p<0.001 as compared to s tandard.

### 3.4.2. Evaluation of Biochemical parameters

Paracetamol treatment caused serious liver damage, which was shown by a significant increase in liver enzyme levels including SGOT, SGPT, ALP, total bilirubin, and total cholesterol.

When the animals were treated with EERA, these liver enzymes were reduced in both preventive and curative studies. The higher dose (400 mg/kg) showed better liver-protective activity compared to that of 200 mg/kg dose. The hepatoprotective effect of EERA was comparable to that of the standard drug, Silymarin. Table 2 shows the detailed biochemical changes observed in different groups.

**Table 2: Biochemical response to EERA treatment in curative and preventive models of paracetamol induced hepatotoxicity**

**Curative Studies**

| Animal Group | Treatment | SGOT | SGPT | ALP | Total Bilirubin | Total Protein | Total Cholesterol |
|---|---|---|---|---|---|---|---|
| Normal | Vehicle (Distilled water, p.o.) | 0.783 ± 0.03 | 8.183 ± 0.168 | 71.67 ± 3.584 | 0.783 ± 0.03 | 8.183 ± 0.168 | 71.67 ± 3.584 |
| Positive Control | PCM 2 g/kg, p.o. | 3.3 ± 0.139 | 4.33 ± 0.088 | 163.8 ± 3.772 | 3.3 ± 0.139 | 4.33 ± 0.088 | 163.8 ± 3.772 |
| Standard | Silymarin 100 mg/kg, p.o. | 1.117 ± 0.06*** | 7.967 ± 0.071*** | 78 ± 5.967*** | 1.117 ± 0.06*** | 7.967 ± 0.071*** | 78 ± 5.967*** |
| | | | | | | | |
| EERA – Low Dose | 200 mg/kg, p.o. | 2.95 ± 0.076 | 5.983 ± 0.101*** | 120.3 ± 3.639*** | 2.95 ± 0.076 | 5.983 ± 0.101*** | 120.3 ± 3.639*** |
| EERA – High Dose | 400 mg/kg, p.o. | 2.25 ± 0.07*** | 7.267 ± 0.049*** | 84.83 ± 3.24*** | 2.25 ± 0.07*** | 7.267 ± 0.049*** | 84.83 ± 3.24*** |

**Preventive Studies**

| Animal Group | Treatment | SGOT | SGPT | ALP | Total Bilirubin | Total Protein | Total Cholesterol |
|---|---|---|---|---|---|---|---|
| Normal | Vehicle (Distilled water, p.o.) | 36.98 ± 3.028 | 42.21 ± 2.98 | 223.3 ± 2.397 | 0.783 ± 0.03 | 9.137 ± 0.563 | 79.4 ± 1.837 |

| Animal Group | Treatment | SGOT | SGPT | ALP | Total Bilirubin | Total Protein | Total Cholesterol |
|---|---|---|---|---|---|---|---|
| Positive Control | PCM 2 g/kg, p.o. | 121.8 ± 5.094 | 143.7 ± 9.87 | 318.3 ± 6.839 | 2.1 ± 0.18 | 3.428 ± 0.079 | 157.6 ± 2.194 |
| Standard | Silymarin 100 mg/kg, p.o. | 38.68 ± 1.892*** | 45.84 ± 5.929*** | 230.2 ± 3.422*** | 1.03 ± 0.07*** | 7.712 ± 0.178*** | 87.09 ± 3.85*** |
| EERA – Low Dose | 200 mg/kg, p.o. | 61.56 ± 1.924*** | 97.5 ± 4.08*** | 264.6 ± 3.327*** | 1.91 ± 0.086 | 5.613 ± 0.196*** | 114.3 ± 4.154*** |
| EERA – High Dose | 400 mg/kg, p.o. | 42.92 ± 5.71*** | 71.12 ± 8.151*** | 236.3 ± 7.614*** | 0.972 ± 0.07*** | 6.765 ± 0.296*** | 97.15 ± 1.905*** |

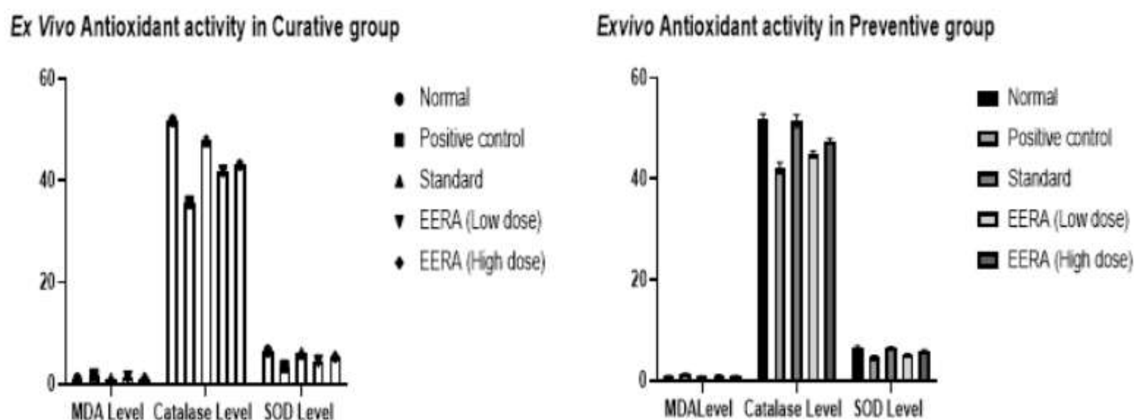
Values expressed as Mean ± SEM, n=6 in each group. One way ANOVA followed by Tukey's multiple comparison test
*p<0.05 , **p<0.01, ***p<0.001 as compared to positive control ,$^{z}$ p< 0.05, $^{y}$ p<0.01, $^{x}$ p<0.001 as compared to standard

**3.4.3. Ex vivo study**

Administration of Paracetamol increased lipid peroxidation and reduced levels of antioxidant enzyme levels such as catalase and superoxide dismutase. Treatment with EERA, particularly at 400 mg/kg, significantly reduced lipid peroxidation and restored antioxidant enzyme levels. Figure 3 depicts the comparative antioxidant enzyme activity in curative and preventive groups.

**Figure 3: Estimation of *Ex vivo* parameters in both Curative and Preventive groups.**

Values expressed as Mean ± SEM, n=6 in each group. One way ANOVA followed by Tukey's multiple comparison test *p<0.05 , **p<0.01, ***p<0.001 as compared to positive control $^{z}$ p< 0.05, $^{y}$ p<0.01, $^{x}$ p<0.001 as compared to standard.




**3.4.4. Histopathological analysis**

Histopathological evaluation indicated severe liver damage in the paracetamol-treated group, characterized by centrilobular necrosis and hemorrhage, while the normal control group exhibited a well-preserved hepatic architecture. Treatment with EERA (400 mg/kg) led to a clear improvement in liver histology, with reduced necrosis and inflammation, showing effects comparable to Silymarin. Figure 4 illustrates these histopathological changes in the curative study. Similarly, in the preventive study, EERA demonstrated notable protective effects, with improved liver architecture and reduced tissue damage, as shown in Figure 5.

**Figure 4: Liver histopathology of wistar rats in the curative study.**

**(A)** Normal control showing preserved hepatic lobular pattern with distinct central vein.
**(B)** Paracetamol control revealing centrilobular necrosis, hemorrhage, and lymphocytic infiltration.
**(C)** Silymarin treated group showing restored hepatic architecture with mild vascular changes.
**(D)** EERA (200 mg/kg) showing partial recovery with reduced necrosis and congestion.
**(E)** EERA (400 mg/kg) showing nearly normal hepatic structure, indicating strong hepatoprotection.

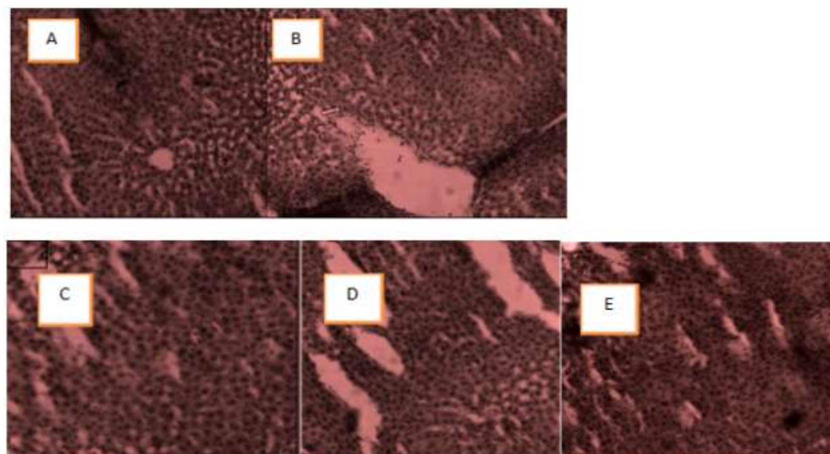
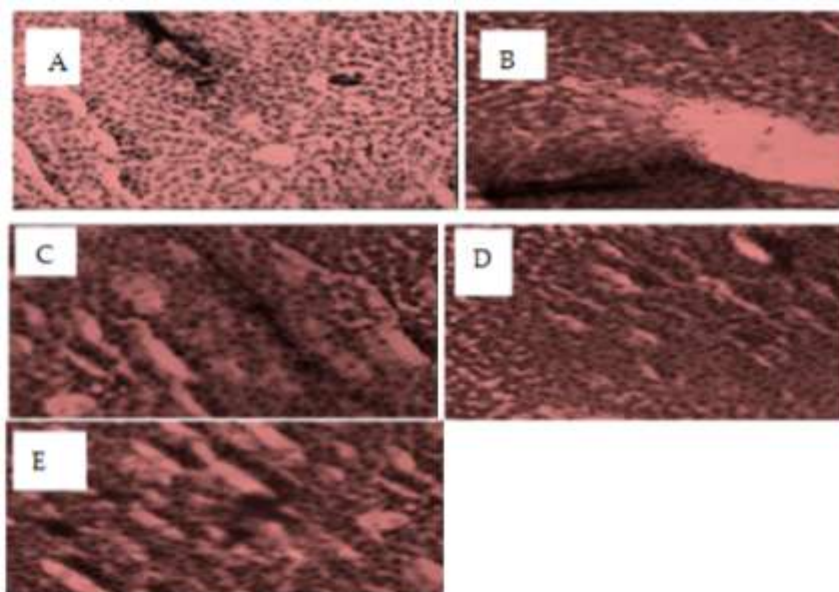



**Figure 5: Liver histopathology of wistar rats in the preventive study.**

**(A)** Normal control showing preserved lobular pattern with distinct central vein and intact hepatocytes.
**(B)** Paracetamol treated group exhibiting extensive necrosis, haemorrhage and inflammatory infiltration.
**(C)** Silymarin 100 mg/kg showing normal hepatic morphology comparable to the control group.
**(D)** *EERA* 200 mg/kg showing partial structural recovery with mild inflammatory changes.
**(E)** *EERA* 400 mg/kg showing marked restoration of hepatic architecture and minimal tissue damage.




## 4. Discussion

The major significant global health burden is liver disorders, accounting for approximately 2 million deaths annually worldwide, with drug-induced liver injury (DILI) serving as a leading cause of hepatic damage [36]. Paracetamol (acetaminophen) overdose is responsible for nearly 50% of all acute liver failure cases in the United States and Europe [37]. The present study investigated the hepatoprotective efficacy of the ethanolic extract of *Rotula aquatica* Lour against paracetamol-induced hepatotoxicity in rats, with particular emphasis on its antioxidant mechanisms and cytoprotective properties. Our findings demonstrate that EERA possess significant dose-dependent hepatoprotection, and could be a promising option for future drug development.

Paracetamol induced liver injury is commonly used as a model to study hepatoprotective drugs. At therapeutic doses, glucuronidation and sulfation are the two main pathways were paracetamol gets metabolized in liver (less than 10%). It is metabolized in liver by the enzyme CYP2E1, which produces a toxic substance called NAPQI (N-acetyl-p-benzoquinone imine) [38]. Under certain conditions, NAPQI the toxic compound is quickly neutralized by a natural antioxidant in the liver called glutathione (GSH), which is safely removed from the body. However, during a paracetamol overdose, the two main pathways gets saturated [39]. As a result, more NAPQI is produced, which uses up the glutathione stores in the liver. When glutathione levels become less, the excess NAPQI formed cannot be detoxified. Instead of detoxifying they will get bind to liver proteins, causing mitochondrial damage, oxidative stress, and hepatocellular necrosis [40]. Recent studies show that acetaminophen overdose is a major problem, which accounts for approximately 78,000 emergency department visits annually in the United States alone, with substantial healthcare costs exceeding $1.06 billion per year [41]. The narrow therapeutic index of paracetamol combined with its widespread availability creates a persistent public

health challenge that necessitates the development of effective hepatoprotective strategies. Our utilization of this established model ensures the translational relevance of our findings to clinical scenarios of acute liver injury.

The hepatoprotective efficacy observed with EERA is comparable to that reported for other plant-derived interventions for paracetamol-induced liver damage. In the present study, administration of paracetamol developed hepatic damage

This was shown by significant increase in serum SGOT (AST), SGPT (ALT), ALP, bilirubin, and cholesterol concentrations, along with the reduced total protein levels. These biochemical alterations indicate hepatocellular membrane damage, impaired liver functions, and cholestatic injury [42].

The protective effect of EERA extract was similar to that of the standard drug silymarin. It also shows significant dose-dependent effects. These effects have also been observed with other herbal compounds for example, quercetin, a flavonoid commonly found in many medicinal plants, has been reported to reduce paracetamol-induced increases in liver enzymes such as SGPT and SGOT and to decrease inflammatory markers like NF-κB and TNF-α in rats [43]. Similarly, some plant extracts, like *Kummerowia striata*, protects paracetamol induced liver damage by acting on specific cell pathways (S1P/Nrf2/Keap1 pathway), showing that herbal medicines may work through different protective mechanisms [44].

Comparing EERA with other recent studies shows both shared and distinct features by which plant compounds protect the liver. Quercetin mainly works as an antioxidant and reduces inflammation, while *Kummerowia striata* protects the liver by affecting sphingolipid metabolism and Nrf2 signaling [45,46]. EERA, on the other hand, protects the liver by restoring antioxidant enzymes and stabilizing cell membranes. In our study, silymarin, from *Silybum marianum*, was used as a standard because of its hepatoprotective effects. Recent research shows that silymarin upregulates irisin (a protective protein) which reduce oxidative stress markers like MDA, TOS, and OSI, and protect against both liver and cardiac damage caused by paracetamol. They reduce lipid damage, restoring antioxidant enzymes, and lowering inflammatory markers such as COX-2 and iNOS [47,48]. Phytochemical screening of EERA revealed the presence of various chemical constituents mainly flavonoids**,** known for their hepatoprotective effects. This result agrees with previous studies on *Rotula aquatica Lour* which have reported several important bioactive compounds such as phytol, 9,12,15-octadecatrienoic acid, squalene, stigmasterol, β-sitosterol, and α/β-amyrin [49]. α and β-amyrin pentacyclic triterpenes in the EERA contributes anti-inflammatory, antioxidant antinociceptive, gastroprotective, and hepatoprotective properties [50]. These compounds may play an important role in hepatoprotection. Flavonoids exert hepatoprotective activity through following mechanisms: (1) free radical scavenging ( reactive oxygen species (ROS) and reactive nitrogen species (RNS)); by DPPH, nitric oxide, and hydrogen peroxide assays (2) Binding metal ions like iron or copper so they cannot produce harmful free radicals; (3) production of antioxidant enzymes including superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase; and (4) inhibition of pro-inflammatory signaling pathways [51].Recent studies in quercetin protects from paracetamol-induced liver damage by reducing oxidative stress, inflammation, and cell death, and by regulating proteins like BCL2 and caspase-3. When comparing with EERA, they also exhibit similar bioactive compounds, it could be a promising way to prevent or treat paracetamol drug-induced liver injury (DILI) [52].

The antioxidant property of EERA protects the liver from paracetamol induced damage. Paracetamol induced liver damage results high oxidative stress, losing its natural antioxidants, damaging fats in cells (lipid peroxidation), and mitochondrial damage. In our study, treatment with EERA significantly increased various antioxidant enzymes like SOD and CAT and reduced lipid peroxidation, and free radical scavenging protecting from oxidative stress [53].

Nrf2/Keap1 pathway play a pivotal role in controlling in cellular antioxidant responses [54]. Under normal conditions, Nrf2 is held by Keap1 and undergoes proteasomal degradation. During oxidative stress, Nrf2 gets dissociated from Keap1, and this is translocated to the nucleus, which activates transcription of antioxidant response element (ARE)-driven genes encoding phase II detoxification enzymes and antioxidant proteins [55]. Recent studies reported that *Kummerowia striata* extract inhibited Keap1 expression and restored Nrf2 signaling, thereby mitigating mitochondrial oxidative damage and ferroptosis in paracetamol-induced liver injury [56]. While our study did not directly act in the Nrf2/Keap1 modulation, but the observed restoration of antioxidant enzymes suggests potential involvement of this pathway in EERA's mechanism of action. Furthermore, recent investigations estimated SPHK1/S1P/S1PR2/4 axis or targets in paracetamol hepatotoxicity [57]. Another pathway called Sphingosine-1-phosphate (S1P) signaling helps in controlling the inflammation and cell survival in hepatic injury [58].

The comparative efficacy of EERA in restoring antioxidant enzymes warrants particular attention. Silymarin's hepatoprotective effects have been extensively attributed to its capacity to scavenge oxygen free radicals, decrease lipid peroxidation, and accelerate hepatocyte regeneration [59]. In triptolide-induced liver injury, silymarin pretreatment reversed reductions in SOD, CAT, GST, and GSH-Px activities while normalizing MDA production [60]. The parallel restoration of antioxidant enzymes observed with EERA suggests comparable mechanistic efficacy, potentially mediated by similar radical scavenging and enzyme-inducing properties.

EERA's role in elevating the antioxidant enzymes is very important. The standard drug in our study silymarin**,** a well-known hepatoprotective agent, neutralizes free radicals, reduces lipid peroxidation, and helps liver cells to regenerate**.** In studies of triptolide induced liver injury, silymarin restored antioxidant enzymes like SOD, CAT, GST, and GSH-Px, and also reduced biochemical markers of cell damage (MDA). This suggests that it may protect the liver through free radical scavenging and boosting antioxidant enzymes.

The histopathological findings of our study revealed the liver-protecting effect of EERA. Treatment with EERA, especially at the higher dose (400 mg/kg), reduced tissue damage and maintained normal tissue structure when compared with the paracetamol drug-treated control group. EERA also improved biochemical markers of liver function and antioxidant levels. This confirms that the extract has hepatoprotective activity. Recent studies reported that treatment with quercetin significantly reduced liver damage in rats that received a high dose of paracetamol. Histopathological studies revealed a reduction in centrilobular necrosis and chemotaxis [61]. In another study, treatment with silymarin also reduced hepatic cell death and inflammation, and immunohistochemical analysis showed decreased expression of certain inflammatory markers which includes COX2, iNOS, and SOD2 [62,63]. The histological improvements observed with EERA in our study are similar to these findings.
EERA's ability to protect the liver is important for treating hepatic disorders. *Rotula aquatica* Lour grows widely in tropical and subtropical regions, and it has been traditionally used to treat hepatic problems in many indigenous medical systems. Showing its effectiveness through scientific studies in a supports these traditional uses with scientific evidence. The demonstration of scientific efficacy against drug-induced liver injury (DILI) model provides evidence-based support for these traditional applications. In recent years, researchers were able to understand how paracetamol causes liver damage. N-acetylcysteine (NAC) is the main medical treatment for paracetamol overdose. However, NAC is effective when it is given within 8–24 hours after the overdose, if treatment is delayed, its effectiveness decreases [64]. Although the study showed promising hepatoprotective activity of EERA, certain limitations remain. Molecular mechanisms and inflammatory cytokines were not evaluated, and CYP2E1 estimation was not performed. In addition, the active compounds responsible for the activity were not isolated, and pharmacokinetic studies were not carried out. Further studies are needed to clarify these aspects.
Future studies should focus on elucidating the mechanisms by which EERA functions at different molecular levels. This includes studying several pathways and observing their effects mainly Nrf2/Keap1 signalling, sphingolipid metabolism, and mitochondrial protection, which are important for protecting liver cells. Along with the mechanistic pathways studies, understanding the pharmacokinetics will also help determine the correct dose and support its use in clinical treatment. Furthermore, it is necessary to identifythe chemical constituents in EERA that are responsible for its liver-protecting effects. These may include flavonoid-rich fractions or triterpenoid compounds such as α-amyrin and β-amyrin.

## 5. Conclusion

In conclusion, the present study shows that the ethanolic extract of *Rotula aquatica* Lour possesses significant dose-dependent hepatoprotective activity against paracetamol-induced liver injury in rats. The extract when compared to that of the reference standard reference silymarin is attributable to its antioxidant capacity, restoration of endogenous enzymatic defenses, and cytoprotective effects on hepatic architecture. These findings prove the traditional use of *Rotula aquatica* Lour in hepatic disorders and support its potential development as a natural hepatoprotective agent. The multiple mechanism of action, involving both direct free radical scavenging and enhancement of cellular antioxidant capacity, suggests that EERA may offer advantages in complex hepatic pathologies characterized by oxidative stress and inflammation. Further research into the specific molecular targets and active constituents of EERA will helps understand how they protect liver and support evidence-based therapies.

### 6. Abbreviations

EERA- ethanolic extract of *Rotula aquatica* Lour
DILI- Drug induced liver injury
DPPH- 2,2-diphenyl-1-picrylhydrazyl
SOD-Super oxide dismutase
CAT- Catalase
NAC- N-acetylcysteine
ROS-Reactive oxygen species
RNS- Reactive nitrogen species
GST- Glutathione S-Transferase
GSH- Glutathione
MDA- Malondialdehyde
MTT- 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
SGOT- Serum Glutamic Oxaloacetic Transaminase
SGPT- Serum Glutamic Pyruvic Transaminase
ALP- Alkaline Phosphatase
DMSO- Dimethyl Sulfoxide
CCL4- Carbon Tetrachloride
NF-κB – Nuclear Factor kappa-light-chain
TNF-α – Tumor Necrosis Factor-alpha
GPT – Glutamate Pyruvate Transaminase
GOT – Glutamate Oxaloacetate Transaminase
OSI – Oxidative Stress Index

TOS – Total Oxidant Status
NAPQI (N-acetyl-p-benzoquinone imine)

**Informed consent statement**: Not applicable.

**Data Availability Statement:** The original contributions presented in this study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

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