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ORIGINAL ARTICLE

In vitro and *In vivo* Hepatoprotective Studies on Methanolic Extract of Aerial Parts of *Ludwigia hyssopifolia* G. Don Exell

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ABSTRACT

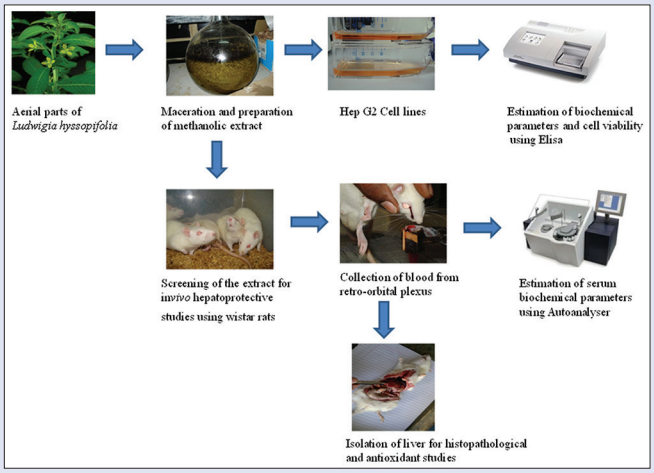
Background: *Ludwigia hyssopifolia* of family Onagraceae is traditionally used in the treatment of jaundice. There were no reports on the plant for both *in vivo* and *in vitro* hepatoprotective studies. **Objective:** The current study was aimed to evaluate the methanolic extract of aerial parts of L. h*yssopifolia* (LHME) for its *in vitro* and *in vivo* hepatoprotective activity against ethanol-induced oxidative damage in HepG2 cell lines and Wistar rats, respectively, and *in vivo* hepatoprotective activity against paracetamol and antihepatotoxic activity against D-galactosamine in rats. **Materials and Methods:** The antioxidant potential of the extract was investigated by employing different *in vitro* methods. The *in vitro* hepatoprotective activity of the extract was assessed by estimating cell supernatant for aspartate aminotransferase (AST), Alanine aminotransferase (ALT), and lactate dehydrogenase (LDH) while the *in vivo* hepatoprotective activity of the extract was assessed on the basis of improvement in the altered level of various serum biochemical parameters and in the changes occurred in the histology of liver of the rats. **Results:** Among the three test doses of LHME, 100 µg/kg and 200 mg/kg were found to be the effective doses in *in vitro* and *in vivo* hepatoprotective methods, respectively. **Conclusion:** The extract, LHME, exhibited significant ($P < 0.01$) hepatoprotective activity in both *in vitro* and *in vivo* models, which may be attributed to its antioxidant property revealed in both *in vitro* and *in vivo* studies.

Key words: Antioxidant enzymes, cytotoxicity, D-galactosamine, HepG2, *Ludwigia hyssopifolia*

SUMMARY

- The findings of the study suggest that the extract has antioxidant principles such as flavonoids and phenolic compounds which reduce the inflammatory cytokines mediated in liver disease contributing to the hepatoprotective activity of the extract.

Abbreviation used: DPPH: 1,1 Diphenyl-1-picryl hydrazyl; ALT: Alanine transaminase; ALB: Albumin; ALP: Alkaline phosphatase; AST: Aspartate transaminase; CHOL: Cholesterol; DB: Direct bilirubin; FBS: Fetal bovine serum; GAE: Gallic acid equivalents; GGT: Gamma glutamyl transferase; GLU: Glucose; LDH: Lactate dehydrogenase; MEM: Modified Eagle's Medium; NBT: Nitro blue tetrazolium; PMS: Phenazine methosulphate; RE: Rutin equivalents; LHME: The methanolic extract of Ludwigia hyssopifolia; TBA: Thiobarbituric acid; TB: Total bilirubin; TP: Total protein; TCA: Trichloro acetic acid.



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INTRODUCTION

Liver disorders are recognized as one of the serious global health problems which are occurring due to the adoption of modern food styles, exposure to many environmental pollutants, and excessive intake of some drugs. According to the World Health Organization report on liver disease mortality 2013, about 50 million people die every year worldwide from cirrhosis and liver cancer. Viral hepatitis and other liver diseases have been recognized as substantial contributors to global mortality.[1] Synthetic drugs used in the treatment of liver diseases have undesirable side effects. In the absence of reliable liver protective drug in the modern system of medicine, traditional herbal medicine has begun to gain popularity worldwide for promoting health care as well as disease prevention and been used as conventional or complementary medicines for both treatable and incurable diseases all over the world.[2] In spite of the availability of more than 300 preparations for the treatment of jaundice and chronic liver diseases in Indian systems of medicine, only few plants have been scientifically elucidated while adhering to the internationally acceptable scientific protocols. Therefore, there is a great need for identification of such plants for scientific pharmacological investigation.

Ludwigia hyssopifolia (Colsm.) Pennell of family Onagraceae is extensively grown in Bangladesh, all parts of India, and Ceylon.[3] The plant is considered

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as astringent, anthelmintic, carminative, and diuretic. Decoction of the plant is used in diarrhea and dysentery, jaundice, flatulence, leukorrhea, and spitting of blood.[4] The plant was reported to contain isoflavonoids, namely, vitexin, isovitexin, orientin, and isoorientin[5] and alkaloid piperine.[6]

MATERIALS AND METHODS

Animals

Wistar albino rats weighing 150–200 g were purchased from Mahaveer Agencies, Uppal, Hyderabad, India, with a prior permission from our institutional animal ethical committee (1820/GO/Re/S/15/CPCSEA Date: January 09, 2015) and used for the studies. The animals were caged under constant environmental and nutritional conditions (14:10 h light and dark cycle; at an ambient temperature of 25°C ± 5°C; 35%–60% of relative humidity). They had free access to food and water *ad libitum*.

Drugs and chemicals

HepG2 Cell lines were obtained from National Centre for Cell Science, Pune, India. The drugs and chemicals were purchased from various companies, and the details are as follows: silymarin and D-galactosamine (D-GaIN) – Sigma Aldrich, Spruce Street, St. Louis, China; fetal bovine serum, trichloroacetic acid – Merck Specialities Private Limited, Mumbai, India; 1,1-diphenyl-1-picrylhydrazyl (DPPH) and thiobarbituric acid – Himedia, Mumbai, India; deoxyribose, Griess reagent, H_2O_2, nitro blue tetrazolium, and phenazine methosulfate (PMS) – Sigma, Germany; ethanol – Changshu Yangyuan Chemicals, China. All other chemicals and solvents used were of analytical grade.

Collection and preparation of extracts

The aerial parts of the plant *L. hyssopifolia* were collected in the month of July 2013, from cotton fields of Bayyaram, Khammam district, Telangana state, India, after the authentication of the plant by Prof. V. S. Raju, Department of Botany, Kakatiya University, Warangal. The aerial parts of the plant were washed under tap water and shade dried, coarsely powdered (800 g), and macerated with methanol in a round bottom flask for 7 days with intermittent stirring and filtered after 7 days and concentrated under reduced pressure to yield a green semi-solid mass. The percentage yield of the extract was found to be 9.8%.

Detection of phytoconstituents

The methanolic extract of *L. hyssopifolia* (LHME) was subjected to chemical tests for detection of various phytoconstituents such as saponins, steroid/triterpenoidal, flavonoidal compounds and their glycosides, alkaloids, phenolics, and tannins.[7]

Determination of total phenolic content

The total phenolic content in LHME was determined using Folin–Ciocalteu reagent according to the method of Marinova *et al.*[8] with minor modifications. It was carried out in triplicate and expressed as gallic acid equivalents (GAEs) in mg per gram of extract.

Determination of total flavonoid content

The total flavonoid content in LHME was determined using aluminum chloride assay according to the method of Marinova[8] and was performed in triplicate and expressed as rutin equivalents (REs) in mg per gram of extract.

Determination of *in vitro* antioxidant activities

The test extract, LHME, was screened to assess its antioxidant property by DPPH radical,[9] superoxide,[10] nitric oxide,[11] and hydroxyl[12] radical assay methods and also by reducing power assay.[13]

Determination of *in vitro* cytotoxic activity

The 50% cytotoxic concentration (CTC_{50}) was determined by estimating mitochondrial synthesis using tetrazolium assay.[14] HepG2 cells (5.0×10^3 cells/well) were maintained in 96 well-culture plates for 72 h in the presence of 100 µl of LHME at the concentrations of 100, 300, 1000, and 3000 µg/ml. At the end of incubation period, the drug solutions in the wells were discarded, and 50 µl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) prepared in Modified Eagle's Medium without phenol red was added in each well. The plates were gently shaken and incubated for 3 h at 37°C in 5% CO_2 atmosphere. After 3 h, the supernatant was removed. Later on, 50 µl of propanol was added and the plates were gently shaken to solubilize the formed formazan followed by 30 min incubation at room temperature with constant shaking. Absorbance (optical density [OD]) was read at 540 nm using microplate reader (BioTek Instruments, Inc., Winooski, VT, USA). The percentage growth inhibition was calculated using the following formula:

% Growth inhibition = 100 − (Mean OD of individual test group/Mean OD of control group) × 100

A dose-response curve was generated using % growth inhibition on Y-axis and the extract concentration (µg/ml) on X-axis. The CTC_{50} value is calculated from dose-response curve.

Evaluation of hepatoprotective activity of methanolic extract of *Ludwigia hyssopifolia in vitro*

The hepatoprotective activity of LHME was evaluated using well-maintained HepG2 cells. Ethanol was used as hepatotoxicant and silymarin was used as a standard positive control. The toxic concentration of ethanol taken was 100 mM. The doses of LHME and standard were chosen based on the results of the MTT assay. The experimental groups were carried out in triplicate as follows:

- Group I (control)
  1. Normal control: The cells were treated with 100 µl of serum-free culture medium for 24 h
  2. Dimethyl sulfoxide (DMSO) control: The cells were treated with 100 µl of serum-free culture medium containing DMSO (0.3% v/v) for 24 h
  3. Silymarin control: The cells were treated with 100 µl of serum-free culture medium containing silymarin (200 µg/ml) for 24 h
  4. LHME control: The cells were treated with 100 µl of serum-free culture medium containing LHME (200 µg/ml) for 24 h
- Group II (toxin treatment): The cells were treated with 100 µl of serum-free culture medium containing 100 mM ethanol for 24 h
- Group III (silymarin treatment): The cells were treated with 100 µl of serum-free culture medium containing 100 mM ethanol with silymarin at a concentration of 50, 100, and 200 µg/ml for 24 h
- Group IV (LHME treatment): The cells were treated with 100 µl of serum-free culture medium containing 100 mM ethanol with LHME at a concentration of 50, 100, and 200 µg/ml for 24 h.

Later, cell viability, AST, ALT, and lactate dehydrogenase (LDH) leakage assays were performed using standard methods using Ecoline diagnostic kits.[14]

Acute toxicity study

Acute toxicity study was carried out for LHME according to the Organization for Economic Co-operation and Development 423 guidelines.[15] All animals were observed for toxic symptoms and mortality for 72 h.

Assessment of hepatoprotective activity of methanolic extract of *Ludwigia hyssopifolia* against ethanol-induced hepatotoxicity in rats (prophylactic study)

The assessment of hepatoprotective activity of LHME was performed according to the method given in the literature with minor modifications.[16] The animals were pretreated for 10 days with single daily dose of vehicle, silymarin, and the test extracts in different doses as described in paracetamol-induced hepatotoxicity experiment. On the 10th day, food was discontinued, and 1 h after the daily treatment, the animals of all the groups leaving Group I were intoxicated with an acute oral dose of ethanol (5 g/kg. b.w) in distilled water (6:4 v/v). Eighteen hours after administration of ethanol, the blood and liver samples were collected under ether anesthesia for numerical estimation of various serum biochemical parameters and histological studies, respectively.

Assessment of hepatoprotective activity of methanolic extract of *Ludwigia hyssopifolia* against paracetamol-induced hepatotoxicity in rats (prophylactic study)

The experiment was performed according to the method given in the literature with minor modifications.[17] The rats were divided into six groups comprising six in each. 2% gum acacia was used as vehicle for suspending the standard drug and the extract. Group I was kept as control and received single daily dose of vehicle (2% gum acacia 1 ml/kg. b.w. p.o.) for 7 days. Groups II, III, IV, V, and VI were given orally daily dose of vehicle (2% gum acacia 1 ml/kg. b.w. p.o.), silymarin (100 mg/kg b.w.), LHME (100 mg/kg b.w), and LHME (200 mg/kg b.w) and LHME (400 mg/kg) once a day for 7 days, respectively. On the 8th day, a dose of paracetamol (3 g/kg) was administered to the animals of all groups leaving Group I. Then, blood and liver samples were collected from the animals of all groups 24 h after administration of paracetamol for estimation of various biochemical parameters and histopathological studies, respectively.

Assessment of antihepatotoxic activity of methanolic extract of *Ludwigia hyssopifolia* against D-galactosamine-induced hepatotoxicity in rats (curative study)

The antihepatotoxic activity of LHME against D-GaIN-induced hepatotoxicity was carried out according to the procedure given in the literature with minor modifications.[18] The rats were randomly divided into four groups of six animals each. Group I served as normal and received the vehicle (1 mL/kg b.w. p.o of 2% gum acacia in water) for 3 days. On the 1st day, D-GaIN (400 mg/kg i.p) was given to Groups II, III, and IV. Vehicle (2% gum acacia 1 mL/kg b.w. p.o.), silymarin (100 mg/kg), and LHME (200 mg/kg) were given to the animals of Groups II, III, and IV, respectively, for three times at the time point of 2, 24, and 48 h after the administration of D-GaIN. The blood and liver samples were collected from the animals 1 h after the last treatment for estimation of various biochemical parameters and histopathological studies, respectively. The liver was quickly removed and perfused immediately in ice cold saline (0.9% NaCl). A portion of the liver was homogenized using chilled sodium phosphate buffer (0.1 M, pH 7.4) using a Potter Elvehjem Teflon homogenizer. The homogenate obtained was centrifuged in a cooling centrifuge at 12,000 g or 30 min at 4°C to obtain a postmitochondrial supernatant (PMS) which was used for enzyme analysis. The contents of glutathione[19] (GSH), catalase[20] (CAT), and malondialdehyde[21] (MDA) were determined by the methods given in the literature.

Determination of prothrombin time

The prothrombin time (PT) was determined by collecting blood in normal capillary tubes and breaking it into pieces until a thread is observed. Time was noted between the collections of blood to the appearance of thread.[22]

Assessment of serum biochemical parameters

The collected blood was allowed to clot for 30 min, and serum was separated at 4000 rpm for 15 min and assayed for serum levels of alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP), total bilirubin (TB), direct bilirubin (DB), albumin (ALB), total protein (TP), cholesterol (CHOL), glucose (GLU), gamma-glutamyltransferase (GGT), and LDH.

Histological studies

The livers from all the animals were isolated and fixed in formalin solution and processed for histopathological examination.

Evaluation of anti-inflammatory activity of methanolic extract of *Ludwigia hyssopifolia*

The inhibitory effect of selected test dose on carrageenan-induced rat paw edema was determined according to the method described in the literature.[23] The rats were randomly divided into three groups of six animals each. Group I served as toxic control (negative) and received the vehicle (1 ml/kg b.w. p.o of 2% gum acacia in water), and Group II served as standard and received 20 mg/kg of diclofenac sodium by oral route. Group III received the test dose of LHME 200 mg/kg b.w. p.o. One hour after the aforesaid treatment, 0.05 ml of 1% w/v carrageenan solution in normal saline was injected into the subplantar tissue of the left hind paw of the rat and the right hind paw was served as the control. The paw volume of the rats was measured before the injection of carrageenan and at the end of 1, 2, 3, and 4 h after injection of carrageenan using plethysmometer.

The percentage inhibition was calculated by the following formula:

$I\% = \{1 - (dt/dc)\} \times 100$.

Statistical analysis

The results were expressed as mean ± standard deviation. Statistical analysis was carried out by one-way analysis of variance followed by Dunnett's *t*-test, using Graph pad Prism 4.0, San Diego, California, USA. $P < 0.05$ was considered to be significant.

RESULTS

Phytochemical analysis

Preliminary phytochemical investigation on the extract, LHME, was found to contain saponins, steroid/triterpenoidal, flavonoidal compounds and their glycosides, phenolic compounds, and tannins. The total phenolic and flavonoid contents of LHME were found to be 29.6 ± 1.23 and 70.15 ± 1.16 mg of GAE and RE per gram of extract, respectively.

In vitro antioxidant studies

The test extract, LHME, has shown a concentration-dependent *in vitro* free radical scavenging activity. The CTC_{50} of LHME and the standard are shown in Table 1. The extract also showed a concentration-dependent reducing power. The reducing power of the extract, LHME, is expressed

in terms of ascorbic acid equivalents (AAEs) and was found to be 49.41 ± 1.36 mg of AAE/g of extract.

In vitro cytotoxicity activity

The results of the study are shown in Figure 1. LHME showed a CTC_{50} value of 1870.45 ± 8.31 µg/ml in HepG2 cell line.

Hepatoprotective activity of methanolic extract of *Ludwigia hyssopifolia in vitro*

The results of the study are depicted in Table 2. There was a significant ($P < 0.01$) decrease in cell viability and a significant ($P < 0.01$) increase in the levels of LDH, ALT, and AST in the Group II, i.e., treated with 100 mM ethanol as compared with normal control (Group I). HepG2 cells when treated with different concentrations of LHME (50, 100, and 200 µg/ml) showed a significant restoration of the altered levels of hepatic enzymes and improved cell viability which was comparable to that of standard drug silymarin. Of all the doses tested, LHME at 100 µg/ml showed better cytoprotective activity.

Acute toxicity study

The extract, LHME, did not cause any adverse effects and mortality up to a dose level of 2000 mg/kg b.w. p.o and was considered as safe. Hence, three doses, i.e., 100, 200, and 400 mg/kg b.w were selected for hepatoprotective study.

Hepatoprotective activity of methanolic extract of *Ludwigia hyssopifolia* against ethanol-induced hepatotoxicity in rats

The results of the study are presented in Table 3 and Figure 2. The standard LHME 100, 200, and 400 mg/kg groups significantly ($P < 0.01$) brought down the elevated serum (ALT, AST, and ALP), LDH, GGT, TB, DB, CHOL, and PT and increased the reduced serum TP and ALB levels as compared to the toxic group. LHME 200 mg/kg showed a better hepatoprotective activity than the other two test doses as was evident from the percentage recovery. The effect shown by LHME 200 mg/kg was well comparable to that of the standard drug, silymarin (100 mg/kg). The histopathological examination of liver sections showed significant signs of amelioration of ethanol-induced liver injury which was evident from the reduction of accumulation of fatty lobules and necrosis. The recovery effect of LHME 200 mg/kg was comparable to silymarin (100 mg/kg).

Assessment of hepatoprotective activity of methanolic extract of *Ludwigia hyssopifolia* against paracetamol-induced hepatotoxicity in rats

Paracetamol intoxication in normal rats significantly ($P < 0.01$) elevated the level of hepatospecific enzymes (AST, ALT, and ALP), TB, DB, and LDH and decreased the level of TP and ALB in serum [Table 4]. It indicates acute hepatocellular damage and biliary obstruction which was endorsed by the histopathological examination of the liver sections of rats showing centrilobular necrosis, dilatation of sinusoidal spaces, and bleeding in hepatic lobes [Figure 3]. The rats treated with extract and silymarin showed a significant ($P < 0.01$) protection, against paracetamol-induced hepatic damage by normalizing serum biochemical parameters and by minimizing the histopathological abnormalities. Among the test doses, percentage protection shown by extract at 200 mg/kg was well comparable to that of silymarin (100 mg/kg).

Table 1: *In vitro* antioxidant studies on the extract, methanolic extract of *Ludwigia hyssopifolia*

Free radical	IC50 value of the extract LHME in µg/ml	IC50 value of the standard in µg/ml
DPPH	48.3±1.6	0.39±0.42 (rutin)
Superoxide	467.31±4.5	3.52±0.28 (rutin)
Nitric oxide	59.31±2.8	6.87±0.44 (ascorbic acid)
Hydroxyl radical	281.37±3.1	3.83±0.13 (mannitol)

Data expressed as mean±SD, *n*=3. LHME: Methanolic extract of *Ludwigia hyssopifolia*; SD: Standard deviation

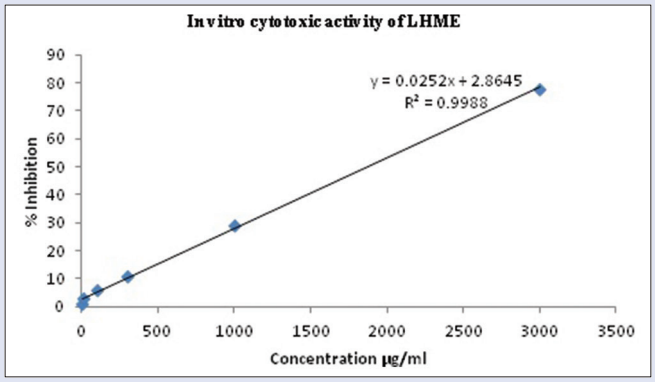

Figure 1: *In vitro* cytotoxic activity of methanolic extract of *Ludwigia hyssopifolia*

Table 2: Hepatoprotective activity of methanolic extract of *Ludwigia hyssopifolia in vitro*

Groups	Cell viability (%)	LDH	ALT	AST
Group I (control)				
Normal control	98.51±1.1	133.33±4.11	8.61±1.14	11.61±1.03
DMSO control (0.1% v/v)	95.61±1.04	141.11±3.34	9.33±1.24	13.14±1.02
Silymarin control (100 µg/ml)	97.24±1.02	138.36±4.16	8.93±1.25	12.18±1.11
LHME control (200 µg/ml)	96.11±1.08	140.31±3.76	9.12±2.11	13.06±1.24
Group II (toxin treatment)				
100 mM ethanol	31.27±1.02[a]	230.6±5.42	36.38±2.15	49.31±2.54
Group III (silymarin treatment)				
100 mM ethanol + silymarin (50 µg/ml)	78.31±1.81[a]	156.31±3.16[a]	15.17±2.14[a]	20.14±2.17[a]
100 mM ethanol + silymarin (100 µg/ml)	86.72±1.35[a]	144.23±4.32[a]	13.31±1.19[a]	18.36±1.33[a]
Group IV (LHME treatment)				
100 mM ethanol + LHME (50 µg/ml)	62.15±1.01[a]	189.3±1.26[a]	24.61±2.35[a]	29.31±1.27[a]
100 mM ethanol + LHME (100 µg/ml)	70.16±1.52[a]	174.21±3.14[a]	19.64±2.31[a]	26.34±2.05[a]
100 mM ethanol + LHME (200 µg/ml)	69.33±2.03[a]	182.24±4.43[a]	22.4±2.18[a]	31.36±2.67[a]

Data represent mean±SD (*n*=6). *P* value: Normal control versus other groups; [a]*P*<0.01. LHME: Methanolic extract of *Ludwigia hyssopifolia*, DMSO: Dimethyl sulfoxide; SD: Standard deviation

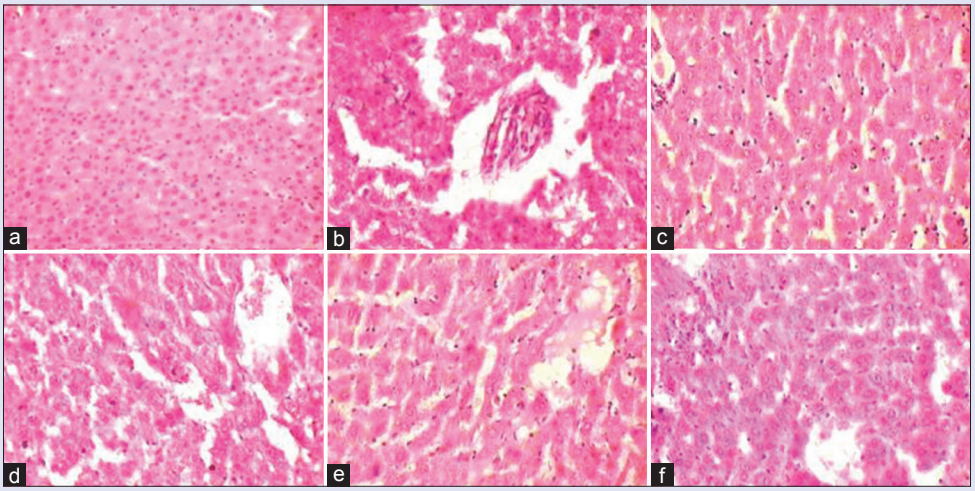

Figure 2: Effect of methanolic extract of *Ludwigia hyssopifolia* on histology of liver of rats against ethanol-induced hepatotoxicity. Effect of methanolic extract of *Ludwigia hyssopifolia* on liver pathologic analysis by hematoxylin-eosin stain after ethanol treatment in rats. methanolic extract of *Ludwigia hyssopifolia* on liver pathologic analysis and ethanol were given as described in "Methods." (a) Normal group; (b) ethanol treated toxic group; (c) ethanol and 100 mg/kg silymarin-treated group; (d) ethanol and 100 mg/kg methanolic extract of *Ludwigia hyssopifolia* on liver pathologic analysis-treated group; (e) ethanol and 200 mg/kg methanolic extract of *Ludwigia hyssopifolia* on liver pathologic analysis-treated group; (f) ethanol and 400 mg/kg methanolic extract of *Ludwigia hyssopifolia* on liver pathologic analysis-treated group (×40)

Table 3: Effect of methanolic extract of *Ludwigia hyssopifolia* on different serum biochemical parameters in ethanol-induced hepatotoxicity in rats

Groups	Normal	Toxic	Silymarin (100 mg/kg)	LHME (100 mg/kg)	LHME (200 mg/kg)	LHME (400 mg/kg)
SGOT (IU/ml)	64.81±3.1	142.9±8.7	77.0±1.70[a] (83.75%)	104.35±2.3[a] (53.29%)	85.63±2.84[a] (73.9%)	89.35±3.29[a] (61.2%)
SGPT (IU/ml)	65.2±5.8	185.7±5.1	85.3±5.5[a] (83.80%)	136.55±1.6[a] (69.69%)	102.3±5.25[a] (70.42%)	105.28±4.2[a] (66.12%)
ALP (IU/ml)	451.6±12.3	961.2±20.2	542.0±12.5[a] (85.82%)	621.7±18.2[a] (66.16%)	599.9±15.1[a] (70.78%)	610.3±12.6[a] (68.1%)
DB (mg/dl)	0.03±0.001	1.21±0.24	0.61±0.02[a] (83.69%)	0.47±0.11[a] (62.23%)	0.34±0.05[a] (73.57%)	0.39±0.25[a] (69.23%)
TB (mg/dl)	0.15±0.06	2.95±0.25	0.61±0.07[a] (83.33%)	1.32±0.13[a] (57.05%)	0.90±0.06[a] (72.44%)	1.12±0.36[a] (65.71%)
TP (g/l)	8.31±1.47	5.21±1.35	7.71±0.15[a] (83.9%)	6.86±0.25[a] (62.87%)	7.31±0.15[a] (77.42%)	6.98±0.21[a] (66.55%)
ALB (g/l)	3.58±0.24	1.73±0.31	3.32±0.10[a] (84.84%)	2.73±0.11[a] (54.20%)	3.07±0.12[a] (72.25%)	2.96±0.31[a] (66.21%)
LDH (IU/ml)	221.67±4.4	371.4±7.5	243.5±3.75[a] (85.97%)	288.6±3.75[a] (55.46%)	261.6±8.22[a] (73.48%)	271.38±6.8[a] (66.22%)
GGT (IU/ml)	5.59±1.88	39.5±2.69	103±0.75[a] (85.41%)	18.21±3.73[a] (60.67%)	16.80±1.11[a] (68.14%)	17.11±2.13[a] (66.21)
PT (s)	12.6±1.11	145.6±4.5	35.3±1.52[a] (82.41%)	58.1±93.01[a] (65.70%)	44.37±3.05[a] (75.52%)	49.38±0.26[a] (72.77%)

Data represent mean±SD (n=6). Values in parenthesis indicate percentage recovery. P value: Normal versus other groups; [a]P<0.01. LHME: Methanolic extract of *Ludwigia hyssopifolia*, SGOT: Serum glutamate-oxaloacetate transaminase; SGPT: Serum glutamate-pyruvate transaminase; ALP: Alkaline phosphatase; DB: Direct bilirubin; TB: Total bilirubin; TP: Total protein; ALB: Albumin; LDH: Lactate dehydrogenase; GGT: Gamma-glutamyltransferase, PT: Prothrombin time; SD: Standard deviation

Table 4: Effect of methanolic extract of *Ludwigia hyssopifolia* on different serum biochemical parameters in paracetamol-induced hepatotoxicity in rats

Groups	Normal control	Toxic	Silymarin (100 mg/kg)	LHME (100 mg/kg)	LHME (200 mg/kg)	LHME (400 mg/kg)
SGOT (IU/ml)	65.1±4.12	133.9±9.47	71.0±1.37[a] (84.75%)	91.36±2.30[a] (62.32%)	84.63±2.1[a] (71.9%)	88.26±3.9[a] (66.6%)
SGPT (IU/ml)	66.6±3.47	137.60±6.35	76.3±1.5[a] (85.78%)	101.93±4.2[a] (50.0%)	85.3±1.62[a] (73.12%)	91.21±2.4[a] (64.68%)
ALP (IU/ml)	567.2±15.11	1078.0±13.31	668±13.5[a] (80.83%)	789.83±9.1[a] (56.19%)	731.9±15[a] (67.9%)	754.3±12.4[a] (63.4%)
DB (mg/dl)	0.03±0.007	1.33±0.47	0.48±0.02[a] (80.08%)	0.8±0.05[a] (62.56%)	0.74±0.06[a] (70.4%)	0.71±0.22[a] (66.8%)
TB (mg/dl)	0.071±0.024	2.32±0.11	0.55±0.07[a] (82.89%)	0.93±0.13[a] (61.38%)	0.68±0.05[a] (68.7%)	0.79±0.11[a] (68.6%)
TP (g/l)	7.92±0.15	5.03±0.34	7.43±0.05[a] (81.10%)	6.86±0.15[a] (62.17%)	7.11±0.15[a] 70.42%	6.98±0.24[a] (66.55%)
ALB (g/l)	3.72±0.14	1.96±0.28	3.35±0.20[a] (78.54%)	3.11±0.005[a] (65.68%)	3.2±0.12[a] (68.25%)	3.18±0.31[a] (67%)
LDH (IU/ml)	168.57±5.29	340.43±11.68	195.32±9.1[a] (85.24%)	241.7±12.4[a] (56.08%)	228.2±8.2[a] (64.48%)	234.38±6.8[a] (61.22%)
GGT (IU/ml)	4.39±0.05	34.16±3.56	15.86±2.3[a] (80.16%)	19.26±3.6[a] (63.60%)	16.3±1.11[a] (73.14%)	18.11±2.13[a] (67.21%)
PT (s)	13.13±2.41	168.17±3.15	29.33±3.78[a] (84.29%)	58.0±2.0[a] (68.21%)	53.3±0.05[a] (74.52%)	56.38±0.26[a] (70.77%)

Data represent mean±SD (n=6). Values in parenthesis indicate percentage recovery. P value: Normal versus other groups; [a]P<0.01. LHME: Methanolic extract of *Ludwigia hyssopifolia*; SGOT: Serum glutamate-oxaloacetate transaminase; SGPT: Serum glutamate-pyruvate transaminase; ALP: Alkaline phosphatase; DB: Direct bilirubin; TB: Total bilirubin; TP: Total protein; ALB: Albumin; LDH: Lactate dehydrogenase; GGT: Gamma-glutamyltransferase; PT: Prothrombin time; SD: Standard deviation

Curative study

The results of the study are presented in Table 5 and Figure 4. The effective dose of the extract, LHME 200 mg/kg, was selected to assess antihepatotoxic activity against D-GaIN-induced hepatotoxicity in rats. Pretreatment of rats with D-GaIN caused hepatotoxicity which was confirmed by estimating the hepatic enzymes, bile pigments, proteins, and GLU in serum and in the histology of liver of rats. Treatment with reference standard (silymarin 100 mg/kg) and the extract, LHME (200 mg/kg b.w. p.o.), showed a significant (P < 0.01) protection, against D-GaIN-induced liver

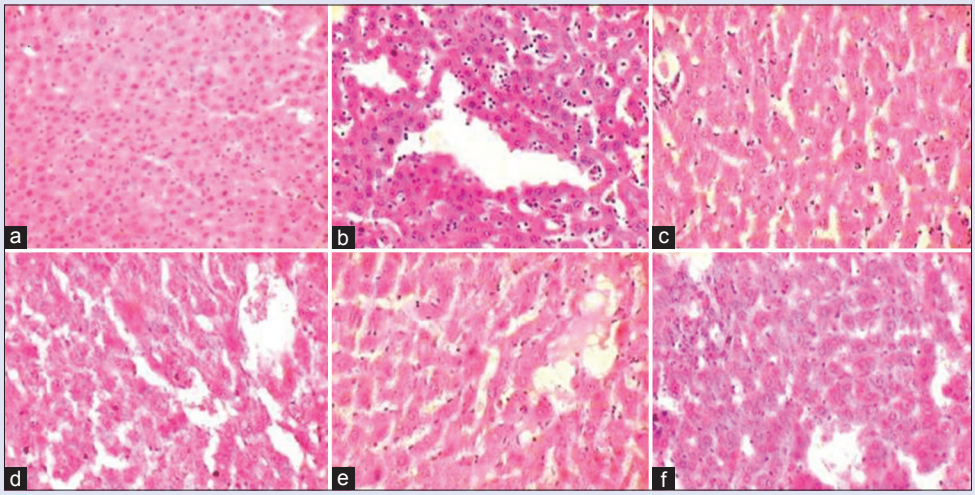

Figure 3: Effect of methanolic extract of *Ludwigia hyssopifolia* on histology of liver of rats against paracetamol-induced hepatotoxicity. Effect of methanolic extract of *Ludwigia hyssopifolia* on liver pathologic analysis by hematoxylin-eosin stain after paracetamol treatment in rats. Methanolic extract of *Ludwigia hyssopifolia* on liver pathologic analysis and paracetamol were given as described in "Methods." (a) Normal group; (b) paracetamol-treated toxic group; (c) paracetamol and 100 mg/kg silymarin-treated group; (d) paracetamol and 100 mg/kg methanolic extract of *Ludwigia hyssopifolia* on liver pathologic analysis-treated group; (e) paracetamol and 200 mg/kg methanolic extract of *Ludwigia hyssopifolia* on liver pathologic analysis-treated group; (f) paracetamol and 400 mg/kg methanolic extract of *Ludwigia hyssopifolia* on liver pathologic analysis-treated group (×40)

Table 5: Effect of methanolic extract of *Ludwigia hyssopifolia* on different serum biochemical parameters in D-galactosamine-induced antihepatotoxicity in rats

Groups	Normal control	Toxic	Silymarin (100 mg/kg)	LHME (200 mg/kg)
GLU (mg/dl)	87.1±2.4	20.5±1.5	66.41±3.41 (68.9%)	51.12±2.5 (45.45%)
SGOT (IU/ml)	66.10±5.98	174.23±8.41	78.0±3.37[a] (88.59%)	97.46±2.20[a] (70.32%)
SGPT (IU/ml)	48.36±3.98	194.60±9.35	69.93±4.2[a] (85.0%)	83.30±5.95[a] (76.87%)
ALP (IU/ml)	484.2±15.19	1062.0±103.31	612.83±9.10[a] (77.19%)	662.83±9.4[a] (69.78%)
DB (mg/dl)	0.06±0.005	1.88±0.49	0.48±0.05[a] (76.56%)	0.62±0.04[a] (69.73%)
TB (mg/dl)	0.073±0.015	2.52±0.10	0.66±0.07[a] (75.89%)	0.98±0.13[a] (62.38%)
TP (g/l)	7.62±0.1	4.13±0.30	7.22±0.05[a] (81.10%)	7.03±0.15[a] (78.17%)
ALB (g/l)	3.61±0.10	1.42±0.26	3.15±0.20[a] (78.94%)	2.93±0.005[a] (69.68%)
LDH (IU/ml)	139.57±6.79	229.43±18.68	154.32±9.1[a] (83.94%)	162.7±12.4[a] (74.08%)
GGT (IU/ml)	6.02±0.04	41.16±4.36	12.86±2.3[a] (79.66%)	15.26±3.6[a] (73.60%)
PT (s)	10.33±1.42	159.67±3.15	33.33±3.78[a] (81.79%)	50.0±2.0[a] (68.21%)
MDA (mM/mg)	1.56±0.05	3.77±0.09	1.93±0.04[a] (75.69%)	2.23±0.15[a] (69.45%)
GSH (nM/mg)	6.34±0.14	1.42±0.04	5.36±0.15[a] (79.66%)	4.43±0.25[a] (69.67%)
CAT (U/mg)	10.06±0.1	3.05±0.08	8.7±0.2[a] (88.91%)	7.62±0.16[a] (65.65%)

Data represent mean±SD (n=6). Values in parenthesis indicate percentage recovery. P value: Normal versus other groups; [a]P<0.01. LHME: Methanolic extract of *Ludwigia hyssopifolia*; GLU: Glucose; SGOT: Serum glutamate-oxaloacetate transaminase; SGPT: Serum glutamate-pyruvate transaminase; ALP: Alkaline phosphatase; DB: Direct bilirubin; TB: Total bilirubin; TP: Total protein; ALB: Albumin; LDH: Lactate dehydrogenase; GGT: Gamma-glutamyltransferase; PT: Prothrombin time; MDA: Malondialdehyde; GSH: Glutathione; CAT: Catalase; SD: Standard deviation

damage with good recovery from the altered level of aforesaid serum biochemical parameters and by curtailing the histological abnormalities. The MDA level was decreased, and GSH and CAT levels were significantly increased in LHME 200 mg/kg-treated group.

Evaluation of anti-inflammatory activity of methanolic extract of *Ludwigia hyssopifolia*

The results of the study are given in Table 6. After subplantar administration of carrageenan in rats, a time-dependent increase in paw edema was observed at 1 h and was maximal at 2 h after administration of carrageenan in the toxic control group. Pretreatment with LHME 100, 200, and 400 mg/kg b.w, diclofenac sodium 20 mg/kg produced varying levels of significant inhibition of paw edema induced by carrageenan. The anti-edematous effect shown by LHME 200 mg/kg was comparable to that of reference standard diclofenac sodium (20 mg/kg) at any time point of the study ($P < 0.01$).

DISCUSSION

Free radicals such as superoxide, nitric oxide, H_2O_2, and hydroxyl radicals are harmful and cause oxidative damage to DNA, lipids, proteins, etc., leading to several degenerative disorders such as cardiovascular, respiratory, and liver disorders.[24] The findings from the *in vitro* antioxidant studies revealed that the extract, LHME, has shown a concentration-dependent DPPH, superoxide, nitric oxide, and hydroxyl radical scavenging activities and reducing power. The antioxidant activity exhibited by the extract, LHME, may be attributed to the flavonoids and phenolic compounds present in it.[25] HepG2 cells are a suitable *in vitro* model system for the study of human liver diseases that are caused by xenobiotic metabolism and other chemicals that cause toxicity to the liver.[26] The doses (50, 100, and 200 µg/ml) were selected based on the results of cytotoxicity assay (CTC_{50} value). When HepG2 cells are incubated with 100 mM ethanol for 24 h, there is a significant decrease in cell viability and increase in hepatic enzymes such as LDH, AST,

and ALP which might be due to cellular leakage and loss of functional integrity of hepatic membrane architecture.[27] Treatment with standard silymarin and LHME preserved the structural integrity of cell membrane and prevented the leakage of these enzymes and thus improved the cell viability.

In *in vivo* hepatoprotective activity, ethanol/paracetamol intoxication in rats leads to the change in the level of hepatic enzymes such as ALT, AST, ALP, GGT, and LDH in serum. High levels of AST and ALT are the crucial parameters to detect the liver damage.[28] The membrane-bound enzyme ALP and bilirubin levels are related to the status and function of hepatic cells. Increased serum ALP is due to increased synthesis in the presence of increasing biliary pressure.[12] GGT is microsomal brush border enzyme found notably in liver. Serum GGT, ALP, TB, and DB levels are considered as a better index to biliary tract damage. In liver injury, other biochemical parameters such as TP and ALB in serum are also significantly altered due to inhibition of protein synthesis. Ethanol/paracetamol intoxication also prolonged the PT. Treatment with LHME caused significant improvement in the level of these parameters toward normalization which could be due to by-protecting the structural integrity of plasma membrane of liver cells preventing the leakage of cytosolic contents into the blood and by increasing the synthetic capacity of the liver. The extract, LHME, has shown a definite sign of protection against injury at all test doses. Among the three test doses, LHME at 200 mg/kg exhibited a remarkable protection from both altered serum biochemical parameters and histopathological lesions of the liver.

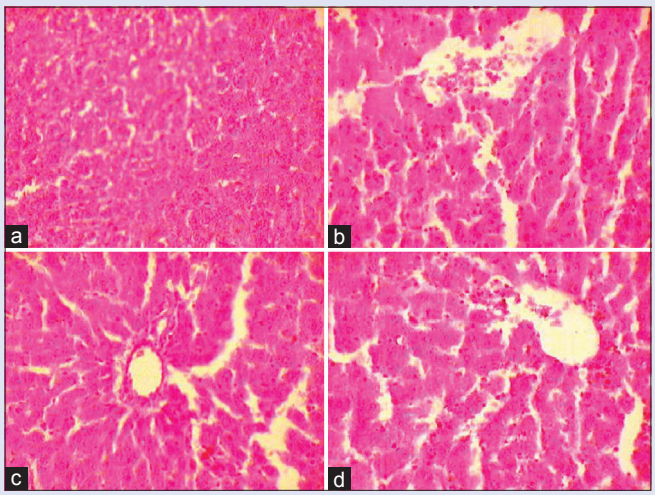

Figure 4: Effect of methanolic extract of *Ludwigia hyssopifolia* on histology of liver of rats against D-galactosamine induced hepatotoxicity. Effect of methanolic extract of *Ludwigia hyssopifolia* on liver pathologic analysis by hematoxylin-eosin stain after D-galactosamine treatment in rats. Methanolic extract of *Ludwigia hyssopifolia* on liver pathologic analysis and D-galactosamine were given as described in "Methods." (a) Normal group; (b) D-galactosamine-treated toxic group; (c) D-galactosamine- and 100 mg/kg silymarin-treated group; (d) D-galactosamine and 200 mg/kg methanolic extract of *Ludwigia hyssopifolia* on liver pathologic analysis-treated group (×40)

The hepatotoxicity induced by D-GaIN resembles that of human viral hepatitis both in metabolic and morphological variations[29] and hepatocyte apoptosis.[30] The hepatotoxicity induced by D-GaIN is due to the acquisition of uridine di-phosphate (UDP)-GaIN derivatives in the liver and depletion of hepatic uridine tri-phosphate (UTP), thus resulting in the inhibition of mRNA and protein synthesis leading to activation of various signaling pathways consequently causing apoptotic cellular death.[31] Treatment with LHME 200 mg/kg decreased the level of serum ALT, AST, ALP, GGT, and LDH suggesting extract's ability to scavenge reactive oxygen species generated from D-GaIN intoxication and hence prevented damage to the hepatic membrane. Administration of LHME 200 mg/kg decreased the serum bilirubin level indicating the extract's ability to repair the damaged hepatocytes. In addition, it also normalized the reduced levels of TP, ALB, and PT which may be attributed to the extract's ability to stabilize endoplasmic reticulum and trigger protein synthesis. Galactosamine-1-phosphate inhibits the uridine diphosphate glucose (UDPG)-pyrophosphorylation reaction which is used in synthesis of GLU, glycogen, heteropolysaccharides, and glucuronides. The extract also increased the serum GLU level by eliminating the toxic metabolite, galactosamine-1-phosphate. D-GaIN reduces the activity of antioxidant enzymes and causes hepatopathy.[32] The increase in MDA or decrease in GSH and CAT levels indicates the lipid peroxidation. MDA is one of the end products resulting from the peroxidation of biological membrane composing of polyunsaturated fatty acids.[33] GSH is a nonenzymatic antioxidant which prevents damage to important cellular components caused by reactive oxygen species such as free radicals and peroxides.[20] CAT is known to breakdown H_2O_2 to H_2O and O_2 and can be found in the peroxisome and mitochondria, especially in liver. The groups received LHME and standard before D-GaIN administration exhibited significant protection against lipid peroxidation and enzymatic antioxidants, namely, GSH and CAT indicating the antioxidant potential of LHME. The significant antioxidant effect of LHME 200 mg/kg revealed that it has the ability to ameliorate oxidative stress and preserve hepatic function against free radicals produced by D-GaIN intoxification.

Hepatotoxicity induced by paracetamol resembles other kinds of acute inflammatory liver disease with prominent increase of AST, ALT, and ALP levels. Furthermore, the mechanism responsible for effects of alcohol is mediated by cytokines which are secreted by liver and other parts of the body. In the liver, persistent cytokine secretion results in chronic inflammation leading to conditions such as hepatitis, fibrosis, and cirrhosis.[34] Hence, the extract LHME was also evaluated for anti-inflammatory activity against carrageenan-induced paw edema. The extract, LHME (200 mg/kg), exhibited a significant ($P < 0.01$) anti-inflammatory activity which was well comparable with the standard drug, diclofenac sodium (20 mg/kg). The activity may be attributed to the antioxidant compounds present in the extract, LHME, and thus substantiated the hepatoprotective activity.

Table 6: Anti-inflammatory activity of methanolic extract of *Ludwigia hyssopifolia* on carrageenan-induced rat paw edema model

Groups	Rat hind paw volume (mean±SD) in mL			
	1 h	2 h	3 h	4 h
Control	2.48±0.02	2.39±0.02	2.32±0.04	2.29±0.008
Diclofenac sodium 20 mg/kg	1.86±0.16[a] (80.52%)	1.83±0.004[a] (84.4%)	1.79±0.21[a] (89.6%)	1.75±0.09[a] (94.9%)
LHME 100	2.28±0.02[b] (25.31%)	2.16±0.01[a] (33.25%)	2.03±0.05[a] (41.27%)	1.97±0.04[a] (48.67%)
LHME 200	1.99±0.03[a] (44.3%)	1.74±0.2[a] (70.64%)	1.69±0.15[a] (85.02%)	1.64±0.05[a] (89.02%)
LHME 400	2.05±0.03[a] (40.12%)	1.98±0.06[a] (45.24%)	1.86±0.05[a] (60.21%)	1.72±0.03[a] (72.14%)

Data expressed as mean±SD, n=6, values in parenthesis indicate percentage recovery. P: [b]<0.05; [a]<0.01

CONCLUSION

The findings of the study suggest that the extract has antioxidant principles such as flavonoids and phenolic compounds which reduce the inflammatory cytokines mediated in liver disease contributing to the hepatoprotective activity of the extract. Further, the study also substantiates the usage of the plant in traditional medicine for the treatment of jaundice.

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Conflicts of interest

There are no conflicts of interest.

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