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Ameliorative effect of ethanolic extract of *Limnophila rugosa* (Scrophulariaceae) in paracetamol- and carbon tetrachloride-induced hepatotoxicity in rats

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Abstract

Background: *Limnophila rugosa* (Scrophulariaceae) is a perennial aquatic plant used as a diuretic and digestive tonic as well as in the treatment of diarrhea, dysentery, dyspepsia and urinary ailments. Genus *Limnophila* has been reported as hepatoprotective. The present study was undertaken to evaluate the hepatoprotective activity of the ethanolic extract of *L. rugosa* aerial part in paracetamol- and carbon tetrachloride-induced (CCl_4) hepatotoxicity in albino Wistar rats. Ethanolic extract was subjected to high-performance liquid chromatography (HPLC) analysis for the estimation of phenolic and flavonoid compounds and gas chromatography–mass spectrometry (GC–MS) analysis for phytochemical analysis. The in vitro antioxidant activity was carried out by 2,2-diphenyl-1-picrylhydrazyl, nitric oxide radical and hydrogen peroxide assay. Hepatoprotective potential of *L. rugosa* was studied in paracetamol (750 mg/mg)- and CCl_4 (1.25 ml/kg)-induced liver damage in albino rats at dose 200 and 300 mg/kg using silymarin (100 mg/kg) as standard. Lipid peroxidation, superoxide dismutase (SOD), catalase (CAT) and glutathione (GSH) were determined in liver tissue homogenate. Serum biochemical and histopathological examination was performed. Molecular docking analysis was performed to understand the molecular mechanism of hepatoprotective activity.

Results: HPLC analysis revealed predominance of rutin. GC–MS analysis revealed camphor as principal component. Ethanolic extract exhibited significant concentration-dependent scavenging efficacy. The altered biochemical chemical parameters: aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, bilirubin, cholesterol, albumin, globulin and total protein, were significantly improved at 200 and 300 mg/kg in experimental rats. Extract signified hepatoprotective by decreasing lipid peroxidation and upregulating SOD, CAT and GSH. The findings were well supported by histological analysis. 2-Butyl-2, 7-octadien-1-ol (-5.8) and camphor (-4.8) gave the highest docking score on the transforming growth factor-β1.

Conclusions: The ameliorative effect of *L. rugosa* in the rat model of hepatotoxicity could be attributed to its antioxidant potential and bioactive principles such as betulin, 5-hydroxy-6,7,4′-trimethoxyflavone (salvigenin), betulinic acid, ursolic acid, 3-octanol, acetophenone, anisylacetone, caryophyllene, *cis*-anethole and the compounds camphor and 2-butyl-2,7-octadien-1-ol identified from GC–MS analysis.

Keywords: HPLC, GC–MS, Paracetamol, CCl_4, Molecular docking

Background

Liver is considered a vital organ of the human body involved in important functions. Any injuries or impairment to the liver leads to several complication of

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individual's health. It preserves the body against foreign particle by elimination through the excretion process [1]. Some drugs and chemicals may cause hepatotoxicity. Arsenic, phosphorus, copper and iron are the inorganic compounds which are accountable for generating hepatotoxicity.

The change in normal physiological function of the liver attributed to the accumulation of some toxic chemicals (thioacetamide, alcohol) in the liver tissue leads to generation of free radicals. The reactive oxygen species (ROS) produced in the process of oxidative stress reacts with cell membranes, leading to lipid peroxidation or inflammation. The ROS has been considered as an important pathological mediator of many clinical diseases (heart, diabetes, kidney, liver, hepatic necrosis, gout, hepatitis, cirrhosis and cancer) [2]. The imbalance between the levels of pro-oxidants and antioxidants can cause oxidative stress, which can damage the body [3]. The synthetic drugs currently available for the treatment of liver disease are inadequate and are known to have various side effects. Previous scientific reports have shown that the use of plant phenols minimizes the risk of hepatic disease by acting as an antioxidant [4]. Recently, there has been an increasing interest in plants that are wealthy in antioxidants and healthy phytochemicals. It is believed that antioxidants play a vital role in the body's defense system against ROS. Many plants usually contain a lot of antioxidants, such as vitamins C and E, carotenoids, flavonoids and tannins. Administration of antioxidants might reduce oxidative damage to organs. Further, there has been research to suggest that the antioxidants naturally found in herbs can also reduce oxidative damage to various organs. Plants possess flavonoids, tannins, carotenoids, steroids and other compounds that may help treat liver disease [5].

Limnophila rugosa (Scrophulariaceae) is a perennial aquatic plant encountered throughout India. This plant is used as diuretic, stomachic, as well as a perfume for the hair [6]. It is also used to treat elephantiasis as a combination with coconut oil. Besides this, it is used to treat diarrhea, dysentery and dyspepsia, as a carminative and as a tonic. In pestilent fever, the juice of the plant is massaged over the body [7]. Leaf infusions are employed in the islands of the Philippines and India as diuretics, stomachics, digestion enhancers and tonics [7–9]. Tulsi leaves along with leaf paste made from *L. rugosa* were traditionally used in Odisha as a remedy for urinary burning [10]. The plant is also found useful in liver disorder [11]. The genus of *Limnophila* (*L. heterophylla* and *L. repens*) has already been reported for hepatoprotective activity [12, 13]. However, no such type of study is reported yet for *L. rugosa*. Free radical scavenging activity of *L. rugosa* was studied against 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) free radical and nitro blue tetrazolium (NBT) color reduction test [14]. As a potential antioxidant, *L. rugosa* can also have a hepatoprotective effect. Therefore, the aim of the present study was to investigate the hepatoprotective activity of the ethanolic extract of *L. rugosa* in hepatotoxicity that is induced in albino Wistar rats using paracetamol and CCl_4.

Experimental animals

Sixty Wistar albino rats of either sex (8–10 weeks old weighing about 150–170 gm) were procured from M/S Chakraborty Enterprises (reg. no.: 1443/PO/Bt/s/11/CPCSEA, Kolkata) for hepatoprotective study and were housed in cages. A 12-h day and night schedule at a temperature of 22 ± 3 °C was used for acclimatization of the rats to laboratory conditions for 7 days before the commencement of experiment. Standard pellet diets and water ad libitum were provided for the animals. During the experimental period, the cages were kept cleaned at an interval of three days and maintained with hygienic condition prior to avoid any infection. Proper care was provided to the animals in compliance with the ethical guideline of International Standards for the use of laboratory animals. The design protocol was approved by Animal Ethics Committee (1376/ac/10/CPCSEA).

A study conducted by Maes et al. [15] revealed that an acute overdose of paracetamol in mice best reproduces the pathophysiology of liver injury in humans compared to other relevant species. However, several studies have demonstrated the successful induction of hepatocellular damage or necrosis by higher doses of paracetamol in rodents including experimental rats. Moreover, rats with greater body mass are more convenient for experimentation than mice, particularly when blood parameters are needed to be analyzed. In our studies also, paracetamol successfully induced hepatotoxicity, proved by liver function test as well as histopathological studies. This justified our use of rat in paracetamol-induced hepatotoxicity model for evaluation of hepatoprotective activity of *L. rugosa* [16–19].

Methods

Chemicals and reagent

The chemicals used were petroleum ether (Merck), ethanol (Merck), 2,2-diphenyl-1-picrylhydrazyl (Sigma–Aldrich), ascorbic acid (Sigma–Aldrich), carbon tetrachloride (Merck), carboxymethyl cellulose (Sigma–Aldrich), gallic acid (Merck), hydrogen peroxide (Merck), paracetamol (Glaxo SmithKline), quercetin (Sigma–Aldrich), rutin (Sigma–Aldrich) and silymarin (Micro-Lab). All the chemicals were analytical grade.

Collection and authentication of *Limnophila rugosa*

The plant was collected from Barpali, Bargarh, Odisha, and identified by Botanist Dr. Surya Kumar Barpanda, Shree Ram College, Rampur, Sonepur. The voucher specimen TPC/COL/21/012 was deposited in Pharmacology Department of the Pharmaceutical College, Barpali, for future reference. The aerial parts (leaf, stem, fruit and flower) were dried under shade, powdered and stored in an airtight container.

Extraction of plant material

The dried aerial parts were defatted with petroleum ether (60–80 °C) to remove waxy material, followed by extraction with ethanol in a Soxhlet apparatus and concentrated using a rotary evaporator at 40 °C. The percentage yield was recorded and the dried extract stored in the refrigerator (4 °C). Qualitative phytochemical analysis of the extracts of *L. rugosa* was performed according to standard procedures [20]. Ethanolic extract of *L. rugosa* (ELR) was taken for the experimental purpose.

Identification of phenolic and flavonoids by HPLC analysis

High-performance liquid chromatography (HPLC) analysis was carried out on ELR for the quantification of phenolic (gallic acid) and flavonoid (quercetin and rutin) compounds using an auto sampler and a photodiode array detector. Each 4 mg of gallic acid, quercetin and rutin was dissolved in 60 ml HPLC–methanol and gently heated in a 100-ml volumetric flask to prepare standard stock solution. A methanolic solution containing 60 ml of ELR was sonicated for 10 min and boiled on a water bath for 5 min at 70–80 °C. The solution was allowed to cool at room temperature and the volume adjusted to 100 ml with methanol. A 0.45-µm membrane filter paper was used to filter the standard and test solutions. Greater resolution of the compounds was accompanied by gradient elution of buffer concentration (solvent A) and acetonitrile (solvent B) (Additional file 1: Table S1). Twenty microliters each of test and reference sample was injected and run for 6 min. The gallic acid was noted at 270 nm, while quercetin and rutin were observed at 370 nm [21].

GC–MS analysis

The gas chromatography–mass spectrometry (GC–MS) analysis of ELR was performed as per the method described by Meher et al. [22]. A Thermo Trace 1300 GC was used in conjunction with the Thermo TSQ 800 triple quadrupole MS, following condition of TG 5MS (30 m × 0.25 mm ID × 0.25 µm). 5% phenyl methylpolysiloxane is used as a column. A constant flow rate of 1.0 ml/min of helium (carrier gas) was used with an injection volume of 1.0 µl. A temperature increase of 10 °C per min to 280 °C was programmed for an oven from 60 °C. A mass spectrometer (MS TSQ 8000) was used to look at fragments between 40 and 550 Da. Mass spectrometry data from the National Institute of Standards and Technology (NIST) library were used to identify phyto-components.

Estimation of total phenolic and flavonoid content

Total phenolic and flavonoid content of ELR was performed by following the methods of Danilewicz et al. and Park et al. [23, 24], respectively. Gallic acid (20–100 µg/ml) was used as a standard for the estimation of the total phenolic contents and expressed as milligram gallic acid equivalents per gram (mg GAE/g). Calibration curve for standard (quercetin) was constructed at concentration 5–100 µg/ml for quantification of total flavonoids, and the values were expressed as milligram of quercetin equivalent per gram (mg QE/g).

Assay of in vitro antioxidant activity

The free radical scavenging property of ELR (100–500 µg/ml) was investigated by DPPH assay, nitric oxide radical scavenging and hydrogen peroxide (H_2O_2) assay as per the method described by Meena et al. [25] and Green et al. [26]. Ascorbic acid (100–500 µg/ml) was used as standard. The percentage inhibition was calculated by using the following formula:

$$\%\text{ofInhibition} = \frac{A - B}{A} \times 100$$

where A is the absorbance of control and B is the absorbance of sample at different concentrations.

All the assay methods were performed in triplicate.

Determination of IC_{50}

A calibration curve drawn from percentage inhibition against concentration was used to determine the IC_{50} values of extracts.

Acute oral toxicity study

Acute oral toxicity study was performed following the guideline of Organization for Economic Cooperation and Development (OECD) 423 (Annexure 2b) [27]. Twelve female rats were randomly divided into four groups of three rats each. The ELR was administered to the female rats under overnight fasting. Group I received 1% carboxy methyl cellulose (CMC) and treated as normal control. Group II received ELR 50 mg/kg body weight dissolved in 1% CMC. The rats were observed for general behavioral changes after treatment for the first 4 h and then over a period of 48-h. Group III received the next higher dose of ELR 300 mg/kg body weight dissolved in 1% CMC and observed for 48-h. There were no signs of

toxicity or mortality observed after 48 h of treatment. Group IV received the next higher dose of ELR 2000 mg/kg with 1% CMC. All animals were observed at least once during the first 30 min in the first 24 h with great consideration given for the first 4 h following vehicle and ELR administration and then once a day for 14 days. This observation was done to check the onset of clinical or toxicological symptoms according to the OECD guideline. All observations included changes in skin, eyes and mucous membranes, and behavioral patterns (food intake, body weight, temperature, breathing, drowsiness, sedation, coma and mortality) were systematically recorded and maintained with an individual record. ELR at dose of 200 and 300 mg/kg was considered safest and taken for the hepatoprotective activity.

Effect of ELR on paracetamol-induced hepatotoxicity in rats

The rat liver toxicity caused by paracetamol was determined by the method of Araya et al. [28]. Thirty animals were randomly divided into five groups, each of six animals as follows:

Group-I: Served as normal control, received 1% CMC, 2 ml/kg/day, orally, daily for seven days.
Group-II: Received paracetamol (750 mg/kg) and treated as negative control
Group-III: Received standard drug (silymarin, 100 mg/kg/day), orally, daily for seven days and treated as positive control.
Group-IV: Received 200 mg/kg of ELR orally daily for seven days.
Group-V: Received 300 mg/kg of ELR orally daily for seven days.

On day 7, except for Group I, all rats received oral paracetamol suspension at a dose of 750 mg/kg body weight.

Effect of ELR on carbon tetrachloride-induced hepatotoxicity in rats

CCl_4-induced hepatotoxicity was performed by intraperitoneally injecting 1.25 ml/kg b. w. CCl_4 dissolved in liquid paraffin in ratio 1:1 after animals were denied food for 18 h. Thirty animals were randomly divided into five groups of six rats each [29].

Group-I: Served as normal control, received 1% CMC, 2 ml/kg/day, orally, daily for seven days.
Group-II: CCl_4-induced hepatotoxicity, received 1% CMC, 2 ml/kg/day, orally and treated as negative control.
Group-III: CCl_4-induced hepatotoxic rats received 100 mg/kg of silymarin for 7 days and treated as positive control.
Group-IV: CCl_4-induced hepatotoxic rats received 200 mg/kg of ELR for 7 days.
Group-V: CCl_4-induced hepatotoxic rats received 300 mg/kg of ELR for 7 days.

Serum biochemical examination

Twenty-four hours after the last dose, the rats were anesthetized with ether and killed by cervical dislocation. Blood samples were collected by cardiac puncture and centrifuged at 3000 rpm (rotation per minute) for 10 min. The serum was collected and subjected to serum biological examination. Alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), albumin, bilirubin, globulin and total protein were determined by using biochemical diagnostic kits (BIO-LATEST, USA).

Preparation of tissue homogenate

The liver taken from the killed animal was minced with a scalpel and homogenized in phosphate-buffered saline [30]. The tissue homogenate was centrifuged at 800 rpm for 10 min at 4 °C. The resultant supernatant liquid confirmed the presence of maximum release of the enzymes for biochemical examination.

Evaluation of in vivo antioxidant enzymes

Liver tissue homogenate was subjected to evaluation of in vivo antioxidant enzyme: lipid peroxidation (LPO), catalase activity (CAT), superoxide dismutase (SOD) and glutathione (GSH) following the method of Ohkawa et al., Beers and Sizer, Kakkar et al. and Ellman [31–34], respectively, with slight modification.

Histological analysis

Histological examination was carried out on liver of experimental animals. The tissues were fixed in 10% formalin and dehydrated with graded alcohol. It was then fixed into paraffin blocks after stained with hematoxylin and eosin. The manifestations of liver pathological changes were studied under a microscope [35].

Study of molecular docking

Molecular docking analysis of the phyto-constituents identified from GC–MS analysis of ELR with the protein (Crystal Structure of a Naphthyridine Inhibitor of Human TGF-beta Type I Receptor and Crystal structure of the complex PPARalpha/AL26-29) was carried out by Autodock Vina (version 1.1.2) software, following the method of Das et al. [36], taking silymarin as standards.

The binding affinity of the best fitting configurations to the target protein was expressed in Kcal/mol. The crystal structure of transforming growth factor β1 was obtained from Research Collaboratory for Structural Bioinformatics (RCSB) protein Data Bank.

Statistical analysis

Statistical analysis was carried out by one-way ANOVA followed by post hoc Tukey HSD test (GraphPad Prism 6.0) and values expressed as mean ± S.E.M (standard error mean). 'P' value less than 0.05 was regarded as significant.

Results

HPLC analysis for the identification of phenolic and flavonoid compounds

HPLC analysis of ELR revealed phenolic (gallic acid) and flavonoids (quercetin and rutin) compounds with rutin as highest concentration (4.66% w/w) (Additional file 1: Table S2). HPLC chromatograms of standard and ELR are presented in Fig. 1.

GC–MS analysis of ethanolic extract of *L. rugosa*

GC–MS analysis revealed four compounds: camphor (88.06%), 2-butyl-2, 7-octadien-1-ol (6.75%), 12, 15-octadecadiynoic acid, methyl ester (2.78%) and 10-heptadecen-8-ynoic acid, methyl ester, (E)- (2.40%) (Fig. 2 and Table 1).

Total phenolic and flavonoid content

The content of total phenolics and flavonoid of *L. rugosa* was found as 61.23 ± 1.31 and 53.11 ± 0.23 mg GAE/g and mg QE/g of extract, respectively (Table 2).

Evaluation of in vitro antioxidant activity

The antioxidant potential of ELR at 100, 200, 300, 400 and 500 µg/ml against free radicals was studied by DPPH, hydrogen peroxide and nitric acid radicals which exhibited maximum percentage of inhibition of 55.19 ± 0.45, 56.23 ± 0.81 and 65.23 ± 0.23 with corresponding IC_{50} values of 69.19 ± 0.58, 102.23 ± 0.25 and 54.12 ± 0.23 at 500 µg/ml. The data were comparable to standard ascorbic acid (Table 2).

Effect of ELR on biochemical markers in paracetamol-induced hepatotoxicity in rats

The rats in which hepatotoxicity was induced with paracetamol exhibited remarkable ($p < 0.001$) injuries to liver, indicated by elevated biochemical markers: ALT, AST, DBL, TBL, ALP and TC as compared with normal controls. Albumin, globulin and TP in negative control group were significantly reduced in contrast to normal healthy rats. However, treatment of ELR at 200 and 300 mg/kg was shown to exhibit remarkable decline in increased biochemical parameters and notable augmentation of ALB, globulin and TP in paracetamol hepatotoxicity rats compared to disease control group. Group treated with silymarin also significantly normalized the altered biochemical parameters (Tables 3, 4).

Effect of ELR on biochemical markers in carbon tetrachloride-induced hepatotoxicity in rats

Hepatotoxicity induced by CCl_4 was characterized by significant elevation of serum AST, ALT, ALP, DBL, TBL and TC and reduction in ALB, globulin and TP as compared to normal healthy rats. However, oral administration of ELR at 200 and 300 mg/kg appreciably restored the altered biochemical parameters compared to negative control rats. Also, group treated with silymarin exhibited significant improvement in the parameters discussed above in contrast to vehicle-treated CCl_4-induced rats (Tables 5, 6).

Antioxidant enzyme activities in paracetamol-induced hepatotoxicity

Rats subjected to paracetamol-induced hepatotoxicity exhibited significant increase in malondialdehyde (MDA) concentration as compared to healthy rats. Groups treated with ELR 200 and 300 mg/kg significantly eliminate the increased level of MDA. Administration of silymarin at 100 mg/kg significantly reduces the level of MDA compared to negative control rats. Rats subjected to paracetamol-induced hepatotoxicity showed significant decline in the levels of SOD, CAT and GSH in contrast to the healthy animals. Administration of rats with doses (200, 300 mg/kg) of ELR and silymarin (100 mg/kg) significantly ($p^{\wedge}0.001$) augmented these levels in contrast to Group II rats (Fig. 3).

Antioxidant enzyme activities in CCl_4-induced hepatotoxicity

In the present investigation, CCl_4-administered rats exhibited significantly higher levels of MDA than normal rats. In groups treated with ELR, MDA levels were found significantly reduced than those in Group II. In comparison with a normal control group, the CCl_4 treatment caused remarkable decline in the levels of tissue antioxidant enzymes (SOD and CAT). Administration of ELR (200, 300 mg/kg) caused significant upregulation of these endogenous antioxidant enzymes compared to negative control group. The activity of ELR was found comparable to that of silymarin (100 mg/kg) when administered at 300 mg/kg. A reduction in glutathione has been suggested to play an important role in protecting cellular membranes against peroxidative damage. The glutathione level in the CCl_4 administered animals was declined in

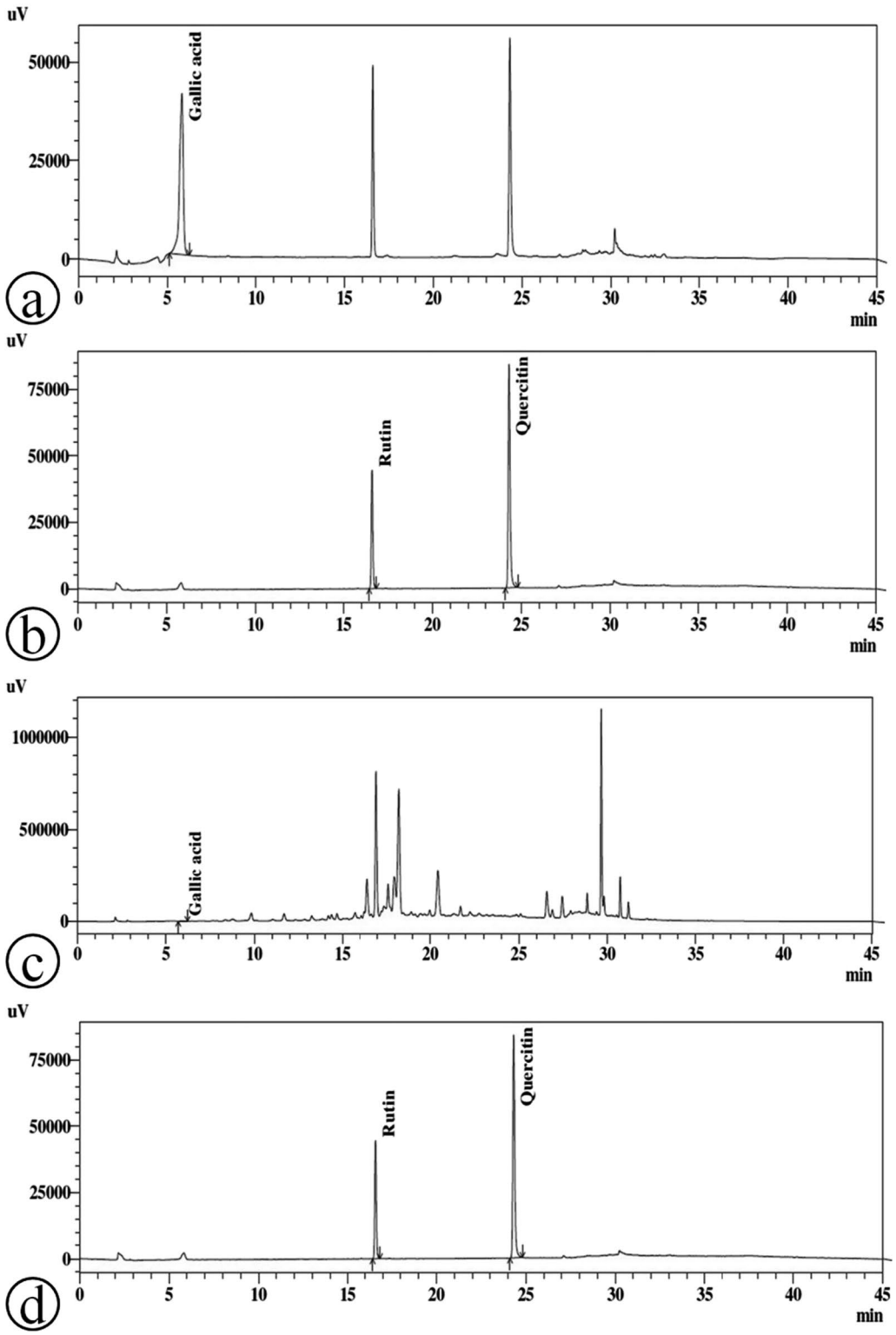

Fig. 1 HPLC chromatogram of **a** standard gallic acid; **b** standard rutin and quercetin; **c** gallic acid in ethanolic extract of *L. rugosa*; **d** rutin and quercetin in ethanolic extract of *L. rugosa*

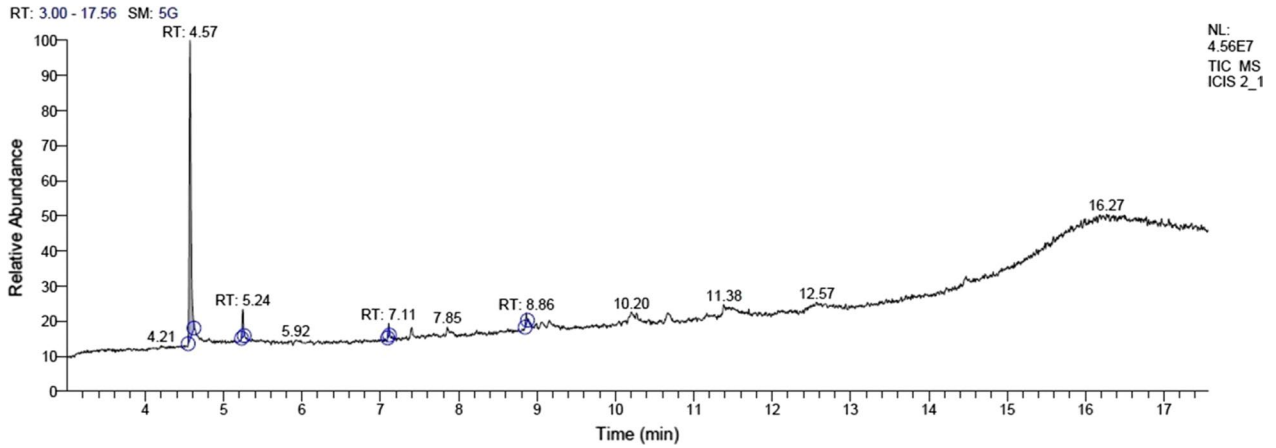

Fig. 2 GC–MS spectrum of ethanolic extract of *L. rugosa*

Table 1 GC–MS spectral analysis of ethanolic extract of *L. rugosa*

Sl. No	RT	Name of the component	M. F	M. W	Peak area %	Chemical nature
1	4.57	Camphor	$C_{10}H_{16}O$	152.23	88.06	Ketone
2	5.24	2-Butyl-2,7-octadien-1-ol	$C_{12}H_{22}O$	182.30	6.75	Alcohol
3	7.11	12,15-Octadecadiynoic acid, methyl ester	$C_{19}H_{30}O_2$	294.5	2.78	Ester
4	8.86	10-Heptadecen-8-ynoic acid, methyl ester, (E)-	$C_{18}H_{30}O_2$	278.4	2.40	Ester

Sl: serial number, RT: retention time, M.F: molecular formula, M.W: molecular weight

Table 2 Determination of total phenolic, flavonoid content, in vitro antioxidant activity and corresponding IC_{50} value of the ethanolic extract of *L. rugosa*

Plant extracts and standard	Total phenolic content (mg GAE/g)	Total flavonoid content (mg QE/g)	In vitro antioxidant		
			DPPH %	Hydrogen peroxide %	Nitric oxide %
Ascorbic acid	–	–	83.45 ± 0.51	75.81 ± 0.46	86.23 ± 0.63
IC_{50} (µg/ml)	–	–	57.12 ± 0.17	47.29 ± 0.72	49.13 ± 0.33
L. rugosa	61.23 ± 1.31	53.11 ± 0.23	55.19 ± 0.45*	56.23 ± 0.81*	65.23 ± 0.23*
IC_{50} (µg/ml)	–	–	69.19 ± 0.58	102.23 ± 0.25	54.12 ± 0.23

GAE: gallic acid equivalent, QE: quercetin equivalent

Values are expressed as mean ± SEM, $n = 3$ and were estimated by one-way ANOVA followed by Dunnet test. $^{*}p < 0.05$ considered as significant

Table 3 Effect of ethanolic extract of *L. rugosa* on liver function enzymes in paracetamol-induced hepatotoxicity in rats

Treatment	AST	ALT	ALP
Group-I (normal saline)	18.07 ± 0.32	49.66 ± 0.08	39.87 ± 0.08
Group-II (paracetamol 750 mg/kg)	83.56 ± 0.34**	101.08 ± 0.08**	98.09 ± 0.11**
Group-III (100 mg/kg Silymarin)	24.09 ± 0.45***	55.76 ± 0.93***	44.10 ± 0.34**
Group-IV (200 mg/kg)	51.09 ± 0.13*	72.03 ± 0.13**	68.42 ± 0.29*
Group-V (300 mg/kg)	39.15 ± 0.54*	67.32 ± 0.09**	53.76 ± 0.03*

AST: aspartate aminotransferase; ALT: alanine aminotransferase; ALP: alkaline phosphatase

Values are expressed as mean ± SEM, $n = 6$ and were estimated using one-way ANOVA followed by post hoc Tukey HSD test. Comparisons are made between (1) Group-I versus Group-II, (2) Group-II versus Group-III, IV and V. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ considered as significant

Table 4 Effects of ethanolic extract of *L. rugosa* on some liver function indices in paracetamol-induced hepatotoxic rats

Treatment	ALB	DBL	TBL	Globulin	TP	TC
Group-I (normal saline)	48.09 ± 0.26	22.03 ± 0.34	19.54 ± 0.87	8.05 ± 0.34	56.72 ± 0.87	9.11 ± 0.42
Group-II (paracetamol 750 mg/kg)	19.65 ± 0.12**	47.23 ± 0.87**	59.98 ± 0.12**	5.11 ± 0.23**	18.09 ± 0.11**	19.65 ± 0.71**
Group-III (100 mg/kg Silymarin)	42.23 ± 0.78***	24.11 ± 0.75***	27.32 ± 0.90**	7.97 ± 0.54**	53.22 ± 0.87***	8.23 ± 0.98***
Group-IV (200 mg/kg)	25.65 ± 0.87*	37.87 ± 0.05*	42.77 ± 0.55*	6.01 ± 0.76*	39.08 ± 0.45**	15.09 ± 0.04*
Group-V (300 mg/kg)	37.02 ± 0.82**	29.77 ± 0.32**	35.56 ± 0.11**	6.98 ± 0.09*	49.67 ± 0.34**	10.11 ± 0.98**

ALB: albumin; DBL: direct bilirubin; TBL: total bilirubin; TP: total protein; TC: total cholesterol

Values are expressed as mean ± SEM, $n = 6$ and were estimated using one-way ANOVA followed by post hoc Tukey HSD test. Comparisons are made between (1) Group-I versus Group-II, (2) Group-II versus Group-III, IV and V. *$p < 0.05$,**$p < 0.01$,***$p < 0.001$ considered as significant

Table 5 Effect of ethanolic extract of *L. rugosa* on liver function enzymes in CCl_4-induced hepatotoxicity in rats

Treatment	AST	ALT	ALP
Group-I (normal saline)	22.47 ± 0.47	53.46 ± 0.88	44.07 ± 0.98
Group-II (CCl_4 1.25 ml/kg)	103.62 ± 0.60**	133.63 ± 0.48**	177.06 ± 0.90**
Group-III (100 mg/kg silymarin)	36.46 ± 0.49**	62.85 ± 0.80***	49.17 ± 0.19**
Group-IV (200 mg/kg)	76.54 ± 0.70*	93.96 ± 0.80**	114.07 ± 1.20**
Group-V (300 mg/kg)	56.28 ± 0.82**	82.59 ± 0.57**	96.18 ± 0.93**

AST: aspartate aminotransferase; ALT: alanine aminotransferase; ALP: alkaline phosphatase

Values are expressed as mean ± SEM, $n = 6$ and were estimated using one-way ANOVA followed by post hoc Tukey HSD test. Comparisons are made between (1) Group-I versus Group-II, (2) Group-II versus Group-III, IV and V. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ considered as significant

Table 6 Effects of ethanolic extract of *L. rugosa* on some liver function indices of CCl_4-induced hepatotoxic rats

Treatment	ALB	DBL	TBL	Globulin	TP	TC
Group-I (normal saline)	56.59 ± 1.06	16.71 ± 0.42	15.60 ± 0.56	6.66 ± 0.24	60.03 ± 0.52	6.81 ± 0.35
Group-II (CCl_4 1.25 ml/kg)	14.80 ± 0.23**	67.18 ± 0.16**	76.58 ± 0.41**	3.72 ± 0.09**	16.43 ± 0.41**	21.34 ± 0.65**
Group-III (100 mg/kg Silymarin)	45.62 ± 0.69***	28.09 ± 0.67***	22.76 ± 0.64**	5.64 ± 0.29*	54.46 ± 1.29***	9.05 ± 0.20***
Group-IV (200 mg/kg)	29.16 ± 0.62*	49.21 ± 0.79*	56.30 ± 1.40**	4.86 ± 0.54 ^{ns}	35.88 ± 1.07**	17.57 ± 0.26**
Group-V (300 mg/kg)	39.32 ± 0.52**	37.62 ± 0.58**	37.43 ± 1.16**	4.53 ± 0.24 ^{ns}	45.87 ± 0.58**	11.20 ± 0.12**

ALB: albumin; DBL: direct bilirubin; TBL: total bilirubin; TP: total protein; TC: total cholesterol

Values are expressed as mean ± SEM, $n = 6$ and were estimated using one-way ANOVA followed by post hoc Tukey HSD test. Comparisons are made between (1) Group-I versus Group-II, (2) Group-II versus Group-III, IV and V. ns: nonsignificant, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ considered as significant

the present study. A significant increase in glutathione levels was seen after treatment with ELR compared to treatment with CCl_4 (Fig. 4).

Effect of ELR on Histopathological profile of hepatotoxicity rats

Hepatocytes in the livers of normal healthy animals exhibited well-preserved cytoplasm, nuclei and distinct sinusoidal spaces, as revealed by histological examination (Figs. 5a, 6a). In rats from the paracetamol toxicity, marked hepatic deterioration occurred with periportal hypertrophy, leukocyte infiltration and hepatic parenchyma destruction (Fig. 5b). Liver tissue cells from CCl_4-treated group exhibited severe injury due to necrosis (Fig. 6b). Groups treated with silymarin (100 mg/kg) showed mild inflammation and less deterioration of hepatic parenchyma as shown in Figs. 5c and 6c. Dilation of sinusoidal capillary, as well as hypertrophy and necrosis of periportal tissue, had been noted in the paracetamol and CCl_4 toxicity group. After treatment with ELR 200 mg/kg, reduced inflammation and periportal hypertrophy were noted, and sinusoidal capillary dilation was observed (Figs. 5d and 6d). However, animals treated with 300 mg/kg had remarkable reduction in periportal hypertrophy, central vein hypertrophy and steatosis in paracetamol and CCl_4 toxicity rat (Figs. 5e, 6e). Administration of ELR at a dose of 300 mg/kg was found more significant compared to 200 mg/kg. Liver sections of rats treated with silymarin exhibited almost normal livers with no evidence of necrosis. Moreover, the results

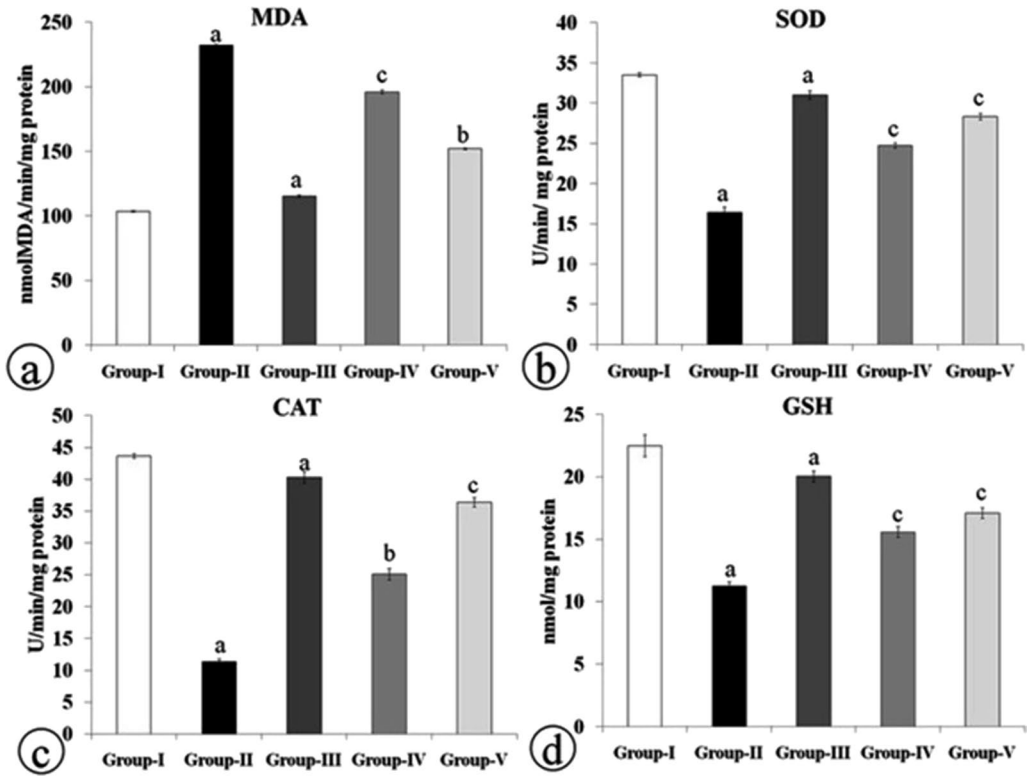

Fig. 3 Effects of ethanolic extract of *L. rugosa* on antioxidant enzymes activity and liver malonaldehyde content of paracetamol toxicity rats. Values are expressed as mean ± SEM, $n = 6$ and were estimated using one-way ANOVA followed by post hoc Tukey HSD test. Comparisons are made between (1) Group-I versus Group-II, (2) Group-II versus Group-III, IV and V. $^{c}p < 0.05$, $^{b}p < 0.01$, $^{a}p < 0.001$ considered as significant

of histopathological studies support those of biochemical studies and demonstrated that paracetamol and CCl_4 were less effective at damaging livers in animals treated with ELR.

Molecular docking analysis

The compounds, camphor and 2-butyl-2, 7-octadien-1-ol identified from GC–MS analysis exhibited docking score -4.8 and -5.8 against transforming growth factor-β1 (TGF-β1), respectively. Interestingly, 2-butyl-2, 7-octadien-1-ol showed higher docking scores than silymarin (-5.1) (Fig. 7 and Table 7).

Discussion

Hepatotoxicity is one of the damages caused by free radicals. This study demonstrated the antioxidant potential and hepatoprotective activity of ELR on paracetamol- and CCl_4-induced liver injury in rats. The phenolic compounds found in plant products act as antioxidants and are getting recognition from various studies due to their health benefits [37]. In this study, the ELR exhibited concentration-dependent scavenging activity when examined against DPPH, hydrogen peroxide and nitric acid radicals. The antioxidant properties of phenols and flavonoids could be accountable for the liver protection of plant extracts, which strongly minimize oxidative stress and other inflammatory reactions [38, 39]. Therefore, identification of gallic acid, rutin and quercetin in ELR by HPLC was believed to be associated to their reducing capacity.

Exposure of the liver cell membrane to hepatotoxic substances immediately initiates a series of events to trigger lipid peroxidation and destruction of the hepatocyte cell, which ultimately leads to the destruction of membrane structure and the escape of liver cell enzyme markers into the blood [40, 41]. Intemperate utilization or overdoses of paracetamol can lead to hepatic injury. Generation of reactive metabolite *N*-acetyl-*p*-benzoquinone imine (NAPQI) by the operation of cytochrome P450 (CYP) enzyme on paracetamol contributes to the hepatic damage. NAPQI is an oxidative product of paracetamol which covalently binds to the sulfhydryl groups of protein and instigates oxidative stresses which cause cell necrosis and lipid peroxidation [42]. The liver damage caused by carbon tetrachloride (CCl_4) is mainly related to the liberation of free radicals by the CYP system during the

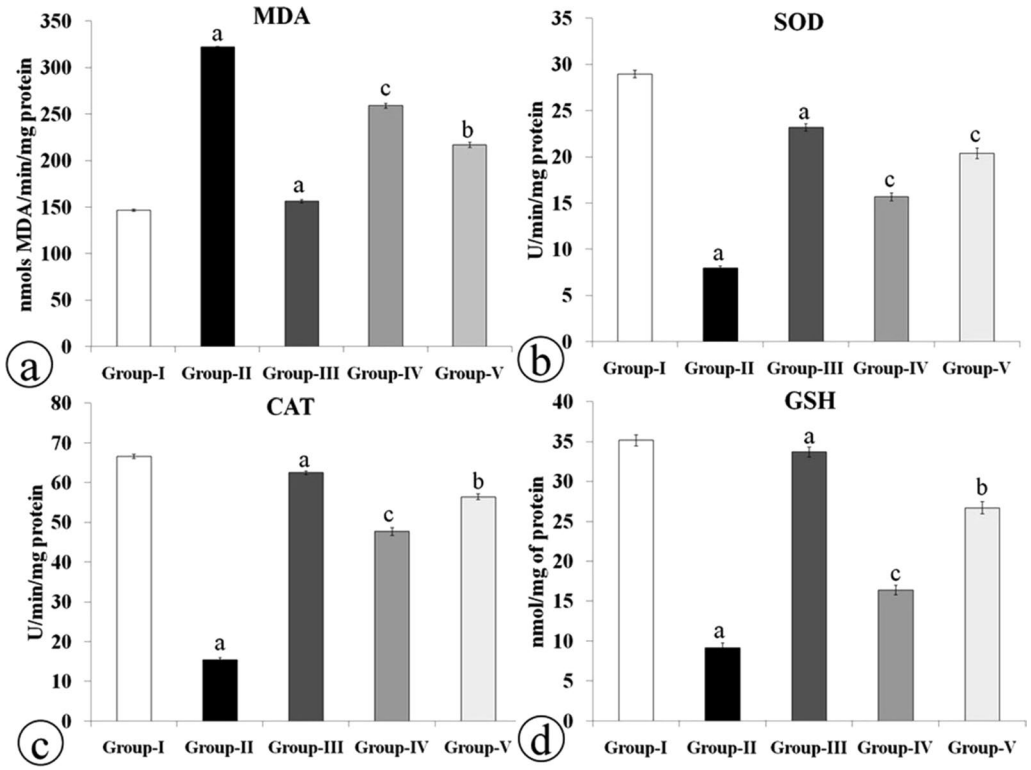

Fig. 4 Effects of ethanolic extract of *L. rugosa* on antioxidant enzymes activity and liver malonaldehyde content of CCl_4 toxicity rats. Values are expressed as mean ± SEM, $n = 6$ and were estimated using one-way ANOVA followed by post hoc Tukey HSD test. Comparisons are made between (1) Group-I versus Group-II, (2) Group-II versus Group-III, IV and V. $^{c}p < 0.05$, $^{b}p < 0.01$, $^{a}p < 0.001$ considered as significant

metabolism of CCl_4. Activation of liver CYP by the CCl_4 poisoning leads to formation of trichloromethyl (-CCl_3) and peroxytrichloromethyl (-$OOCCl_3$) radicals. The generated free radicals covalently bind to the cell protein and cause membrane lipid peroxidation and ultimately cell necrosis [43, 44]. The assessment of liver damage can be done by evaluating plasma biochemical parameters such as AST, ALT, ALP, ALB, DBL, TBL, globulin, TP and cholesterol. These substances enter into the bloodstream in liver disease and confirm the degree of liver damage [45]. Elevated levels of lipid peroxide free radicals can lead to an increase in cytosolic AST, ALT and ALP in the body system and are used to evaluate drug-induced liver toxicity [46]. ALP is an enzyme marker of the plasma membrane and endoplasmic reticulum. The high levels of these biochemical parameters directly reflect the changes in the structural integrity of the liver. The levels of total protein, bilirubin and ALP are related to the liver function of hepatocytes [47]. Bilirubin and ALP levels are the most useful clinical indicators of necrosis severity and biliary pressure. Any abnormal increase in total bilirubin reflects hepatobiliary disease and a severe change in hepatocellular architecture, while the increase in ALP is due to increased synthesis with increased biliary pressure [48]. The significant increase in serum bilirubin concentration in hepatotoxic rats may be related to paracetamol- and CCl_4-mediated hepatotoxicity, which is caused by impaired bilirubin uptake and secretion into bile [49]. Rats treated with paracetamol and CCl_4 alone showed a significant increase in TC values. These results indicate that paracetamol and CCl_4 increased the accumulation of cholesterol, which may negatively affect the liver tissue and cardiovascular system [50]. In the current study, the increased activities of these enzymes were found in paracetamol and CCl_4 hepatotoxicity rat. However, after administration of ELR at 200 and 300 mg/kg dose significantly checked the altered level of liver enzymes in experimental hepatotoxicity induced with paracetamol and CCl_4. Silymarin-treated rats also showed significant reduction in these liver enzymes in contrast to negative control group.

Serum albumin, globulin and bilirubin concentrations are a well-known index for determining the secretory and synthetic function of the liver and can be used to determine the types of liver damage [51]. In the present investigation, the animals induced with liver damage using paracetamol and CCl_4 were observed with notable depletion in serum albumin and globulin which

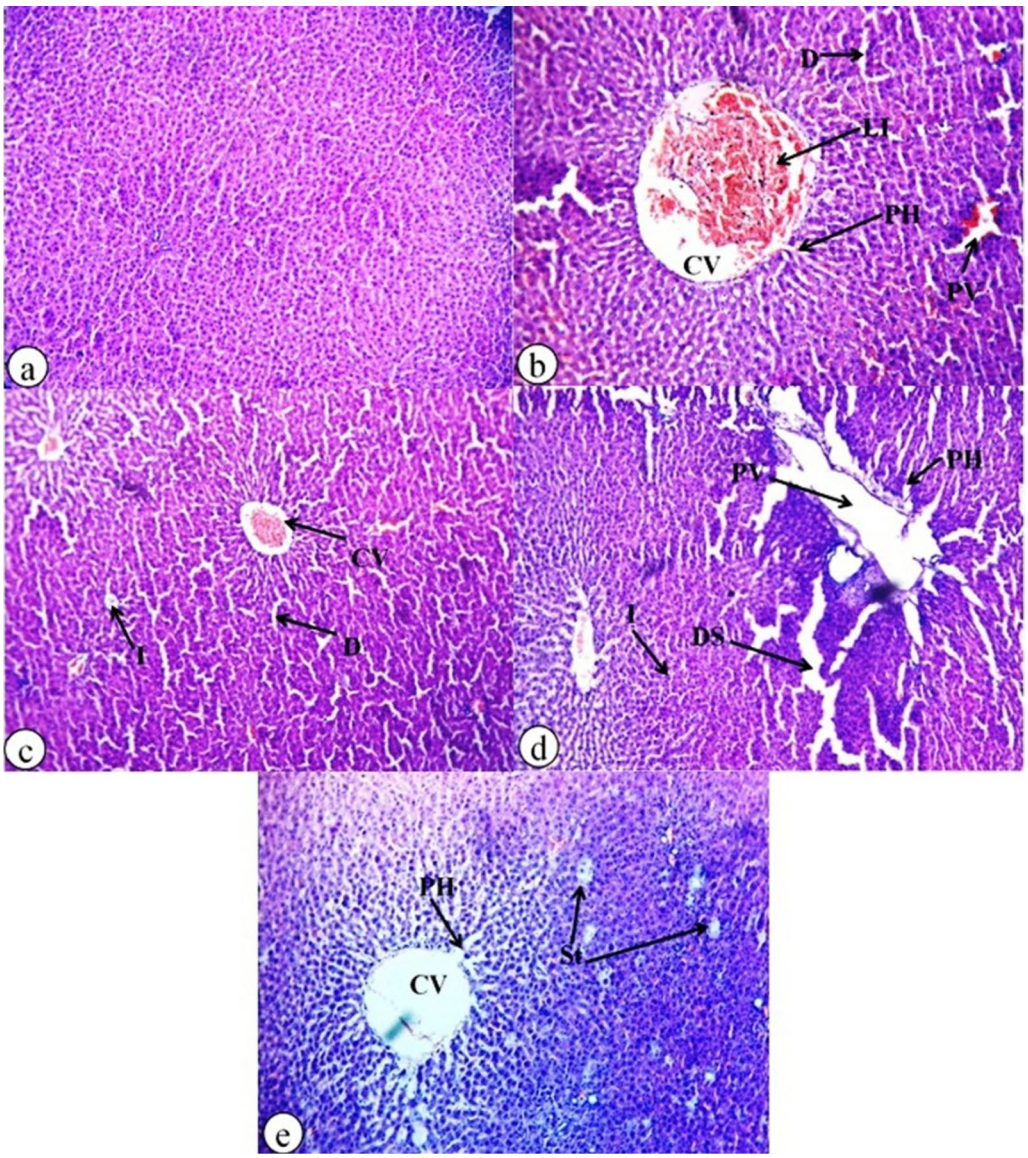

Fig. 5 Photomicrograph of the effect of administration of ethanolic extract of *L. rugosa* on the histology of the liver; **a** Normal control; **b** paracetamol hepatotoxic control; **c** paracetamol hepatotoxic treated with silymarin; **d** paracetamol hepatotoxic treated with 200 mg/kg b.w LR; **e** paracetamol hepatotoxic treated with 300 mg/kg b.w LR. I: inflammation; PH: periportal hypertrophy; D: deterioration of hepatic parenchyma; DS: dilation of sinusoidal capillary; LI: leukocyte infiltration; CV: central vein; PV: portal vein, St: steatosis

was significantly counteracted by ELR (200 and 300 mg/kg), indicating in the improvement of liver cell synthesis, secretion and excretion. The reduction of total protein level can be regarded as a useful indicator of the severity of cell dysfunction in chronic liver disease, and the stimulation of protein synthesis has been regarded as a liver protectant, a mechanism that promotes the formation of hepatic cells [52]. Compared with normal control rats, the TP was significantly reduced in Group II rats. However, after administration with plant extracts rats showed a significant increase in the level of TP, indicating that ELR stimulated the protein biosynthesis and accelerated the formation and production of hepatic cell. The silymarin-treated group is also marked with significant elevation in TP as compared to disease control group.

Increased activity of transaminase such as ALT and AST is a known marker of ROS-mediated liver cell damage caused by oxidative stress [53]. It has been shown that lipid peroxidation is a destructive process of liver damage caused by ingestion of paracetamol and

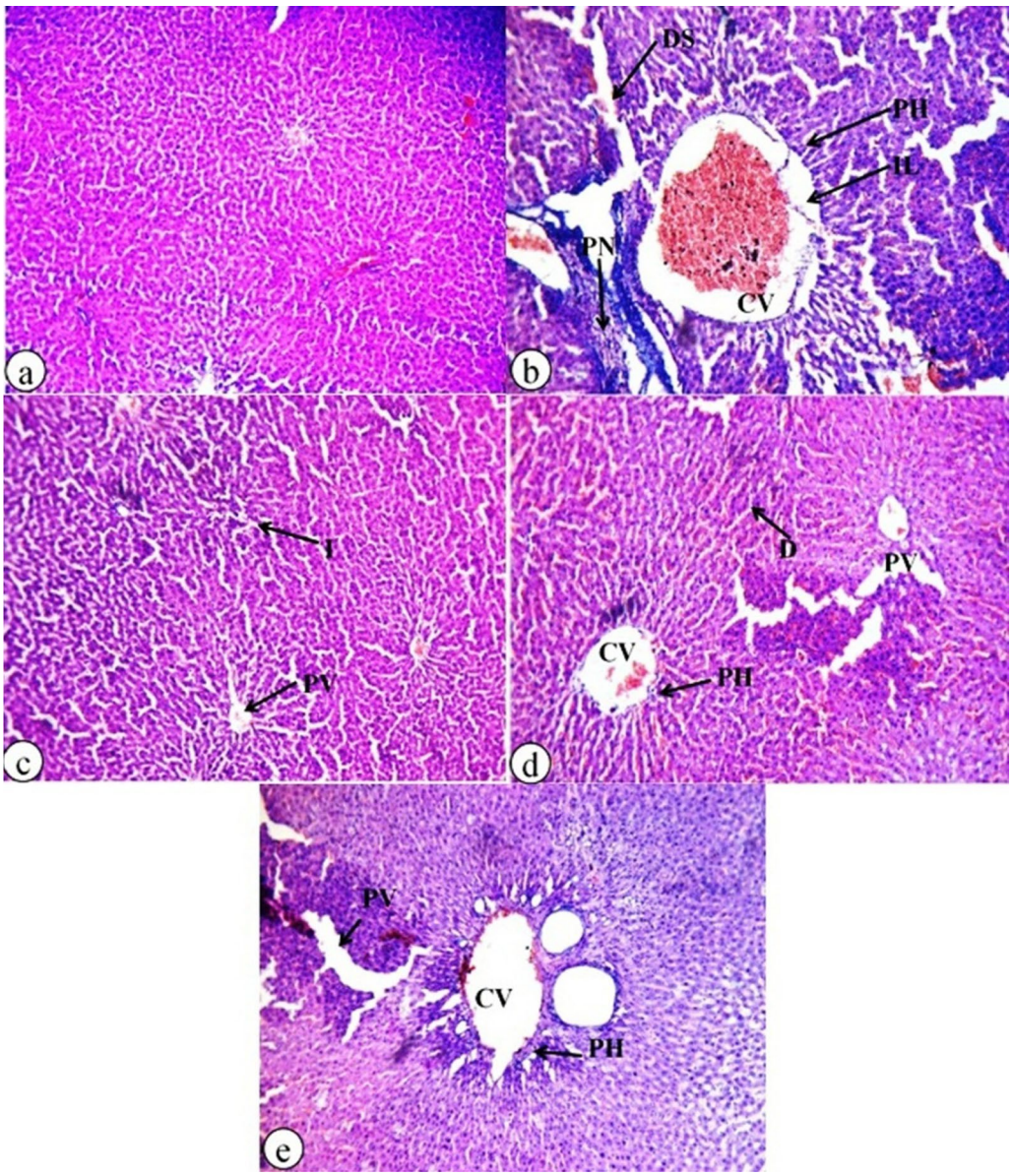

Fig. 6 Photomicrograph of the effect of administration of ethanolic extract of *L. rugosa* on the histology of the liver; **a** normal control; **b** CCl_4 hepatotoxic control; **c** CCl_4 hepatotoxic treated with silymarin; **d** CCl_4 hepatotoxic treated with 200 mg/kg b.w LR; **e** CCl_4 hepatotoxic treated with 300 mg/kg b.w LR. I: inflammation; PH: periportal hypertrophy; IL: infiltration of leukocyte; D: deterioration of hepatic parenchyma; PN: periportal necrosis; DS: dilation of sinusoidal capillary; CV: central vein; PV: portal vein

CCl_4. MDA, the final product of lipid peroxidation, was elevated in liver cells, which indicates that the lipid peroxidation has occurred due to lack of antioxidant protection [54]. The excessive production of NAPQI due to overdose of paracetamol breaks the SH group of proteins, nucleic acids and membranes. This results in loss of the architectural integrity and functions of liver cells, increasing lipid peroxidation and leading to necrosis or membrane damage and altered calcium homeostasis after depletion of the GSH, SOD and CAT concentration [48]. The present investigation demonstrated that rats treated with paracetamol and CCl_4 showed a significant increase in MDA levels as compared to normal healthy rats. On the other hand, animals treated with ELR 200 and 300 mg/kg had a significant reduction in MDA. Similarly, Group III treated with silymarin had markedly reduced MDA levels.

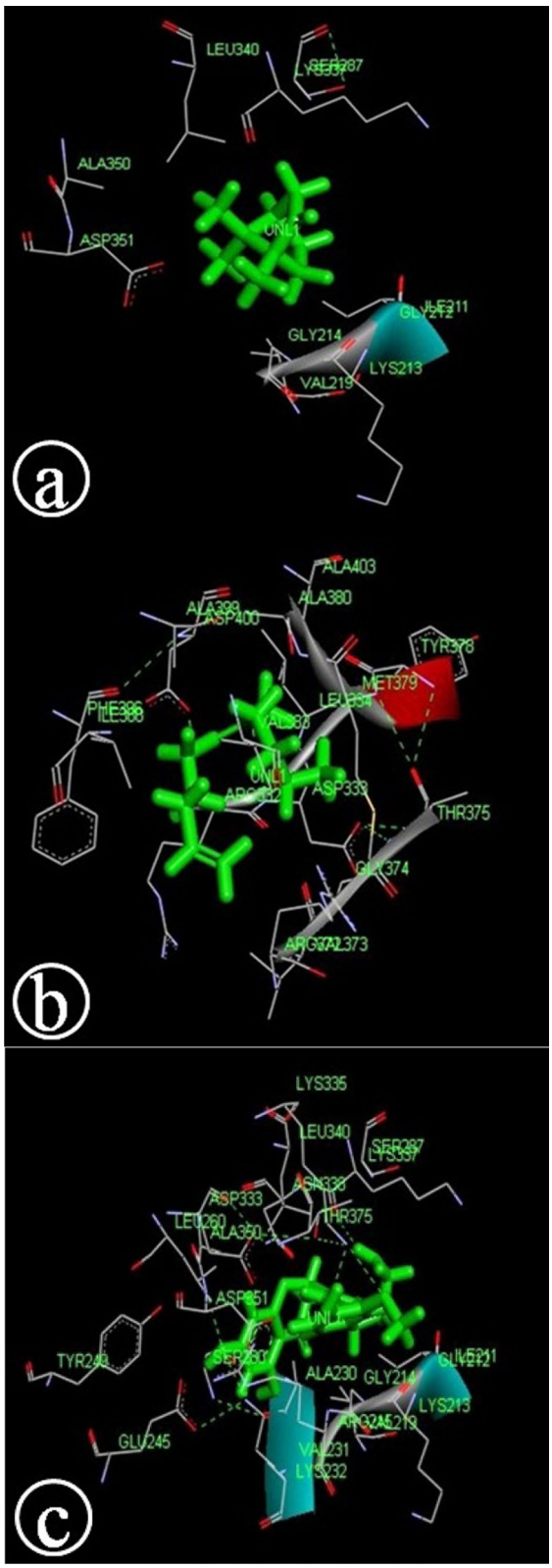

Fig. 7 Predicted docked structure of **a** camphor; **b** 2-butyl-2,7-octadien-1-ol; **c** silymarin for TGF-β1 (PDB ID: 1VJY)

Table 7 Binding affinity of ethanolic extract of *L. rugosa* for TGF-β1 (PDB ID: 1VJY)

Compound	Best score (Kcal/mol) TGF-β1 (PDB ID: 1VJY)
Camphor	−4.8
2-Butyl-2,7-octadien-1-ol	−5.8
Standard	
Silymarin	−5.1

The administration of ELR indicated its antioxidant capacity, which could be explained by the extract's ability to eliminate ROS in the liver. Hepatoprotective properties of phenolics and flavonoids were attributed to their antioxidant properties. Oxidative stress and other inflammatory responses are effectively reduced by these compounds [38, 39]. The presence of flavonoids, phenols, alkaloids and carbohydrates might be attributed to the antioxidant and liver protective properties of the ELR. Several bioactive principles were identified from whole plant; aerial part, root and essential oil of *L. rugosa* which exhibited significant antioxidant activity. These include: 5-hydroxy-6, 7, 4'-trimethoxyflavone (Salvigenin) [55], betulinic acid [56], ursolic acid [57], 3-octanol [58], acetophenone [59], anisylacetone [60], caryophyllene [61] and *cis*-anethole [62]. However, betulin has been reported as hepatoprotective by Kazakova et al. [63].

In many studies, the hepatoprotective activity also attributed to the presence of camphor as a principal component, in *Achillea wilhelmsii*, *Ajuga iva*, *Artemisia capillaries*, *Coriandrum sativum* L, *Hyptis crenata* and *Rosmarinus officinalis* [64]. Therefore, detection of camphor at high percentage (88.06%) by GC–MS analysis in ELR could be responsible for hepatoprotective activity. However, molecular docking score of camphor (-4.8) was also comparable to silymarin (-5.1). Interestingly, compound 2-butyl-2, 7-octadien-1-ol exhibited greater docking score (-5.8) than silymarin. Thus, we can conclude that the ameliorative potential of *L. rugosa* could be attributed to the presence of these compounds and also appreciable quantities of phenolic and flavonoid compounds that scavenge the free radicals and thus protect against liver toxicity caused by paracetamol and CCl_4.

The observations of this study were also supported by histological examination, which show that ELR gradually restores normal liver tissue structure in a dose-dependent manner and has hepatoprotective effects on paracetamol- and CCl_4-induced hepatotoxicity. Administration of both paracetamol and CCl_4 caused hepatotoxicity, as evidenced by extensive destruction of hepatocytes as well as loss of hepatocyte integrity. However, hepatotoxicity

is effectively managed with ELR treatment, as indicated by histological results. It is thought that the ELR may have resulted in liver tissue regeneration and recovery with hepatocytes showing close to normal cell structure. Based on these findings, it is clear that the histopathological findings are consistent with the observed biochemical analysis. Histological examination of animals treated with silymarin was also marked with normal hepatocytes. Silymarin is a flavonolignan that has been used as a liver protecting agent due to the antioxidant activity. The hepatoprotective potential of ELR was also found comparable with hepatoprotective efficacy of *Limnophila heterophylla* [12] and *Limnophila repens* [13] against paracetamol- and CCl_4-induced hepatic damage in rats.

Conclusions

The antioxidant properties of the constituents and highest docking scores observed for 2-butyl-2, 7-octadien-1-ol and camphor could be a potential mechanism of the hepatoprotective effect of *L. rugosa* aerial parts against paracetamol- and CCl_4-induced hepatotoxicity. Further, research is needed to isolate the bioactive components accountable for the hepatoprotective properties of this plant.

Plant authentication

The plant was collected from Barpali, Bargarh, Odisha, and identified by Botanist Dr. Surya Kumar Barpanda, Shree Ram College, Rampur, Sonepur. The voucher specimen TPC/COL/21/012 was deposited in Pharmacology Department of The Pharmaceutical College, Barpali, for future reference.

Abbreviations

ABTS: 2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid); ALP: Alkaline phosphatase; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; CAT: Catalase; $–CCl_3$: Trichloromethyl radicals; CCl_4: Carbon tetrachloride; CMC: Carboxymethyl cellulose; CYP: Cytochrome P450; DPPH: 2,2-Diphenyl-1-picrylhydrazyl; ELR: Ethanolic extract of *L. rugosa*; GC–MS: Gas chromatography–mass spectrometry; GSH: Glutathione; H_2O_2: Hydrogen peroxide; HPLC: High-performance liquid chromatography; LPO: Lipid peroxidation; MDA: Malondialdehyde; mg GAE/g: Milligram gallic acid equivalents per gram; mg QE/g: Milligram of quercetin equivalent per gram; NAPQI: *N*-Acetyl-*p*-benzoquinone imine; NBT: Nitro blue tetrazolium; NIST: National Institute of Standards and Technology; OECD: Organization for Economic Cooperation and Development; $–OOCCl_3$: Peroxytrichloromethyl; ROS: Reactive oxygen species; Rpm: Rotation per minute; S.E.M: Standard error mean; SOD: Superoxide dismutase; TGF-β1: Transforming growth factor-β1.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s43094-021-00397-y.

Additional file 1: Table S1: Gradient conditions of solvent for HPLC analysis. **Table S2:** Quantitative estimation of phenolic and flavonoid compounds in ethanolic extract of *L. rugosa* by HPLC analysis.

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Authors' contributions

RNH contributed to methodology, data collection and writing original graft preparation. BKN contributed in supervision, conceptualization, study design, writing, review and final editing. BRB contributed to formal analysis and investigation. AB contributed to data collection. All authors have read and approved the manuscript.

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Availability of data and materials

All the data and materials will be provided on request.

Declarations

Ethics approval and consent to participate

The study protocol was approved by Animal Ethics Committee (1376/ac/10/CPCSEA) and confirm to the guidelines for care and use of animals in scientific research.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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