BJP

Bangladesh Journal of Pharmacology

Research Article

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A Journal of the Bangladesh Pharmacological Society (BDPS) **Bangladesh J Pharmacol 2014; 9: 383-388**
Journal homepage: www.banglajol.info
Abstracted/indexed in Academic Search Complete, Agroforestry Abstracts, Asia Journals Online, Bangladesh Journals Online, Biological Abstracts, BIOSIS Previews, CAB Abstracts, Current Abstracts, Directory of Open Access Journals, EMBASE/Excerpta Medica, Global Health, Google Scholar, HINARI (WHO), International Pharmaceutical Abstracts, Open J-gate, Science Citation Index Expanded, SCOPUS and Social Sciences Citation Index
ISSN: 1991-0088

Anti-oxidant and hepatoprotective activity of ethanolic and aqueous extracts of *Limonia elephantum* leaves

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Article Info

Received: 25 July 2014
Accepted: 6 August 2014
Available Online: 21 August 2014

DOI: 10.3329/bjp.v9i3.19704

Cite this article:
Kamalakannan K, Balakrishnan V. Anti-oxidant and hepatoprotective activity of ethanolic and aqueous extracts of *Limonia elephantum* leaves. Bangladesh J Pharmacol. 2014; 9: 383-88.

Abstract

Limonia elephantum (wood apple) has been widely used in an Indian folk medi -cine system. In the present study, the acute toxicity studies were carried out to determine the safety of the compounds in liver diseases. The antioxidant and the hepatoprotective properties of the *L. elephantum* are evaluated against paracetamol induced hepatic damage in rats. Liver superoxide dismutase, lipid peroxidation, glutathione peroxidase, catalase levels and serum biochemical profile such as serum glutamate oxalate transaminase, serum glutamate pyruvate transaminase, alkaline phosphatase, bilirubin and total protein were examined for the anti-oxidant and hepatoprotective effects of these treatments. The results of the present studies strongly indicate that the higher dose (400 mg/kg) of ethanolic extract of *L. elephantum* proved against hepatoprotective effects and also the anti-oxidant properties.

Introduction

Herbal drugs are generally plays a vital role in the management of different liver disorder, most of which fastens the natural healing processes of the liver without any side effects. For example, *Alcea rosea* (Hussain et al., 2014), *Cestrum nocturnum* (Qadir et al., 2014a), *Chenopodium murale* (Saleem et al., 2014a), *Malva parviflora* (Mallhi, 2014), *Rumex dentatus* (Saleem et al., 2014b), *Thymus linearis* (Alamgeer et al., 2014) *Achyranthes aspera* (Kamalakannan and Balakrishnan, 2010) and *Viola odorata* (Qadir et al., 2014b) showed hepatoprotective effect.

Limonia elephantum, commonly known as Vilamaram, Vilangai (Tamil) is the largest and principal representtative genus of the Rutaceae family. Traditionally Vilamaram has been used to treat intestinal infections, indigestions, heart diseases, hiccough and dysentery. Many reports revealed that Vilamaram was found to posses antibacterial, antifungal, hypoglycemic, cardiotonic, expectorant, antihyperlipidemic, hepatoprotective, anti-inflammatory and anti-oxidant activity.

In recent decades, the extracts of leaves, fruits, barks and gums of *L. elephantum* have been extensively studied for many medicinal uses. The present study has attempted to assess and evaluate the possible hepatopro -tective activity of aqueous and ethonolic extract of *L. elephantum* leaves in paracetamol-induced hepatotoxicity in rats.

Materials and Methods

Collection and authentication of the plant materials

The leaves of *L. elephantum* were collected in the month of March 2010 from Tropical area of Western Ghats region of Coimbatore district. The plant was authenticated at the Botanical Survey of India (BSI) Coimbatore, where the voucher specimens were deposited (Herbarium voucher no BSI/SC/5/23/09-10/Tech-266). The samples were shade dried at room temperature.

Preparation of plant extract

The shade dried leaves of *L. elephantum* were pulverized to coarse powder and extracted by continuous hot percolation process by using Soxhlet apparatus with methanol as a solvent. The methanolic extract were obtained through hot percolation process were subjected to solvent evaporation through vacuum distillation method to obtain dried methanolic extract.

The aqueous extract of leaves of *L. elephantum* was obtained by simple maceration process. In this process the marc obtained through hot continuous extraction process were utilized for the experimental purpose. The marc was weighed and placed into the maceration flask and added with sufficient quantity of water and macerate for 24 hours, during the process the first six hours the flask were subjected to gentle agitation and set aside for remaining 18 hours. After 24 hours the solvent was filtered and clarified to get clear filtrate. The clear filtrate was evaporating to obtain the dried extract. Both extracts were stored in desiccators, which was used for further experimental purpose.

Experimental animals

Male albino rats of Wistar strain weighing about 180 and 220 g were housed indivi-dually in polypropylene cages and fed on standard pellet diet (Hindustan liver). The animals were obtained from the animal house, Perundurai Medical College, Erode, Tamil Nadu, India. Water was given *at libitum.* The animals were maintained at standard laboratory conditions (temperature 24 ± 2°C, relative humidity 30-70% and 1:1 dark and light cycle).

Acute toxicity studies

Healthy adult male albino mice (18-22 g) were subjected to acute toxicity studies as per guidelines (AOT 425) subjected by the organization for economic co-operation and development (OECD-2001). The selected animals were used to determine the dose. The animals were divided into seven groups of six in each. The animals were fasted over night prior to the acute experimental procedure. The Karber's method (Ghosh and Kale, 2005) was used to determine the dose; gum acacia (2% w/v) was used as vehicle to suspend the extracts and administered intraperitoneally. The control group was received 2 mL/kg of the vehicle intra peritoneally. The other group received the extract as test drug in one of the following doses– 100, 200, 400, 800, 1,000, 2,000 and 3,000 mg/kg in a similar manner. Immediately after dosing, the animals were observed continuously for first four for behavior changes and for mortality at the end of 24, 48 and 72 hours respectively. The toxicity studies shows that the ethanolic and aqueous extract of drug at minimum dose of 200 mg/kg onwards shows the reaction in experimental animals. However, no mortality was reported even after 72 hours. This indicates that the alcoholic extract was safe up to a single dose of 3 g/kg body weight.

Experimental design

Paracetamol-induced hepatotoxicity in rats (acute model)

Animals were randomized and divided into seven groups of six animals in each group. Group I served as untreated control and fed orally with normal saline 5 mL/kg body weight daily for seven days. Group II rats were similarly treated as Group I. Group III and IV were treated with 200 mg and 400 mg/kg body weight of the aqueous extract of *L. elephantum* and Group V and VI were treated with the ethanolic extract of *L. elephantum* orally daily for seven days respectively (Hiroshini et al., 1987). Animals of Group VII were fed with standard drug silymarin 25 mg/kg (Veereshwarayya and Thiruvengadam, 2004) p.o daily for seven days. On the seventh day, paracetamol suspension was given by oral route, in a dose of 750 mg/kg body weight to all rats except the rats in Group I. The biochemical parameters were estimated after an 18 hours fast following the last dose.

Biochemical studies

The blood was obtained from all animals by puncturing retro-orbital plexus. The blood samples were allowed to clot for 45 min at room temperature. Serum was separated by centrifugation at 2,500 rpm at 30°C for 15 min and utilized for the estimation of various biochemical parameters namely SGOT, SGPT (Retimen and Frankel, 1957) SALP (King and Armstrong, 1934), serum bilirubin (Malloy and Evelyn, 1937) and total protein (Gornall et al., 1949).

After collection of blood samples the rats in different groups were sacrificed and their livers were excised immediately and washed in ice cold normal saline, followed by 0.15 M Tris-HCl (pH 7.4) blotted dry and weighed. A 10% w/v of homogenate was prepared in 0.15 M Tris-HCl buffer and processed for the estimation of lipid peroxidation (Ohkawa et al., 1979). A part of homogenate after precipitating proteins with trichloro-acetic acid was used for estimation of glutathione (Ellman, 1959). The rest of the homogenate was centrifuged at 1,500 rpm for 15 min at 40°C. The supernatant thus obtained was used for estimation of SOD and CAT activities (Kakkar et al., 1984; Aebi, 1974).

Serum hepatospecific markers

Activities of serum glutamate oxaloacetate transaminase (SGOT) and serum glutamate pyruvate transaminase (SGPT) were estimated by the method of Retimen and Frankel (1957). 0.05 mL of serum with 0.25 mL of substrate (aspartate and α-ketoglutarate for SGOT; alanine and α-keto glutarate for SGPT, in phosphate buffer pH 7.4) was incubated for an one hour in case of SGOT and 30 min for SGPT. 0.25 mL of DNPH solution was added to arrest the reaction and kept for 20 min in

room temperature. After incubation one mL of 0.4N NaOH was added and absorbance was read at 505 nm in uv-vis spectrophoto-meter. Activities were expressed as IU/L.

Based on the method of King and Armstrong (1934) alkaline phosphatase activity was assayed by using disodium phenyl phosphate as substrate. The color was developed and read at 510 nm in uv-vis spectrophotometer after 10 min. Activities of ALP was expressed as IU/L. Serum total bilirubin level was estimated based on the method of Malloy and Evelyn (1937). Diazotised sulphonilic acid (0.25 mL) reacts with bilirubin in diluted serum (0.1 mL serum + 0.9 mL distilled water) and forms purple colored azobilirubin, which was measured at 540 nm in UV-VIS spectrophotometer. Activities of total bilirubin were expressed as mg/dL. Serum total protein level was estimated based on the method of Gornall et al. (1949). Biuret reagent (1.0 mL) reacts with serum (10 µL) and the color was developed and read at 578 nm in uv-vis spectrophotometer. Activities of total protein were expressed as mg/dL.

Assay of superoxide dismutase (SOD)

Superoxide dismutase (SOD) activity was determined by the method of Kakkar et al. (1984). The assay mixture were contained 0.1 mL of sample, 1.2 mL of sodium pyrophosphate buffer (pH 8.3, 0.052M), 0.1 mL of phenazine metho-sulfate (186 µm), 0.3 mL of nitro blue tetrazolium (300 µm), 0.2 mL of NADH (750 µm). Reaction was started by addition of NADH. After incubation at 30°C for 90 sec, the reaction was stopped by the addition of 0.1 mL of glacial acetic acid. The reaction mixture was stirred vigorously with 4.0 mL of *n*-butanol. The mixture was allowed to stand for 10 min, centrifuged and butanol layer was separated. The color intensity of the chromo-gen in butanol layer was measured at 560 nm against *n*-butanol and concentration of SOD was expressed as units/g of liver tissue. Absorbance values were compared with a standard curve generated from known SOD.

Assay of catalase (CAT)

Catalase was assayed according to the method of Aebi (1974). The estimation was done spectrophotometrically following the decrease in absorbance at 230 nm. The liver tissue was homogenized in M/150 phosphate buffer (pH 7.0) at 1-40°C and centrifuged at 5,000 rpm. The reaction mixture contain-ed 0.01 M phosphate buffer (pH 7.0), 2 mM H_2O_2 and the enzyme extract. The specific activity of catalase was expressed in terms of units/gram of liver tissue. Absorbance values were compared with a standard curve generated from known CAT.

Assay of glutathione peroxidases (GP_X)

Glutathione peroxidases activities (total and Se-dependent) were measured at 340 nm following NADPH oxidation in the presence of excess glutathione reductase, reduced glutathione and corresponding peroxide (Lawrence and Burk, 1976). Cumene hydroperoxide and H_2O_2 were used as substrates for the determination of total and Se-dependent peroxidase activity. The difference in the absorbance per unit of time (the rate of blank reaction was subtracted from the total rate) was taken as the measure of GPx activity (C =−6.22 mM^{-1} cm^{-1}). The GPx activities in the gills and mantle of *B. azoricus* are expressed as µmol/min/mg of total protein concentrations.

Lipid peroxidation

The method was used and designed to evaluate malondialdehyde (MDA) and 4-hydroxy-alkenals (4-HNE) concentrations upon the decomposition by polyunsaturated fatty acid peroxides (Erdelmeier et al., 1998). This procedure is based on the reaction of two moles of *N*-methyl-2-phenylindole, a chromogenic reagent, with one mole of either MDA or 4-HNE at 45°C for 60 min to yield a stable chromophore that has a maximum absorbance at 586 nm. Therefore, 10 µL of 0.5M butylated hydroxytoluene, 650 µL of a mixture of 6 mL of methanol with 18 mL of 10.3 mM *N*-methyl-2-phenylindole and 150 µL of 15.4M methane-sulfonic acid were added to 200 µL of the first cytosolic fraction of the homogenate of the gills or mantle of *B. azoricus* and incubated at 45°C for 60 min.MDA + 4-HNE concentrations were estimated by measuring the maximum absorbance of the formed chromophore at 586 nm using malondialdehyde bis-tetrametoxypropan (SIGMA) as a standard. Lipid peroxidation is expressed as nmoles of MDA and 4-HNE g^{-1} of total protein concentrations.

Statistical analysis

All the results were expressed as mean ± SEM. Statistical analysis was performed using GraphPad Prism version 5. The data were statistically analyzed by one-way analysis of variance (ANOVA) between plant extract treated groups and disease control group. P values <0.05 were considered to be significant.

Results

The effects of *L. elephantum* on serum transaminase, alkaline phosphatase, bilirubin and total protein levels in paracetamol-induced liver damage in rats summaryzed in Table I. Administration of paracetamol (750 mg/kg; body weight), after 18 hours of intoxication resulted a significant ($p<0.05$) elevation of hepatospecific serum markers SGOT, SGPT, SALP, bilirubin and total protein in paracetamol-treated group, in comparison with the normal control group. On administration of *L. elephantum* (Group III to VI) and silymarin at the dose of 25 mg/kg (Group VII) the level of these enzymes were found retrieve towards normal control.

Table I

Effect of *L. elephantum* extract on various biochemical parameters in paracetamol-induced hepatotoxicity

Group	Dose (mg/kg)	SGOT (IU/L)	SGPT (IU/L)	SALP (IU/L)	Bilirubin (mg/dL)	Total protein (mg/dL)
GP-I NC	0.5%CMC	45.1 ± 2.4	57.3 ± 2.3	16.1 ± 1.3	1.6 ± 0.1	7.9 ± 0.4
GP-II-PARA- CON	750	118.5 ± 1.7	114.0 ± 2.6	32.2 ± 1.9	6.2 ± 0.6	5.0 ± 0.2
G-III-AQ-200	200	88.3 ± 3.0[c]	81.4 ± 2.5[c]	28.5 ± 1.9[b]	3.7 ± 0.4[c]	6.1 ± 0.4[b]
G-IV-AQ-400	400	70.2 ± 2.9[c]	74.4 ± 3.0[c]	24.2 ± 2.2[b]	3.6 ± 0.5[c]	6.5 ± 0.3[b]
G-V-ET-200	200	80.9 ± 2.4[c]	69.3 ± 2.3[b]	22.3 ± 2.1[b]	2.8 ± 0.3[b]	7.0 ± 0.2[a]
G-VI-ET-400	400	59.3 ± 2.7[b]	56.1 ± 1.8[a]	15.8 ± 1.3[a]	2.3 ± 0.1[a]	7.6 ± 0.3[a]
G-VII-SILYMARIN	25	56.5 ± 1.6[a]	56.4 ± 2.2[a]	15.3 ± 1.6[a]	2.0 ± 0.2[a]	7.9 ± 0.7[a]

Value are expressed as mean ± SEM; n = 6 rats in each group; [a]p<0.001, [b]p<0.01, [c]p<0.05 was considered as statistically significant when compared with Group I

Table II

Effect of *L. elephantum* extract on LPO, SOD, CAT and GP_X in paracetamol-induced hepatotoxicity

Groups	LPO	SOD	CAT	GPX
GP-I NC	8.9 ± 1.6	558.5 ± 1.0	105.9 ± 1.4	11.6 ± 1.1
GP-II-PARA –CON	13.2 ± 1.4	314.5 ± 1.9	63.3 ± 1.6	19.4 ± 2.4
G-III-AQ-200	10.6 ± 1.0[c]	428.3 ± 19.8[c]	87.2 ± 2.4[c]	14.1 ± 1.3[c]
G-IV-AQ-400	10.4 ± 1.0[b]	446.3 ± 18.5[b]	88.2 ± 2.8[b]	13.8 ± 1.3[b]
G-V-ET-200	10.1 ± 1.7[b]	465.7 ± 71.6[b]	93.0 ± 1.3[b]	12.4 ± 1.3[a]
G-VI-ET-400	9.9 ± 1.1[a]	495.5 ± 48.9[a]	97.3 ± 5.4[a]	12.3 ± 1.1[a]
G-VII-SILYMARIN (25 mg/kg)	9.0 ± 2.1[a]	557.9 ± 1.2[a]	106.2 ± 1.3[a]	11.8 ± 1.4[a]

Value are expressed as mean ± SEM; n = 6 rats in each group; [a]p<0.001, [b]p<0.01, [c]p<0.05 was considered as statistically significant when compared with Group I

The levels of SOD and CAT recorded a significant decline and the level of lipid peroxidation and GPx were increased in paracetamol treated animals when compared with control group of animals (Table I and II), while in *L. elephantum* treated rats the activities of these enzymes attained a near-normal control (p<0.05).

Discussion

The pretreatment with aqueous and ethanolic extract of *L. elephantum*, both at the dose of 200 and 400 mg/kg, significantly attenuated the elevated levels of the serum markers. The normalization of serum markers by aqueous and ethanolic extract of *L. elephantum* suggests that they are able to condition the hepatocytes so as to protect the membrane integrity against paracetamol induced leakage of marker enzymes into the circulation. The above changes can be considered as an expression of the functional improvement of hepatocytes, which may be caused by an accelerated regeneration of parenchyma cells. Serum ALP and bilirubin levels, on the other hand are related to hepatic cell damage. Increase in serum level of ALP is due to increased synthesis in presence of increasing billiary pressure (Moss and Butterworth, 1974). Effective control of bilirubin level and alkaline phosphatase activity points towards an early improvement in the secretory mechanism of the hepatic cell.

Lipid peroxidation has been postulated to the destructive process of liver injury due to acetaminophen administration. In the present study, the elevations in the levels of end products of lipid peroxidation in the liver of rat treated with paracetamol were observed. The increase in malondialdehyde (MDA) levels in liver suggests enhanced lipid peroxidation leading to tissue damage and failure of antioxidant defense mechanisms to prevent formation of excessive free radicals. Treatment with aqueous and ethanolic extract of *L. elephantum* significantly reversed these changes. Hence it may be possible that the mechanism of hepatoprotection by aqueous and ethanolic extract of *L. elephantum* is due to its antioxidant effect.

The non-enzymic antioxidant, glutathione is one of the most abundant tripeptides present in the liver. Its functions are mainly concerned with the removal of free radical species such as hydrogen peroxide, superoxide

radicals, alkoxy radicals and maintenance of membrane protein thiols and as a substrate for glutathione peroxidase and GST (Prakash et al., 2001). In the present study the decreased level of GSH has been associated with an enhanced lipid peroxidation in paracetamol-treated rats. Administration of aqueous and ethanolic extract of *L. elephantum* significantly increased the level of glutathione in a dose-dependent manner.

The enzymatic antioxidant defense system is the nature protector against lipid peroxidation. SOD, CAT and GPx enzymes are important scavengers of superoxide ion and hydrogen peroxide. These enzymes prevent generation of hydroxyl radical and protect the cellular constituents from oxidative damage (Scott et al., 1991). In the present study, it was observed that the aqueous and ethanolic extract of *L. elephantum* significantly increased the hepatic SOD activity in paracetamol induced liver damage in rats. This shows that the aqueous and ethanolic extract of *L. elephantum* can reduce reactive free radicals that might lessen the oxidative stress to the tissues and improves the activity of hepatic anti-oxidant enzyme.

Catalase (CAT) is an enzymatic antioxidant widely distributed in all animal tissues and the highest activity is found in the red cells and in the liver. CAT decomposes hydrogen peroxide and protects the tissue from highly reactive hydroxyl radicals (Chance et al., 1952). Therefore the reduction in the activity of these enzymes may result in a number of deleterious effects due to the accumulation of superoxide radicals and hydrogen peroxide. Administration of aqueous and ethanolic extract of *L. elephantum* increased the activities of CAT in paracetamol-induced liver damage in rats to prevent the accumulation of excessive free radicals and protected the liver from paracetamol intoxication.

The observations in paracetamol-treated rats showed severe necrosis. This could be due to the formation of highly reactive radicals because of oxidative threat caused by paracetamol. All these changes were very much reduced in rats treated with aqueous and ethanolic extract of *L. elephantum*. Based on the above results, it could be concluded that aqueous and ethanolic extract of *L. elephantum* exerts significant hepatoprotection against paracetamol-induced toxicity.

Ethical Issue

Ethical clearances for the handling of experimental animals were obtained from Institutional Animal Ethical Committee (NCP/IAEC/Ph.D/2010-001) for the present investigation (Wagh and Gangadhar, 2010).

Acknowledgement

The authors are grateful to the management, Principal for providing necessary laboratory facilities. The author acknowledge to Mr. M. Suresh, Lab Assistant of Nandha College of Pharmacy, Erode for providing the valuable support to carry out this research work.

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