

Brief Communication

Hepatoprotective and antioxidant activity of *Leea asiatica* leaves against acetaminophen-induced hepatotoxicity in rats

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ABSTRACT

Leea asiatica (L.) Ridsdale, a folk medicinal plant is used by the ethnic people of North East India for the treatment of hepatic disorder. In this study, we have investigated the hepatoprotective and antioxidant activity of *L. asiatica* leaves against acetaminophen induced hepatotoxicity. Methanol extract of *L. asiatica* (150 and 300 mg/kg/day, p.o.) were administered to rats for three consecutive days followed by single acetaminophen (3000 mg/kg, p.o.) administration on 3rd day. After 48 h of acetaminophen administration animals were sacrificed and biochemical estimation of serum, *in vivo* antioxidant activity using liver tissue were carried out. High levels of serum glutamate oxaloacetate transaminase, serum glutamate pyruvate transaminase, serum alkaline phosphatase, total bilirubin, direct bilirubin, total cholesterol and triglycerides were observed in disease control group, which found near to normal in extract treated groups. Higher dose exhibited significant hepatoprotective activity against acetaminophen induced toxicity. Level of superoxide dismutase, catalase, glutathione peroxidase in liver tissue, and reduced glutathione in liver and blood were also significantly increased in extract (300 mg/kg) treated animals compare to disease control group. In this study we found that leaves of *L. asiatica* exhibited potent hepatoprotective activity against acetaminophen induced hepatic damage in experimental animals which justify the folklore claim, and the possible mechanism of this activity may be due to strong antioxidant activities of extract.

Keywords *Leea asiatica*, leaves, acetaminophen, hepatoprotective, antioxidant

INTRODUCTION

Acetaminophen (APAP) is a most common non steroidal analgesic and antipyretic drug used worldwide. APAP exerts few side effects in therapeutic doses, but hepatotoxicity is the frequent consequence of APAP overdose (Olaleye et al., 2010). APAP overdose is responsible for generation of a highly reactive metabolite *N*-acetyl-*p*-benzoquinoneimine (NAPQI). NAPQI causes covalent modification, reduction of enzyme activity, inhibition of protein oxidation, free radical generation, lipid peroxidation, DNA fragmentation, mitochondrial dysfunction, alteration of innate immunity, deregulation of Ca²⁺ homeostasis, depletion of glutathione thus leads to hepatotoxicity (James et al., 2003; Ray et al., 1996).

Traditional herbal drugs and folk medicinal plants are considered as a key source of new drug molecule or therapy which has attracted considerable attention of researchers. Free radical induced oxidative stress is considered as a key reason for hepatotoxicity, thus a free radical scavenger could be useful to prevent APAP induced liver toxicities (Sen and Chakraborty, 2011). The species *Leea* possess several biological and

antioxidant activities (Saenjum et al., 2007). *Leea asiatica* L. Ridsdale (family: Leeaceae), a folk medicinal plant is used for the treatment of a broad spectrum of diseases including worm infection, wound, eye diseases, bone fracture, diabetes and gastrointestinal disorders. We have conducted a ethnomedicinal survey in Tripura, India and found that the plant is used to treat liver disorder by the ethnic people (Bhandary et al., 1995; Prasad et al., 2008; Sen et al., 2011). In our previous investigation we reported that leaves of *L. asiatica* possess *in vitro* antioxidant, anthelmintic and nephroprotective activity (Sen et al., 2011, 2012). However to date there is no scientific evidence to support the hepatoprotective effect of *L. asiatica*. The present study aim to investigate the *in vivo* antioxidant and hepatoprotective activity of methanol extract of *Leea asiatica* leaf against acetaminophen induced liver toxicity.

MATERIALS AND METHODS

Plant material and extraction

Leaves of *Leea asiatica* were collected from Agartala, Tripura, India. The plant material was authenticated by Dr. BK Datta, Department of Botany, Tripura University, Tripura, India. A voucher specimen (TU/BOT/HEB/SS23072011c) has been deposited in the herbarium of Plant Taxonomy & Biodiversity Laboratory, Tripura University.

The fresh leaves were collected and cleaned to remove unwanted materials. The leaves were air dried under shade, pulverized into coarse powder and extracted with methanol

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Table 1. Hepatoprotective activity of methanol extracts of *L. asiatica* leaves

Treatment	SGOT (U/l)	SGPT (U/l)	ALP (U/l)	Total bilirubin (mg/dl)	Direct bilirubin (mg/dl)	Total cholesterol (mg/dl)	Triglycerides (mg/dl)
Normal control	101.50 ± 6.01	38.04 ± 2.09	105.66 ± 5.19	0.39 ± 0.03	0.31 ± 0.03	32.29 ± 1.88	30.62 ± 3.19
Negative control	391.40 ± 16.00 ^z	173.60 ± 6.88 ^z	430.84 ± 15.01 ^z	2.34 ± 0.32 ^z	1.00 ± 0.11 ^z	47.83 ± 2.09 ^z	60.07 ± 6.00 ^z
Silymarin (25 mg/kg)	148.77 ± 13.05 [#]	55.34 ± 4.44 [#]	149.09 ± 6.18 [#]	0.63 ± 0.08 [#]	0.38 ± 0.08 [#]	35.02 ± 1.45 [#]	35.22 ± 3.11 [#]
Methanol extract (150 mg/kg)	285.63 ± 9.17 [#]	74.00 ± 7.05 [#]	244.33 ± 6.00 [#]	0.99 ± 0.09 [#]	0.51 ± 0.10 [#]	40.12 ± 2.42	41.1 ± 5.91 ^ε
Methanol extract (300 mg/kg)	210.32 ± 7.86 [#]	54.88 ± 6.73 [#]	201.04 ± 5.33 [#]	0.70 ± 0.06 [#]	0.41 ± 0.09 ^ε	37.10 ± 1.98 ^ε	37.88 ± 2.55 [#]

Each value represents mean (n = 6) ± SEM. Statistical analysis was carried out by ANOVA followed by the Tukey test. ^zp < 0.001 when negative control group compared with normal control group. [#]p < 0.001, ^εp < 0.001, ^γp < 0.05 when drug treated group compared with negative control group.

using Soxhlet apparatus. The methanol extract was concentrated to dryness under reduced pressure to obtain the methanol extract of *L. asiatica* leaves.

Experimental model and treatments

Healthy *Wistar* rats (150 - 200 g) of 2 - 3 months were used for the study. Animals were kept under controlled environmental conditions with ad libitum access to standard rodent chow and water. The study was approved by the Institutional Animal Ethical Committee (Reg. No. 1305/ac/09/CPCSEA).

Thirty rats of either sex were taken and divided into five groups (n = 6) in following manner,

- Group I – Healthy control (saline)
- Group II – Negative control or disease control (saline)
- Group III – standard drug (silymarin 25 mg/kg, p.o.)
- Group IV – lower dose of methanol extract (extract 150 mg/kg, p.o.)
- Group V – higher dose of methanol extract (extract 300 mg/kg, p.o.)

Animals were treated with respective test or standard drug or vehicle for consecutive three days once daily. Animals of all groups except healthy control group received single dose of acetaminophen (3000 mg/kg) on 3rd day, thirty minutes after the administration of respective drug treatment (Manokaran et al., 2008; Singh et al., 1995). After 48 h of acetaminophen administration, the blood was collected under light ether anaesthesia and serum was separated by centrifugation of blood at 4000 × g, which was used for the biochemical estimations.

Biochemical estimation

Biochemical estimation of several serum parameters like serum glutamic oxaloacetate transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT), alkaline phosphatase (ALP), total bilirubin, direct bilirubin, total cholesterol, total triglycerides were carried by using commercial biochemical kit obtained from Agapee Diagnostic Ltd., Kerala.

In vivo antioxidant activity

After blood collection animals were sacrificed through cervical dislocation method. The liver of all animal was dissected out and perfused separately with cooled 0.15 M KCl. The liver was centrifuged with 0.15 M KCl - 10 mM potassium phosphate buffer (pH 7.4) to prepare 10% liver homogenate (Kesiova et al., 2006). Liver tissue homogenate was used to estimate the concentration of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and reduced glutathione (GSH).

SOD activity was estimated by the inhibition of auto-oxidation of epinephrine to adrenochrome at 480 nm in the presence of liver homogenate (Huo et al., 2011; Misra and Frisovich, 1979). Briefly, mixture contain 0.1 ml of liver homogenate, 0.5 ml carbonate buffer (pH 10.2), 0.5 ml EDTA solution. The volume of mixture was adjusted to 2.5 ml. Epinephrine solution (0.5 ml) was mixed to initiate the reaction.

Auto oxidation of epinephrine to adrenochrome was carried out in a control tube without the homogenate. The SOD activity was calculated using a molar extinction coefficient of $4.02 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$, and expressed as $\mu\text{M}/\text{min}/\text{mg}$ protein.

CAT activity was determined by measuring the decomposition of H_2O_2 to H_2O (Huo et al., 2011). Briefly, 0.1 ml of supernatant was mixed with 1.9 ml of 50 mM potassium phosphate buffer (pH 7.0) and the reaction was initiated by the addition of freshly prepared 0.1 ml of 30 mM H_2O_2 . The rate of H_2O_2 decomposition was determined spectrophotometrically at 240 nm. The result was calculated using a molar extinction coefficient of 43.6 M/cm and expressed as $\mu\text{M H}_2\text{O}_2/\text{mg}$ protein/min.

Briefly GPx was estimated by using taking 100 μl tissue homogenate solution and 800 μl 100 mM/l potassium phosphate buffer (pH 7.4) containing 1 mM EDTA, 1 mM sodium azide, 0.2 mM NADPH, 1 U/ml glutathione reductase, 1 mM GSH. After 5 min incubation 100 μl 2.6 mM H_2O_2 was added and the absorbance change at 340 nm in 3 min was recorded at 37°C (Hsu et al., 2007). Activity of GPx was calculated using the molar extinction coefficient of NADPH $6220 \text{ M}^{-1} \text{ cm}^{-1}$ and expressed as $\mu\text{M NADPH}$ oxidized/min/mg protein at 37°C.

GSH content in liver was estimated based on the reaction of GSH with 5,5'-dithiobis (2 nitro benzoic acid) (DTNB) to produce a compound that absorbs at 412 nm (Raphael, 2004). Liver homogenate (0.5 ml) was mixed with 125 μl of 25% TCA. The test tubes containing the mixture were kept on ice for 5 min. Exactly, 0.6 ml of 5% TCA was mixed with the previous mixture and centrifuged at 1500 rpm for 10 minute. After centrifugation, 0.3 ml of supernatant was mixed with 0.7 ml of phosphate buffer (0.2 M, pH 8) and 2.0 ml DTNB solution (0.6 mM in 0.2 M sodium phosphate buffer, pH 8). The absorbance of solution was measured after 10 min 412 nm. The GSH content was determined using a standard curve varying from 5 - 100 nm in 5% TCA for assay, and results were expressed as nmol/mg protein.

GSH content in blood was estimated by the method of Khyriam and Prasad (Khyriam and Prasad, 2001). Blood sample (0.1 ml) was mixed with 0.9 ml of water and 1.5 ml of precipitating solution (1.67 g glacial metaphosphoric acid, 0.2 g sodium EDTA, 30.0 g NaCl in 100 ml water), and the mixture was incubated at room temperature. After 5 min of incubation period, the mixture was centrifuged at 3000 × g at 4°C for 15 min. Exactly 0.5 ml of clear supernatant was withdrawn and mixed thoroughly with 0.3 M/l phosphate solution (2.0 ml), 0.2% DTNB in 1% sodium citrate solution (0.25 ml). Blank solutions prepared with 1.0 ml 0.3 M/l phosphate solution, 1.0 ml water, 0.5 ml precipitating solution and 0.25 ml DTNB solution. Absorbances of mixtures were determined against water at 412 nm. Concentration of glutathione by using following formula, Concentration of GSH (mg GSH/ 100 ml blood) = $(A \times 2.75 \times 2.75 \times 307 \times 100)/(13.6 \times 0.1 \times 0.5 \times 1000)$ Absorbance (A) = Absorbance of sample - Absorbance of blank; Extinction

coefficient = 13.6 and molecular weight of glutathione 307.

Statistical analysis

The data were expressed as mean \pm SEM (n = 6) and subjected to analysis of variance (ANOVA). Statistical analysis was carried out by analysis of variance followed by Tukey tests, using SPSS (Statistical Package for Social Sciences) version 10.0 software. A level of $p < 0.05$ was used as the criterion for statistical significance.

RESULTS AND DISCUSSION

Hepatoprotective activity

Treatment with toxic dose of acetaminophen resulted significant increase in serum SGOT, SGPT, ALP, total and direct bilirubin, total cholesterol and triglyceride level in animals of disease control group, which indicated that paracetamol in toxic dose impairs hepatic function. Higher dose of methanol extract produced significant beneficial effect. Level of above serum biochemical parameter found near to normal in extract (300 mg/kg) treated group (Table 1).

Serum level of SGPT and SGOT are increases significantly in drug, toxin and chemic induced liver damage condition and considered as the most commonly used relied biomarker of hepatotoxicity. SGOT, SGPT, ALP releases into the extracellular space due to hepatocyte damages, which ultimately enter into circulation and thereby increase serum levels these enzyme in negative control group (Olaleye et al., 2010; Singh et al., 2011). In extract treated group the level of these enzyme was found less compare to negative control group, and in higher dose extract was found to maintained the level of these enzymes near normal which indicated that extract may preserved the integrity of hepatocellular membrane from the acetaminophen induced damage.

Hepatocyte damage or obstruction of excretory ducts of the liver spoils the capacity of liver to excrete normal amounts of bilirubin. Elevated levels of bilirubin may indicate severe dysfunction of liver. Total bilirubin considered as combination of indirect (nonhepatic) and direct (hepatic) bilirubin (Goel et al., 2012; McConnachie et al., 2007; Olaleye et al., 2010). This investigation showed that level of total and direct bilirubin were increased by the acetaminophen administration, but in extract treated group the level of direct and total bilirubin in the serum found significantly ($p < 0.01$) less that negative control group. Increased in serum cholesterol and triglyceride levels in negative control group indicated impaired lipoprotein, fat and cholesterol metabolism due to hepatic damage (Goel et al., 2012; McConnachie et al., 2007; Olaleye et al., 2010). Treatment with both dose of extract brought down the triglyceride level in normal, while only higher dose of methanol extract exhibited hypocholesrerolemic effct. These results suggested that extract produced potent hepatoprotective

activity.

In vivo antioxidant activity

Administration of acetaminophen causes depletion of liver GSH and blood GSH content. SOD, CAT, GPx level also reduced significantly ($p < 0.001$) in hepatotoxic animals compare to normal animal. Treatment with higher dose methanol extract of *L. asiatica* significantly ($p < 0.01, 0.001$) ameliorated the level of enzymatic and non enzymatic antioxidant levels. Lower dose of extract found effective only in increasing CAT level significantly (Table 2).

SOD, CAT and GPx are the endogenous antioxidant enzymes that important to avert oxidative stress situation. SOD catalyzes the dismutation of superoxide to H_2O_2 and O_2 , while CAT converts H_2O_2 to water and molecular oxygen. GPx present in the cell cytoplasm eliminates H_2O_2 by coupling its reduction to H_2O with oxidation of GSH (Sen and Chakraborty, 2011). Oxidative stress considered as one of the important factor of liver toxicity. NAPQI can increase the generation of different free radicals, reduces antioxidant enzyme level and causes lipid peroxidation which leads to oxidative stress (McConnachie et al., 2007; Olaleye et al., 2010). Extract treatment significantly increases level of endogenous antioxidant enzyme levels suggesting that extract possess significant *in vivo* antioxidant activity that is beneficial in preventing acetaminophen induced liver toxicity.

Metabolism of acetaminophen primarily occur in liver, where a small portion of acetaminophen (5 - 10%) in therapeutic dose metabolized via the hepatic cytochrome P450 enzyme to produce *N*-acetyl-*p*-benzo-quinoneimine, a highly reactive alkylating metabolite (Setty et al., 2007; Yen et al., 2007). NAPQI covalently bind to cellular macromolecules and causes lipid peroxidation that results the tissue damage. NAPQI also causes depletion of glutathione which further deteriorate the situation (Yen et al., 2007). GSH is an endogenous non-enzymatic antioxidant important to maintain thiol group and tissue integrity. GSH also capable of scavenging ROS directly or enzymatically via GPx and protects liver against chemical/drug induced hepatic damage and oxidative stress. Over production of NAPQI due to toxic dose of acetaminophen results hepatic GSH depletion, that can considered as a key mechanism of hepatocellular dysfunction (McConnachie et al., 2007; Olaleye et al., 2010). In the present study pretreatment with extract restored GSH levels near to normal. Therefore, it is suggested that extract have potent beneficial effects in preventing oxidative stress and acetaminophen induced hepatotoxicity. Silymarin, a flavonolignan obtained from 'milk thistle' (*Silybum marianum*) plant. Four flavonolignan isomers, like silybin, isosilybin, silydianin and silychristin are the ingredients of silymarin. A number of studies proved that silymarin is a potent antioxidant and also found to possess strong hepatoprotective activity (Burczynski et al., 2012; Pradhan and Girish, 2006). In this study *L. asiatica* extract also

Table 2. *In vivo* antioxidant activity of methanol extract of *L. asiatica* leaves

Treatment	GSH in liver (μ M GSH/gm Liver tissue)	GSH in blood (μ M GSH/ml Blood plasma)	SOD (μ M/min/mg protein)	CAT (μ M/min/mg protein)	GPx (μ M/min/ mg protein)
Normal control(Normal saline)	4.04 \pm 1.10	21.33 \pm 1.60	6.66 \pm 0.61	21.33 \pm 2.11	44.52 \pm 4.44
Negative control (PCM)	6.91 \pm 0.62 [*]	7.104 \pm 0.85 [*]	1.97 \pm 0.18 [*]	8.01 \pm 0.93 [*]	14.00 \pm 3.01 [*]
Silymarin (25 mg/kg)	12.08 \pm 1.02 ^e	17.66 \pm 1.35 [#]	6.01 \pm 0.47 [#]	17.99 \pm 1.39 ^e	38.18 \pm 2.29 [#]
Methanol extract (150 mg/kg)	8.40 \pm 0.72	8.53 \pm 0.77	3.02 \pm 0.52	14.61 \pm 1.11 ^y	19.36 \pm 1.53
Methanol extract (300 mg/kg)	11.91 \pm 0.68 [#]	13.32 \pm 1.03 ^e	5.06 \pm 0.47 ^e	16.32 \pm 1.55 [#]	37.99 \pm 1.98 [#]

Each value represents mean (n = 6) \pm SEM. Statistical analysis was carried out by ANOVA followed by the Tukey test. ^{*} $p < 0.001$ when negative control group compared with normal control group. [#] $p < 0.001$, ^e $p < 0.001$, ^y $p < 0.05$ when drug treated group compared with negative control group.

possess strong hepatoprotective activity. Ethnomedicinal information from ethnic people always served as goldmine to investigate new drug and therapy. This present study was undertaken to validate the claim about its medicinal value and results of the study confirmed the same. This study unlocks the possibility to investigate this leaf extract further to develop a new hepatoprotective drug.

CONCLUSION

Present work justifies the folk medicinal uses *Leea asiatica* leaves in the treatment of liver disorders. The possible mechanism of this activity may be due to strong antioxidant activities of extract. Though further study are required but the plant extract or their phytoconstituents present in the extract may be considered as a lead of future that could prevent the drug induced toxicity in liver by their concurrent uses.

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CONFLICT OF INTEREST

The authors have no conflicting financial interests.

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