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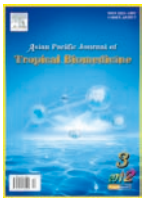

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Anti-hepatotoxic potential of *Hedyotis corymbosa* against D-galactosamine-induced hepatopathy in experimental rodents

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ABSTRACT

Objective: To evaluate hepatoprotective potential of the methanolic extract of *Hedyotis corymbosa* against D-galactosamine-induced hepatopathy in experimental animals. **Methods:** In the present study, in- vivo hepatoprotective effect of 50% methanolic extract of *Hedyotis corymbosa* (HCE, 100 and 200 mg/kg body weight) was evaluated using experimental models D-Galactosamine (D-GalN) (200 mg/kg, body weight i.p.) induced hepatotoxicity in experimental animals. The hepatoprotective activity was assessed using various biochemical parameters like aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatise (ALP), γ-glutamyl transferase (γ-GT) and total bilirubin. Meanwhile, in vivo antioxidant activities as lipid peroxidation (LPO), reduced glutathione (GSH), superoxide dismutase (SOD) and catalase (CAT) were screened along with histopathological studies. **Results:** Obtained results demonstrated that the treatment with HCE signi-cantly (P<0.05-P<0.001) and dose-dependently prevented chemically induced increase in serum levels of hepatic enzymes. Furthermore, HCE signi-cantly (up to P<0.001) reduced the lipid peroxidation in the liver tissue and restored activities of defence antioxidant enzymes GSH, SOD and catalase towards normal levels. Histopathology of the liver tissue showed that HCE attenuated the hepatocellular necrosis and led to reduction of in ammatory cells in-ltration. **Conclusions:** The results of this study strongly indicate the protective effect of HCE against acute liver injury which may be attributed to its hepatoprotective activity, and there by scienti-cally support its traditional use.

1. Introduction

Liver diseases are one of the most serious health problems in the world today but, despite tremendous advances in modern medicine, their prevention and treatment options still remain limited. However, the pathogenesis of hepatic diseases as well as the role of oxidative stress and inflammation therein is well established[1,2], and accordingly, blocking or retarding the chain reactions of oxidation and inflammation process could be a promising therapeutic strategy for prevention and treatment of liver injury. Detoxification reactions (phase I and phase II) metabolize xenobiotics with the aim of increasing substrate hydrophilicity for excretion. Drug-metabolizing enzymes detoxify many xenobiotics but bioactivate or increase the toxicity of others. In the case of bioactivation, liver is the first organ to be exposed to the damaging effects of the newly formed toxic substance. Therefore, protective mechanisms relevant to the liver are of particular interest. Effectively, herbal products are widely used in the treatment of hepatic disorders all over the world[3].

Hedyotis corymbosa (L.) Lam. Syn. Oldenlandia corymbosa (L.) Lam. (Rubiaceae) is a weedy herb, widely distributed throughout India. It is commonly known as 'Parppatakapullu' in traditional medicine of Kerala. There are approximately 180 species recorded of which 35 were identified in Malaysia[4]. *Hedyotis corymbosa* is extensively used in modern Chinese practice for the treatment of viral infections, cancer, syndromes involving "toxic heat", acne, boils, skin ailments, appendicitis, hepatitis, eye diseases and bleeding[5]. They call it 'Peh-Hue-Juwa-Chi-Cao'[6]. The plant is used for treating venomous bites. It is bitter, acrid, cooling, febrifugal, pectoral, anthelmintic, diuretic, depurative, diaphoretic, expectorant, and digestive and has stomachic properties[7]. It is given in jaundice, and other diseases of the liver, heat eruptions, vitiated conditions of

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pitta, hyperdypsia, giddiness, dyspepsia, flatulence, colic, constipation, helminthiasis, leprosy, skin diseases, cough, bronchitis, necrosis, nervous depression caused by deranged bile and hepatopathy[8].

Three new iridoid glycosides[8], nine iridoid and lignin glycosides and rutin have been isolated from the whole plant[9]. To the best of our knowledge there was lack of scientific reports available in support of its traditional claim of hepatoprotective potential. So far, there has been only two research reported on hepatoprotective effect against carbon tetrachloride[10] and paracetamol[11] induced liver damage in rats has been investigated Therefore, present study was designed to demonstrate the effect of *Hedyotis corymbosa* extract (HCE) against D- galactosamine induced hepatic damage in experimental animals.

2. Materials and methods

2.1. Chemicals

All the chemicals used were of analytical grade and procured from Sigma chemicals Co., USA and Qualigens fine chemicals, Mumbai, India.

2.2. Preparation of plant extract

The whole plants of *Hedyotis corymbosa* were collected from campus garden of National Botanical Research Institute, Lucknow, India in December 2010. The plant material was identified and authenticated and the voucher specimen number NAB-79026 was deposited in the institutional herbarium. The whole plants of *Hedyotis corymbosa* were washed thoroughly in tap water, shade dried and powdered. The powder (100 g) was successively extracted with 1000 ml of methanol overnight with constant stirring. The filtrate was then concentrated and the solvent was evaporated under reduced pressure in a rotary evaporator. The yield of the extract was found to be 0.42% (w/v). This crude extract was referred to as HCE. The extract obtained was further subjected to pharmacological investigation. For administration, the crude extract was suspended in 1% Tween- 80, to required concentrations.

2.3. Animals

Wistar rats weighing (150-170 g) and Swiss albino mice (25-30 g) of either sex were procured from CDRI, Lucknow. They were kept in departmental animal house in well cross ventilated room at 22 ± 2 ℃ with light and dark cycles of 12 h for 1 week before and during the experiments. Animals were provided with standard rodent pellet diet (Amrut, India) and the food was withdrawn 18-24 h before the experiment though water was given ad libitum. All studies were performed in accordance with the guide for the care and use of laboratory animals, as adopted and promulgated by the Institutional Animal Care Committee, CPCSEA, India (Reg. No. 222/2000/CPCSEA).

2.4. Behavioural and toxicological effects

Two groups of 10 mice were treated with graded doses of the HCE (100 and 200 mg/kg, p. o.). One group was maintained as control and was given 0.5% Tween-80. They were observed continuously for 1 h for any gross behavioural changes and death, if any, and then, intermittently for the next 6 h, and then again at 24 h after dosing with HCE[11].

2.5. D-Galactosamine (D-GalN)-induced hepatotoxicity

The rats were randomly divided into five groups of six animals each. Group I (control) animals were administered a single daily dose of sodium carboxymethyl cellulose (0.3%, 5 ml/kg body weight, p.o. body weight). Group II was served as D-GalN treated control and received the vehicle. Groups III-IV was treated with HCE at the dose levels of 100 and 200 mg/kg body weight. Group V was treated with standard drug silymarin at 100 mg/kg body weight. All these treatments were given orally for 8 days. On the last day of the treatment, the animals of groups II-V received a single dose of D-GalN in distilled water at 200 mg/kg body weight intraperitoneally after 1 h of the vehicle, HCE or standard silymarin treatments. On the 9th day, the animals were anesthetized by anesthetic ether the liver samples were dissected and blood was collected[12].

2.6. Assessment of hepatoprotective activity

The collected blood was allowed to clot and serum was separated at 2500 rpm for 15 min and the biochemical parameters like serum enzymes: aspartate aminotransferase (AST, U/L), alanine aminotransferase (ALT, U/L)[13], alkaline phosphatase (ALP, U/L)[14], total bilirubin (mg/dL)[15] and gamma glutamyl transferase (γ -GT) using assay kits[16].

2.7. Assessment of antioxidant parameters

2.7.1. Assessment of lipid peroxidation (LPO)

The dissected out liver samples were washed immediately with ice cold saline to remove as much blood as possible. Liver homogenized (5%) in ice cold 0.9% NaCl with a Potter- Elvenhjem glass homogenizer. The homogenate was centrifuged at 800 for 10 min and the supernatant was again centrifuged at 12,000 for 15 min and the obtained mitochondrial fraction was used for the estimation of LPO[17]. A volume of the homogenate (0.2 ml) was transferred to a vial and was mixed with 0.2 ml of a 8.1% (w/v) sodium dodecyl sulphate solution, 1.5 ml of a 20% acetic acid solution (adjusted to pH 3.5 with NaOH) and 1.5 ml of a 0.8% (w/v) solution of thiobarbituric acid (TBA) and the final volume

was adjusted to 4.0 ml with distilled water.

Each vial was tightly capped and heated in a boiling water bath for 60 min. The vials were then cooled under running water. Equal volumes of tissue blank or test samples and 10% trichloroacetic acid were transferred into a centrifuge tube and centrifuged at 1000g for 10 min. The absorbance of the supernatant fraction was measured at 532 nm (Beckman DU 650 spectrometer). Control experiment was processed using the same experimental procedure except the TBA solution was replaced with distilled water[18]. Malonyldialdehyde (MDA) is an end product of lipid peroxidation, which reacts with thiobarbituric acid to form pink chromogen thiobarbituric acid reactive substance. 1, 1, 3, 3-tetra ethoxypropan was used as standard for calibration of the curve and is expressed as nmole/mg protein.

2.7.2. Assessment of catalase and superoxide

The liver tissue was homogenized (5%) and mitochondrial fraction was prepared as described above. Decomposition of H_2O_2 in presence of catalase (CAT) was followed at 240 nm[19]. One unit (U) of catalase was defined as the amount of enzyme required to decompose 1 μ mol of H_2O_2 per min, at 25 ℃ and pH 7.0. Results are expressed as units (U) of CAT activity/mg protein. Superoxide dismutase (SOD) activity was estimated by the inhibition of nicotinamide adenine dinucleotide reduced-phenazine methosulphate-nitrobluetetrazolium reaction system as described by Nishikimi *et al*.[20] and as adapted by Kakkar *et al*.[21]. One unit of the enzyme is equivalent to 50% inhibition in the formazan formation in 1 min at room temperature (25 ± 2℃) and the results have been expressed as units (U) of SOD activity/mg protein.

2.7.3. Assessment of reduced glutathione (GSH) activity

The concentration of GSH was determined by the method of Anderson[22] based on the development of a yellow colour when 5, 5-dithiobis (2-nitrobenzoic acid) is added to compounds containing sulfhydryl groups. The reaction mixture contained equal volumes of 4% sulfosalicylic acid and tissue samples homogenized in 4 volume of ice cold 0.1 m/l phosphate buffer (pH 7.4). The method used for estimating GSH in this study also measures non-protein sulfhydryl concentration inclusive of GSH. However, 80-90% of the non-protein sulfhydryl content of the cell represents free endogenous GSH. Enzyme activity was expressed as milligram per hundred grams[23].

2.8. Histopathological studies

For histological studies, the liver tissues were fixed with 10% phosphate buffered neutral formalin, dehydrated in graded (50-100%) alcohol and embedded in paraffin. Thin sections (5 M) were cut and stained with routine hematoxylin and eosin stain for photo microscopic assessment. The initial examination was qualitative, with the purpose of determining histopathological lesions in liver tissue.

2.9. Statistical analysis

The values were represented as mean ± S.E.M. for six rats. Analysis of variance (ANOVA) test was followed by individual comparison by Newman-Keuls test using Prism Pad software (Version 3.0) for the determination of level of significance. The values of $P<0.05$ was considered statistically significant.

3. Result

3.1. Behavioural and toxicological effects

Hedyotis corymbosa produces no any behavioural and toxicological effects at 100 and 200 mg/kg. Therefore, 200 mg/kg dose of extract were selected as therapeutic highest dose and just half of its as lowest dose (100 mg/kg) respectively, in this study.

3.2. Effect of HCE on AST, ALT, ALP, γ-GT and total bilirubin against D-GalN induced liver injury in rats

The effect various doses of HCE were studied on serum marker enzymes and total bilirubin in D-GalN intoxicated animal. Hepatic injury induced by D-GalN caused significant changed in marker enzyme as AST by 172.35%, ALT by 188.04%, ALP by 119.9%, γ-GT by 112.33% and total bilirubin by 57.73% compared to control group. The percentage protection in marker enzyme of treated group at 100 mg/kg as AST 7.83 ($P< 0.01$), ALT 12.47 ($P< 0.05$), ALP

Table 1.
Effect of HCE on serum AST (U/L), ALT (U/L), ALP (U/L), Total Bilirubin (TBL) level (mg/dl), and γ-GT (U/L) against D-galactosamine induced liver toxicity in rats.

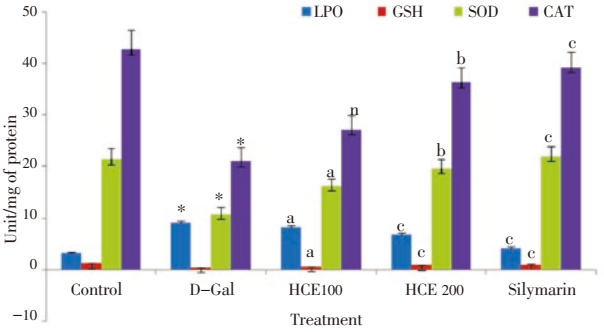
Groups	AST	ALT	ALP	TBL	γ-GT
Control	72.28 ± 3.2	32.38 ± 2.7	98.27 ± 2.2	0.67 ± 0.02	1.54 ± 0.09
D-GalN	213.20 ± 5.24*	93.27 ± 4.6*	216.1 ± 5.8*	1.03 ± 0.04*	3.27 ± 0.23*
HCE 100	196.50 ± 4.2b	81.67 ± 4.2a	197.8 ± 4.1b	0.92 ± 0.03b	3.04 ±0.2n
HCE 200	131.35 ± 3.8c	62.81 ± 3.8c	136.28 ± 3.2c	0.78 ± 0.02c	2.41 ± 0.19b
Silymarin	93.08 ± 3.5c	49.65 ± 3.1c	116.81 ± 2.9c	0.7 ± 0.02c	2.01± 0.17c

Values are mean ± S.E.M. of 6 rats in each group
n : no significant
P values: *<0.001 compared with respective control group I
P values: a<0.05, b<0.01, c<0.001 compared with group II (D-Gal)

8.4 (P< 0.01), γ-GT 7.03 (ns) and total bilirubin 10.67(P< 0.01) as compared to toxic group while maximum percentage protection in marker enzyme at the dose of 200 mg/kg and silymarin (100mg/kg) as AST 38.38 (P< 0.001), 56.34 (P< 0.001), ALT 32.65 (P< 0.001), 46.76 (P< 0.001), ALP 36.93 (P< 0.001), 45.94 (P< 0.001), γ-GT 26.3 (P< 0.01), 38.53 (P< 0.001), and total bilirubin 24.27 (P< 0.001), 32.03 (P< 0.001) which is almost comparable to the group treated with silymarin, a potent hepatoprotective drug used as reference standard (Table 1).

3.3. Estimation of LPO, GSH, SOD and CAT against D-GalN induced liver injury in rats

The results in Figure 1 showed clear significant percentage change in the levels of LPO in D-GalN intoxicated rats as 175.52 (P< 0.001) compared to control group. Treatment with HCE at the doses of 100 and 200 mg/kg significantly prevented this heave in levels and the percentage protection in LPO were 10.19 (P< 0.05) and 24.45 (P< 0.001) respectively. The GSH, SOD and CAT content had significantly increased in HCE treated groups whereas D-GalN intoxicated group had shown significant decrease in these parameters compared to control group. The percentage changed of GSH, SOD and CAT in D-GalN intoxicated group were as 61.98 (P< 0.001), 49.41 (P< 0.001) and 50.78 (P< 0.001) respectively. The percentage protection in GSH as 32.6 (P< 0.05), 86.95 (P< 0.001) and SOD 50.87 (P< 0.05), 81.96 (P < 0.01) while in CAT 28.99 (ns), 72.66 (P < 0.01) at the doses levels 100 and 200 mg/kg, respectively. In different doses level of HCE, 200 mg/kg has shown maximum protection which was almost comparable to those of the normal control and silymarin.



Groups	LPO	GSH	SOD	CAT
Control	3.31 ± 0.17	1.21 ± 0.08	21.37 ± 2.1	42.81 ± 3.8
D-Gal	9.12 ± 0.32*	0.46 ± 0.02*	10.81± 1.3*	21.07 ± 2.6*
HCE 100	8.19 ± 0.41a	0.61 ± 0.03a	16.31± 1.41a	27.18 ± 2.8n
HCE 200	6.89 ± 0.27c	0.86 ± 0.04c	19.67 ± 1.8b	36.38 ±2.9b
Silymarin	4.21 ± 0.21c	1.02 ± 0.06c	21.98 ± 2.0c	39.32 ± 3.0c

Values are mean ± S.E.M. of 6 rats in each group
n: no significant
P values: *<0.001 compared with respective control group I
P values: a<0.05, b<0.01, c<0.001 compared with group II (D-Gal)
Figure 1. Effect of HCE on liver LPO (MDA nmole/min/mg of protein), GSH (nmole/mg of protein), SOD (unit/mg of protein) and CAT (units/mg of protein) against D-galactosamine induced liver toxicity in rats.

3.4. Histopathological observations

The histological observations basically support the results obtained from serum enzyme assays. Liver section in normal control rats showed central vein surrounded by hepatic cord of cells while in D-GalN treated rats showing massive fatty changes along with congestion in central vein, necrosis, and the loss of cellular boundaries.Whereas the HCE treated groups showed absence of cell necrosis, but with minimal inflammatory conditions. The HCE 200 mg/kg, p.o. treated group showed regeneration of hepatocyte around central vein with near normal liver architecture possessing higher hepatoprotective action (Figure. 2).

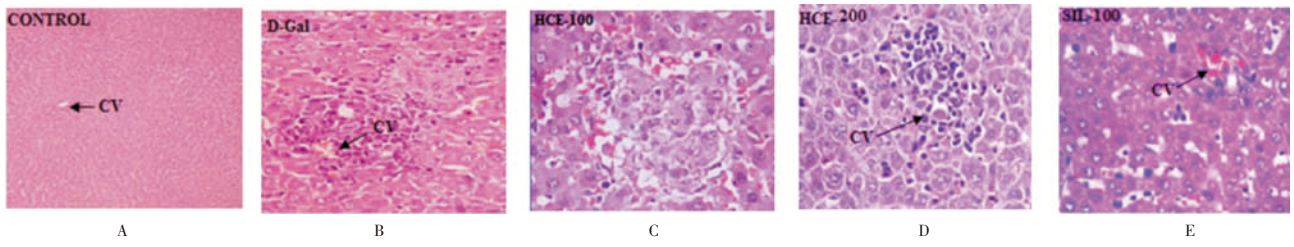


Figure 2. Histopathology of liver tissues. (A) Liver section of normal control rat shows central vein surrounded by hepatic cord of cells (normal architecture). (B) Liver section of D- Gal treated rats showing massive fatty changes along with congestion in central vein, necrosis, and the loss of cellular boundaries. (C) Liver section of rats treated D-Gal and 100 mg/kg of HCE showing focal necrosis with sinusoidal dilatation. (D) Liver section of rats treated D-Gal and 200 mg/kg of HCE showing less in ammatory cells around central vein and mild necrosis. (E) Liver section of rats treated D-Gal and 100 mg/kg of silymarin showing normal liver architecture.

4. Discussion

As a result of considerable attention that has been devoted to the liver diseases during the last two decades, the vast majority of studies have been concerned with the liver of the experimental animals and the characteristics of animal model's normal liver have been well defined. In the absence of reliable liver-protective drugs in modern medicine, a large number of medicinal plants with long and well-established traditional use have been recommended for treatment of liver disorders[24]. However, their application requires detailed in-vivo pharmacological characterization[25], and clinical studies the reafter. In the present investigation, *Hedyotis corymbosa* (HCE) was

evaluated for the hepatoprotective activity using D-GalN hepatotoxicity in rat.

D-GalN is also a well-established hepatotoxicant, inducing a liver injury witch closely resembling human viral hepatitis in its morphologic and functional features and, therefore, it is very useful for evaluation of hepatoprotection[26,27]. D-GalN hepatotoxicity is considered as an experimental model of acute hepatitis and it does not affect other organs[28]. D-GalN has great liver specificity because hepatocyte have high levels of galactokinase and galactose-1-uridyltransferase, and it disrupts the synthesis of essential uridylate nucleotides. Depletion of these nucleotides ultimately impairs the synthesis of protein and glycoprotein, leads to progressive damage of cellular membranes resulting in a change in permeability of the cellular membrane, and finally with enzyme leakage from the cells[29]. Liver damage induced by D-GalN generally reflects disturbances of liver cell metabolism which lead to characteristic changes in the serum enzyme activities[30]. The increased levels of AST, ALT, ALP, and γ-GT in this study may be interpreted as a result of the liver cell destruction or changes in the membrane permeability indicating the severity of hepatocellular damage induced by D-GalN, which is in accordance with previous reports[29]. Determination of serum bilirubin represents an index for the assessment of hepatic function and any abnormal increase in the levels of bilirubin in the serum indicate hepatobiliary disease and severe disturbance of hepatocellular function[31]. Pre-treatment with HCE extract (at different doses level 100 and 200 mg/kg) attenuated the increased activities of these enzymes (AST, ALT, ALP and γ-GT) in serum caused by D-GalN. Recovery towards normalisation suggests that HCE extract causes parenchymal cell regeneration in liver, thus protecting membrane fragility, thereby, decreasing enzyme leakage and extract mediated suppression of the increased bilirubin level suggests the possibility of the extract being able to stabilise biliary dysfunction. Oxidative stress has been reported as one of the major causes of DGa1N-induced liver damage, excessive production of free radicals resulting from oxidative stress can damage macromolecules as lipids and the D-Ga1N injection decreased liver GSH, SOD and CAT; these results were in agreement with Najmi *et al*.[32] who indicated that D-Ga1N-intoxicated rats showed an increased TBARS (Thio barbituric acid reactive substance) level, a typical parameter of lipid peroxidation. Also, Zhou *et al*.[33] indicated that treatment with DGa1N decreased antioxidative enzyme activities. The current results showed that HCE increasing liver antioxidant parameters and decreased oxidative stress which appeared in decreasing the mean value of liver TBARS and compared to D-GalN. The reduced activities of GSH, SOD and catalase observed point out the hepatic damage in the rats administered with D-GalN but the treated with 100 and 200 mg/kg of HCE groups showed significant increase in the level of these enzymes and normalise the level of LPO, which indicates the antioxidant activity of the *Hedyotis corymbosa*. The hepatoprotective effect of the HCE was further accomplished by the histopathological examinations. HCE at different dose levels offers hepatoprotection, but 200 mg/kg is more effective than all other lower doses. As demonstrated in our study, administration of D-GalN significantly elevated serum levels of hepatic enzymes, indicating considerable hepatocellular damage. Our study confirmed the protective effect of HCE against D-GalN in rats. In rat, HCE hepatoprotective activity is quite similar to silymarin, a reference hepatoprotective agent.

Conflict of interest statement

We declare that we have no conflict of interest.

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