



Hepatoprotective Potentiality of Various Fractions of Ethanolic Extracts of *Lawsonia Inermis* (Henna) Leaves Against Chemical-Induced Hepatitis in Rats

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Abstract: Liver is a metabolically active organ responsible for many vital life functions. More than 900 drugs, toxins, and herbs have been reported to cause liver injury. In spite of tremendous advances in modern medicine, there are hardly any reliable drugs that protect the liver from damage and/or help in regeneration of hepatic cell. It is, therefore, necessary to search for effective and safe herbal drugs for the treatment of liver disease. In the present study, we investigated the hepatoprotective activity of ethanolic extracts of various fractions (Ethyl acetate, Petroleum ether and Chloroform) of *Lawsonia inermis* leaves in carbon tetrachloride (CCl₄)-induced hepatitis rats. Hepatotoxicity was induced in rats by intraperitoneal injection of CCl₄ on alternate days for 7 days. After 7 days of pretreatment of test extracts, the biochemical markers such as Serum bilirubin, Serum Glutamic Pyruvic Transaminase (SGPT), Serum Glutamic Oxaloacetic Transaminase (SGOT) and Alkaline Phosphatase (ALP), were examined. The extracts at the doses of 200 mg/kg p.o. significantly ($P < 0.05$) reduced the elevated levels of Serum bilirubin, SGPT, SGOT and SALP compared to the CCl₄-treated group alone. The findings of the study indicate that the leaf extract of *L. inermis* showed a potential hepatoprotective activity and the protective action might be manifested by reducing the oxidative stress as the leaf extracts possess flavonoid and phenolic compounds which have free radical scavenging and antioxidant activity. However, further study is necessary uncovering the exact molecular mechanism of hepatoprotective activity.

Keywords: Antioxidant Activity, Carbon Tetrachloride, Ethanolic Extracts, Hepatoprotective Activity

1. Introduction

Liver is one of the largest organs in human body and the chief site for intense metabolism and excretion. So it has a surprising role in the maintenance, performance and regulating homeostasis of the body. It is involved with almost all the biochemical pathways to growth, fight against disease, nutrient supply, energy provision and reproduction [1]. Thus, to maintain a healthy liver is a crucial factor for overall health and well being. Liver diseases are mainly caused by toxic chemicals, excess consumption of alcohol infections and autoimmune disorders. Most of the hepatotoxic

chemicals damage liver cell mainly by inducing lipid peroxidation and other oxidative damages [2, 3]. Adverse hepatic events caused by drug can be considered to be either predictable or unpredictable. They may associate with serious morbidity and mortality [4]. Despite considerable progress in the treatment of liver diseases by oral hepatoprotective agents search for newer drugs continues because the existing synthetic drugs have several limitations. Hence crude drugs or natural food diet which possesses antioxidant or free radical scavenging activity has become a central focus for research designed to prevent or ameliorate tissue injury and may have a significant role in maintaining health [5]. *Lawsonia inermis* (Lythraceae) commonly known as 'Henna'

is a well-known plant used in the traditional medicine. Various parts of this plant have been used in traditional medicine. Therefore, this work was aimed to study the hepatoprotective effect of *L. inermis* leaves ethanolic extracts on carbon tetrachloride (CCl₄)-induced hepatotoxicity in rats by evaluating various biochemical parameters such as serum bilirubin, serum glutamic pyruvic transaminase (SGPT), serum glutamic oxaloacetic transaminase (SGOT), serum alkaline phosphatase (SALP) etc. We also investigated antioxidant phytochemicals in order to correlate the hepatoprotective mechanism of *L. inermis*.

2. Materials and Methods

2.1. Reagents and Chemicals

Sylimarin standard drug powder was generous gift sample from Square pharmaceuticals Ltd., Pabna, Bangladesh. 0.9% NaCl solution (Beximco Infusion Lab., Dhaka, Bangladesh), SGOT, SGPT and ALP wet reagent diagnostic kits (AMP Medizintechnik GmbH; Austria), gallic acid standard (GE Health care, Buckinghamshire, UK) α , α -diphenyl- β -picrylhydrazyl (DPPH), sodium phosphate, ammonium molybdate, ascorbic acid, ethanol and carbon tetrachloride (Sigma Aldrich, St. Louis, USA), were obtained from the sources noted. All employed chemicals and solvents were of analytical grade.

2.2. Plant Materials

Fresh leaves of *L. inermis* (Local name: Henna) was collected from medicinal plant garden, Department of Pharmacy, University of Rajshahi, Rajshahi and various parts of the locality. The plants were taxonomically identified by Mr. A. F. M Ali Haider, Assistant Professor, Department of Botany, University of Rajshahi and the voucher specimen has been preserved there. After thorough washing the leaves were dried under mild sun and subsequently in oven at 50°C temperatures for complete dryness. The dried materials were ground in electric grinder into a coarse powder.

2.3. Extraction and Fractionation of Plant Materials

The dried and pulverized plant material was cold extracted by ethanol as described previously [6] and the ethanolic extract was successively partitioned with ethyl acetate (EA), petroleum ether (PE) and chloroform (CF) using modified Kupchan partitioning method [7]. The resultant fractions were then evaporated by roto-dryer at low temperature (40-50°C) to dryness. The fractions were preserved at -20°C until use. Dimethyl sulfoxide was used as a solvent for the preparation of dose of various fractions.

2.4. Chemical Analysis of the Various Fractions of Ethanolic Extract of *L. inermis*

2.4.1. Determination of Total Phenolic (TP) Content

The total phenolic (TP) content of the various fractions (PE, CF, EA) of ethanolic extract of *L. inermis* were determined by Folin-Ciocalteu Reagent (FCR) according to

the method of Kumar *et al.*, [8] with slight modification. Briefly, the solution of each extract (0.5 ml, 1 mg/ml) was diluted to 10 ml with distilled water in a volumetric flask. FCR (1 ml) was added and mixed thoroughly, and then sodium carbonate solution (3 ml, 2%) was added. After 2h incubation at room temperature, absorbance was measured at 760 nm. The total phenolic content was determined by comparison with the standard calibration curve of gallic acid, and results are presented as mg of gallic acid equivalents (mg of GAE) per gram dry weight of extracts. All tests were conducted in triplicate.

2.4.2. Determination of Total Flavonoid (TF) Content

The total flavonoid content of each fraction of the extract was estimated by Zhishen *et al.* [9]. Briefly, 0.5 ml (1 mg/ml) of each sample was mixed with 2 ml of distilled water and subsequently with 0.15 ml of NaNO₂ solution (15%). After incubation for 6 min, 0.15 ml of AlCl₃ solution (10%) was added and allowed to stand for another 6 min. Then 2 ml of NaOH solution (4%) was added to the mixture and adjusted the final volume to 5 ml by distilled water. The mixture was then mixed thoroughly and allowed to stand for another 15 min. The absorbance of the final solution was determined at 510 nm. The total flavonoid content was determined by comparison with the standard calibration curve of gallic acid, and results are presented as mg of gallic acid equivalents (mg of GAE) per gram dry weight of extracts.

2.5. In Vitro Antioxidant Assay

2.5.1. DPPH Radical Scavenging Assay

The plant extracts were tested for the scavenging effect on DPPH radical according to the method of Pan *et al.*, [10]. Accordingly, 0.2 ml of extract solution in ethanol (95%) at different concentrations (1, 2, 4, 8, 16, 32 and 64 μ g/ml, respectively) was added to 8 ml of 0.004% (w/v) stock solution of DPPH in ethanol (95%). The scavenging activity on the DPPH radical was determined by measuring the absorbance at 517 nm until the reaction reached the steady state, using a UV-visible spectrophotometer (Shimadzu, Tokyo, Japan). Ascorbic acid was used as a positive control. The DPPH radical scavenging activity (S %) was calculated using the following formula:

$$S\% = [(A_{\text{control}} - A_{\text{sample}}) / A_{\text{control}}] \times 100$$

2.5.2. Total Antioxidant Activity Assay

The total antioxidant activity of the ethanolic extract and its various fractions of *L. inermis* were assessed by phosphomolybdenum method as described previously [11]. Briefly, 0.5 ml sample solution of each fraction was mixed with 3 ml of phosphomolybdenum solution comprising: 0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate. The mixture was then incubated at 95°C for 90 min followed by cooling at room temperature. The absorbance of the solution was measured at 695 nm against blank. Ascorbic acid was used as a positive control.

2.6. Experimental Animals

Male Wister rats weighing about 110–120 g were purchased from the animal resource branch of International Centre for Diarrheal Diseases and Research (ICDDR), Bangladesh. The animals were kept under standard environmental conditions (temperature $23 \pm 2^\circ\text{C}$, relative humidity $55 \pm 10\%$, and 12-h light/dark cycle). The animals were fed with standard rat pellets (formulated by ICDDR, Bangladesh) and water *ad libitum* and acclimatized to laboratory conditions for 7 days before conducting experiments. The investigation was conducted on experimental animals in accordance with the international principles for laboratory animals' use and care as found in the guidelines [12]. The study protocol was approved by the Ethical Review Committee, Faculty of Biological Science, University of Rajshahi.

2.7. Hepatoprotective Activity Test

The hepatoprotective activity was evaluated according to the method described by Mahmud *et al.*, [13] with minor modification. Twenty-four rats were randomly selected and divided into six groups of four animals each. Group I served as normal control and received only the vehicle (1% Tween-80 in normal saline) at a dose of 1 mL/kg body weight daily. Group II served as the CCl_4 -treated control group/negative controls and received the vehicle (1 mL/kg/day) and CCl_4 diluted with olive oil. Group III animals were administered with the standard drug silymarin at a dose of 50 mg/kg/day. Groups IV, V and VI received the PE, EA and CH fractions of the ethanolic extract of *L. inermis* at a dose of 200 mg/kg/day, respectively. The vehicle or test drugs were administered orally for successive 7 days. To induce hepatotoxicity, carbon tetrachloride diluted with olive oil (1:1) was given intraperitoneally (i.p.) [14] at a dose of 1 mL/kg body weight to all the rats except the rats in Group I on alternate days for a period of 7 days while olive oil (0.5 mL/kg i.p.) was injected into group I animals. After 24 h of the last dose of CCl_4 , all the rats were sacrificed by cervical decapitation; blood samples were collected through retro-orbital plexus and allowed to clot for 30 min at room temperature. The clear serum was separated by centrifugation at 4000 rpm for 10 min and serum samples were stored at -40°C until use for the determination of biochemical parameters.

2.8. Assessment of Liver Function

The functional state of the liver was determined by estimating the biochemical parameters such as Serum Glutamic Oxaloacetic Transaminase (SGOT), Serum Glutamic Pyruvic Transaminase (SGPT), Serum Alkaline Phosphatase (SALP), Serum bilirubin concentration etc. SGOT and SGPT were estimated by enzymatic UV kinetic methods based on the reference method of International Federation of Clinical Chemistry [15-17]. Alkaline

phosphatase (ALP) was estimated by the method described by McComb and Bowers [18]. Serum total bilirubin is measured as an end point chemical reaction using diazotization to produce azobilirubin. Increases in absorbance generated by blue colored azobilirubin and measured at 546 nm is proportional to the concentration of total bilirubin in the sample [19].

2.9. Statistical Analysis

The result are expressed as mean \pm SEM using Graph pad prism (version 5) computer program (Graph pad software San Diego, CA, USA). We used a one-way analysis of variance (ANOVA), followed by Scheff's post-hoc test or students paired or un paired *t*-test where appropriate. The statistical method applied in each analysis was described in each figure. Results were considered to be significant when *p* values were less than 0.05 ($p \leq 0.05$).

3. Results and Discussion

3.1. Chemical Analysis of Total Phenol and Flavonoid Contents of the Various Fractions of *L. inermis*

Table 1. Determination of total phenol and flavonoid contents of the various fractions of *L. inermis*.

| Sample | Total phenol | Total flavonoid |
|-------------|--------------------------|--------------------------|
| | GAE/gm of dried extracts | GAE/gm of dried extracts |
| EA fraction | 30.80 ± 1.90 | 79.16 ± 2.72 |
| PE fraction | 39.39 ± 2.46 | 51.39 ± 1.37 |
| CH fraction | 58.40 ± 1.96 | 32.35 ± 1.74 |

The phenolic and flavonoid compounds are considered as potential antioxidants and free radical scavengers. Here, we investigated the contents of total phenol (TP) and total flavonoid (TF) compounds in the various fractions (EA, PE and CF) of ethanolic extracts of *L. inermis*. The results are shown in Table 1. The highest amount of TP and TF contents were found in CHF and EAF, respectively.

3.2. In Vitro Antioxidant Activity Analysis of Various Fractions of *L. inermis*

The Fig. 1A shows that there was significant scavenging of DPPH free radicals on various fractions of *L. inermis*. Maximum scavenging of $79.16 \pm 0.98\%$ was observed by PE fractions in a similar extent to that of standard ascorbic acid ($78.073 \pm 1.2\%$), followed by EA (73.77 ± 0.97 and CH fractions ($72.61 \pm 0.98\%$), respectively. Similarly, Fig. 1B shows the total antioxidant capacity of different fractions of *L. inermis*. Different fractions show difference of antioxidant activity with the order of: EAF > CHF > PEF. The various fractions of leaves were found to increase the total antioxidant capacity with the increasing concentration of samples. The difference in the amount of antioxidant of these samples may be attributed to the differences in the amount and kind of antioxidant compounds in them.

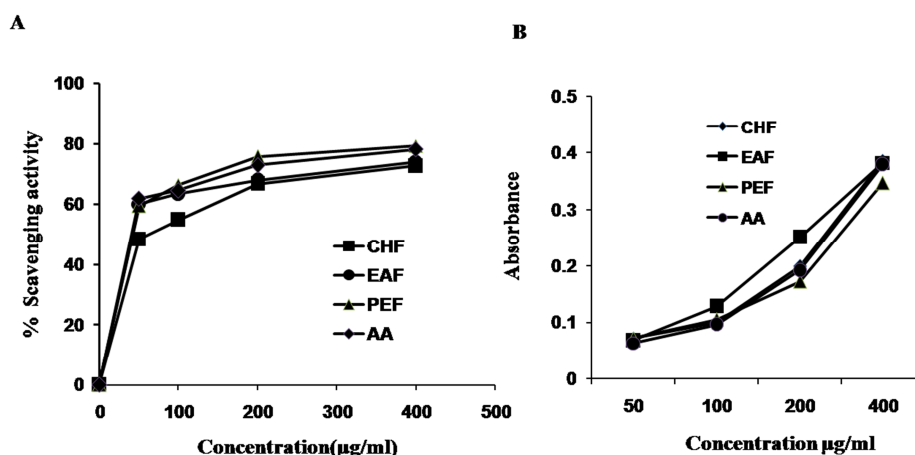


Figure 1. In vitro antioxidant activity of the various fractions of ethanol extracts of *L. inermis*: (A) DPPH radical scavenging activity of various fractions of *L. inermis*. (B) Total antioxidant activity of various fractions of *L. inermis*. Ascorbic acid (AA) was used as standard scavenger of the oxidant as well as standard antioxidant agent.

3.3. Effects of Different Fractions of *L. inermis* on S. bilirubin, SGPT, SGOT and SALP Concentrations in CCl₄-Induced Hepatitis Rats

Hepatic intoxication by CCl₄ caused significant elevation of serum bilirubin level (normal control vs CCl₄; 0.40 ± 0.06 mg/dl vs 1.20 ± 0.21 mg/dl) which was reduced significantly to 0.50 ± 0.06 , 0.63 ± 0.09 , 0.67 ± 0.03 and 0.73 ± 0.09 mg/dl (Fig. 2A) by the administration of sylimarin standard drug, EAF, PEF and CHF, respectively. The fractions were administered at a dose of 200 mg/kg body weight while sylimarin at a dose of 50 mg/kg. Similarly, CCl₄ intoxication increased SGPT concentrations from 41.33 ± 5.78 U/L (normal control group) to 104.67 ± 12.79 U/L (CCl₄ group). Administration of various fractions of *L. inermis* decreased the CCl₄-induced elevated SGPT concentrations significantly to 51.00 ± 3.61 , 58.67 ± 2.60 , 52.33 ± 3.53 and 51.67 ± 5.90 for sylimarin, EAF, PEF and CHF, respectively (Fig. 2B). Intraperitoneal injection of CCl₄ increased SGOT concentrations from 39.33 ± 2.33 U/L (normal control group) to 144.00 ± 8.08 U/L (CCl₄ group). Administration of various fractions of *L. inermis* decreased the CCl₄-induced elevated SGOT concentrations significantly to 60.67 ± 0.89 , 84.00 ± 4.56 , 91.33 ± 4.48 and 91.00 ± 1.00 for sylimarin, EAF, PEF and CHF, respectively (Fig. 2C). There was an increased concentration of SALP from 24.67 ± 2.91 U/L (normal control group) to 110.33 ± 5.78 U/L (CCl₄ group). Administration of various fractions of *L. inermis* decreased the CCl₄-induced elevated SALP concentrations significantly to 35.33 ± 2.40 , 46.00 ± 5.69 , 55.00 ± 4.16 and 64.15 ± 7.21 for sylimarin, EAF, PEF and CHF, respectively (Fig. 2D). Because of its functional roles in the body, liver is the major target organ of toxicity. Injury to the liver may affect the integrity of hepatocytes leading to the release of liver enzymes such as ALT (GPT), AST (GOT), ALP etc., since these enzymes are confined to hepatocytes and released into the blood following liver injury. Hence, these enzymes are commonly used as markers of hepatic injuries [20, 21]. In the current study, damage of the liver caused by CCl₄ was

evident by the alteration in serum marker enzymes. Administration of CCl₄ (Group II) significantly increased the serum levels of liver enzymes; GPT, GOT, and ALP. This result indicates liver cell damage; leakage of enzymes from cells and loss of functional integrity of cell. This is in consistent with the work done by Mahmud ZA et al., [13] that indicated IP administration of rats with CCl₄ caused significant liver damage. Serum GPT and GOT are elevated in nearly all liver diseases, but are particularly high in conditions that cause extensive cell necrosis, such as severe viral hepatitis, toxic injury, and prolonged circulatory collapse. GPT is a metabolic enzyme expressed primarily in the liver. Increase in serum GPT activity is typically associated with hepatocellular membrane damage and leakage of enzyme from hepatocytes [20]. Damage to the liver causes the release of GPT into the blood. Elevation of GPT levels is an indication of liver damage and has been associated with liver injury. The increase in serum ALP activity is also associated with a pathological damage occurrence in the liver [22]. When damage to heart or liver cells occurs, intracellular enzymes, such as GOT, are released into the peripheral blood. Since GOT is located in the parenchymal hepatic cells and heart muscle, this enzyme is used to assess damage to these areas. Increases in GOT can be seen in hepatitis, liver necrosis, cirrhosis, and liver metastasis [23]. Total bilirubin results are comprised of the conjugated and unconjugated forms of bilirubin. Hyperbilirubinemia can occur in three areas as bilirubin is addressed by the body. In the Prehepatic phase, increased bilirubin levels are caused by an increase in heme degradation and hemolysis. In the hepatic phase, increase levels are due to defective transport to the liver or defective conjugation by the liver. In the post hepatic phase, increase levels are due to defects transporting the conjugated bilirubin and bile out of the liver. Therefore, total bilirubin measurements are used to diagnose and treat liver, hemolytic, hematological, and gallbladder obstructive disorders [24].

In the present study, as shown in the Fig. 2 (A,B,C,D) administration of various fractions of the ethanolic extracts of

L. inermis significantly reduced the CCl₄-induced elevated serum bilirubin, SGPT, SGOT, and SALP levels, respectively. In vitro phytochemical tests confirm the presence of significant amount of phenolic and flavonoid compounds (Table 1) with DPPH-free radical scavenging and total antioxidant activity (Fig. 1 A,B) in the ethanolic extracts of various fractions (EAF, PEF, CHF) of *L. inermis*. Among the various phytoconstituents, flavonoids and phenolic compounds have beneficial effects in the inflammatory condition. Flavonoids are particularly reported for significant antioxidant, vasculoprotector, antihepatotoxic, antiallergic, anti-inflammatory and anti tumor activity [25]. This protective effect of plant extracts could be the result of direct free radical scavenger properties. These plant extracts could also react with membrane phospholipid bilayers to break the chain reaction initiated by ROS. However, further study necessitates uncovering the detailed molecular mechanism.

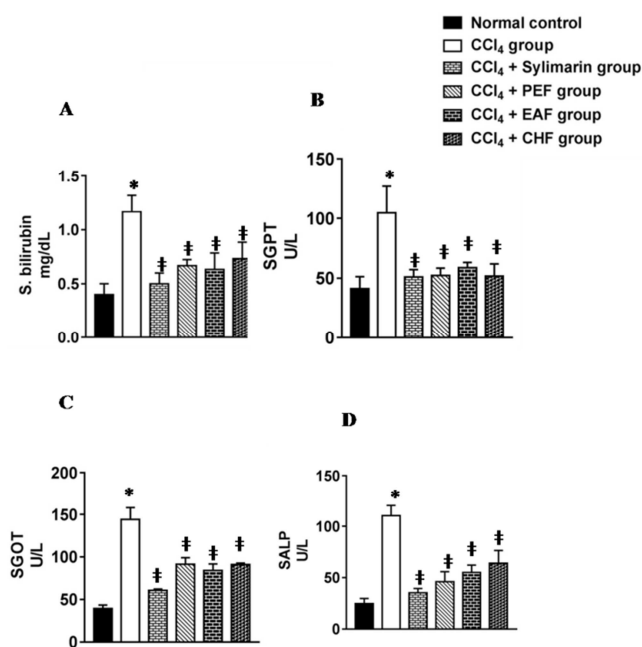


Figure 2. Effect of syllimarin and different fractions of *L. inermis* on various biochemical parameters of liver disease markers (*S. bilirubin*, *SGPT*, *SGOT*, *SALP* etc.) in normal control and CCl₄-induced hepatitis rats. The results are expressed as means \pm SEM of three experiments. *: Significantly different ($p < 0.05$) from without CCl₄ treatment. #: Significant ($p < 0.05$) difference of treatment group from the CCl₄ group alone. Data were analyzed by paired or unpaired *t*-test followed by Scheffe's post-hoc tests.

4. Conclusion

This study demonstrated that ethanolic extracts of various fractions (EAF, PEF, and CHF) of *L. inermis* leaves can protect against CCl₄-induced acute hepatotoxicity. Possible mechanism of hepatoprotective activity of *L. inermis* may be due to its free radical scavenging and antioxidant activity as the result of the presence of flavonoids and phenolic compounds in the extracts. This study will help researchers to search for a new lead compound or blend it to a formulation which could be an effective treatment for various liver diseases. However, further study is necessary to clarify the

exact molecular mechanism of hepatoprotective activity.

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