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Original Article

Antioxidants and Total Phenolics of *Ludwigia repens* rubin Partially Improve the Hepatic Damage Induced by Carbon Tetra Chloride in Animal Model

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ABSTRACT: This research investigated the antioxidative and hepatoprotective properties of *Ludwigia repens* methanol extract. Antioxidative effects were measured by DPPH radical scavenging method and $FeCl_3$ reducing power assay method. Total phenolic and total flavonoid were measured by established *in vitro* methods. Hepatoprotective potential was investigated by carbon tetrachloride (CCl_4) induced hepatotoxic model of Wistar Albino rat. Data were analyzed by using statistical software SPSS. The extract showed a dose-dependent antioxidative activity in all *in vitro* experiments. Free radical scavenging effect in terms of IC_{50} value of the extract was found to be 75 µg/ml. Reducing power was gradually increased with the increase of concentration of the extract. Total phenolic content was 906.38 ± 15.95 mg gallic acid /g of dry weight of the extract and total flavonoid was 661.21 ± 2.20 mg of querecetine equivalent/g of *L. repens* extract. The hepatoprotective action of the plant extract at the dose of 300 mg/kg BW was evidenced by lowering of ALT, AST, ALP, bilirubin, total cholesterol, low-density lipoprotein (LDL) and triglycerides level. An increase level of protein synthesis and high-density lipoprotein (HDL) compared to CCl_4 control group was also evident in treatment group. The study results suggested that oral intake of *L. repens* leaves extract may enhance the antioxidant defense status in the body and may reduce the risk of hepatic damage.

KEYWORDS: *Ludwigia repens*, oxidative stress, antioxidative, hepatoprotective.

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INTRODUCTION

Liver is one of our most important organs to protect from various injurious substances, toxic metabolites, and xenobiotics absorbed from intestinal tract. Illness to liver causes cirrhosis, hepatic fibrosis, inflammatory responses and viral hepatitis.[1] These hepatopathies become aggravated through oxidative stress which ultimately leads to hepatic damage. Treatment options for common liver diseases are problematic to some extent because the effectiveness of synthetic drugs such as interferon, colchicines, corticosteroids and penicillamine are inconsistent and side-effects are profound.[2]

Because of significant role of oxidative stress in hepatic diseases, natural antioxidants have been very popular as alternative therapy. They are very effective, harmless and economic.[3] Antioxidants are also evident to protect induced hepatic injury through reducing cellular oxidative stress.[4-5] A number of herbal medicines, especially silymarin and glycyrrhizin, are potent antioxidants to show excellent hepatoprotective activity.[6] Natural antioxidants-rich foods have also been offered as a tool for prevention and cure of liver damage.[7] Due to strong correlation between antioxidative effects and healing of liver diseases, hepatoprotective effects of *Ludwigia repens* plant sample has been investigated upon the determination of the antioxidant capacity of the plant in carbon tetrachloride (CCl_4) induced acute hepatotoxic model of albino rat .[8]

Ludwigia repens belonging to Onargaceae family is an evergreen herb available in Southern part of North America. It is also distributed in Asia, Africa, and Australia.[9] It is a flowering plant locally known as Kashordum. It is familiar as an aquatic weed and found in the shallow waters of streams and lakes and freshwater.

Some other species of *Ludwigia* are used as ornamental aquarium plants, vegetables, pollen source for honey-bees, fish feed and medicinal purposes.[10-13] Sedative and analgesic action of the methanolic leaf extract of this plant has been reported very recently.[14] However, the plant has not yet been studied for its antioxidative effects as well as hepatoprotective effects. This research attempts to investigate the antioxidative and hepatoprotective effect of *Ludwigia repens* methanolic extract. Total phenolic content and total flavonoid contents were also determined due to their role as antioxidative metabolites.

MATERIALS AND METHODS

Chemicals and reagents

All chemicals and reagents used in this study were of analytical grade. N-hexane (99.5%) and Methanol (99.9%) were purchased from Merck India. Carbon tetrachloride, Folin-Ciocalteu reagent, 2,2-Diphenyl-1-picrylhydrazyl (DPPH) and other chemicals were purchased from Sigma chemicals (St. Louis, MO, USA). Silymarin was collected from the local source (Square Herbal and Neutraceuticals, Bangladesh).

Plant collection

The plant *Ludwigia repens* was collected from Hajee para village in Chittagong, Bangladesh in September, 2011. The plant was taxonomically identified and authenticated by Dr. Sheikh Bokhtear Uddin, Taxonomist and Professor, Department of Botany, University of Chittagong. A voucher specimen of the plant has been deposited in Chittagong University Herbarium as the accession number K 4856.

Preparation of extract

The collected plant leaves were oven-dried for a week at 35 °C and ground in electric grinder (three in one electric grinder, Moulinex, China). The powder obtained (500 g) was defatted with n-hexane and successively extracted in methanol at room temperature (25 ± 2)°C. The solvent was dried by using rotatory evaporator (RE 200, Bibby Sterling, UK) under reduced pressure. A total of 22.5 g (4.5%, w/w) of semisolid crude extract was found to be preserved at 4 °C until further use.

Experimental animals

Six to seven weeks old male albino rats weighing 120-150g were procured from the animal house of the International Centre for Diarrheal Disease and Research, Bangladesh (icddr, B). The animals were individually placed in polycarbonated cages and maintained under standard laboratory conditions (relative humidity 55-60%, room temperature 23.0 ± 2.0 °C and 12 h light: dark cycle). The animals were fed with standard pellet diets for rodents and water *ad libitum*. Animals were handled according to the Helsinki protocol and all the experimentations were made under an approval of an institutional animal ethics review board.

Assay of Antioxidative effects

DPPH free radical scavenging assay

The free radical scavenging effect of the extract was analyzed by the DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging assay described by Gupta et al.[15] Briefly, 2 ml of the extract (concentrations ranging from 10 µg/ml to 500 µg/ml) was mixed with 1 ml of DPPH solution (0.004% w/v in methanol). The absorbance was taken at 517 nm after 30 min of incubation in the dark at room temperature. Ascorbic acid was used as the reference antioxidative agent. A typical solution contained all reagents except plant extract or standard solution was used as blank. The experiment was done as triplicate. The percentage (%) scavenging effect was calculated from the following equation:

$$\% I = \{(A_0 - A_1)/A_0\} \times 100$$

Where,

A_0 is the absorbance for the control (freshly prepared DPPH solution), and

A_1 is the absorbance for the extract/standard.

Then % inhibitions (% I) were plotted against concentration to calculate IC_{50} from the graph.

Reducing power assay

Reducing power of *L. repens* extract was investigated by established method as described by Oyaizu (1886).[16] Briefly, different concentrations of *L. repens* extract (125, 250, 500, 1000 µg/mL) in 1 ml of distilled water were mixed with phosphate buffer (2.5 mL, 0.2 M, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (2.5 ml, 1% w/v). The mixture was incubated at 50 °C for 20 min. After incubation, 2.5 mL of 10% trichlorocacetic acid (TCA) solution was added to each of the tubes to centrifuge the mixture at 3000 rpm for 10 min. Separated supernatant (5 ml) was mixed with distilled water (5 ml) and 1 ml of $FeCl_3$ solution (0.1% w/v) to take the absorbance at 700 nm. A blank solution containing the same solution mixture without extract or standard was prepared for measuring the reducing activity. Ascorbic acid was used as reference standard in this experiment. Increasing absorbance of reaction mixture indicated increase reducing power.

Percentage of reducing power increase

$$= \left[\frac{A_{test}}{A_{control}} - 1 \right] \times 100$$

Where, A_{test} is absorbance of test solution; $A_{control}$ is absorbance of control.

Determination of total phenolic content

Total phenol content of *L. repens* extract was estimated by modified Folin-Ciocalteu method.[17] A 0.5 ml extract (200 µg/mL) was mixed with 2.5 mL Folin-Ciocalteu reagent (diluted with water for 10 times) and 2.5 mL of 7.5% Na_2Co_3. The test tube was incubated at 25 °C for 20 min for the completion of the reaction. The absorbance was recorded at 760 nm using a spectrophotometer (1201 UV-VIS, Shimadzu Corporation, Japan) against blank solution. The phenolic compound content was determined as gallic acid equivalents using the linear equation based on the calibration curve : C = (c - V)/m, where, C = total phenolic contents (mg/g plant extract in GAE), c = gallic acid concentration obtained from calibration curve (mg/ml), V = the volume of the sample solution (ml), m = weight of the sample (g).

Determination of total flavonoid content (TFC)

Ludwigia repens extract was determined for its total flavonoid content by colorimetric assay based on the procedures described by Nooman et al.[18] One ml of plant extract (200 µg/ml) was mixed with 200 µl of 10% $AlCl_3$ solution and 200 µl of 1M potassium acetate solution to make 10 ml with distilled water. After 30 min incubation, absorbance was measured against blank at 420 nm at room temperature. Total flavonoid content was determined plotting a standard curve of querecetine (12.5-100 µg/ml). The values were expressed as mg of querecetine equivalent (QE/g of extract). TFC of the extract was expressed as querecetine (12.5-100 µg/ml) equivalents (QE) after calculation using the following equation: C=(c-V)/m, where, C =total flavonoid contents, mg/g plant extract in QE, c = concentration of querecetine obtained from calibration curve (mg/ml), V =the volume of the sample solution (ml), m= weight of the sample (g).

Acute toxicity test

Acute toxicity test was conducted prior to hepatoprotective intervention to assess whether there is any immediate effect of the extract. Acute toxicity test was carried out in Wistar Albino rats maintained under standard laboratory condition (temperature 23°C, humidity 56-6-%). Six rats received a single oral dose of 1.0, 2.0 and 3.0 g/kg body weight of the extract. Animals were fasted over-night prior to administration. Once the extract was administrated, food was withdrawn for next 3 to 4 h. Animals were monitored once during the first 30 min after dosing, periodically during the first 24 h (first 4 h was paid for special attention) and daily thereafter for a total of 14 days. Cage side was observed once daily paying attention to the changes in fur and skin, mucous membrane and eyes, respiratory and circulatory rate, autonomic and CNS.[19] The effective therapeutic dose was taken 300 mg/kg BW as one tenth of the median lethal dose (LD50 >3.0 g/kg).[20]

Assay of hepatoprotective effect

The hepatoprotective effects of *L. repens* extract was investigated using CCl_4 induced liver damaged rodent model. The animals were acclimatized for seven days to divide them into four groups in communal cages each comprising of five rats and treatment was carried out for 7 days as follows:

Normal control group received only saline 1 ml/kg, p.o, hepatic control group received only CCl_4 (CCl_4: liquid paraffin 1:2; 1 ml/kg i.p.) and left free of treatment, reference control group received silymarin 25 mg/kg body weight, p.o and test control group received plant extract 300 mg/kg body weight, p.o. All the groups except normal control group received CCl_4 (CCl_4: liquid paraffin 1:2; 1 ml/kg i.p.) once in every 72 hrs. After 24 hr of the last dose, the animals were anaesthetized (sodium phenobarbital anesthesia) to collect blood through heart puncture. Blood samples were subject to centrifuge at 2500 rpm for 10 min to separate serum which was further used to assay the liver biomarkers viz. serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), bilirubin, total protein and lipid profile (cholesterol, LDL, HDL, TG) by using commercially available kits.

Statistical Analyses

Results are expressed as average ± SEM. Data were statistically analyzed by One way analysis of variance (ANOVA) followed by post hoc Dunnett's test using the statistical software SPSS (statistical package for social science, version 22.0, IBM corporation, NY). Values with $p < 0.05$ were considered as statistically significant.

RESULTS

DPPH radical scavenging assay

The antioxidant effects of *L. repens* extract was evaluated by through its DPPH radical scavenging capacity expressed in terms of IC_{50} value. The extract showed a dose dependent radical scavenging effect (Figure 1). Numerically, the IC_{50} value of the extract and ascorbic acid (the reference standard) were 75 µg/ml and 7.45 µg/ml, respectively.

Reducing power assay

The leaf extract of *L. repens* showed promising effect which was significant ($p < 0.05$) compared to ascorbic acid. Reducing power was proportionally increased with the increase of extract concentration (Figure 2).

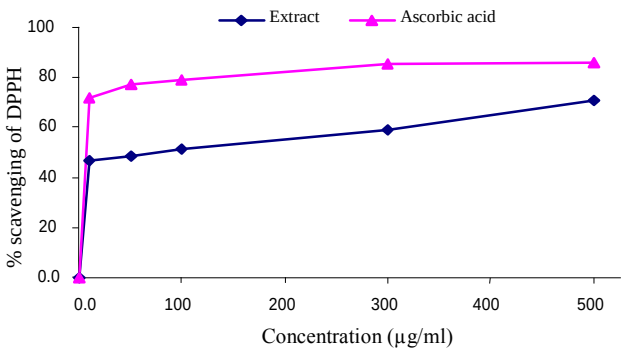

Figure 1. Comparative DPPH radical scavenging activity of *L. repens* methanolic extract ascorbic acid.

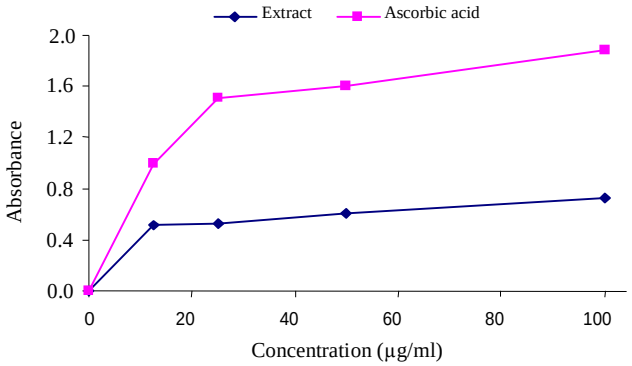

Figure 2. Comparative reducing capacity of *L. repens* methanolic extract and ascorbic acid.

Determination of total phenolic and flavonoid content

The Folin-Ciocalteu reagent was used for total phenolic content estimation in *L. repens* extract and the values were calculated in terms of Gallic acid equivalents (GAE). It was found that total phenolic content of leaf extract,

calculated on the basis of the standard gallic acid curve, was 906.38 ± 15.95 mg GAE /g of the plant extract (Table 1). The total flavonoid content of the extract was 661.21 ± 2.20 mg of Querecetine Equivalent (QE)/g of extract.

Acute toxicity test

The extract was found to be non-toxic in animal study.

Hepatoprotective effect

The results of this study clearly revealed the increase of ALT, AST, ALT, total bilirubin and total protein in the hepatic control group of carbon tetra chloride induced albino rats (Table 2). The administration of *L. repens* significantly ($p < 0.05$) reduced the liver biomarkers ALT, AST, ALP. Total bilirubin and total protein were also restored towards the normal value in test control and reference control groups of carbon tetrachloride intoxicated rats lipid profiles, viz. cholesterol, LDL, triglycerides were markedly increased HDL level was decreased in hepatic control animals compared to normal control group. However, lipid profiles were partially normalized by the intervention of *L. repens* extract and Silymarin (Table 3).

Table 1. Polyphenolic content of *L. repens* leaf extract.

Polyphenols	Quantity
Total phenolic content	906.38 ± 15.95 mg GAE /g of *L. repens* extract
Total flavonoid content	661.21 ± 2.20 mg of Querecetine equivalent/g of *L. repens* extract

Table 2. Effect of methanolic extract of *L. repens* leaves and silymarin on serum biochemical parameters in CCl_4 induced liver damage in rats.

Treated Group	Biochemical Parameters				
	ALT (U/L)	AST (U/L)	ALP (U/L)	Bilirubin (mg/dl)	Total protein (mg/dL)
Normal control	21.66 ± 1.08**	24.33 ± 4.71**	18.67 ± 1.08**	0.50 ± 0.07	5.80 ± 0.28
CCl4 control	97.67 ± 1.78**	275.67 ± 2.86**	934.00 ± 1.41**	1.13 ± 0.22	7.50 ± 0.70
Reference control	30.00 ± 1.41**	148.00 ± 5.34**	516.67 ± 2.48**	0.90 ± 0.07	6.30 ± 0.14
Test control	46.67 ± 2.16**	22.00 ± 2.12**	873.33 ± 1.08**	0.73 ± 0.04	6.03 ± 0.35

Values are expressed as Mean ± SEM for five (5) rats in each group. Data were analyzed by the statistical software SPSS (Statistical package for social science, version 19.0, IBM corporation, NY) using post hoc Dunnett's test. The asterisk values are statistically significant to each other. P values less than or equals to 0.05 were considered as significant.

Table 3. Effect of methanolic extract of *L. repens* leaves and Silymarin on lipid profile in CCl_4 induced liver damage in rats.

Treated Group	Biochemical Parameters			
	Cholesterol (mg/dl)	HDL (mg/dl)	LDL (mg/dl)	TG (mg/dl)
Normal control	106.33 ± 1.47**	114.00±2.83**	55.37 ± 1.46**	84.67 ± 1.79**
CCl4 control	144.00 ± 1.87**	24.67 ± 1.78**	65.00 ± 1.41**	212.33 ± 1.78**
Reference control	118.00 ± 1.41**	70.00 ± 1.41**	26.43 ± 0.52**	106.33 ± 3.89**
Test control	124.33 ± 2.86**	45.33 ± 1.78**	42.20 ± 0.67**	112.00 ± 4.24**

Values are expressed as Mean ± SEM for five (5) rats in each group. Data were analyzed by the statistical software SPSS (Statistical package for social science, version 19.0, IBM corporation, NY) using post hoc Dunnett's test. The asterisk values are statistically significant to each other. P values less than or equals to 0.05 were considered as significant.

DISCUSSION

It has long been recognized that naturally occurring substances in plants have antioxidant activity. Among those substances, the phenolics and flavonoids widely distributed in plants have the ability to scavenge free radicals. Along with the total phenolics and flavonoids, we determined the antioxidative effects of *L. repens* because antioxidative capability has been used as one of the potential indexes for defense mechanisms. And antioxidative defense among a series of mechanisms is likely the best understood way for cellular protection. Antioxidants scavenge excess free radicals and protect the cells from toxic effects contributing disease prevention. Therefore, hepatoprotective effects of *L. repens* extract has been supported by the antioxidative effects of the extract.[21]

DPPH assay is a direct and reliable method for determining the radical scavenging action. The DPPH radical contains an odd electron, which is responsible for the absorbance at 515-517 nm and also for a visible deep purple color. When DPPH accepts an electron donated by an antioxidant compound, the DPPH is decolorized, which can be quantitatively measured from the changes in absorbance. The lowest absorbance was observed for the sample containing highest concentrated extract. Inhibition concentration (IC_{50}) values of the extract suggested very promising free radical scavenging effect through a comparison with that of reference antioxidative agent, ascorbic acid. However, the stronger DPPH activity could be correlated with the presence of higher contents of plant phenolics which causes the faster decrease in absorbance of DPPH radical.[22]

The reducing capacity of a compound serves as an important indicator of its potential antioxidant activity.[23] The reducing property is generally associated with the presence of reductones which are evident to exert antioxidative action causing the breakage of free radical chain reaction by the donation of a hydrogen atom.[24] Reductones are also found to react with certain precursors of peroxide, thus preventing peroxide formation. The

reducing capacity of *L. repens* was investigated by a Fe^{3+} to Fe^{2+} transformation reaction where the Fe^{3+}/ ferricyanide complex was transformed to the Fe^{2+} through the reductones. Formation of Fe^{2+} is usually monitored by visible Perl's Prussian blue at the wavelength 700 nm. This research showed a moderate but dose dependent reducing power compared to the ascorbic acid. As the researchers hypothesized a direct correlation between antioxidative effect and reducing power of plant extracts,[25] it could be due to the polyphenols which are already established as rich source of antioxidative agent.

Damage of liver cell is reflected by an increase in the levels of hepatospecific enzymes, which are cytoplasmic and are released in to circulation after cellular damage.[26,27] In this research significant increase in the total bilirubin content and in the AST, ALT and ALP activities in the CCl_4 treated group could be taken as an index of liver damage. Treatment with *L. repens* extract inhibited CCl_4 induced increase in total bilirubin, total protein, AST, ALT and ALP activities as compared with CCl_4 treated group.[28-29] The effect of the extract was comparable to that of reference control Silymarin.

Extract treatment significantly attenuated the acute elevation serum lipids. *L. repens* shows the dose – dependent preventive effect against the deleterious effect of CCl_4 intoxication on the serum lipid profile. The liver is known to be involved in the syntheses of triglyceride and cholesterol which are synthesized from a substrate, acetyl CoA (produced through fatty acid oxidation).[30] The hepatoprotective effects of the oral doses of *L. repens* leaf extract were determined using serum triglyceride, total cholesterol, HDL and LDL as measuring parameters of liver function since they are synthesized *de novo* in the liver. Significant ($P < 0.05$) increase in CHL, TG, and LDL was observed in the CCl4-intoxicated group. Significant ($P<0.05$) decrease in HDL was observed in CCl4- intoxicated group. The inhibition of protein synthesis and disturbance of phospholipids metabolism might be responsible for the abnormal levels of lipoproteins in the serum. Treatment with *L. repens* significantly reversed these changes. This is an indication that the extract preserved hepatic protein synthesis and phospholipids metabolism.[31]

In this study the alcoholic extract of *L. repens* was found to have antioxidant and hepatoprotective activities. It is reported that flavonoids and total phenolics have antioxidant and hepatoprotective actions.[32,33] Chemical examination of the extract of *L. repens* showed the pronounced presence of flavonoids and total phenolics. These suggest that the antioxidant activity and protective effects against CCl_4 induced hepatic cell injury of the extract could be due to the flavonoids and total phenolics present in *L. repens*. Further studies are required to establish the phytoconstituents responsible for the antioxidant and hepatoprotective actions.

CONCLUSION

This study clearly showed the promising antioxidative effects reflected in the free radical scavenging assay and reducing power assay of *L. repens* leaf extract. Analyses of biochemical and enzymatic properties focused the protective effects of *L. repens* leaves on chemically induced damage of hepatocyte cells in animal tissue. Observed effects were promoted by the antioxidative effects due to total phenolics and flavonoids of *L. repens*. However, further studies with a dose response relationship along with the histological inclusion may confirm the obvious hepatoprotective mechanism of *L. repens* extract.

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