SUPPLEMENTARY MATERIAL

Antioxidant and Hepatoprotective Activity of *Piper retrofractum* Against Paracetamol-Induced Hepatotoxicity in Sprague-Dawley Rat

Kamanashis Mahaldar\textsuperscript{a}, Amir Hossain\textsuperscript{b}, Fahrima Islam\textsuperscript{c}, Sharmin Islam\textsuperscript{a}, Md. Amirul Islam\textsuperscript{a}, Masum Shahriar\textsuperscript{d}, Md. Mustafizur Rahman\textsuperscript{a*}

\textsuperscript{a}Pharmacy Discipline, Life Science School, Khulna University, Khulna-9208, Bangladesh  
\textsuperscript{b}Department of Pharmacy, ASA University Bangladesh, Dhaka, Bangladesh  
\textsuperscript{c}Department of Pharmacy, Primeasia University, Dhaka, Bangladesh  
\textsuperscript{d}Department of Pharmacy, Jahangirnagar University, Savar, Dhaka, Bangladesh

**Abstract:** The ethanol extracts of *Piper retrofractum* Vahl were investigated for antioxidant and hepatoprotective activity. Hepatoprotective activity against paracetamol-induced acute hepatotoxicity was estimated in Sprague-Dawley rat. In DPPH free radical assay the root and stem extracts showed IC\textsubscript{50} values at 133 and 91µg/mL, respectively, while ascorbic acid at 14µg/mL. Extracts also exhibited hydroxyl radical scavenging activity and reducing power. HPLC-DAD analysis indicated the presence of some polyphenolic compounds. Treatment of extracts significantly reduced the elevated serum levels of GPT (*P* < 0.01), GOT (*P* < 0.01) and bilirubin (*P* < 0.001). Both extracts restored the reduced level of total proteins and albumin. A significant increase in HDL-c but decrease in LDL-c level was observed compared to induced control. In histopathological study of liver sections, both extracts showed minimal to mild multifocal and diffuse granular degeneration and mild to moderate lobular disarray compared to control group. Results suggest that both extracts can prevent paracetamol induced hepatotoxicity.

**Keywords:** Sprague-Dawley strain rat; Hepatic markers; Total proteins; Serum lipid profile; Liver histopathology; HPLC-DAD.
SUPPLEMENTARY MATERIAL

Supplementary material relating to this article is available online, alongside Figures S1-S4.

1. Experimental

1.1 Plant materials

*P. retrofractum* plant was collected from Jessore, Bangladesh and identified by Bangladesh National Herbarium (BNH) located in Mirpur-1, Dhaka-1216, (Accession number: DACB-40675) and a voucher specimen was deposited there. After collection, two different parts (root and stem) were separated from undesirable materials and washed with tap water. After that shade drying was used to dry the separated parts to facilitate grinding and grinding was performed using a suitable grinder (Capacitor start motor, Wuhu motor factory, China). The powdered plant materials were macerated with 95% ethanol for four days at room temperature (~25 °C) and then filtered. The obtained filtrates were dried with the help of a rotary vacuum evaporator at the temperature of 50°C to get crude extracts. The gummy concentrates were designated as EtPCS (ethanol stem extract of *P. retrofractum*) and EtPCR (ethanol root extract of *P. retrofractum*). The extracts were stored in refrigerator at 4°C and were diluted with normal saline prior to pharmacological screening.

1.2 Animals

For the present study Adult Sprague-Dawley strain male rats weighing 100-150 g were collected from the Pharmacy Department of Jahangirnagar University, Savar, Dhaka-1342. The animals were randomly selected and the divided primarily into two groups, normal and experimental groups. The research was carried out according to the rules that govern laboratory animals’ use and the experimental protocol was approved by the Animal Ethics Committee, Khulna University.

1.3 Chemicals and reagents

Ascorbic acid, sodium monobasic phosphate, sodium dibasic phosphate, trichloroacetic acid (TCA), Thiobarbituric acid (TBA), ferric chloride, EDTA, potassium ferricyanide, were obtained from Merck, Germany. DPPH (2,2-diphenyl-1-picrylhydrazyl), H_2O_2 and 2-deoxy 2- ribose were obtained from Sigma Chemical Co. Ltd., (St. Louis, MO, USA).

1.3 DPPH free radical scavenging assay

Plant extracts and ascorbic acid (standard drug), were weighed 3 times and dissolved in ethanol to make the required concentrations by dilution technique. From the stock solution different concentrations (1, 2, 4, 8, 16, 32, 64, 128, 256 and 512µg/mL) of standard and sample were prepared. From each concentration
one ml of both plant extracts and standard was taken in each volumetric flask followed by the addition of 3 ml of 0.004 % DPPH solution. After 30-minute incubation at room temperature, absorbance was taken at 517nm against blank using UV Spectrophotometer 1650 Shimadzu, Japan. All the observations were made in triplicate and the average values were recorded. The following formula was used to calculate the Percentage of scavenging activity: Scavenging activity = \( \frac{(A_0 - A_1)}{A_0} \times 100 \% \) where, \( A_0 \) is the absorbance of control and \( A_1 \) is the absorbance of sample or standard. The IC\(_{50}\) value was calculated from the % inhibition vs. log concentration graph (Sharma & Bhat 2009; Hossain et al. 2016).

1.4 Hydroxyl radical activity test

In this test, 0.5ml 2-deoxy 2-ribose solution (2.8mM) was mixed with 12.5µL of different concentrations (6.25, 12.5, 25, 50, 100, 200, 400 & 800 mg/L) of sample extracts or standard. Then 1mL of 200 µM FeCl\(_3\), 1mL of 1.04mM EDTA, 0.5mL of 1mM H\(_2\)O\(_2\) and 0.5mL of 1Mm of ascorbic acid were added to prepare the reaction mixture. After 1-hour incubation at 37\(^\circ\)C, 3.75mL of 2.8% TCA and 3.75mL of 1% TBA were added and kept at 100\(^\circ\)C for 20 minutes. The absorbance was taken at 530nm. Blank was prepared simultaneously containing all the reagents except extract and standard. The percentage of hydroxyl radical scavenged by the extracts and standard compounds was calculated as follows: % Scavenged (H\(_2\)O\(_2\)) = \( \frac{(A_0 - A_1)}{A_0} \) x 100. Where \( A_0 \) is the absorbance of the control and \( A_1 \) is the absorbance in the presence of the sample of extract and standard (Halliwell et al. 1987; Sumi et al. 2016).

1.5 Reducing power assay

Sample was prepared at the concentrations of 500, 250,125, 62.5, 31.25, and 15.62mg/L by serial dilution of stock solution. 1 mL of the sample solution of each concentration was mixed with, 2.5mL potassium ferricyanide (K\(_3\)Fe(CN)\(_6\), 1%) and 2.5mL phosphate buffer (200mmol/L, pH 6.6) having continuous shaking. The mixture was incubated for 20 minutes at 50°C to allow reactions to occur. Then 2.5mL tri-chloroacetic acid (CCl\(_3\)COOH, 10%) was added and the mixture was centrifuged for 10 min at 1006×g. after that, 2.5mL supernatant was mixed with 0.5mL ferric chloride (FeCl\(_3\), 0.1%). After five minutes, absorbance was measured at 700nm. Extracts’ reducing power were compared with standard by drawing absorbance versus concentration curve (Oyaizu 1986).

1.6 HPLC-DAD analysis of phenolic compounds

This analysis was performed for the detection and quantification of particular phenolic compounds present in ethanol extracts of \( P. \) retrofractum following a modified method (Jahan et al. 2014). It was accomplished on a Dionex UltiMate 3000 system equipped with a quaternary rapid separation pump (LPG-3400RS) and photodiode array detector (DAD-3000RS). Separation was performed at 30°C using Acclaim\@ C18 (5µm) Dionex column (4.6 x 250 mm) where the flow rate and the injection volume were
1mL/min and 20µL respectively. The mobile phase made up of acetonitrile indicating solvent A while, solvent B represented acetic acid solution (pH 3), and methanol (solvent C) with the gradient elution program of 5%A/95%B (0-5 minutes), 10%A/90%B (6-9 minutes), 15%A/75%B/10%C (11-15 minutes), 20%A/65%B/15%C (16-19 minutes), 30%A/50%B/20%C (20-29 minutes), 40%A/30%B/30%C (30-35 minutes) and 100%A (36-40 minutes). The UV detector was fixed to 280nm for 22.0 minutes, changed to 320nm for 28.0 minutes, again change to 280nm for 35 minutes and finally to 380nm for 36 minutes and detained for the rest of the analysis period while the diode array detector was set at an acquisition range from 200nm to 700nm. For the preparation of calibration curve, a standard stock solution was prepared in methanol containing Arbutin, (-)-Epicatechin (5µg/ml each), Hydroquinone, Gallic acid, Rosmarinic acid, Vanillic acid, Myricetin (4µg/ml each), Syringic acid, Caffeic acid, Vanillin, p-Coumaric acid, Quercetin, trans-Ferulic acid (3 µg/mL each), Kaempferol (2µg/mL each), Ellagic acid (10µg/mL each), (+)-Catechin hydrate, trans-Cinnamic acid (1µg/mL), Benzoic acid (8µg/mL) and Rutin hydrate (6µg/mL). A solution of the extract was prepared in ethanol containing Arbutin, (-)-Epicatechin (5µg/ml each), Hydroquinone, Gallic acid, Rosmarinic acid, Vanillic acid, Myricetin (4µg/ml each), Syringic acid, Caffeic acid, Vanillin, p-Coumaric acid, Quercetin, trans-Ferulic acid (3 µg/mL each), Kaempferol (2µg/mL each), Ellagic acid (10µg/mL each), (+)-Catechin hydrate, trans-Cinnamic acid (1µg/mL), Benzoic acid (8µg/mL) and Rutin hydrate (6µg/mL). A solution of the extract was prepared in ethanol (10mg/ml). Prior to HPLC analysis, All the solutions (sample, mixed standards and spiked solutions) were filtered through 0.20µm syringe filter (Sartorius, Germany) and then degassed for 15 min in an ultrasonic bath (Hwashin, Korea).

1.7 Hepatoprotective activity test

Animals were selected in an arbitrary way and separated into five distinct groups and each group containing six rats. Plant extracts, paracetamol suspension and silymarin as a standard drug were given by an intra-gastric tube. A dose of 400mg/kg of plant extracts was taken according to pilot study. Group I was maintained as control receiving 0.1% Tween-80 in distilled water as a daily dose for about nine days. Group II was for induced control and on 8th day rats received paracetamol suspension (2g/kg single dose). In group III, rats received silymarin (100mg/kg) simultaneously for nine days + paracetamol single dose on 8th day. Rats from group IV and V received the same concentrations of EtPCS and EtPCR (400mg/kg) respectively, for nine days + paracetamol single dose on 8th day. After 48 hours of paracetamol administration, rats of all the groups were anaesthetized by ketamine injection and blood samples were collected from post vena cava. Serum was separated from the collected blood immediately after centrifugation at 3000 rpm for 10 min. Serum collected from each group were then examined for GPT, GOT, total bilirubin, total protein, albumin, as well as total cholesterol, HDL and LDL cholesterol. Biochemical analysis was performed by Dimension RXL (Max)/vittros-250 auto analyzer using SPAN diagnostics kit. Whole livers were carefully dissected after sacrificing the animal and fixed in 10% formalin for histopathological examination. The tissues were entrenched and sectioned in a paraffin, having stained with hematoxylin and eosin and were inspected under light microscope (Baheti et al. 2006; Arsad 2014) The histopathological assessments were performed by a pathologist. A photomicroscope
(Motic, Canada) provided with Motic Images Plus 2.0 software was used to take the photomicrographs of the microscopic sections.

**1.8 Statistical analysis**

The data was statistically evaluated by ANOVA(one-way analysis of variance) followed by Turkey multiple comparisons test (Graph Pad software Inc., Version 6.0.0). Where \( n = 6 \) mean values \( \pm \) SEM calculated for each parameter. Level of significance was kept at \( P < 0.05 \). In case of antioxidant study the values were triplicated and the results were tabulated \( \pm \) SD (Standard deviation). HPLC data acquisition, calibrations and peak integration were calculated with DionexChromeleon software (Version 6.80 RS 10).

**Reference**

Arsad SS. 2014. Histopathologic Changes in Liver and Kidney Tissues from Male Sprague Dawley Rats Treated with *Rhaphidophora decursiva* (Roxb.) Schott Extract. J Cytol Histol. 4: 1-6.

Baheti JR, Goyal RK, Shah GB. 2006. Hepatoprotective activity of Hemidesmus indicus R. Br. in rats. Indian J Exp Biol. 44: 399-402

Halliwell B, Gutteridge JMC, Aruoma OI. 1987. The deoxyribose method: A simple “test-tube” assay for determination of rate constants for reactions of hydroxyl radicals. Anal Biochem. 165: 215-219

Hossain A, Islam F, Saifuzzaman M, Saeed MAS, Islam MK, Murshid GMM, Rahman MM. 2016. Bioactivity of *Boehmeria macrophylla* (Urticaceae) leaf extract. Orient Pharm Exp Med. 16(3): 233–241

Jahan N, Islam MA, Alam F, Gan SH, Khalil MI. 2015. Prolonged heating of honey increases its antioxidant potential but decreases its antimicrobial activity. Afr J Tradit Complement Altern Med. 12(4):134-144

Oyaizu M. 1986. Studies on products of browning reaction. Antioxidative activities of products of browning reaction prepared from glucosamine. Japanese J Nutr Diet. 44: 307-315

Sharma OP, Bhat TK. 2009. DPPH antioxidant assay revisited. Food Chem. 113: 1202-1205

Sumi SA, Siraj MA, Hossain A, Mia MS, Afrin S, Rahman MM. 2016. Investigation of the Key Pharmacological Activities of *Ficus racemosa* and Analysis of Its Major Bioactive Polyphenols by HPLC-DAD. Evidence-based Complement Altern Med. Volume 2016: 9
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Figure S2: Hydroxyl radical (OH-) scavenging assay of *P. retrofractum*.

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Figure S4: HPLC chromatogram: A) Standard mixture of polyphenolic compounds. Peaks: 1, arbutin; 2, gallic acid; 3, hydroquinone; 4, (+)-catechin; 5, vanillic acid; 6, caffeic acid; 7, syringic acid; 8, (−)-epicatechin; 9, vanillin; 10, p-coumaric acid; 11, trans-ferulic acid; 12, rutin hydrate; 13, ellagic acid; 14, benzoic acid; 15, rosmarinic acid; 16, myricetin; 17, quercetin; 18, trans-cinnamic acid; 19, kaempferol;

B) EtPCS. Peaks: 1, gallic acid; 2, (+)-catechin; 3, vanillin; 4, rutin hydrate; 5, benzoic acid; and

C) EtPCR. Peaks: 1, gallic acid; 2, vanillin; 3, rutin hydrate; 4, benzoic acid.
**Figure S1:** DPPH-scavenging assay of *P. retrofractum*.

**Figure S2:** Hydroxyl radical (OH·) scavenging assay of *P. retrofractum*. 
Figure S3: Reducing powers of *P. retrofractum*. 

![Graph showing absorbance at 700 nm against concentration (mg/L) for different samples: Ascorbic acid, EtPCR, and EtPCS.](image-url)
Figure S4: HPLC chromatogram: A) Standard mixture of polyphenolic compounds. Peaks: 1, arbutin; 2, gallic acid; 3, hydroquinone; 4, (+)-catechin; 5, vanillic acid; 6, caffeic acid; 7, syringic acid; 8, (−)-epicatechin; 9, vanillin; 10, p-coumaric acid; 11, trans-ferulic acid; 12, rutin hydrate; 13, ellagic acid; 14, benzoic acid; 15, rosmarinic acid; 16, myricetin; 17, quercetin; 18, trans-cinnamic acid; 19, kaempferol; B) EtPCS. Peaks: 1, gallic acid; 2, (+)-catechin; 3, vanillin; 4, rutin hydrate; 5, benzoic acid; and C) EtPCR. Peaks: 1, gallic acid; 2, vanillin; 3, rutin hydrate; 4, benzoic acid.