Integrative Medicine Research

journal homepage: www.imr-journal.com

Original Article

Protection of hepatotoxicity using *Spondias pinnata* by prevention of ethanol-induced oxidative stress, DNA-damage and altered biochemical markers in Wistar rats

*Shoaib Shadab Iqbal, Md. Mujahid, Sayed Mohammad Kashif, Mohammad Khalid, Badruddeen, Muhammad Arif, Paramdeep Bagga, Juber Akhtar, Md. Azizur Rahman**

Bioactive Research Laboratory, Faculty of Pharmacy, Integral University, Uttar Pradesh, India

ARTICLE INFO

Article history:
Received 1 March 2016
Received in revised form
2 April 2016
Accepted 4 May 2016
Available online 17 May 2016

Keywords:
antioxidant
DNA fragmentation
hepatoprotectant
Spondias pinnata

ABSTRACT

Background: Traditional systems of medicine use herbal drugs for hepatoprotection. Thus, the study was designed to evaluate the hepatoprotective and antioxidant effects of *Spondias pinnata* bark extracts against ethanol-induced liver injury in Wistar rats.

Methods: Group I animals were treated with 1 mL/kg 0.3% carboxymethyl cellulose and Group II with 12 mL/kg 50% ethanol for 8 consecutive days. Groups III–VII animals were first treated with 400 mg/kg petroleum ether extract, chloroform extract, acetone extract (AE), ethanol extract (EE), and 100 mg/kg silymarin, and then 12 mL/kg 50% ethanol orally after 2 hours pretreatment each day for 8 consecutive days. Six hours after the last dose, blood was withdrawn. The hepatoprotective activity was assessed by several biochemical and antioxidant parameters. It was accomplished by the histopathology and DNA fragmentation study of liver tissues.

Results: Treatment with *S. pinnata* extracts, mainly AE and EE significantly ($p < 0.05$–0.01) and dose-dependently prevented the ethanol-induced increase in serum levels of aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, lactate dehydrogenase, cholesterol, bilirubin, and malondialdehyde, and decrease in reduced glutathione, catalase, superoxide dismutase, and albumin. They also attenuated the ethanol-induced DNA damage. Hepatoprotective potential of the extract was less than that of standard drug silymarin. Results of the study were well supported by the histopathological observations.

Conclusion: S. pinnata extracts AE and EE possess a potent hepatoprotective effect against ethanol-induced liver injury in Wistar rats, and protect them from hepatotoxicity by prevention of ethanol-induced oxidative stress, DNA-damage and altered biochemical markers.

* *Corresponding author*. Bioactive Research Laboratory, Faculty of Pharmacy, Integral University, Lucknow, Uttar Pradesh 226026, India.
E-mail address: marahman@iul.ac.in (Md.A. Rahman).

http://dx.doi.org/10.1016/j.imr.2016.05.002

1. Introduction

Alcohol has been the most frequently abused drug for centuries and is the third most common cause of death after smoking and hypertension, but it was not until the 1960s that it was recognized as a direct hepatotoxin.[1] Liver-related mortality due to alcohol contributes to 4% of mortality and 5% of disability-adjusted life globally, with highest impact in Europe, where these same figures are 7% and 12%, respectively.[1] Long-term excess alcohol exposure leads to a global health problem of fatty liver and alcoholic liver diseases without effective therapeutic approaches.[2]

Spondias pinnata (family: Anacardiaceae), commonly known as Bile tree or Hog plum and Amrata in Ayurveda, is an evergreen to deciduous tree that is distributed throughout India, Sri Lanka, Burma, China (South, Hainan), Malaysia, and Thailand.[3] The stem bark contains phytoconstituents like alkaloids, flavonoids, and polyphenols.[4] The stem bark contains minerals, ellegitannins, and lignoceric acid glucosides of β-sitosterol.[5] Root, bark, leaves, and fruits of the tree are useful and are used in traditional systems of medicines.[3,6] The bark has been used for its diuretic properties in Thailand.[7] The bark is used as a refrigerant, antidyssenteric, antidiarrheal, antiemetic, antiseptic, tonic, and astringent by different ethnic communities of India, and the paste or lotion of the bark extract when rubbed onto the skin provides relief from sprain and strain in articular and muscular rheumatism.[8] Bark of the tree is known to have hypoglycemic, ulcer-protective, anti-cancer, cytotoxic, thrombolytic, antipyretic, antimicrobial, central nervous system depressant and several other pharmacological activities.[9–12] Stem wood extract of the tree has been reported to reduce hepatotoxicity induced by carbon tetrachloride in experimental rats.[13] There is no scientific documentation available about the hepatoprotective effect of *S. pinnata* bark against ethanol-induced liver injury, which is clinically relevant. Hence, we attempted to evaluate the hepatoprotective effect of *S. pinnata* bark extracts against ethanol-induced liver injury in Wistar rats.

2. Methods

2.1. Chemical reagents, drugs, and equipment

All the chemicals used were of analytical grade: 0.9% normal saline (Albert David, India), silymarin (Sigma Chemicals, St Louis, MO, USA), chloroform (Rankem, India), ethanol (Changshu Yangyuan Chemical, China), petroleum ether (Fisher Scientific, India), acetone (Fisher Scientific), formaldehyde (Fisher Scientific), ethyl acetate (Himedia Chemicals, India), methanol (Fisher Scientific), diethyl ether (SD Fine Chemicals, India), acetic acid (SD Fine Chemicals), formic acid (Rankem). The equipment was: UV spectrophotometer (PharmaSpec UV-1700; Shimadzu, Japan), micropipette (Superfit, India), centrifuge (Spinnwell, India), digital balance (AUX220 Unibloc; Shimadzu), refrigerator (Intello Cool LG, India), and gel electrophoresis (Gel Doc XR+; Bio-Rad, USA).

2.2. Collection and authentication of plant specimens

S. pinnata bark was obtained from Banaras of Uttar Pradesh in April and authenticated by the botanists, authentication office, Faculty of Pharmacy, Integral University Lucknow, Lucknow, India. A voucher specimen of *S. pinnata* (IU/PHAR/HRB/15/12) was deposited there for further reference.

2.3. Preparation of plant extracts and evaluation of extractive values

The freshly collected bark was dried in the shade, ground to coarse powder, evenly packed in a Soxhlet extractor, and successively extracted by Soxhlet extraction method at a temperature not exceeding 45–55 °C, using different solvents in ascending order of their polarity, namely, petroleum ether, chloroform, acetone, and ethanol to achieve different crude extracts. One hundred grams of the powdered material was used in Soxhlet extraction using 1 L of each solvent. Appearance of colorless solvent in the siphon tube was taken as the termination of extraction. The different crude extracts obtained were filtered while hot and concentrated separately in a rotary evaporator (Buchi Rotavapor-R; Labco, India) under reduced pressure and controlled temperature to dryness and the percentage yield was calculated. The petroleum ether extract (PEE), chloroform extract (CE), acetone extract (AE), and ethanol extract (EE) were kept in an air tight container in a refrigerator below 4 °C and used for pharmacological evaluation.[14]

2.4. Experimental animals

Rats (150 ± 20 g) were obtained from the animal house of the Central Drug Research Institute (CDRI), Lucknow, India. They were kept in the departmental animal house, Integral University Lucknow. The animals were housed separately in polypropylene cages for acclimatization at a temperature of 23 ± 2 °C and relative humidity of 50–60% with a 12-hour light/dark cycle for 1 week before and during the commencement of the experiment. Animals were kept on standard pellet diet and provided drinking water *ad libitum* throughout the housing period. All the experiments were conducted in accordance with the guidelines of the Committee for the Purpose of Control and Supervision of Experimental Animal. Ethical clearance for the project was obtained from Institutional Animal Ethics Committee of the Faculty of Pharmacy at the Integral University Lucknow (Approval No. IU/PHARM/M.PHARM/IAEC/15/10).

2.5. Experimental protocol

The animals were randomly divided into seven groups each consisting of five rats. Group I served as a normal control and received 1 mL/kg vehicle (0.3% carboxymethyl cellulose) throughout the study. Group II served as an acute toxic control and received 12 mL/kg orally (po) 50% ethanol orally throughout the study. Groups III–VI received 400 mg/kg po of different bark extracts of *S. pinnata* orally, and Group VII received standard drug silymarin, 100 mg/kg/d po.[15] All groups

of animals were administered 12 mL/kg po of 50% ethanol after 2 hours pretreatment each day, except rats in the normal control group, for 8 consecutive days.[16] Six hours after the last dose, the rats were anesthetized and blood was collected via the retro-orbital plexus followed by heart puncture and allowed to clot. The serum was separated by centrifugation at 3,000 rpm (845.32 g) at 4 °C for 20 minutes for analysis of various biochemical parameters. After withdrawal of blood, the animals were killed. The hepatoprotective activity was assessed by several biochemical and antioxidant parameters. The isolated liver tissues were washed twice with ice-cold saline, blotted, dried, and weighed. The relative liver weight was calculated as the percentage ratio of the liver weight to the body weight. It was also accomplished by histopathological examination. A small portion of the tissue was fixed in formalin for DNA fragmentation study by gel electrophoresis.

2.6. *Analysis of in vivo antioxidant activity*

2.6.1. *Estimation of tissue reduced glutathione*

Determination of tissue reduced glutathione (GSH) was based on the method of Ellman, in which, 5, 5′-dithiobis-(2-nitrobenzoic acid) (DTNB) was reduced by thiol (-SH) group to form 1 mol 2-nitro-5-mercaptobenzoic acid anion with intense yellow color.[17] Four hundred and fifty milligrams of liver tissue was homogenized in 7 mL 0.02 M EDTA and then 4 mL cold distilled water was added to it. After mixing well, 1 mL 50% trichloroacetic acid was added and shaken intermittently for 10 minutes using a Vortex mixer, Bionics Scientific, India. After 10 minutes, the contents were transferred to centrifuge tubes rinsed in EDTA and centrifuged at 6,000 rpm(3,381.3 g) for 15 minutes. Following centrifugation, 2 mL of the supernatant was mixed with 4 mL 0.4 M Tris buffer (pH 8.9). The whole solution was mixed well and 0.1 mL 0.01 M DTNB was added to it. The absorbance was read within 5 minutes of the addition of DTNB at 412 nm against a reagent blank. The blank was prepared the same as for the test, except that 0.02 M EDTA was added in place of tissue homogenate. Tissue GSH was calculated from the equation:

$$\mathrm{GSH}(\mu g/mg\ \text{protein}) = (\mathrm{OD} \times 50 \times 3.5 \times 2.25 \times 1)/(0.337 \times 2 \times \text{mg protein}). \quad (1)$$

where OD is optical density.

2.6.2. *Estimation of catalase*

The decomposition of H_2O_2 was followed directly by a decrease in absorbance at 240 nm. Liver tissue was homogenized in 50 μM/mL potassium phosphate buffer (pH 7.4) with a ratio of 1:10 w/v. The homogenate was centrifuged at 10,000 rpm (9,392.44 g) at 4 °C in a cooling centrifuge for 20 minutes. Catalase activity was measured in the supernatant obtained after centrifugation. Supernatant (0.05 mL) was added to a cuvette containing 2.95 mL 19 μM/mL solution of H_2O_2 prepared in potassium phosphate buffer. The change in absorbance was monitored at 240 nm wavelength at 1-minute intervals for 3 minutes. Catalase decomposes H_2O_2, leading to a decrease in absorbance. The difference in absorbance (ΔA) per unit time was a measure of catalase activity.[18] Catalase activity was calculated from the equation:

$$\mathrm{CAT}\ (\eta\text{moles}H_2O_2\text{consumed/min/mg protein}) = (\Delta A/\text{min} \times \text{volume of assay})/(0.081 \times \text{volume of homogenate} \times \text{mg protein}). \quad (2)$$

2.6.3. *Estimation of superoxide dismutase*

The supernatant was assayed for superoxide dismutase (SOD) activity by following the inhibition of pyrogallol auto-oxidation.[19] Cytosolic supernatant (0.01 mL) was added to Tris HCl buffer (pH 8.5). The final volume of 3 mL was adjusted with the same buffer. At least 0.025 mL 24 mM pyrogallol was added and changes in absorbance at 420 nm were recorded at 1-minute intervals for 3 minutes. The increase in absorbance at 420 nm after the addition of pyrogallol was inhibited by the presence of SOD. Data were expressed as SOD units/mg protein. One unit of SOD was described as the amount of enzyme required to cause 50% inhibition of pyrogallol auto-oxidation per 3 mL of assay mixture and was calculated from the equation:

$$\text{Unit of SOD per ml of sample} = [(A - B) \times 100]/[A \times 50], \quad (3)$$

where, A is the difference of absorbance in 1 minute in the control, and B is the difference of absorbance in 1 minute in the test sample.

2.6.4. *Estimation of thiobarbituric acid reactive substances*

The dissected liver samples were washed immediately with ice-cold saline to remove as much blood as possible. Liver was homogenized in ice-cold 0.9% NaCl with a glass homogenizer in 5% concentration and the homogenate was centrifuged at 800 rpm (60.11 g) for 10 minutes. The supernatant was centrifuged at 12,000 rpm (13,525.12 g) for 15 minutes and the mitochondrial fraction was used for the estimation of malondialdehyde (MDA), an end-product of lipid peroxidation that reacts with thiobarbituric acid (TBA) to form the pink chromogen TBA reactive substance (TBARS).[20] Homogenate (0.2 mL)was transferred to a vial and mixed with 0.2 mL 8.1% w/v sodium dodecyl sulfate solution, 1.5 mL 20% acetic acid solution (adjusted to pH 3.5 with sodium hydroxide) and 1.5 mL 0.8% w/v solution of 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 1 hour. The vials were 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 1,000 rpm (93.92 g)for 10 minutes. The absorbance of the supernatant fraction was measured at 532 nm. The control experiment was processed using the same experimental procedure except that TBA solution was replaced with distilled water. 1,1,3,3-Tetraethoxypropan was used as standard for calibration of the curve and was expressed as nmol/mg protein.

2.7. *Analysis of hepatoprotective activity*

Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), bilirubin, total cholesterol, albumin, and lactate dehydrogenase (LDH) were determined as per standard kit methods.

2.7.1. *Estimation of ALT*

Reagent 2 was added to Reagent 1 in a ratio of 1:4 to prepare working ALT reagent (ALT test kit, modified UV-IFCC kinetic assay; Span Diagnostics, India). One milliliter of ALT reagent was added to 0.1 mL serum and mixed. The rate of the oxidation of reduced NADH was measured kinetically by monitoring the decrease in the absorbance at 340 nm using purified water as a blank at an interval of 30 seconds for four successive readings, as per the equation:

$$\text{ALT activity (mU/mL)} = \Delta A/\text{min} \times \text{kinetic factor} \quad (4)$$

where ΔA/min is the change in absorbance per minute and kinetic factor is 1768.

2.7.2. *Estimation of AST*

Reagent 2 was added to Reagent 1 in a ratio of 1:4 to prepare working AST reagent (AST test kit, modified UV-IFCC kinetic assay; Span Diagnostics). One milliliter of AST reagent was added to 0.1 mL serum and mixed. The rate of oxidation of NADH was measured kinetically by monitoring the decrease in the absorbance at 340 nm using purified water as a blank at an interval of 30 seconds for four successive readings, as per the equation:

$$\text{AST activity (mU/mL)} = \Delta A/\text{min} \times \text{kinetic factor} \quad (5)$$

where ΔA/min is the change in absorbance per min and kinetic factor is 1768.

2.7.3. *Estimation of LDH*

Reagent 2 was added to Reagent 1 in a ratio of 1:4 to prepare working LDH reagent (Infinite liquid LDH, Infinite LDH UV kinetics assay; Accurex Biomedical, India). One milliliter of LDH reagent was added to 0.02 mL serum and mixed. The catalytic concentration was determined from the rate of decrease of $NADH^{+}$ measured at 340 nm using distilled water as a blank at an interval of 30 seconds for four successive readings, as per the equation:

$$\text{LDH activity(mU/mL)} = \Delta A/\text{min} \times \text{kinetic factor} \quad (6)$$

where, ΔA/min is the change in absorbance per min and kinetic factor is 8109.

2.7.4. *Estimation of ALP*

Reagent 2 was added to Reagent 1 in a ratio of 1:4 to prepare working ALP reagent (ALP test kit, Kinetic assay, Merck Specialities, India). One milliliter of ALP reagent was added to 0.02 mL serum and mixed. In presence of magnesium ion (Mg^{2+}) and diethanolamine as phosphatase acceptor, *p*-nitrophenylphosphate was transformed by ALP to phosphate and nitrophenol (yellow compound), whose absorbance was measured at 405 nm at an interval of 30 seconds for four successive readings, as per the equation:

$$\text{ALP activity (mU/mL)} = \Delta A/\text{min} \times \text{kinetic factor} \quad (7)$$

where, ΔA/min is the change in absorbance per min and kinetic factor is 2750.

2.7.5. *Estimation of albumin*

One milliliter of Reagent 1 was added to 0.01 mL serum and to 0.01 mL standard Reagent 2 (Albumin test kit, modified UV-IFCC kinetic assay, Span Diagnostics). It was mixed and incubated at 37 °C for 1 minute. At pH 3.68, albumin acts as a cation and binds to the anionic dye bromocresol green, forming a green complex whose color intensity is proportional to albumin concentration in the sample. The absorbance of final color in test and standard mixtures was measured at 630 nm using a reagent blank as blank, and albumin concentration was measured, as per the equation:

$$\text{Albumin (mg/mL)} = \text{absorbance of test} \times 40/\text{absorbance of standard.} \quad (8)$$

2.7.6. *Estimation of cholesterol*

One milliliter of Reagent 1 was added to 0.01 mL serum and to 0.01 mL 2 mg/mL standard Reagent 2 (Total cholesterol test kit, CHOD-PAP; Span Diagnostics). It was mixed and incubated at 37 °C for 10 minutes. Cholesterol esters are hydrolyzed by cholesterol esterase to yield free cholesterol. In a subsequent reaction, cholesterol oxidase oxidizes the 3-OH group of free cholesterol to liberate cholest-4-en-3-one and H_2O_2. In the presence of peroxidase, H_2O_2 couples with 4-aminoantipyrin and phenol to produce red quinoneimine dye whose absorbance is measured at 505 nm using Reagent 1 as a blank. Cholesterol concentration was measured as per the equation:

$$\text{cholesterol(mg/mL)} = \text{absorbance of test}/(\text{absorbance of standard} \times 100). \quad (9)$$

2.7.7. *Estimation of total bilirubin*

One milliliter of working reagent was added to 0.1 mL Reagent 2 for the sample blank and to a mixture of 0.05 mL each of Reagent 1 and Reagent 2 for the test sample. Then, 0.05 mL serum was added to each sample blank and test sample (Bilirubin test kit, Jenrassik and Grof; Span Diagnostics). It was mixed and incubated at room temperature for 5 minutes. The absorbance of the sample blank and test sample was measured at 546 nm (λ_1) and 630 nm (λ_2). Sulfanilic acid reacts with sodium nitrite to produce diazotized sulfanilic acid (diazo). Total bilirubin couples with diazo and caffeine to produce colored azobilirubin whose intensity is directly proportional to the amount of total bilirubin present in the sample. The total bilirubin concentration was measured as per the equation:

$$\text{Total bilirubin(mg/mL)} = (AT_1 - AB_1) \times \text{factor}/100 \quad (10)$$

where AB_1 is the absorbance of sample blank, AT_1 is the absorbance of test for total bilirubin and kinetic factor is 26.312.

2.7.8. *Histopathological analysis of liver tissues*

At the end of each schedule, the control as well as treated rats were killed under anesthesia after blood withdrawal from retro-orbital plexus and cardiac puncture. For histopathological studies, the isolated livers of different groups of rats were preserved in 10% formalin solution for further examination. The tissues were mounted by embedding in paraffin wax and 6-mm slices were cut. The slices were stained by the dyes eosin and hematoxylin. The prepared slides were observed under a light microscope for visualization of centrilobular necrosis and lymphocyte infiltration.[21]

2.8. *DNA fragmentation analysis of hepatic tissues by gel electrophoresis*

Lysis buffer was prepared by mixing 50 mL each of 1 M Tris HCl and 0.5 M Na-EDTA. The 0.613 mL lysis buffer was added to 0.03 mL 20% sodium lauryl sulfate to form lysis buffer solution, and then, 10 mg homogenized tissue and 0.007 mL 100 μg/mL protein kinase were added. It was incubated at 55 °C for 13 hours in a water bath. The 0.375 mL of 5 M NaCl was added and inverted several times to mix. It was left for 30 minutes and centrifuged at 14,000 rpm for 30 minutes at room temperature. Supernatant was carefully transferred to each tube using a pipette and then, 0.75 mL chloroform was added to each tube. The tubes were inverted several times to mix and centrifuged at 12,000 rpm (13,525.12 g) for 10 minutes at room temperature. The top layer from each tube was transferred. Ribonuclease (0.005 mL) was added and it was incubated at 37 °C for 15 minutes. Isopropanol (0.75 mL) was added to each tube and left at 20 °C for 30 minutes to precipitate DNA. It was centrifuged at 14,000 rpm (18409.19 g) for 30 minutes at 4 °C and the supernatant was drained on tissue paper. The pellets were dried under vacuum for 30 minutes. MQ water (0.1 mL) was added to each tube at room temperature, which were left for 30 minutes to allow DNA pellets to dissolve.[22]

The mixture of tris buffer, glacial acetic acid and EDTA (1 L) was prepared by dissolving 48.4 g Tris buffer, 11.4 mL glacial acetic acid, and 3.7 g Na-EDTA, and then, 1% agarose gel was prepared in 100 mL TAE. It was boiled for 50 seconds in a microwave oven and 0.005 mL ethylene bromide was added and shaken for few seconds. It was poured into a mold and left for 30 minutes. Then, 50 mL TAE was poured into the mold. Now, 0.004 ml of barium phenol dyes in DNA appendroff's tube were added. Then, micropipette was used and DNA sample taken and put in well. Current of 50 V was supplied to run sample. After small run, the current was increased at 100 V to reach the DNA solution into half mold. The final body weight of the rats of hepatotoxic group II was significantly ($p < 0.01$) decreased to 143.4 ± 5.10 g compared to their initial body weight of 175.6 ± 5.85 g.

2.9. *Statistical analysis*

One-way analysis of variance followed by Dunnett's multiple comparison test were performed using Prism version 3.0 (GraphPad Software, San Diego, CA, USA) for determination of level of significance. The data were expressed as mean ± standard error and $p < 0.05$ was considered statistically significant.

3. Results

3.1. *Evaluation of extractive value*

Extractive value for the extracts PEE, CE, AE, and EE was 1.6% w/w, 1.73% w/w, 4.69% w/w, and 17.2% w/w, respectively.

3.2. *Analysis of in vivo antioxidant activity*

The effects of different extracts of *S. pinnata* bark on the levels of different specific variables GSH, catalase, SOD, and TBARS (MDA) in hepatic homogenate in control and experimental groups of animals are presented in Table 1. Oral 50% ethanol (12 mL/kg; hepatotoxic control) significantly ($p < 0.01$) decreased the levels of hepatic GSH, catalase, and SOD from 29.8 ± 2.78 μg/mg tissue protein, 16.44 ± 1.20 μmol H_2O_2/mg tissue protein and 4.85 ± 0.50 U/mg tissue protein to 18.6 ± 0.812 μg/mg tissue protein, 7.74 ± 0.41 μmol H_2O_2/mg tissue protein and 2.112 ± 0.33 U/mg tissue protein, respectively, and increased the levels of hepatic MDA from 0.20 ± 0.02 nmol/mg tissue to 0.49 ± 0.02 nmol/mg tissue as compared to the normal control group rats. Oral EE (400 mg/kg; Group VI) significantly increased the levels of GSH, catalase and SOD ($p < 0.01$) to 29.8 ± 1.21 μg/mg tissue protein, 13.52 ± 0.50 μmol H_2O_2/mg tissue protein and 3.88 ± 0.25 U/mg tissue protein, respectively, and reduced the level of MDA to 0.27 ± 0.02 nmol/mg tissue as compared to the hepatotoxic control group rats. The levels of these antioxidant markers GSH, catalase and SOD were significantly ($p < 0.01$) increased to 29 ± 1.31 μg/mg tissue protein, 14.78 ± 0.70 μmol H_2O_2/mg tissue protein, and 4.06 ± 0.28 units/mg tissue protein, respectively, while that of MDA was significantly ($p < 0.01$) reduced to 0.22 ± 0.22 n mole/mg of tissue by treatment with 100 mg/kg po silymarin (Group VII) as compared to hepatotoxic control group rats. Treatment with 400 mg/kg po PEE (Group III) and CE (Group IV) did not significantly affect the levels of these antioxidant markers. Treatment with 400 mg/kg po EE (Group VI) showed more protection than 400 mg/kg po PEE (Group III), CE (Group IV), and AE (Group V) but showed less protection than 100 mg/kg po silymarin (Group VII).

3.3. *Analysis of hepatoprotective activity*

The final body weight of hepatotoxic Group II rats following 12 mL/kg po 50% ethanol treatment was significantly ($p < 0.01$) decreased to 143.4 ± 5.10 g compared to initial body weight of 175.6 ± 5.85 g. Treatment with 400 mg/kg po PEE and CE treatment in Groups III and IV showed non-significant increases in final body weight to 158.1 ± 3.43 g and 161.2 ± 5.86 g, respectively, while 400 mg/kg po EE (Group VI) showed a significant ($p < 0.01$) increase in final body weight to 186.1 ± 5.88 g as compared to hepatotoxic Group II rats. Treatment with 12 mL/kg po 50% ethanol significantly ($p < 0.01$) increased relative liver weight to 5.81 ± 0.27/100 g body weight compared to the

Table 1 – Effects of different extracts of *Spondias pinnata* bark on different liver specific variables in control and experimental groups of animals

Treatment groups & liver-specific variables	I Normal control: 0.3% CMC 1 mL/kg	II Hepatotoxic control: 50% ethanol 12 mL/kg po	III PEE 400 mg/kg + 50% ethanol 12 mL/kg po	IV CE 400 mg/kg + 50% ethanol 12 mL/kg po	V AE 400 mg/kg + 50% ethanol 12 mL/kg po	VI EE 400 mg/kg + 50% ethanol 12 mL/kg po	VII Silymarin 100 mg/kg + 50% ethanol 12 mL/kg po
GSH (μg/mg tissue protein)	29.8 ± 2.78	18.6 ± 0.812*	20 ± 2.53	25 ± 2.60	25.2 ± 2.17	29.8 ± 1.21‡	29 ± 1.31‡
Catalase (μmol H_2O_2/mg tissue protein)	16.44 ± 1.20	7.74 ± 0.41*	9.28 ± 0.31	9.40 ± 0.31	10.48 ± 0.52†	13.52 ± 0.50‡	14.78 ± 0.70‡
SOD (units/mg tissue protein)	4.85 ± 0.50	2.112 ± 0.33*	2.68 ± 0.18	2.88 ± 0.20	3.64 ± 0.41†	3.88 ± 0.25‡	4.06 ± 0.28‡
TBARS or MDA (n mole/mg of tissue)	0.20 ± 0.02	0.49 ± 0.02*	0.50 ± 0.03	0.47 ± 0.02	0.37 ± 0.03†	0.27 ± 0.02‡	0.22 ± 0.22‡
AST (mU/mL)	89.91 ± 3.21	338.68 ± 8.35*	320.77 ± 8.44	305.76 ± 30.66	275.1 ± 12.10†	123.55 ± 2.49‡	96.56 ± 2.04‡
ALT (mU/mL)	39.56 ± 0.64	193.53 ± 4.62*	189.93 ± 6.28	176.54 ± 5.04	172.21 ± 8.86†	55.92 ± 1.39‡	43.53 ± 1.43‡
ALP (mU/mL)	75.99 ± 1.95	209.58 ± 4.33*	190.89 ± 5.31	188.43 ± 10.02	181.37 ± 9.96†	90.04 ± 2.64‡	81.82 ± 2.75‡
LDH (mU/mL)	381.14 ± 3.37	709.58 ± 5.52*	686.44 ± 5.42	684.42 ± 12.94	655.22 ± 29.89†	405.26 ± 4.95‡	387.79 ± 5.01‡
Bilirubin (mg/mL)	0.006 ± 0.0002	0.028 ± 0.0008*	0.027 ± 0.0005	0.0257 ± 0.0006	0.0253 ± 0.0006†	0.0087 ± 0.0004‡	0.0085 ± 0.0002‡
Cholesterol (mg/mL)	0.34 ± 0.01	0.79 ± 0.01*	0.72 ± 0.02	0.73 ± 0.03	0.70 ± 0.03†	0.41 ± 0.01‡	0.36 ± 0.01‡
Albumin (mg/mL)	40.7 ± 0.1	28.3 ± 0.2*	29.2 ± 0.4	29.4 ± 0.3	29.6 ± 0.6†	43.9 ± 0.1‡	40.9 ± 0.1‡
Initial BW (g)	166.4 ± 3.48	175.6 ± 5.85	159.8 ± 2.63	162.4 ± 4.21	170.4 ± 3.21	171.4 ± 7.76	161.4 ± 2.08
Final BW (g)	185.1 ± 6.92	143.4 ± 5.10*	158.1 ± 3.43	161.2 ± 5.86	168 ± 5.93†	186.1 ± 5.88‡	181.8 ± 6.39‡
Liver weight (g)	5.20 ± 0.24	8.67 ± 0.31*	8.58 ± 0.08	8.12 ± 0.25	7.03 ± 0.68†	5.82 ± 0.52‡	5.42 ± 0.15‡
Relative liver weight (liver weight/100 g BW)	2.80 ± 0.04	5.81 ± 0.27*	5.43 ± 0.06	5.03 ± 0.05†	4.14 ± 0.32‡	3.09 ± 0.18‡	2.98 ± 0.03‡

Values expressed as mean ± standard error ($n = 5$).

* $p < 0.01$ as compared with respective normal control Group I.

† $p < 0.05$ as compared with respective hepatotoxic control Group II.

‡ $p < 0.01$ as compared with respective hepatotoxic control Group II.

AE, acetone extract; ALP, alkaline phosphatase; ALT, alanine transaminase; AST, aspartate transaminase; BW, body weight; CE, chloroform extract; CMC, carboxymethyl cellulose; EE, ethanol extract; GSH, reduced glutathione; LDH, lactate dehydrogenase; MDA, malondialdehyde; PEE, petroleum ether extract; SOD, superoxide dismutase; TBARS, thiobarbituric acid reactive substance.

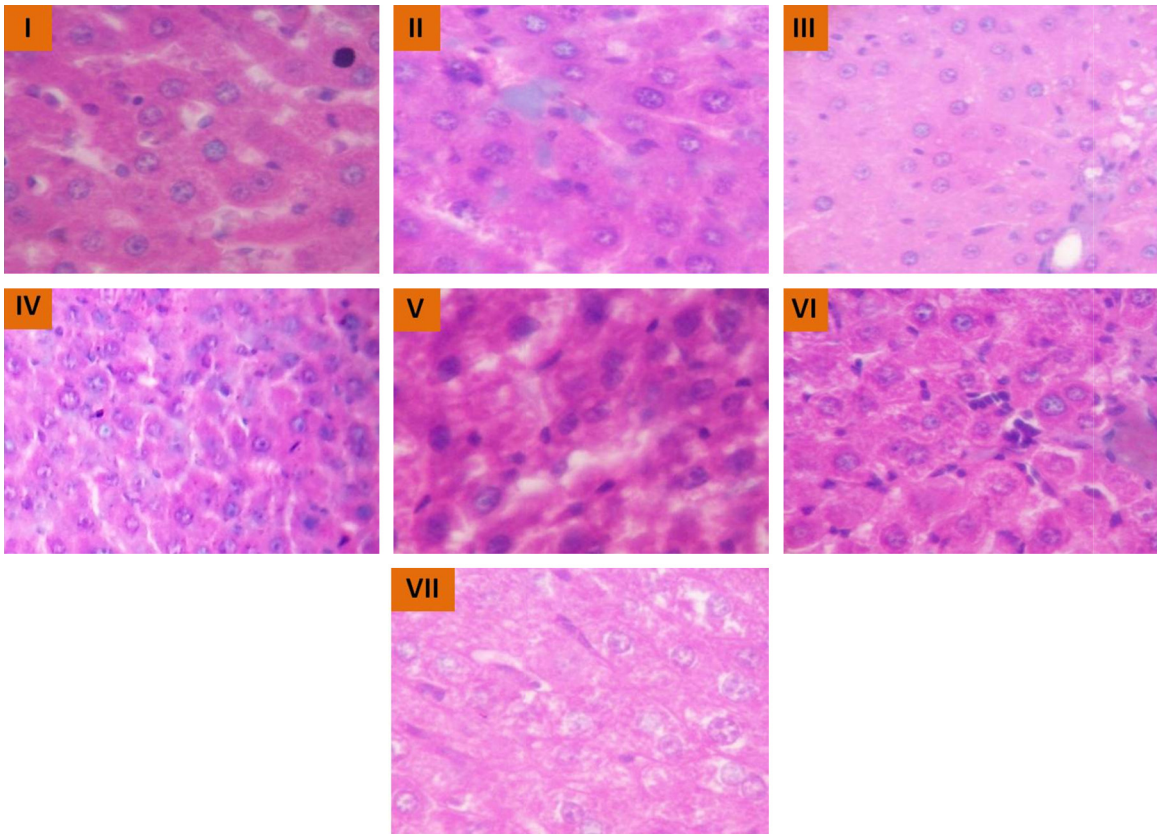

Fig. 1 – Histopathology of liver tissues under 40 × magnification: (A) Normal control showing uniform hepatocytes with small vesicular nuclei and well-maintained liver architecture. (B) Hepatotoxic control showing highly proliferating small hepatocytes, eosinophilic cytoplasm with indistinct cell boundaries, and well-maintained liver architecture with increased vascularity. (C) Petroleum-ether-extract-treated test showing changes in hepatocytes with more increase in size, coarse granularity, indistinct cell boundaries, and increased intercellular space. (D) Chloroform-treated test showing changes in hepatocytes with less increase in size, hyperplasia of Kupffer's cell, well-maintained liver architecture, and increase in interstitial cell and vascularity. (E) Acetone-extract-treated test showing disturbed liver architecture, shrunken hepatocytes with small nuclei, cytoplasm with hyaline changes in a few places, and decreased vascularity. (F) Ethanol-extract-treated test showing more prominent hepatocytes with smaller vesicular nuclei, eosinophilic cytoplasm with indistinct cell boundaries, and disturbed liver architecture with increased vascularity. (G) Silymarin-treated standard showing normal hepatocytes with granular cytoplasm, well-maintained liver architecture, and normal interstitial cells.

normal control group. Treatment with 400 mg/kg po PEE, CE, AE, and EE in Groups III, IV, V, and VI, respectively, reduced relative liver weight to 5.43 ± 0.06/100 g ($p > 0.05$), 5.03 ± 0.05/100 g ($p < 0.05$), 4.14 ± 0.32/100 g ($p < 0.01$), and 3.09 ± 0.18/100 g ($p < 0.01$), respectively, as compared to the hepatotoxic control group. EE-treated Group VI hepatoprotective activity was less than the standard silymarin-treated Group VII rats at the concentration used.

The effects of different extracts of *S. pinnata* bark on different liver specific variables in control and experimental groups of animals are presented in Table 1. Treatment with 12 ml/kg po 50% ethanol in Group II rats showed significantly ($p < 0.01$) increased serum AST (338.68 ± 8.35 mU/mL), ALT (193.53 ± 4.62 mU/mL), ALP (209.58 ± 4.33 mU/mL), LDH (709.58 ± 5.52 mU/mL), cholesterol (0.7921 ± 0.0131 mg/mL), and bilirubin (0.028 ± 0.0008 mg/mL) but decreased serum albumin (0.028 ± 0.0008 mg/mL) as compared to normal control Group I rats (89.91 ± 3.21 mU/mL, 39.56 ± 0.64 mU/mL, 75.99 ± 1.95 mU/mL, 381.14 ± 3.37 mU/mL, 0.34 ± 0.01 mg/mL, 0.006 ± 0.0002 mg/mL, and 40.7 ± 0.1 mg/mL, respectively). Treatment with 400 mg/kg po AE in Group V rats significantly ($p < 0.05$) reduced the above to (275.1 ± 12.10 mU/mL, 172.21 ± 8.86 mU/mL, 181.37 ± 9.96 mU/mL, 655.22 ± 29.89 mU/mL, 0.70 ± 0.03 mg/mL, and 0.0253 ± 0.0006 mg/mL) and increased albumin to 29.6 ± 0.6 mg/mL as compared to the hepatotoxic Group II rats. Treatment with 400 mg/kg po EE in Group VI rats significantly ($p < 0.01$) reduced the above to (123.55 ± 2.49 mU/mL, 55.92 ± 1.39 mU/mL, 90.04 ± 2.64 mU/mL, 405.26 ± 4.95 mU/mL, 0.41 ± 0.01 mg/mL, and 0.0087 ± 0.0004 mg/mL) but increased albumin to 43.9 ± 0.1 mg/mL as compared to the hepatotoxic Group II rats.

Normal controls had uniform hepatocytes with small vesicular nuclei and well-maintained liver architecture, while hepatotoxic controls had proliferating small hepatocytes, eosinophilic cytoplasm with indistinct cell boundaries, and

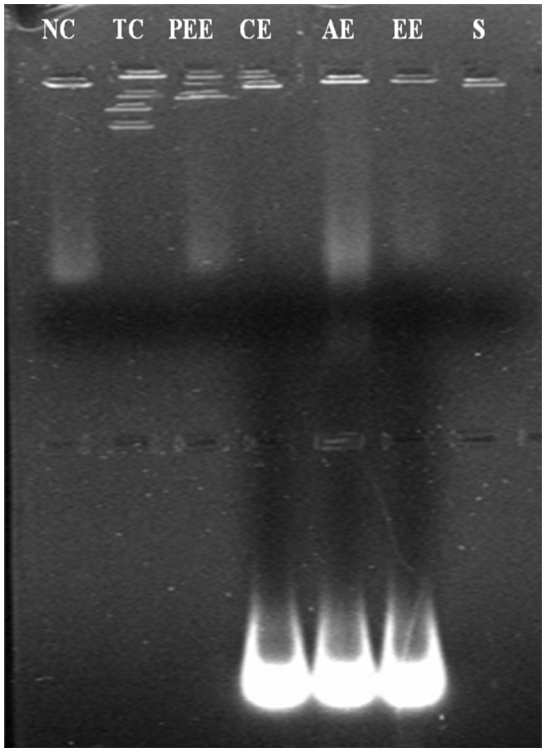

Fig. 2 – Gel electrophoretic pattern of DNA isolated from liver tissues of different groups for the effects of treatment of the extracts on DNA fragmentation of hepatic tissues in rats fed with 50% ethanol for 8 consecutive days. AE, acetone-extract-treated test; CE, chloroform-extract-treated test; EE, ethanol-extract-treated test; NC, normal control; PEE, petroleum-ether-extract-treated test; S, silymarin-treated standard; TC, toxic control.

well-maintained liver architecture with increased vascularity (Fig. 1). The EE-treated rats (Group VI) had more prominent hepatocytes with smaller vesicular nuclei, eosinophilic cytoplasm with indistinct cell boundaries, and well-maintained liver architecture with increased vascularity. Thus, EE treatment prevented hepatotoxicity and this activity was comparable to the standard drug silymarin.

3.4. *DNA fragmentation analysis of hepatic tissues by gel electrophoresis*

DNA fragmentation detected by electrophoresis as a DNA ladder representing a series of fragments (Fig. 2) showed a marked increase in the toxic control group compared to the normal control group and treated groups. Treatment with the extracts resulted in a marked improvement in DNA fragmentation compared to the toxic control group, suggesting the hepatoprotective action of the different fractions of *S. pinnata*.

4. Discussion

The extractive values in different solvents indicated the quantity and nature of the constituents in each extract. The alcohol soluble extractive value was found to be 17.2% w/w, indicating the presence of polar constituents like steroids, phenols, alkaloids, flavonoids, and glycosides, and signifying that a large amount of constituents of the *S. pinnata* bark was soluble in alcohol.[23,24]

Acute toxicity study of the *Spondias mangifera* (*S. pinnata*) by the fixed-dose method of OECD (Organization for Economic Co-operation and Development) guideline number 420 did not exhibit any sign of toxicity up to 2,000 mg/kg.[25] Hence, an optimum dose of 400 mg/kg of *S. pinnata* bark extracts was selected here for the experimental study.

Alcohol was recognized as a direct hepatotoxin in the 1960s.[1] Thus, 12 mL/kg po of 50% ethanol each day for 8 consecutive days was used to induce hepatotoxicity in rats.[16] It is clear from the present study that the 12 ml/kg po of 50% ethanol (hepatotoxic control) significantly ($p < 0.01$) decreased the levels of antioxidant enzymes, namely, hepatic GSH, catalase, and SOD and increased the levels of hepatic MDA as compared to the normal control group rats, leading to oxidative stress. Ethanol consumption leads to the production of excessive reactive oxygen species, because ethanol is extensively metabolized by the microsomal oxidizing system to acetaldehyde and then to acetate by cytochrome P450.[26] These reactive oxygen species oxidize cellular molecules, such as protein and DNA, and initiate membrane peroxidative degeneration in the adipose tissue, resulting in fatty infiltration of hepatocytes and causing impairment of gluconeogenesis and diversion of metabolism to ketogenesis and fatty acid synthesis.[26,27] Similarly, 12 mL/kg po of 50% ethanol significantly ($p < 0.01$) decreased the final body weight and serum albumin, and significantly ($p < 0.01$) increased the relative liver weight, serum AST, ALT, ALP, LDH, cholesterol, and bilirubin as in hepatotoxic control Group II compared to normal control Group I rats.

Treatment with 400 mg/kg po AE and EE normalized the different liver-specific variables altered by 12 mL/kg po ethanol. Results of the liver function tests were well supported by the histopathological changes in liver tissues, and the activity was comparable to the standard drug silymarin. The hepatotoxic control Group II showed marked fragmentation of hepatic DNA while treatment with the extracts resulted in marked improvement in DNA fragmentation compared to the toxic control group, suggesting the hepatoprotective action of the different fractions of *S. pinnata*. EE (Group VI) showed greater activity that was comparable with normal control and silymarin-treated groups.

The hepatoprotection induced by the extracts may be due to antioxidant property of the extract or phytochemicals present in the extracts, like flavonoids and polyphenols, which reduce the oxidative stress imposed by alcohol, and others, like anti-inflammatory and analgesic properties, preventing inflammatory damage.[4,25,26,28]

From the results of the study, it can be concluded that the *S. pinnata* protected Wistar rats from hepatotoxicity via prevention of ethanol-induced oxidative stress, DNA-damage, and altered biochemical markers. Thus, this may provide a novel potent hepatoprotective agent for liver injury, which could be considered for further clinical studies for future drug development.

Conflicts of interest

The authors declare that they have no conflict of interest to disclose.

Acknowledgments

This study did not receive financial support from any funding bodies or organizations. The authors are thankful to the Department of Pharmacy, Integral University for providing all the necessary facilities related to the present research work.

REFERENCES

1. Singal AK, Bashar H, Anand BS. Outcomes after liver transplantation for alcoholic hepatitis are similar to alcoholic cirrhosis: exploratory analysis from the UNOS database. *Hepatol* 2012;55:1398–405.
2. Gao B, Bataller R. Alcoholic liver disease: pathogenesis and new therapeutic targets. *Gastroenterol* 2011;141: 1572–85.
3. Badoni A, Bisht C. Importance and problems in natural regeneration of *Spondias pinnata*. *Report and Opinion* 2009;1:12–3.
4. Chetia B, Gogoi S. Antibacterial activity of the methanolic extract of stem bark of *Spondias pinnata*. Moringa oleifera and Alstonia scholaris. *Asian J Trad Med* 2011;6:163–7.
5. Tandon S, Rastogi RP. Studies on the chemical constituents of *Spondias pinnata*. *Plan Med* 1976;29:192–7.
6. Gardner S, Sidisunthorn P, Anusarnsunthorn V. A field guide to forest trees of northern Thailand. *Kobfai Publishing Project, Thailand* 2000.
7. Wetwitayaklung P, Charoenteeraboon J, Limmatvapirat C, Phaechamud T. Antioxidant activities of some Thai and exotic fruits cultivated in Thailand. *Res J Pharm Biol Chem Sci* 2012;3:12–21.
8. Arif M, Rahman MA, Imran M, Khalid M, Khushtar M. An insight of *Spondias mangifera* Willd: an underutilized medicinal plant with immense nutraceutical and therapeutic potentials. *Int J Res Pharm Sci* 2015;6: 17–26.
9. Acharyya S, Dash GK, Mondal S, Dash SK. Studies on hypoglycaemic activity of the different extracts of *Spondias mangifera* Wild root. *J Pharm Sci Technol* 2010;2:184–90.
10. Ghate NB, Hazra B, Sarkar R, Mandal N. *In vitro* anticancer activity of *Spondias pinnata* bark on human lung and breast carcinoma. *Cytotechnol* 2014;66:209–18.
11. Manik MK, Islam SMA, Wahid MA, Morshed MM, Kamal S, Islam MS, Ahmed KT. Investigation of *in vitro* antioxidant, antimicrobial and thrombolytic activity of the exocarp of *Spondias pinnata* (Anacardiaceae). *Canadian Chem Trans* 2013;1:191–201.
12. Dhar M, Dhawan BN, Prasad CR, Rastogi RP, Singh KK, Tandon JS. Screening of Indian plants for biological activity: Part V. *Indian J Exp Biol* 1974;12:512–23.
13. Rao BG, Raju NJ. Investigation of hepatoprotective activity of *Spondias pinnata*. *Int J Pharm Sci Res* 2010;1:193–8.
14. Rahman MA, Hussain A. Anticancer activity and apoptosis inducing effect of methanolic extract of *Cordia dichotoma* against human cancer cell line. *Bangladesh J Pharmacol* 2015;10:27–34.
15. Mujahid M, Siddiqui HH, Hussain A, Hussain S. Hepatoprotective effects of *Adenanthera pavonina* against anti-tubercular drugs-induced hepatotoxicity in rats. *Pharmacog J* 2013;5:286–90.
16. Wang H, Feng F, Zhuang B, Sun Y. Evaluation of hepatoprotective effect of Zhi-Zi-Da- Huang decoction and its fractions against acute-alcohol induced liver injury in rats. *J Ethnopharmacol* 2009;126:273–9.
17. Anderson ME, Meister A. *Determination of glutathione (Ed.) Method in enzymology*. 113. New York: Academic press; 1985:548–55.
18. Clairbone AL. Assay of catalase. In: Greenwald RA, editor. *Handbook of method of oxygen free radical research*. Boca Raton: CRC press; 1985:283–4.
19. Marklund S, Marklund G. Involvement of super oxide anion radical in the auto oxidation of pyrogallol and a convenient assay for superoxide dismutase. *European J Biochem* 1974;47:469–74.
20. Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal Biochem* 1979;95:351–8.
21. Belur B, Kandaswamy N, Mukherjee KL. *Medical laboratory technology – a procedure manual for routine diagnostic tests (Laboratory techniques in histopathology)*. New Delhi: Tata McGraw Hill; 1990:1124–88.
22. Perandones CE, lllera AV, Peckham D, Stunzl LL, Ashman RF. Regulation of apoptosis *in vitro* in mature murine spleen T-cell. *J Immunol* 1993;151:3521–9.
23. Anonyms. Indian Pharmacopoeia. Vol. 2, Ministry of Health and Family Welfare, New Delhi, India; 1996.
24. World Health Organization (WHO). Quality control method for medicinal plant material. Geneva: WHO; 1998.
25. Arif M, Fareed S, Rahman MA. Stress relaxant and antioxidant activities of acid glycoside from *Spondias mangifera* fruit against physically and chemically challenged albino mice. *J Pharm Bioall Sci* 2016;8:58–63.
26. Joy-Hoskeri H, Krishna V, Vinay-Kumar B, Shridar AH, Ramesh-Babu K, Sudarshana MS. *In vivo* prophylactic effects of oleanolic acid isolated from chloroform extract of *Flaveria trinervia* against ethanol induced liver toxicity in rats. *Arch Pharm Res* 2012;35:1803–10.
27. Gramenzi A, Caputo F, Biselli M, Kuria F, Loggi E, Andreone P, Bernardi M. Alcoholic liver disease-pathophysiological aspects and risk factors. *Alimentary Pharmacol Ther* 2006;24:1151–61.
28. Chaturvedi S, Drabu S, Sharma M. Anti-inflammatory and analgesic activity of *Tamarix gallica*. *Int J Pharm Sci* 2012;4:653–8.