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EXPERIMENTAL RESEARCH

Study Of The Hepatoprotective Activity Of The Ethanolic Extract Of The Pulp Of Eugenia Jambolana (Jamun) In Albino Rats

DAS S *, SARMA G **

ABSTRACT

Objective: To evaluate the hepatoprotective effect of the ethanolic extract of the pulp of *Eugenia jambolana* (EPEJ) on paracetamol (PCM)-induced hepatotoxicity in albino rats.

Materials and Methods: Healthy albino rats (thirty in number) of either sex, weighing 100-150 gms, were randomly divided into five groups of six animals each. Group A (Normal control) and Group B (Paracetamol-treated control) received 5ml/kg/day of 3% gum acacia; Groups C and D received the ethanolic extract of the pulp of *Eugenia jambolana* 100mg/kg/day and 200mg/kg/day respectively and Group E received silymarin 100mg/kg/day. All formulations were given orally for ten days. Hepatotoxicity was induced in Groups B, C, D and E by giving a single dose of paracetamol (2gm/kg) orally on the eighthth day of the experiment. Liver function tests (serum levels of alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, total bilirubin, total protein and albumin) and the histopathological examination (HPE) of the liver was done for all the five groups on the tenth day.

Statistical Analysis: One-way ANOVA, followed by Dunnett's multiple comparison test, were used for statistical analysis. Values of $p < 0.01$ were assumed to be significant.

Results: Group B showed a significant ($p<0.01$) increase in all serum marker enzymes and total bilirubin and a significant ($p<0.01$) decrease in total protein, as compared to Group A. In comparison to Group B, Groups C and D showed significant ($p<0.01$) reduction in the serum levels of all liver enzymes and total bilirubin and an increase in the total protein. HPE studies showed fatty changes, necrosis and fibrosis in Group B, while in Groups C, D and E, histopathology was near normal.

Conclusion: Thus, as revealed by the above study, the ethanolic extract of the pulp of *Eugenia jambolana* at 100 and 200mg/kg/day, possesses significant hepatoprotective activity in rats induced with hepatotoxin paracetamol.

Key Words: serum marker enzymes, paracetamol, hepatoprotective, *Eugenia jambolana*.

Key Message: The fruit of *Eugenia jambolana* is considered to be a 'tonic to the liver' in traditional Yunani medicine. Very few studies have been done to validate this fact and so the present study was undertaken to evaluate its hepatoprotective activity.

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Introduction

Eugenia jambolana Lam. (Syn. *Syzygium cumini* Skeels or *Syzynium jambolana* Dc), belonging to the family Myrtaceae, is a large evergreen tree, up to 30 m high and is widely distributed throughout India, Ceylon-Malaya and Australia [1]. The plant is commonly known as Black plum or Jambol (English), Jambavam (Sanskrit), Jamun (Hindi) and Jamu (Assamese). The bark is digestible and is good for bronchitis and asthma. The seed can be used as an astringent and a diuretic and stops urinary discharges. The fruit is a tonic to the liver, it enriches the blood and strengthens the teeth and gums[2]. The antidiabetic[3][4], antibacterial, antifungal [5],[6], antiinflammatory [7] and CNS depressant activities[8] of the plant have already been established. The anti-diarrhoeal [9] and gastric ulceroprotective activities [10] of the plant have also been reported. The hepatoprotective activity of the methanolic extract of the seeds of *Eugenia jambolana* in streptozotocin-induced diabetic rats has recently been evaluated [11]. The phytochemical analysis of the seeds of *Eugenia jambolana* has revealed the presence of alkaloids, flavonoids, glycosides, phytosterols, saponins, tannins and triterpenoids [12]. Fruits of *Eugenia jambolana* have been reported to contain raffinose, glucose, fructose, citric acid, anthocyanins, mallic acid and gallic acid[1]. Literature reviews indicated that very few studies on the hepatoprotective potential of this plant, on experimentally induced hepatic damage, have so far been undertaken. From this viewpoint, the present study was aimed at evaluating the hepatoprotective activity of the ethanolic extract of the pulp of *Eugenia jambolana* against paracetamol-induced hepatotoxicity in albino rats.

Materials and Methods

Plant Materials

Fresh, ripe fruits of *Eugenia jambolana* Lam. were collected from the local market in Dibrugarh in the months from June to August. The plant material was authenticated by Dr. L.R. Saikia, Reader, Department of Life Sciences, Dibrugarh University, Dibrugarh. A voucher specimen (No.DU/LS/207) was deposited at the Department of Life Sciences, Dibrugarh University, Dibrugarh. The pulp of the fruits was manually separated from the seeds, it was air dried and was finely powdered in an electrical mixer grinder. 500 grams of the powdered pulp was soaked in sufficient quantity of 90% ethanol and was allowed to remain for 15 min in a tightly covered container. The entire solution was then transferred to a percolator and enough of 90% ethanol was added to saturate the powder and leave a stratum above it. The top of the percolator was closed and when liquid was about to drip from the apparatus, the lower orifice of the percolator was also closed and the solution was allowed to macerate for 48 hours. Then, percolation was allowed to proceed slowly with sufficient solvent, at a rate not exceeding 1 ml/min, until the drug was exhausted [13] This procedure was repeated twice after full percolation by adding fresh solvent to the previously used drug powder. The extract obtained from percolation was collected in a flask. The extract was flask evaporated by using controlled temperature (bath temperature 40—50 ^{0}C) until the

solvent part was evaporated [14]. The extract was collected in glass petri dishes, further dried in a vacuum dessicator and was finally stored in air tight glass containers in a refrigerator at 2—8 ^{0}C for use in the experiments. A final yield of 69.5 grams i.e. 13.9% w/w with respect to the original air dried powder was obtained.

Experimental Animals

Healthy adult Wistar albino rats *(Rattus norvegicus)* weighing 100—150 grams each were used for the study. The animals were procured from Chakraborty Enterprise, Kolkata, and were maintained at standard housing conditions in the Central Animal House, Assam Medical College and Hospital, Dibrugarh. Housed individually in clean polypropylene cages, the animals were maintained on a standard animal diet and water was provided *ad libitum* during the entire period of the experiment. The animals were acclimatized to laboratory conditions for five days prior to the experiments. All the animals were taken care of under ethical consideration as per the CPCSEA guidelines [15] with regular inspections of the rats and the laboratory conditions duly undertaken by a registered veterinary practitioner.

Acute Toxicity Study

Acute oral toxicity test for the ethanolic extract of the pulp of *Eugenia jambolana* Lam. was carried out as per OECD Guideline 425 [16]. When administered orally, the seed extract of *Eugenia jambolana* was found to be non-toxic upto the maximum dose of 2000mg/kg body weight[7],[8]. As such, the limit test at 2000 mg/kg, which required the use of only five albino rats, was performed.

One–tenth and one-twentieth of the upper bound dose of the extract from the limit test was decided to be considered for the experiments [17].

Materials Used

- Ethanolic extract of the pulp of *Eugenia jambolana* (EPEJ).
- Paracetamol (Powder obtained from Bharat Chemicals, Tarapur, Gujarat and standardised as per specifications.)
- Silymarin (Powder obtained from Micro Labs Ltd., Bangalore and standardised as per specifications.)
- 3% aqueous suspension of gum acacia.

Evaluation Of Hepatoprotective Activity

The study of the hepatoprotective activity was carried out as described by Chattopadhyay RR *et al* [18]. A total of thirty animals were equally divided into five groups, with six animals in each group. Group A (Normal control) received 3% gum acacia (5ml/kg, orally) for ten days. Group B (Paracetamol-treated control) rats were also given 3% gum acacia (5ml/kg, orally) for ten days and on the eighth day, hepatotoxicity was induced in these rats by giving a single dose of paracetamol (PCM) suspension (2gm/kg, orally) [18] Groups C, D and E were pretreated with EPEJ (100 mg/kg, orally.), EPEJ (200mg/kg, orally.) and Silymarin (100 mg/kg, orally)[19] respectively, for ten days. On the eighth day of the experiment, hepatotoxicity was induced in Groups–C, D, E also, by giving a single dose of paracetamol (PCM) suspension (2gm/kg, orally) [18]. The rats were sacrificed under light ether anaesthesia after overnight fasting on the tenth day of the experiment. Blood samples were collected by direct cardiac puncture, into sterilized centrifuge tubes and were allowed to coagulate at room temperature. The serum was separated by centrifugation at 3000 rpm for five

minutes [20] and by using Qualigens-Diagnostics Kits manufactured by Sigma Diagnostics (India) Pvt. Ltd., Baroda, alongwith instruments like colorimeter and incubator, biochemical analysis to assess the liver function viz, serum transaminases [aspartate aminotransferase (AST), alanine aminotransferase (ALT)][21], alkaline phosphatase (ALP) [22], total bilirubin [23] and total protein (TP) [24].

Histopathological Study

A portion of the liver tissue of all the animal groups was excised and was then washed with normal saline. The liver tissues were fixed in 10% buffered neutral formalin for 48 hours and then with bovine solution for six hours and were then processed for paraffin embedding. By using a microtome, sections of five mm thickness were taken, were processed in alcohol-xylene series, were stained with alum-haematoxylin and eosin and were subjected to histopathological examination under light microscope using a magnification of 100X [19].

Statistical Analysis

One-way analysis of variance (ANOVA) [25], followed by Dunnett's multiple comparison test [26], were used for the statistical analysis of the results. The statistical analysis was done using computerised GraphPad Prism software version 5.00. It was assumed to consider values of $p < 0.01$ to be statistically significant.

Results

Acute Toxicity Test

There was no mortality recorded among the rats upto the maximum dose of 2000 mg/kg (all five animals survived at 2000mg/kg). Hence, the LD_{50} can be said to be above 2000mg/kg. One-tenth and one-twentieth of the maximum dose tested were selected for the experiments.

Biochemical Assessment

There was a significant ($p<0.01$) elevation of serum marker enzymes and decrease in total protein and albumin in Paracetamol-treated rats as compared to the rats in the Normal control group, which is an evidence of the extensive liver damage caused by paracetamol. Pretreatment with test drug *Eugenia jambolana* in both doses (100mg/kg/d and 200mg/kg/d), as well as pretreatment with standard drug Silymarin showed a significant ($p<0.01$) protective effect by reducing the level of enzymes and by increasing the level of total protein and albumin. The reduction in the level of liver enzymes and increase in the levels of total protein and albumin caused by *Eugenia jambolana* was dose dependent, the hepatoprotective effect being more with the 200mg/kg dose than with the 100mg/kg dose [Table/Fig 1] .

(Table/Fig 1) EFFECT OF *EUGENIA JAMBOLANA* ON BIOCHEMICAL MARKERS IN PARACETAMOL INDUCED HEPATOTOXICITY IN RATS

Groups	Treatment	Total Protein (gm%)	Albumin (gm%)	AST (units/L)	ALT (units/L)	Alkaline Phosphatase (KA units)	Total Bilirubin (mg%)
A	Normal Control (3%gum acacia; 5 ml/kg/d)	4.8 ± 0.09	3.4 ± 0.12	46 ± 1.34	24 ± 1.44	13 ± 0.58	0.5 ± 0.12
B	PCM Control (PCM-2 gm/kg/d + 3%gum acacia)	*2.7 ± 0.12	* 1.2 ± 0.13	* 240 ± 1.16	*132 ± 2.05	*50 ± 1.51	*3.2 ± 0.12
D	PCM + EPEJ (200 mg/kg/d)	†4 ± 0.10	†2.8 ± 0.09	†120 ± 1.16	†42 ± 1.00	†20 ± 1.65	†1.8 ± 0.10
E	PCM+ Silymarin (100 mg/kg/d)	†4.6 ± 0.12	†3.2 ± 0.13	†110 ± 1.32	†28 ± 1.29	†16 ± 0.82	†1.2 ± 0.08
One Way Anova	F	67.98	57.87	3361	958.50	28.61	100.50
	df	4, 24	4, 24	4, 24	4, 24	4, 24	4, 24
	p	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01

*p < 0.01 as compared to Group–A; † p < 0.01 as compared to Group–B;

Values are Mean ± SEM; n = 6 rats in each group. ANOVA followed by Dunnett's Multiple Comparison Test.

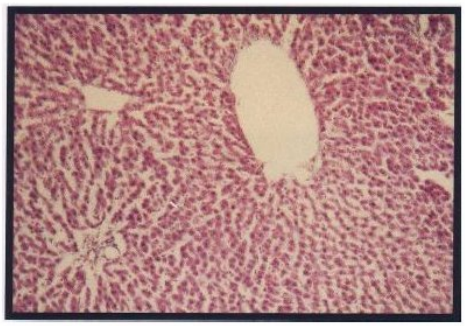
AST- Aspartate transaminase; ALT- Alanine transaminase; PCM- Paracetamol;

EPEJ- Ethanolic extract of pulp of *Eugenia jambolana*

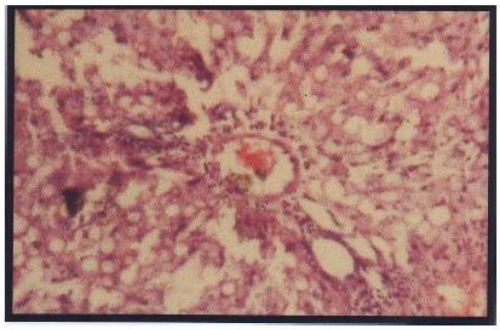
Histopathological Examination

Histopathological examination of the normal control group under light microscopic magnification of 100X, showed normal hepatocytes [Table/Fig

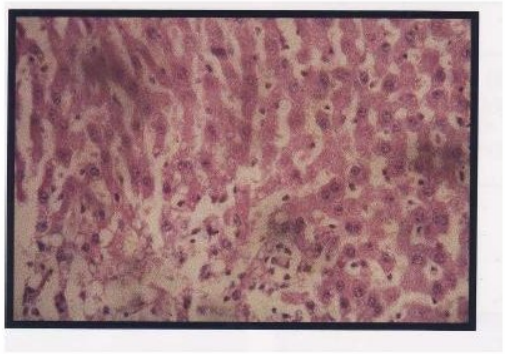
2]. Paracetamol-treated rat liver revealed steatosis, centrilobular necrosis, vacuolisation and fibrosis [Table/Fig 3]. Administration of EPEJ preserved the histological structure of the liver to near normal, though there was congestion and regeneration of the liver tissue [Table/Fig 4]. The sections of the liver taken from the Silymarin treated group showed a hepatic architecture which was similar to that of the normal control group [Table/Fig 5].



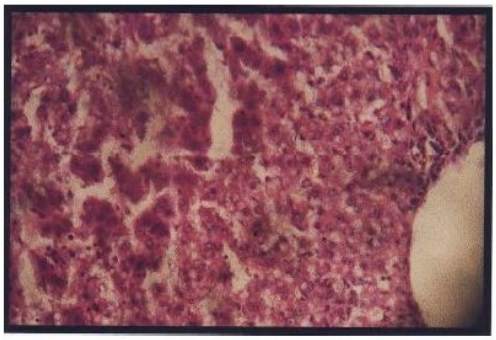
(Table/Fig 2) Section of the liver tissue of Control animal showing normal histology. (100X)



(Table/Fig 3) Section of the liver tissue of animal treated with Paracetamol showing a central hepatic vein , necrosis and fatty change. (100X)



(Table/Fig 4) Section of the liver tissue of ethanolic extract of pulp of *Eugenia jambolana* treated animals showing normal arrangement of hepatocytes, absence of necrosis and mild fatty changes. (100X)



(Table/Fig 5) Section of the liver tissue of Silymarin treated animals showing normal hepatocytes with central hepatic vein. (100X)

Discussion

Paracetamol (Acetaminophen) is a widely used antipyretic and analgesic and produces acute liver damage if overdoses are consumed. The hepatotoxicity of paracetamol has been attributed to the formation of toxic metabolites when a part of the paracetamol is activated by hepatic cytochrome P-450 to a highly reactive metabolite N-acetyl-P-benzoquinoneimine (NAPQI) .NAPQI is initially detoxified by conjugation with reduced glutathione (GSH) to form mercapturic acid, which is excreted in urine [27] After a toxic overdose of paracetamol, the quantity and rate of NAPQI formation may overwhelm the capacity of the liver to replenish it's reduced glutathione stores [28]. The

NAPQI then causes arylation or oxidation of cytosolic and mitochondrial proteins, leading to their inactivation [29]. It also raises the cytosolic calcium levels by inhibiting the calcium-ATPase activity in the plasma membrane and also the mitochondrial calcium uptake and retention [30]. This raised cytosolic calcium is responsible for the degradation of adenine nucleotides and the formation of reactive oxygen species. This leads to further oxidation of protein thiols, lipid peroxidation, DNA fragmentation, cell lysis and thus, cell death [29],[31].

In the assessment of liver damage by paracetamol, the determination of enzyme levels such as aspartate aminotransferase (AST/SGOT) and alanine aminotransferase (ALT/SGPT) is largely used. Serum levels of AST, ALT and alkaline phosphatase are the most frequently utilized indicators of hepatocellular injury [32]. Necrosis or membrane damage releases the enzymes into circulation; and therefore, they can be measured in serum. ALT is more specific to the liver, and is thus a better parameter for detecting liver injury. Elevated levels of serum enzymes are indicative of cellular leakage and loss of functional integrity of the cell membrane in the liver [33]. The mechanism by which alkaline phosphatase reaches the circulation is uncertain; leakage from the bile canaliculi into hepatic sinusoids may result from leaky tight junctions and the other hypothesis is that the damaged liver fails to excrete alkaline phosphatase made in the bone, intestine and the liver [32]. Serum total protein, albumin and bilirubin levels, on other hand, are related to the function of hepatic cells i.e they reveal the functional status of the hepatic cells. Decreased levels of total protein and albumin are indicative of the failure of the biosynthetic function of the hepatocyte, while increased levels of bilirubin indicate defective hepatocellular uptake, conjugation and excretion of bilirubin due to the failure of hepatic cell function [32],[34].

In the present study, the paracetamol-treated rats (Group B) showed a significant ($p<0.01$) elevation in the serum levels of ALT, AST, alkaline phosphatase and total bilirubin, while significantly ($p<0.01$) decreasing the levels of total protein and albumin as compared to the normal control rats (Group A), thereby indicating liver damage. The paracetamol-induced liver damage was confirmed by the histopathological examination of paracetamol-treated rat liver, which revealed steatosis, centrilobular necrosis, vacuolisation and fibrosis. Administration of EPEJ at doses of 100mg/kg and 200mg/kg, significantly ($p<0.01$) prevented the rise in the levels of the marker enzymes and total bilirubin, as well as it significantly ($p<0.01$) prevented the decrease in the serum levels of total protein and albumin. This observation was in conjugation with the histopathological study, which revealed the preservation of the histological structure of the liver to near normal by EPEJ, inspite of slight congestion and regeneration of the liver tissue.

The diminished rise of serum enzymes, together with the diminished fall in the levels of total protein and albumin in the extract (EPEJ)-treated groups, is a clear manifestation of the hepatoprotective effect of the extract. The hepatoprotective effect of *Eugenia jambolana* was dose dependent i.e. it increased with an increase in dose. Histopathological studies have also provided the evidence of the effect of EPEJ as a hepatoprotectant.

These results indicate the stabilizing effect of EPEJ on the plasma membrane

of the hepatocytes, as well as repair of the damaged hepatic tissue, probably brought about by the stimulation of hepatocellular protein synthesis and accelerated regeneration of the hepatocytes. But the major mechanism behind all these effects is probably the diminution of the intensity of oxidative stress induced by paracetamol, which is brought about by the antioxidant activity of EPEJ. The seed kernel of *Eugenia jambolana* has been reported to increase the hepatocellular reduced glutathione content and also to increase the activities of the antioxidant enzymes glutathione peroxidase, superoxide dismutase and catalase in the liver of experimental animals[35]. Since the phytoconstituents of the seed kernel and the pulp of *Eugenia jambolana* are almost the same, the pulp may also be presumed to have similar effects on antioxidant enzymes. The flavonoids, gallic acid and anthrocyanins present in the pulp of Eugenia jambolana, are natural antioxidants [36]. Earlier investigators [37],[38] have screened the hepatoprotective activity of several flavonoid compounds found in plants. Thus, EPEJ, because of the presence of natural antioxidants like flavonoids in it, must have exerted protective action against paracetamol-induced liver damage, probably by increasing the content of reduced glutathione in the blood and liver, which would provide the tissue a better protection against the generation of free radicals, or by increasing the activities of the antioxidant enzymes like superoxide dismutase and thereby scavenging the already generated free radicals. This must have ameliorated the extent of oxidative stress mediated cellular damage caused by paracetamol.

Conclusion

Thus, it can be concluded that the ethanolic extract of the pulp of *Eugenia jambolana* possesses a significant hepatoprotective effect. However, further studies to establish the exact mechanism of the hepatoprotective action have to be undertaken. Also, studies for isolating and elucidating the structure of the active principles responsible for the hepatoprotective action that are present in the pulp of *Eugenia jambolana*, can be undertaken.

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