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STUDIES ON HEPATOPROTECTIVE ACTIVITY OF HYDROALCOHOLIC LEAF EXTRACT OF PONGAMIA PINNATA AGAINST I/R INDUCED HEPATIC REPERFUSION INJURY

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ABSTRACT

Pongamia pinnata (Linn) Pierre commonly known as 'Karanj' contains various phytoconstituents belonging to alkaloids, glycosides, flavonoids, fixed oils and carbohydrates. Leaves of Pongamia pinnata are digestive, laxative, anthelmintic and are good for diarrhoea, leprosy, dyspepsia and cough. A hot infusion of leaves is good for cleaning ulcers and wounds. This study was designed to determine the possible protective effect of Pongamia pinnata (PP) hydro-alcoholic leaf extract, a plant rich in antioxidant, on hepatic ischemia/reperfusion (I/R) injury. Wistar albino rats were subjected to 45 min of hepatic ischemia, followed by a 60 min reperfusion period. Pongamia pinnata hydro-alcoholic leaf extract were administered in doses of 100, 200 and 400 mg/kg/day, orally for 15 days before I/R injury respectively and repeated before the reperfusion period. Liver samples were taken for histological examination or determination of hepatic malondialdehyde (MDA), super oxide dismutase (SOD) and glutathione (GSH) activity. Serum aspartate aminotransferase (AST), alanine aminotransferase (ALT) alkaline phosphatase (ALP) and bilirubin levels were determined to assess liver functions. Lactate dehydrogenase (LDH) was assayed in serum samples for the evaluation of generalized tissue damage. Ischemia/reperfusion caused a significant decrease in hepatic GSH, and significant increases in MDA levels. Serum AST, ALT, ALP and bilirubin levels, as well as LDH activity levels were also elevated in the I/R group. Treatment with PP hydro-alcoholic leaf extract reversed all these biochemical parameters as well as histological alterations induced by I/R. In all the testing, a significant correlation existed between concentrations of the extract and alteration in the biochemical and histological parameters. In conclusion, PP hydro-alcoholic leaf extract at the dose of 400 mg/kg/day reduced I/R-induced organ injury through its ability to balance the oxidant–antioxidant status.

KEYWORDS

Ischemia/reperfusion; Pongamia pinnata (PP) hydro-alcoholic leaf extract; antioxidant

INTRODUCTION

Liver is frequently exposed to IR injury under different clinical conditions like circulating shock, intravascular coagulation, liver transplantation and surgery involving this organ [1, 2]. Hepatic ischemia reperfusion (HIR) injuries cause high morbidity and consume substantial health care capacities in patients with primary hepatic injury and systemic injury [3-5]. Oxygen radicals probably mediate some of the structural and functional alterations associated with reperfusion of ischaemic liver. A number of evidences implicate oxygen-derived free radicals (especially superoxide and hydroxyl radical) and high-energy oxidants (such as peroxynitrite) in the IR injury syndrome, particularly during the reperfusion phase [6]. Thus, agents proposed to be useful in the clinical settings of Hepatic Ischemia Reperfusion damage include antioxidants or free radical scavengers [7]. The main characteristic of an antioxidant is its ability to trap free radicals. Antioxidant compounds like phenolic

acids, polyphenols and flavonoids scavenge free radicals such as peroxide, hydroperoxide or lipid peroxyl and thus inhibit the oxidative mechanisms that lead to degenerative diseases.

The use of herbal drugs as medicines for the treatment of a wide range of diseases can be traced back since ancient time's i.e during the Vedic period in India [8]. Being the outcome of therapeutic experiences of generations of practicing physicians of indigenous systems of medicine for over hundreds of years medicinal plants have played a key role in world health [9, 10]. Hence, in spite of the great advances observed in modern medicine in recent decades, herbal medicines still make an important contribution to health care [11]. Plants phenolics, in particular phenolic acid [12-14] tannins [15, 16] and flavonoids [17] are known to be potent antioxidant and occur in vegetables, fruits, nuts, seeds, roots, barks and leaves. In addition to their antioxidant properties, these compound display a vast variety of pharmacological activities such as anti-inflammatory, anti-carcinogenics, antibacterial or antiviral activities which may explain, at least in part, its use as alternative or supportive treatments in various degenerative diseases [18-21].

Pongamia pinnata (Linn) Pierre (synonyms- Indian beech) commonly known as Karanj belonging to family Fabaceae. It is also called *Pongamia glabra*. It is an indo-Malaysian species, a medium-sized evergreen tree, and common on alluvial and coastal situations from India to Fiji, from sea level to 1200m. Now found in Australia, Florida, Hawaii, India, Malaysia, Oceania, Philippines and Seychelles [22-24]. The plant affords patulitrin, β - sitosterol, spicigerine, aminoacids, albumin, globulin, glutelins, flavonoids such as furanoflavones, furanoflavanols, chromenoflavones, furanochalcones [25]. The plant also contains alkaloids, tannins and carbohydrate [26]. Phenolics, such as flavonoids, flavonols and flavones have potent antioxidant capacity and reports suggest that *Pongamia pinnata* contains flavones, pongamones, furanoflavones, pongamol, pongagalabrone and pongapin, pinnatin and kanjone which have potent antioxidant activity. Potent free radical scavenging capacity of *Pongamia pinnata* can be attributed to different flavones present in the extracts. So the plant may be useful in the management of free radical mediated diseases. The leaves and stem of the plant consist of several flavone and chalcone derivatives such as Pongone, Galbone, Pongalabol, Pongagallone A and B [27]. Punitha and Manohar in 2006 evaluated anti-hyperglycemic and anti-lipid peroxidative effects of ethanolic extract of *Pongamia pinnata* (Linn.) [28]. Effects of *Pongamia pinnata* on lipid peroxidation products and antioxidants in hyper-ammonemic rats with reference to circadian variations were evaluated by Essa and Subramanian [29]. Literature survey of *Pongamia pinnata* revealed its potential being like hepatoprotective and antioxidant [30].However it remains to be scientifically validated. Hence present study titled "Studies on Hepatoprotective Activity of Hydroalcoholic Leaf Extract of Pongamia Pinnata against I/R Induced Hepatic Reperfusion Injury" has been conducted.

MATERIALS AND METHODS

METHOD:

The experimental protocols were conducted with the approval of the Animal Research Committee at Royal College of Pharmacy and Health Sciences, Berhampur. Odisha. All animals were maintained in accordance with the recommendations of the CPCSEA.

Chemicals:

All the chemicals used were of analytical grade. ALT, AST, ALP, bilirubin and LDH Kits were obtained from Crest Biosystems, Bambolim Complex. Goa, India. All drug solutions were freshly prepared in saline before each experiment. The extracts were dissolved in distilled water and administered orally.

Animals:

Male Wistar albino rats (200–250 g) were obtained from the animal house of R.C.P.H.S. and were housed in an air-conditioned room with 12 h light and dark cycles, with constant temperature (22 ± 2 °C) and relative humidity (65–70%) levels. All experimental protocols were approved by the Institutional Animal Ethical Committee (Approval No-07/IAEC/2011). The rats were anesthetized by intraperitoneal injection of sodium pentobarbital (30 mg/kg). All surgical procedures were conducted with clean but not sterile instruments.

Plant collection:

Leaves of *Pongamia pinnata* were collected in the month of December 2011 from its natural habitat from nearby Mohuda village, Berhampur, Ganjam district of Odisha. The plant was authenticated from Department of Botany, Khalikote College, Berhampur, Odisha. The leaves were cleaned and dried under the shade to avoid degradation of volatile oil. The leaves were dried in hot air woven at 55°C for 3 days and at 40°C for the next 4 days.

Preparation of Plant Extracts:

The dried leaves were coarsely powdered and extracted with a mixture of methanol: water (7:3, v/v) by a Soxhlet apparatus at 50°C. The solvent was completely removed and obtained dried crude extract which was used for investigation. Further the extracts were subjected for pharmacological screening.

EXPERIMENTAL PROTOCOL:

Under anesthesia, a midline laparotomy was made using minimal dissection. The abdomen was shaved and a transverse incision was performed. The bowel loops were covered with saline-soaked gauze. Total hepatic ischemia was induced for 45 min by clamping the hepatic artery, the portal vein and the bile duct using a vascular clamp. Then reperfusion was induced for 60 minutes in rats. Abdominal incision was closed in layers with 4-0 dexon and 2-0 nylon during reperfusion stage in order to prevent the loss of body fluid and quantity of heat.

Animals were divided into seven groups consisting of six rats each. *Pongamia pinnata* (PP) hydro-alcoholic leaf extract was dissolved in water and administered to the animals. *Pongamia pinnata* (PP) hydro-alcoholic leaf extract used in this study contains Pongagallone A and B

Group-I: - NAIVE-Normal control-rats in this group did not undergo ischemia or reperfusion and served as the control group.

Group-II: - SHAM-Sham-operated (animals subjected to the identical procedure of surgery without ischemia-reperfusion injury) plus physiologic saline treatment.

Group-III: - I/R-Animals subjected 45 minutes of total hepatic ischemia, followed by reperfusion for 60 mins and served as untreated experimental control.

Group-IV: - PP control- Sham operated plus *Pongamia pinnata* control (400 mg/kg body wt. treatment up to15 days).

Group-V: - PP 100mg/kg + I/R- Hepatic I/R plus *Pongamia pinnata* hydro-alcoholic leaf extract 100 mg/kg body wt. treatment up to 15 days

Group-VI: - PP 200mg/kg + I/R- Hepatic I/R plus *Pongamia pinnata* hydro-alcoholic leaf extract 200 mg/kg body wt. up to 15 days.

Group-VII: - PP 400mg/kg + I/R- Hepatic I/R plus *Pongamia pinnata* hydro-alcoholic leaf extract 400 mg/kg body wt. up to15 days.

None of the animals died during these procedures. At the end of the reperfusion period, animals were decapitated and trunk blood samples were

collected to determine serum alanine aminotransferase (ALT), aspartate amino transferase (AST), alkaline phosphatase (ALP), bilirubin and lactate dehydrogenase (LDH) activity, the indicators of liver functions and generalized tissue damage, respectively. The hepatic tissue samples were stored at −20°C. Afterwards, tissue malondialdehyde (MDA) levels, an end product of lipid peroxidation, superoxide dismutase (SOD), Catalase and glutathione (GSH), key endogenous antioxidants, were measured in these samples. The hepatic tissue samples were also placed in formaldehyde (10%) for histological evaluation.

BIOCHEMICAL MEASUREMENTS

Measurement of serum index of hepatotoxicity

Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), bilirubin and lactate dehydrogenase (LDH) activity was measured using commercial kits (Crest Biosystems, Bambolim Complex. Goa, India) and the results are expressed in international units per liter [31].

Table 1:-Level of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) in Serum

GROUP	TREATMENT	ALT (IU/L) (Mean ± SEM)	AST (IU/L) (Mean ± SEM)	ALP (IU/L) (Mean ± SEM)
GROUP I	Naive	90.40±11.45	196.23±13.32	236.41±21.33
GROUP II	Sham -operated control	97.52±10.79	198.71±14.95	230.56±23.08
GROUP III	Ischemia/reperfusion (I/R)	212.12±26.06[a]	324.21±23.32[a]	337.23±22.12[a]
GROUP IV	PP control	89.68±17.42	196.58±13.53	228.34±40.08
GROUP V	PP 100mg/kg + I/R	194.35±18.45[b]	289.54±21.63[b]	300.04±39.29[b]
GROUP VI	PP 200mg/kg + I/R	168.42±18.29	244.54±18.24	268.71±37.61
GROUP VII	PP 400mg/kg + I/R	95.82±13.25	198.78±17.05	238.04±20.52

[a]P < 0.01 vs. naive, sham and PP groups
[b]P < 0.05 vs. I/R + PP

Table 2:-Level of bilirubin and lactate dehydrogenase (LDH) in Serum

GROUP	TREATMENT	Total Bilirubin (mg/dl) (Mean ± SEM)	LDH ((U/L) (Mean ± SEM)
GROUP I	Naive	0.87±0.09	1197.74±134.58
GROUP II	Sham -operated control	0.92±0.07	1395.47±125.60
GROUP III	Ischemia/reperfusion (I/R)	2.04±0.23[a]	2010.96±140.19[a]
GROUP IV	PP control	0.84±0.29	1168.74±120.13
GROUP V	PP 100mg/kg + I/R	1.72±0.37[b]	1790.31±133.16[b]
GROUP VI	PP 200mg/kg + I/R	1.32±0.31	1498.47±124.48
GROUP VII	PP 400mg/kg + I/R	0.87±0.38	1274.56±123.52

[a]P < 0.01 vs. naive, sham and PP groups.
[b]P < 0.05 vs. I/R + PP.

Table 3:- The renal tissue oxidant and antioxidant enzyme levels of the groups

GROUP	TREATMENT	MDA (nmol/g protein) (Mean ± SEM)	SOD (U/g protein) (Mean ± SEM)	CATALASE (kg/ protein) (Mean ± SEM)	GSH (U/g protein) (Mean ± SEM)
GROUP I	Naive	17.20±3.93	33.65±3.76	24.64±1.24	0.34±0.03
GROUP II	Sham -operated control	18.69±2.08	30.45±3.19	22.45±1.16	0.33±0.04
GROUP III	Ischemia/reperfusion (I/R)	27.70±2.51[a]	20.43±2.44[a]	16.05±0.85[a]	0.18±0.02[a]
GROUP IV	PP control	16.85±1.47	32.65±2.06	24.02±1.02	0.36±0.02
GROUP V	PP 100mg/kg + I/R	25.47±4.29[b]	24.74±2.08[b]	17.96±0.65[b]	0.25±0.03[b]
GROUP VI	PP 200mg/kg + I/R	20.05±2.81	26.38±2.05	20.67±0.97	0.29±0.03
GROUP VII	PP 400mg/kg + I/R	18.28±2.17	28.05±2.07	22.83±1.08	0.33±0.02

[a]P < 0.01 vs. naive, sham and PP groups.
[b]P < 0.05 vs. I/R + PP.

Table 4:- Histological injury scores* of liver tissue

Rat group	Portal inflammation	Necrosis	Vacuolar degeneration	Sinusoidal dilatation	Vascular congestion
Naive	1	0	0	0	0
Sham	0	0	0	0	1
IR	2	3	2	1	2
IR + PP 100mg/kg	1	2	1	1	1
IR + PP 200mg/kg	1	1	0	1	1
IR + PP 400mg/kg	0	0	0	0	1

Injury Scores: None [0], Mild [1], Moderate [2] and Severe [3]

Measurement of hepatic oxidative stress markers

1. Lipid peroxidation was used as an indirect measure of oxidative damage induced by ROS (free radical induced injury). Lipid peroxidation was assayed as the malondialdehyde (MDA) level in liver homogenate by the thiobarbituric acid method using tetraethoxypropane as the standard [32]. A mixture of 8.1% sodium dodecylsulphate (0.2 ml, Merck), 20% acetic acid (1.5 ml), and 0.9% thiobarbituric acid (1.5 ml, Merck) was added to 0.2 ml of 10% tissue homogenate. Distilled water was added to the mixture to bring the total volume to 4 ml. This mixture was incubated (95°C, 1 hr). After incubation, the tubes were placed in cold water and 1 ml of distilled water plus 5 ml of n-butanol/ pyridine (15:1, v/v) was added, followed by mixing. The samples were centrifuged (4,000 x g, 10 min). The organic phase (supernatant) was removed, and absorbances were measured with respect to a blank at 532 nm. 1, 1, 3, 3-Tetraethoxypropane was used as the standard. Lipid peroxide levels were expressed as nmol MDA/g of wet tissue.
2. The hepatic antioxidant activity, superoxide dismutase (SOD), was assessed in the homogenized liver by the method of Sun *et al* [33]. [0.3 mM xanthine, 0.6 mM Na_2EDTA, 0.15 mM nitroblue tetrazolium (NBT), 0.4 M Na_2CO_3, and 1 g/L bovine serum albumin (BSA)] was added 100µl of the tissue supernatant. Xanthine oxidase (50µl, 167 U/L) was added to initiate the reaction and the reduction of NBT by superoxide anion radicals, which are produced by the xanthine-xanthine oxidase system, was determined by measuring the absorbance at 560 nm. Cu, Zn-SOD activity was expressed as units of SOD/mg of tissue protein, where 1 U is defined as that amount of enzyme causing half-maximal inhibition of NBT reduction To 2.45 ml of assay reagent In addition, the total protein content in the liver tissues was determined according to the Lowry's method [34].
3. CAT activity was measured by the method by Aebi [35]. Supernatant (10 µl) was placed in a quartz cuvette and the reaction initiated by adding 2.99 ml of freshly prepared 30 mM H_2O_2 n phosphate buffer (50 mM, pH 7.0). After

rapid mixing, the rate of H_2O_2 decomposition was determined from absorbance changes at 15 and 30 sec at 240 nm. CAT activity was expressed as k/mg of tissue protein, where k is the first order rate constant.

4. GSH in liver tissue was assayed by the method of Tietze and Anderson [36, 37]. Briefly, 100 µl of tissue supernatant was placed in a 3 ml cuvette; 750 µl of 10 mM 5-5'-dithio-bis-2nitrobenzoic acid (DTNB) solution (100 mM KH_2PO_4KH plus 5 mM Na_2EDTA, pH 7.5 and GSHRD, 625 U/L) was added and the mixture was incubated (3 min, room temperature). Then 150 µl of 1.47 mM β -NADPH was added, mixed rapidly by inversion, and the rate of 5-thio-2-nitrobenzoic acid formation (proportional to the sum of reduced and oxidized glutathione) was measured spectrophotometrically for 2 min at 412 nm. The reference cuvette contained equal concentrations of DTNB and NADPH, but no sample; results were expressed as nmol/mg of wet tissue

Histological procedures

Liver specimens from all groups were rapidly taken and fixed in Bouin's solution and processed for light microscopic study using hematoxylin and eosin stain [38]. For light microscopic investigations, hepatic tissue specimens were fixed in 10% formaldehyde, dehydrated in alcohol series, clearing in toluene and embedding in paraffin. Paraffin sections (5 µm) were stained with hematoxylin and eosin (H&E) and examined under a photomicroscope. All tissue sections were examined microscopically for the characterization of histopathological changes by an experienced histologist in blind fashion (Procedures were carried out in NIDAN diagnostics, Berhampur. Odisha. INDIA).

STATISTICAL ANALYSIS

Results are presented as the mean ± SEM. All statistical analyses were performed using Graph Pad Prism Software program (version 5) [39]. Data were analyzed using analysis of variance followed by Bonferroni's post-test. The Kruskal-Wallis 1-way analysis of variance by ranks was used to simultaneously test the pathologic score for the I/R and I/R ± *Pongamia pinnata* groups. A P value of < 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

The present study justifies that temporary blockade of hepatic blood supply yielded structural and functional alterations in the liver. Hydro-alcoholic leaf extract of *Pongamia pinnata* (PP) on the other hand, reduced the severity of injury.

I/R injury is a complex process and evidence suggests that oxygen derived free radicals are involved in the hepatic injury caused by ischemia and reperfusion [40-43]. Thus, therapeutic strategies are designed so as to reduce free radical induced damage, either by intervening in the process by which free radicals are formed or by scavenging the free radicals that have already been formed. Different degrees of protection have been acquired with numerous compounds; however, the structure-activity relationship, bioavailability and therapeutic efficacy of these compounds differ comprehensively. Thus clinical application of these agents is limited in respect of their pharmacokinetic and pharmacodynamics properties.

The present study accentuates three points: (1) The emergence of free oxygen radicals that arise after hepatic IR and cause injury which can be prevented by PP; (2) the increase in AST, ALT, ALP, Total Bilirubin and LDH →an indication of tissue damage in hepatic IR injury →is less after PP treatment; (3) histopathological examination of liver tissue shows considerably less hepatocyte injury in rats with PP treatment.

Free oxygen radicals have a marked mediator role in IR injuries of several organs. Oxygen radicals probably mediate some of the structural and functional alterations associated with reperfusion

of ischaemic liver. Research suggests that antioxidant molecules may provide protection from IR injury. Pongamia pinnata, rich in flavonoids (Pongagallone A and B), is known to be a strong antioxidant, breaking up free radicals. For this reason, it is expected to be protective in hepatic IR injury of rats.

Most procedures of liver ischemia in rats include a portosystemic shunt (usually using an ex vivo bypass between a branch of the mesenteric vein and the jugular vein) with the using of heparin, or segmental rather than total clamping of the hepatic blood supply to prevent mesenteric congestion. Thus partial hepatic ischemia is not a method from which organ viability or animal survival can be assessed. Therefore, in this study surgery was conducted to develop a simple and reproducible method of total hepatic ischemia in rat. Another advantage of the availability of such a simple model is the low cost compared with other models and the lack of a requirement for sophisticated expensive materials and can be instrumental in the research of the mechanisms of liver ischemia-reperfusion injury in different pathophysiological conditions.

In IR injury of the liver during the reperfusion phase, emerging reactive oxygen radicals activate some mediators and can cause inflammatory response and tissue damage. The release of liver enzymes is usually used to assess tissue damage following ischemia-reperfusion. For this reason AST, ALT, ALP, Total Bilirubin and LDH activities may increase [44, 45]. The increase of AST, ALT, ALP, Total Bilirubin and LDH activities in group 3 of our study supports this finding. In the study, it is shown that in group 4, 5, 6 and 7, ALT, AST, ALP, Total Bilirubin and LDH enzymes, markers of liver parenchymal injury, have decreased levels in comparison with group 3 ($p<0.0001$). This finding supports the protective effect of PP treatment on IR injury by justifying that the hydro-alcoholic leaf extract of *Pongamia pinnata* (PP) have shown very significant hepatoprotection against I/R-induced induced hepatotoxicity in wistar albino rats in reducing serum AST, ALT, ALP, Total Bilirubin and LDH levels.

This study showed no significant difference between the biochemical measurements of group 1 and group 2. Hepatic I/R rats (group 3) showed a significant elevation of serum index of hepatotoxicity (ALT). ALT, AST and ALP levels were significantly higher in the I/R group when compared with those of the control group ($p < 0.001$). PP treatment reversed these values significantly. Similarly, in the I/R group, increased lactate dehydrogenase activity, as an index of generalized tissue damage, was reversed significantly by PP treatment ($p < 0.01$) (**Fig.2**)

Oxidative stress occurs particularly in reperfusion after ischemia. Synthesis of proinflammatory cytokines and cell adhesion molecules is activated, and the inflammatory response is increased by oxidative stress. The antioxidant system has an important role in protection from the damage of oxidative stress. Lipid peroxide is an intermediate free radical oxidant that is synthesized during lipid peroxidation [46, 47]. In our study, lipid peroxide levels were found to be significantly higher in group 3 than in group 1. It was also found that treatment with PP in group 4, 5, 6 and 7 decreased the lipid hydroperoxide activity respectively , indicating that the antioxidant effect of PP had prevented the emergence of an oxidant agent.In our study, by measuring Catalase, we obtained information about hepatic IR injury. Catalase is an antioxidant enzyme that catalyses the chage from hydrogen peroxide into water. The concentration of hepatic reduced glutathione decreases progressively during ischaemia[48] with a corresponding increase in oxidized glutathione, which is attenuated by formate, a cell permeable OH scavenger[49]. It is also found that treatment with hydro-alcoholic leaf extract of *Pongamia pinnata* have brought down the elevated levels of LPO and also significantly enhanced the reduced levels of SOD, CAT and GSH

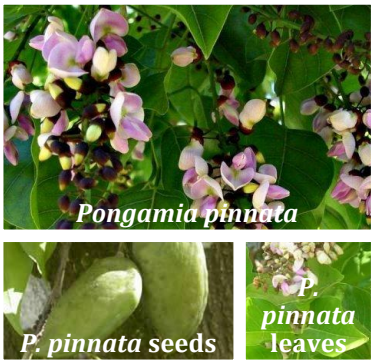


Figure 1:- *Pongamia pinnata* Linn. With permission from B&T world seeds

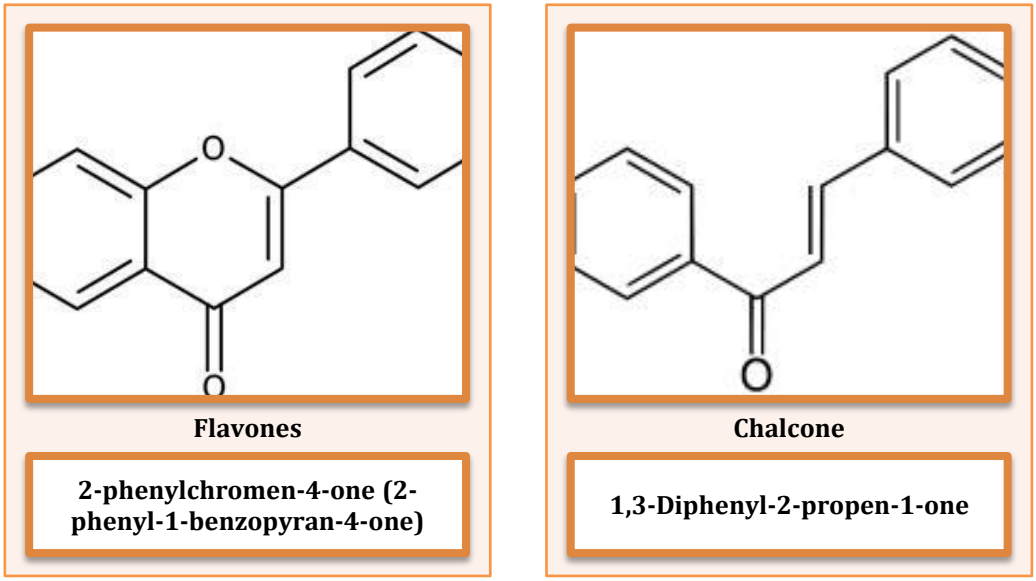
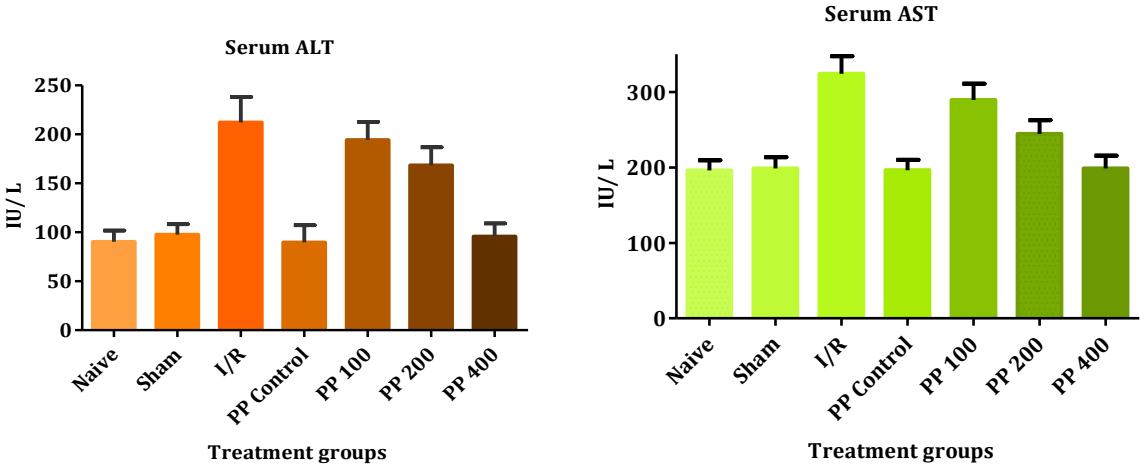
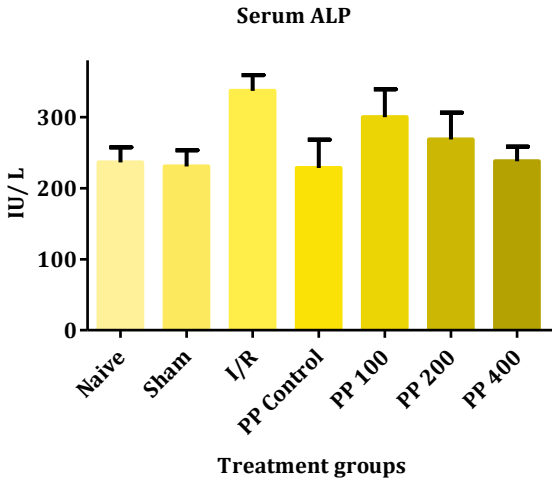


Figure 2:- Flavonoids of *Pongamia pinnata* Linn

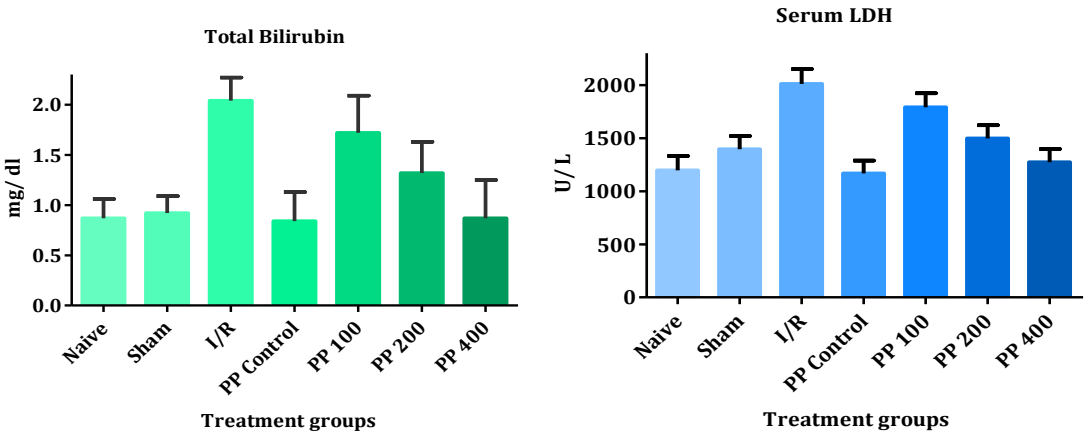


3(A) 3 (B)



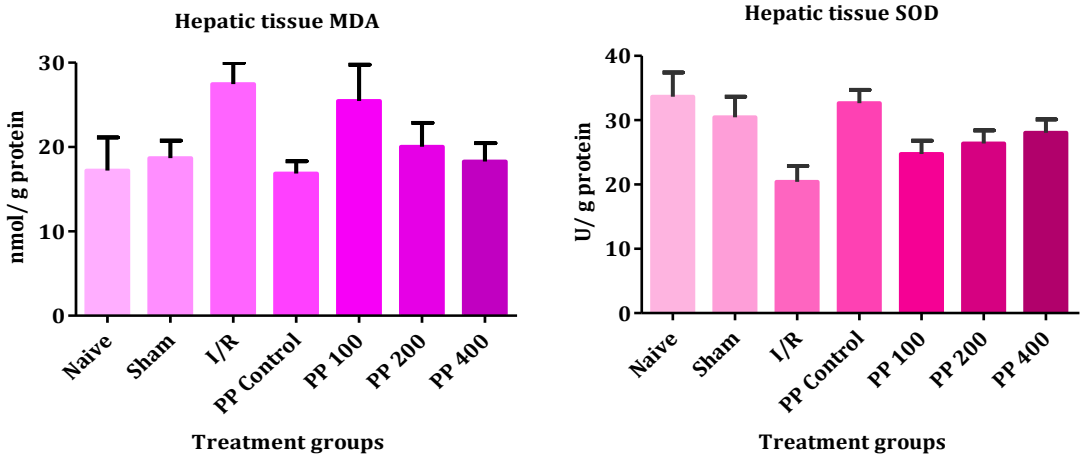
3(C)

Figure 3:- Level of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) in Serum

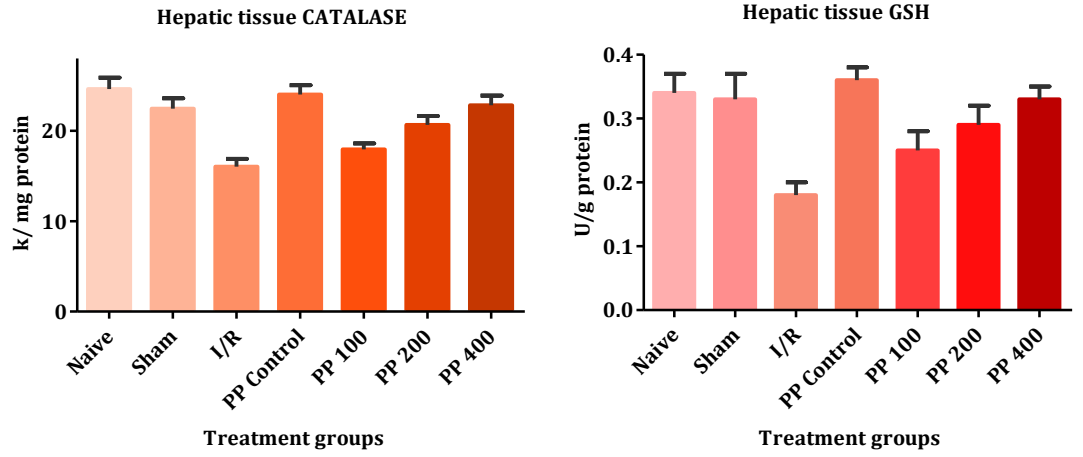


4 (A) 4(B)

Figure 4:-Level of bilirubin and lactate dehydrogenase (LDH) in Serum



5 (A) 5(B)



5 (C) 5(D)

Figure 5:- The renal tissue oxidant and antioxidant enzyme levels of the groups

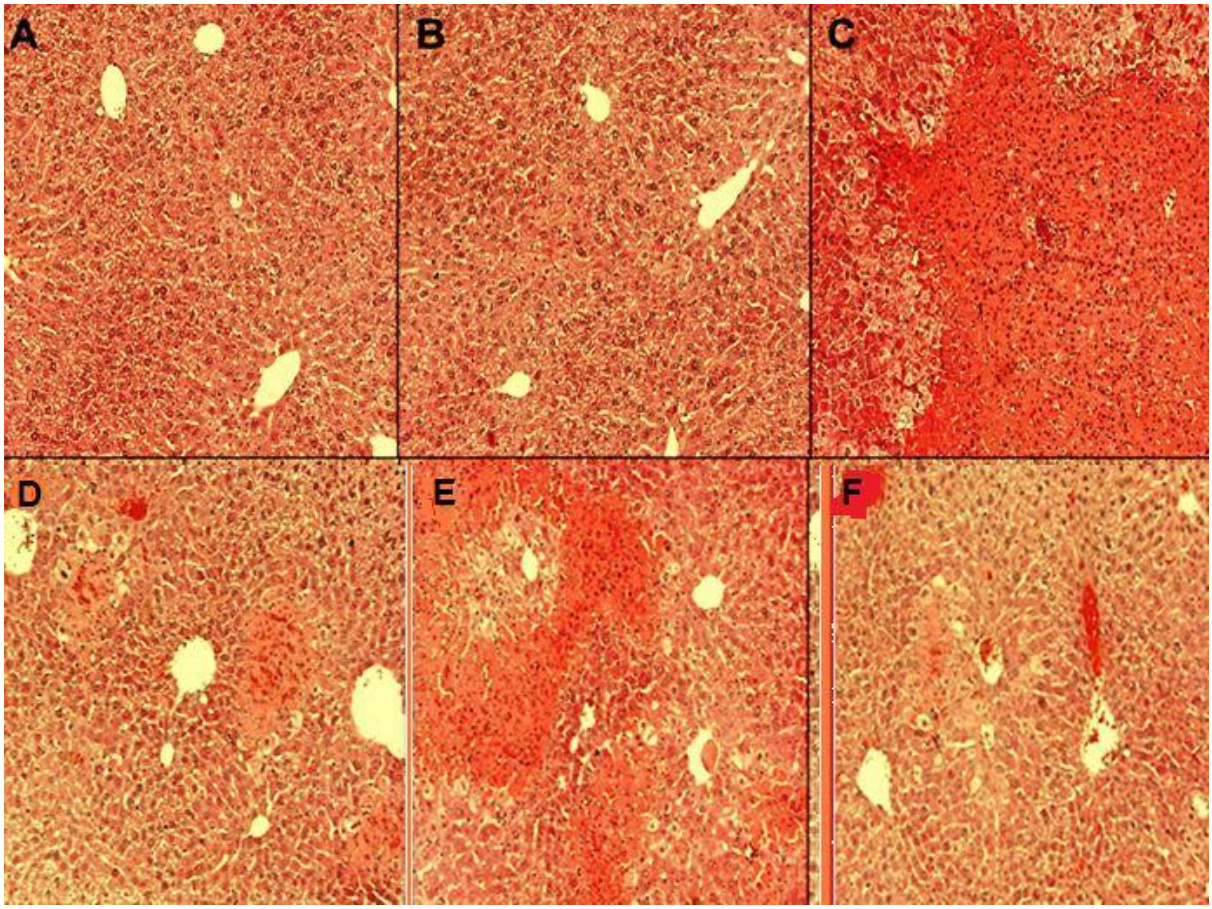


Figure 6: PP pre-treatment prevent histological lesions of the liver following reperfusion injury.
Hematoxylin-eosin-stained sections were used for histopathological evaluation of hepatic injuries.
The sections were examined at 400-fold magnification. Representative livers as follows:

- **A. Normal wistar albino rats**
- **B. Sham-operated rats**
- **C. Sixty minutes ischemia control group**
- **D. PP 100 treated group**
- **E. PP 200 treated group**
- **F. PP 400 treated group**

The liver MDA, which is an index of tissue lipid peroxidation, was found to be significantly higher in the I/R group (27.70±2.51 nmol/g), however treatment with PP decreased the elevated MDA level significantly back to the control level (18.28±2.17 nmol/g protein) (**Fig. 3A**). The levels of liver SOD lowered significantly after hepatic I/R compared with the sham group (SOD: 30.45±3.19 U/g protein vs 20.43±2.44 U/g protein, P = 0.000), after administration of *Pongamia pinnata* hydro-alcoholic leaf extract 100, 200 and 400mg/kg, SOD activity in liver was elevated (20.43±2.44 U/g protein vs 24.74±2.08 U/g protein, 26.38±2.05 U/g protein, 28.05±2.07 U/g protein, P = 0.706, P = 0.014, P = 0.014) (**Figure 3B**). **Figure 3C** shows the plasma Catalase levels of the hepatic injured rat treated with *Pongamia pinnata* hydro-alcoholic leaf extract 100, 200 and 400mg/kg. The administration of PP 100, PP 200 & PP 400, showed excellent effect in suppressing the hepatic injury. Among them, PP 400 showed the greatest inhibitory effect against hepatic reperfusion injury.

The endogenous antioxidant, GSH, level in the hepatic tissue was decreased significantly after I/R (0.18±0.02 U/g protein). On the other hand PP treatment significantly reversed this I/R-induced GSH reduction (0.33±0.02 U/g protein) (**Figure 3D**).

Severe ischemia of organs has metabolic, functional, and structural consequences and if it persists too long, it leads to death of the involved cells. Liver section of hydro-alcoholic leaf extract of *Pongamia pinnata* treated animal group clearly showed normal hepatic cells and central veins thereby confirming hepatoprotective activity. Flavonoids bind to subunit of DNA dependent RNA polymerase I, thus activating the enzyme. As a result, protein synthesis gets increased leading to regeneration and production of hepatocytes [50].

After 60 minutes of continuous ischemia, large confluent areas of tissue lysis with blood congestion in the sinusoids and leukocyte infiltrates were observed (**Figure 4C**). In the liver treated with PP 100, limited and focal areas of hepatocyte necrosis were also observed (**Figure 4D**), whereas the parenchyma was almost normal after PP 200 and PP 400 treatment respectively (**Figures 4E and 4F**).

Light microscopic investigation of the control group (either given saline or PP) revealed a regular morphology of liver parenchyma with intact hepatocytes and sinusoids (**Figure 4A**). In the I/R group, severe sinusoidal congestion and hemorrhage, dilation of central vein, subendothelial edema and degenerated hepatocytes with perinuclear vacuolization were observed (**Figure 4C**). In the PP treated I/R groups, histological analysis demonstrated a well-preserved liver parenchyma. Despite the mild sinusoidal dilatation and hemorrhage, which were in localized areas, the usual appearance of the central vein and hepatocytes was observed in most areas (**Figures 4D, 4E and 4F**).

This study had some limitations. Because of the study protocol, hepatic IR-injured rats were sacrificed just after reperfusion in order to observe the effects of PP. If we could have kept the rats alive, we could also have observed the long-term effects of PP in hepatic IR injury. We expect that further studies on the long-term effects of PP will increase the value of our positive findings. In addition, it is important to examine the possible ability of PP to reverse IR-induced damage in liver tissue and its effects on levels of antioxidant enzymes

CONCLUSION

Reactive oxygen metabolites are probably responsible for the tissue injury observed in hepatic tissues after ischaemia reperfusion. In conclusion, it was found that the treatment with hydro-alcoholic leaf extract of *Pongamia pinnata* (PP) increased the antioxidant ability and decreased oxygen free radicals in hepatic IR injury in rats. Also, evaluation of liver enzymes and histopathological findings of liver tissue indicated that PP had beneficial effects on the liver, thus it can be considered as a preventive treatment agent in hepatic IR injury. As this study does not contain information about the long-term results of PP treatment of hepatic IR injury, further experimental and clinical studies are needed so as to re-examine these views.

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