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Effects of *Pongamia Pinnata* Hydro-Alcoholic Leaf Extract, In I/R Induced Renal Failure in Rats

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ABSTRACT

Toxic oxygen radicals play a role in the pathogenesis of ischemia/reperfusion (I/R) injury in the kidney. The present study was designed to investigate the effects of *Pongamia pinnata* (PP) leaves, a plant rich in flavonoids in I/R induced renal failure in rats. Antioxidant activity of the hydro-alcoholic extract of *Pongamia pinnata* was determined by DPPH free radical, and Hydroxyl radical scavenging assay. The protective effect of *Pongamia pinnata* leaves against the damage inflicted by reactive oxygen species (ROS) during renal I/R was investigated in Wistar albino rats using histopathological and biochemical parameters. Animals were subjected to occlusion of both the renal pedicles for 45min followed by 24h of reperfusion. *Pongamia pinnata* hydro-alcoholic leaf extract (100 mg/kg, 200 mg/kg and 400 mg/kg, p.o.) was administered 8 weeks prior to ischemia. At the end of the reperfusion period, rats were sacrificed. Malondialdehyde (MDA), reduced glutathione (GSH) levels, catalase (CAT), and superoxide dismutase (SOD) activities were determined in renal tissue. Serum creatinine, Serum Cystatin C, serum oxaloacetate transaminase (SGOT), serum pyruvate transaminase (SGPT), blood urea nitrogen (BUN) and Lactate dehydrogenase (LDH) concentrations were measured for the evaluation of renal function. Ischemic control animals demonstrated severe deterioration of renal function, renal morphology and a significant renal oxidative stress. Pretreatment of animals with *Pongamia pinnata* hydro-alcoholic leaf extract markedly attenuated renal dysfunction, morphological alterations, reduced elevated malondialdehyde levels and restored the depleted renal antioxidant enzymes. The findings imply that ROS play a causal role in I/R induced renal injury and *Pongamia pinnata* leaves exert renoprotective effects probably by the radical scavenging and antioxidant activities.

Key words: *Pongamia pinnata*, hydro-alcoholic leaf extract, antioxidant, renal ischemia

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INTRODUCTION

Renal ischemia is a common cause of acute renal failure. Acute renal failure (ARF) is defined as a rapid loss in renal function. Traditionally, the mortality rate of people with intrinsic acute renal failure is 50%, and this figure has not improved over the past several decades. Renal I/R injury are common in several clinical situations, including renal transplantation and shock. Renal ischemia/reperfusion (I/R) injury is the most prominent cause of intrinsic acute renal failure, a primary contributor in delayed graft function, allograft nephropathy and post-transplant hypertension in transplant patients. The term "I/R injury" represents the total damage caused by the initial ischemic episode coupled to the subsequent reperfusion period in which blood flow is reinitiated into the tissue. Therefore, the damage induced by I/R cannot be limited to the ischemic stage, since reperfusion plays an essential role in the process. At present, there is a paucity of data regarding the mechanisms involved in I/R injury, but complex interactions of distinct signaling cascades are known to be involved, resulting in cellular, inflammatory and immune responses. It was demonstrated that reactive oxygen species (ROS) and reactive nitrogen species (RNS) increase in the areas of ischemia and reperfusion, which are responsible for renal damage. Inflammation also plays an important role in the pathogenesis of renal I/R injury, through leukocyte activation and expression of adhesion molecules and cytokines [1-6]. Free radicals and pro-inflammatory cytokines can damage cellular membrane and subcellular structures, which contain large amounts of phospholipids and protein, resulting in lipid peroxidation and sequentially structural and metabolic alterations, leading to cell apoptosis and necrosis.

Pathophysiology of renal I/R injury:

In ischemic acute renal failure, loss of renal blood supply results in tissue hypoxia and leads to a complex cascade of events resulting in renal injury [7, 8]. The series of pathophysiological events induced by I/R resulting in epithelial cell damage and renal function impairment is independent of total blood flow once it is launched. The destructive cascade of events results in a number of pathological changes in renal structure and function. The tissue changes caused by ischemia are well known. Upon depletion of energy rich phosphates (adenosine triphosphate (ATP)), the tissue concentration of their degradation products rises. The return of blood flow to ischemic tissue can result in recovery of normal function, but, paradoxically, the tissue becomes injured during the process of reperfusion [9]. The organ dysfunction that accompanies this condition is generally associated with increased microvascular permeability, interstitial edema, impaired

vasoregulation, inflammatory cell infiltration, and parenchymal cell dysfunction and necrosis [10]. I/R cause abnormalities in auto regulation of local blood flow leading to vasoconstriction and reduced blood flow to the kidney. Especially, blood flow to the outer medullary portion of the kidney markedly decreases following I/R. This reduction is attributed to increased production of vasoconstrictor compounds, like endothelin and TxA_2, by the damaged endothelium, as well as a concomitant reduction of vasodilators, such as nitric oxide and PGI_2. Together, these changes contribute to the overall endothelial dysfunction and irregularities in auto regulation following ischemic insult. In addition to the reduced blood flow, outer medullary congestion caused by I/R further augments hypoxia and lack of nutrition especially in the S3 segment of the proximal tubule and the thick ascending limb of Henle (TALH). This makes the tubular cells of TALH the primary target affected by ischemia, since they are already in an environment with low PO_2 levels even in the physiological conditions. Inadequate blood flow and outer medullary congestion further increase the ischemic injury, which is characterized by a series of structural deformations such as tubular cell membrane blabbing, loss of apical brush border and polarity, swelling of the cells due to increased Na^+ and Cl^- accumulation caused by diminished activity of Na^+ K^+ ATPase pumps, and detachment of tubular cells from the basal membrane. Inside the cell, multiple changes occur due to the ischemia, which include the formation of vacuoles, swelling of the mitochondria and the pyknosis of the nuclei. Subsequent formation of cell debris and proteinaceous substances block the tubular lumen, leading to cast formation. Functionally, the tubular obstruction results in the elevation of back pressures leading to fluid and electrolyte back leak and the formation of edema. The swelling of the tubular cells contributes to the tubular blockage and causes obstruction of vasa recta, which further exacerbates ischemic conditions in the medullary region of the kidney. All of these mentioned structural changes are, in fact, results of disturbances of cellular homeostasis which should receive special attention.

Free radical ablation for the treatment of reperfusion injury has found its first clinical application in the prevention of post ischemic tissue injury following organ transplantation [11, 12.] Thus, agents proposed to be useful in the clinical settings of I/R damage include free radical scavengers. A hydro-alcoholic extract of the leaves of *Pongamia pinnata* L., a mixture mainly composed of avone and chalcone derivatives such as Pongone, Galbone, Pongalabol, pongagallone A and B [13] has been shown to exhibit a variety of pharmacological actions. *Pongamia pinnata* hydro-alcoholic leaf extract has been reported to be a potent free radical scavenger and an antioxidant[14]. *Pongamia pinnata* normalized the levels of ammonia, urea and creatinine during hyperammonemic and nephrotoxic conditions [15].

Based on these reports, this study was designed to determine the possible protective effect of *Pongamia pinnata* leaves against oxidative stress during I/R injury of the kidney, by determining biochemical parameters and histological examination.

To the best of our knowledge, no scientific data regarding the anti-ischemic effect of *P. pinnata* leaves are available except in the treatise of Ayurvedic medicine. Therefore, the present study investigated the possible therapeutic effects of *Pongamia pinnata* hydro-alcoholic leaf extracts on renal I/R injury in rats.

MATERIALS AND METHODS

Animals

Adult Wistar albino rats weighing 200–220 g were given free access to normal rat diet and tap water and maintained in a temperature-controlled room with a 12:12-h light/dark cycle (lights on at 06:00 h). All procedures were performed in accordance with the approval of the Indian Animal Ethics Committee of Royal College of Pharmacy and Health Sciences (Approval No-07/IAEC/2011). The experiments were conducted in accordance with the Committee for the Purpose of Control and Supervision on Experiments on Animals guidelines [16].

Pongamia pinnata leaf extract

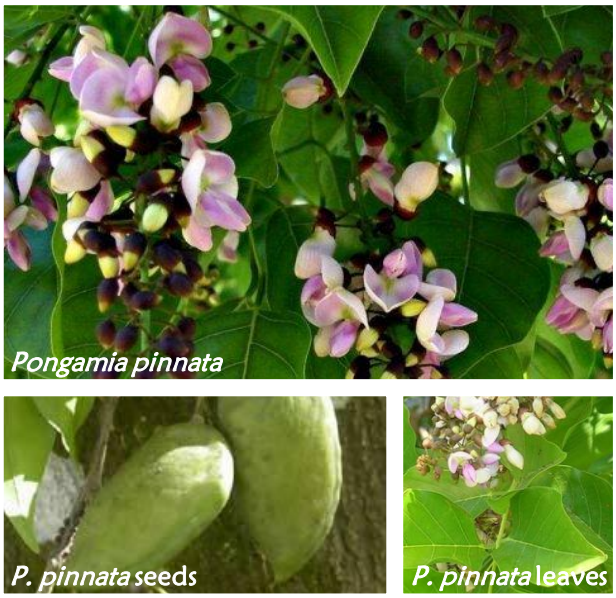


Figure 1:- *Pongamia pinnata* Linn. (With permission from B&T world seeds)

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. 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.

Phytochemical screening

The freshly prepared crude extract was qualitatively tested for the presence of chemical constituents. Phytochemical screening of the extract was performed using the following reagents and chemicals: Alkaloids with Dragendorff's reagent, flavonoids with the use of Mg and HCl; tannins with ferric chloride and potassium dichromate solutions and saponins with ability to produce stable foam and steroids with Libermann- Burchard reagent. Gum was tested using Molish reagent and concentrated sulfuric acid; reducing sugars with Benedict's reagent. These were identified by characteristic color changes using standard procedures.

Determination of antioxidant activity

a) DPPH radical scavenging assay [17]

To the Methanol solution of DPPH (1 mM) an equal volume of the extract dissolved in alcohol was added at various concentrations from 250 to 2000 µg/ml in a final volume of 1.0 ml. An equal amount of alcohol was added to the control. After 20 min, absorbance was recorded at 517 nm. Experiment was performed in triplicate

b) Hydroxyl Radical Scavenging Activity (Deoxyribose degradation assay) [18]

The hydroxyl radicals scavenging activity was measured with Fenton reaction. The reaction mixture contained 60 µl of 1 mM ferric chloride ($FeCl_3$) 90 µl of 1mM 1, 10-phenanthroline, 2. 4 ml of 0.2 M phosphate buffer (pH 7.8), 150 µl of 0.17 M hydrogen peroxide (H_2O_2), and 1.5 ml of PP extracts at various concentrations. After the addition of H_2O_2 all the solutions incubated at room temperature for 05 minutes and the absorbance of the mixture was measured at 532 nm with a spectrophotometer. The hydroxyl radicals scavenging activity was calculated using the following equation

$$\% \text{ Inhibition} = [(A_0 - A_1) / A_0 \times 100]$$

Where, $A_0$was the absorbance of the control (blank) and A1 was the absorbance in the presence different concentrations of the extract

Renal Ischemia/Reperfusion

The animals were anesthetized by intra peritoneal injection of sodium pentobarbital (30 mg/kg) before the surgical procedure and placed in a supine position. The abdominal region of rats were shaved and sterilized with povidone iodine solution. Following surgery preparation, the rats were

placed on a heated table to maintain constant temperature between 36° and 37°C. A midline incision was made, and the renal pedicles were isolated. After laparotomy and dissection of both renal pedicles, bilateral ischemia was induced by occluding the renal pedicles with atraumatic micro-vascular clamp for 45 min followed by followed by 3 h reperfusion. Occlusion was confirmed by a significantly pallid change of the kidney color and a return to a red shade upon reperfusion (During reperfusion, clamps were removed and the blood flow to the kidneys was reestablished with visual verification of blood return). After the surgical procedures, the midline incision was sutured followed by the local application of povidone iodine solution. At the end of the reperfusion period, the animals were euthanized by cervical dislocation.

Experimental Groups

Animals were divided into four groups consisting of six rats each:

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 renal ischemia, followed by reperfusion for 3 hours and served as untreated experimental control.

Group-IV: - **PP control**- Sham operated plus *Pongamia pinnata* control (400 mg/kg body wt. treatment up to 8 weeks).

Group-V: - **PP 100mg/kg + I/R**- Renal I/R plus *Pongamia pinnata* hydro-alcoholic leaf extract 100 mg/kg body wt. treatment up to 8 weeks.

Group-VI: - **PP 200mg/kg + I/R**- Renal I/R plus *Pongamia pinnata* hydro-alcoholic leaf extract 200 mg/kg body wt. up to 8 weeks.

Group-VII: - **PP 400mg/kg + I/R**- Renal I/R plus *Pongamia pinnata* hydro-alcoholic leaf extract 400 mg/kg body wt. up to 8 weeks.

Sham operated animals underwent the same surgical procedures except that the bilateral renal pedicles were not clamped. Twenty-four hours after reperfusion initiation, blood was drawn from the abdominal inferior cava vein immediately before induced death. All of the rats were sacrificed after 24 hours of reperfusion period and both kidneys were harvested for antioxidant and histological analyses. The blood was collected, and was spun at 1000 rpm for 15 min and serum samples were collected. The serum samples were stored at −20°C until serum level determinations were completed for blood urea nitrogen (BUN), serum creatinine, serum cystatin C, lactate dehydrogenase (LDH), serum oxaloacetate and pyruvate transaminases (SGOT &

SGPT). The renal tissue samples were cut in two and immediately placed in Bouin's solution for histological evaluation and for subsequent determination of malondialdehyde (MDA), reduced glutathione (GSH) levels, catalase and super oxide dismutase (SOD).

Kidney Function Study

Blood was collected from the rats by retro-orbital puncture at the time of sacrificing and was allowed to clot for 10 minutes at room temperature. Clots were centrifuged at 2500 rpm for 10 minutes to separate the serum. Serum creatinine, serum cystatin C, urea, serum oxaloacetate and pyruvate transaminases were measured by assay kits (Crest Biosystems, Bambolim Complex. Goa, India) using semiautomatic analyzer (3000 Evolution, Tulip Diagnostics (p) Ltd, Secunderabad, India)

Preparation of Tissue Homogenates

After sacrificing the animals, their kidneys were quickly removed, perfused immediately with ice cold hypertonic saline solution, and homogenized in chilled potassium chloride (1.17%) using a Potter Elvehjem homogenizer (Remi, Mumbai, India). The homogenate was centrifuged at 10500 g or 10500 rpm for 20 minutes at 4°C to get the post-mitochondrial supernatant, which was used to assay superoxide dismutase, catalase, reduced glutathione, and lipid peroxidation activity.

Assessment of Renal Function and Serum Lactate Dehydrogenase Levels

Serum samples were assayed for BUN, serum creatinine, serum LDH using standard diagnostic kits (Crest Biosystems, Bambolim Complex. Goa, India). Serum concentration of LDH was used as a marker of necrosis in tissues. Problems with creatinine (varying muscle mass, recent meat ingestion, etc.) have led to evaluation of alternative agents for estimation of renal function, one of these is Cystatin C, a ubiquitous protein secreted by most cells in the body (it is an inhibitor of cysteine protease). Cystatin C is freely filtered at the glomerulus. After filtration, Cystatin C is reabsorbed and catabolized by the tubular epithelial cells, with only small amounts excreted in the urine. Cystatin C levels are therefore measured not in the urine, but in the bloodstream. Cystatin C concentration was determined with a particle-enhanced nepherometric immunoassay. Renal function was assessed by serum creatinine, BUN concentration, Cystatin C and LDH levels

Determination of serum oxaloacetate and pyruvate transaminases (SGOT & SGPT)

Serum GOT and GPT were determined by the method of Reitman and Frankel[19]. Each substrate (0.5 ml) (2mM α-ketoglutarate and either 200 mM α L-Alanine or L-Aspartate was incubated for 5 min at 37°C in a water bath. Serum (0.1 ml) was then added and the volume was adjusted to 1.0 ml with sodium phosphate buffer. The reaction mixture was incubated for exactly 30 min and

60 min for GPT and GOT, respectively. Then to the reaction mixture, 0.5 ml of DNPH (1mM) was added and left for another 30 min at room temperature. Finally, the colour was developed by addition of 5.0 ml of NaOH (0.4 N) and product read at 505 nm.

Estimation of Antioxidant Enzymes

The antioxidant enzymes were estimated by well-established procedures. Nonprotein sulfhydryl, as a marker for reduced glutathione (GSH), was measured by the method of Jollow and colleagues, [20] and the yellow color developed by the reduction of Ellman's reagent by -SH groups of non-protein sulfhydryl was read at 412 nm. Catalase activity was assayed by the method of Claiborne, [21] and the rate of decomposition of H_2O_2 was followed at 240 nm. Superoxide dismutase (SOD) activity was assessed by the method of Kono[22]. Nitro blue tetrazolium reduction by superoxide anion to blue formazan was followed at 560 nm.

Estimation of Lipid Peroxidation

Malondialdehyde (MDA) content, a measure of lipid peroxidation, was assayed in the form of thiobarbituric acid-reacting substances [23]. In brief, the reaction mixture consisted of 0.2 mL of 8.1% sodium lauryl sulphate, 1.5 mL of 20% acetic acid solution adjusted to a pH of 3.5 with sodium hydroxide, and 1.5 mL of 0.8% aqueous solution of thiobarbituric acid added to 0.2 mL of 10% (w/v) of post-mitochondrial supernatant. The mixture was made up to 4.0 mL with distilled water and heated at 95°C for 60 minutes. After cooling with tap water, 1.0 mL of distilled water and 5.0 mL of the mixture of n-butanol: pyridine (15:1 v/v) was added and centrifuged. The organic layer was taken out and its absorbance was measured at 532 nm. The renal MDA content was expressed as nanomoles of MDA per milligram of protein. Tissue protein was estimated using the Biuret method of protein assay [24]

Histological Analysis

The kidneys was isolated immediately after sacrificing the rats and washed with ice-cold saline. Thereafter, it was fixed in a Bouin's solution and embedded in paraffin wax. Five micrometer-thick sections were cut, deparaffinized, hydrated, and stained with hematoxylin-eosin. The renal sections from all treatments were examined in blind fashion for tubular cell swelling, tubular dilatation, interstitial edema, and moderate to severe necrosis. A minimum of 10 fields for each kidney slide were examined and assigned for severity of changes using scores on a scale of mild (+), moderate (++), and severe (+++) damage.

Statistical *Analyses*

Results are presented as the mean ± SEM. All statistical analyses were performed using Graph Pad Prism Software program (version 5) [25]. Results are expressed as means ± SEM for six rats in

the each group and statistical significant differences between mean values were determined by one way analysis of variance (ANOVA) followed by the Tukey's test for multiple comparisons for antioxidant study. 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

ARF, which is increasing in prevalence, is associated with high mortality in humans[26]. Ischemic ARF frequently occurs in hospitalized patients. The pathophysiology after renal I/R injury is not well established. The mechanisms are most likely multifactorial and interdependent, involving hypoxia, inflammatory responses and free radical damage[27]. The transient discontinuation of renal blood supply is encountered in many clinical situations such as kidney transplantation, partial nephrectomy, renal artery angioplasty, aortic aneurysm surgery, and elective urological operations[28, 29]. This transient discontinuation causes renal I/R injury which results in decreased glomerular filtration and renal blood flow and increased urine output is characterized by natriuresis and impaired concentrating ability. Acute renal failure produced by ischemia and reflow is histopathologically characterized by extensive tubular damage, tubular cell necrosis, glomerular injury, and signs of tubular obstruction with cell debris[30, 31]. Much of this tubular and glomerular dysfunction has been postulated to occur during the reperfusion period following anoxia, and generation of ROS has been postulated as one of the major factors contributing to this reperfusion injury. In renal I/R injury, ROS are capable of reacting with lipids leading to lipid peroxidation of biological membranes, which in turn impacts enzymatic processes, such as ion pump activity, inhibiting transcription and repair of DNA. If lipid peroxidation remains unchecked, it will ultimately result in cell death[32, 33]. Recently, studies have focused on the role of ROS in I/R injury, and oxidative stress has been implicated in the pathogenesis of ischemic ARF[34, 35]. A number of drugs or chemicals have been used to prevent I/R kidney injury, vitamin E 36, montelukast[37], angiotensin-converting enzyme inhibitor [38], cyclosporine [39] and leflunomide [40] and an endothelin-A receptor antagonist 4[1] have been found to be effective in the prevention of lipid peroxidation and general damage. Ischemia is also a stimulus for the release of chemotactic factors for neutrophils. During the reperfusion phase, renal tissue is further destroyed by the release of free radicals and toxic enzymes by neutrophils that have adhered to and traversed the endothelium [35]

Phytochemical screening of the extracts revealed the presence of alkaloids, flavonoids, saponins, steroids and tannins. Several reports indicate that *Pongamia pinnata* may exert antioxidant effects (Table-1)

Table 1:- Phytochemical analysis of *Pongamia pinnata* leaves

Sr Num.	Phytoconstituents	Methanol	Aqueous
1	Alkaloid	+ ++ +	+ - + +
2	Carbohydrate	+ - -	+ - -
3	Glycoside (cardiac glycoside)	+	+
4	Tanins and phenolics	+ + -	+ +
5	Protein & amino acid	- - -	- - -
6	Gum and mucilage	+++	+ + +
7	Flavones & flavonoids	++	+ +
8	Saponins	+	+
9	Steroids & sterols	+	+
10	Triterpenoids	+	+

+ = presence, – = absence

In our earlier study, we showed that *Pongamia pinnata* can provide protection against injury caused by hydrogen peroxide[14] and similar report has been generated in this study by the Hydroxyl Radical Scavenging Activity (Hydroxyl radical is the most reactive oxygen species among all reactive oxygen species owing to its strong ability to react with various biomolecules). *Pongamia pinnata* can reduce ROS activity and protects vascular smooth muscle cells from injury (Table-2; Figure-2). The statement is quite justified as this study shows that the hydro-alcoholic leaf extract of *Pongamia pinnata* have the proton-donating ability and can serve as free radical inhibitor or scavenger, acting possibly as primary antioxidant by the results obtained from DPPH radical scavenging assay (Table-3; Figure-3). In agreement with that study, the results of this study confirm that *Pongamia pinnata* protects kidneys against I/R injury; however, the novel findings from this study are its effects on oxidative stress, the antioxidant system

Table 2:- Study on DPPH scavenging activity in *Pongamia pinnata* leaves

Concentration (µg/ml)	Ascorbic acid (% scavenging activity)	*P. pinnata* (% scavenging activity)
0	0	0
250	90.2±0.004	43 ±0.005
500	91 ±0.009	68.7± 0.004
1000	92.4 ±0.005	71±0.003
2000	93±0.007	76±0.007

Values are mean ± SEM of three separate experiments; Statistical comparison has been done by student‘s t- test

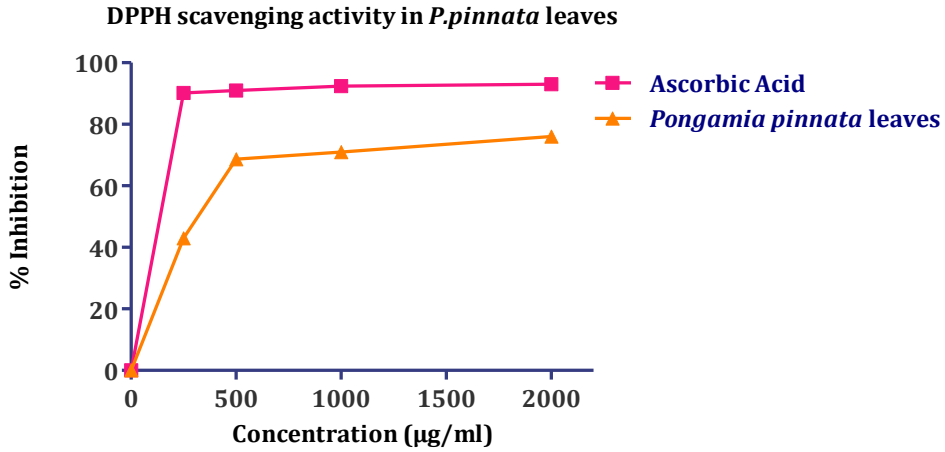


Figure 2:- Study on DPPH scavenging activity in *Pongamia pinnata* leaves at 517 nm

Table 3:- Study on Hydroxyl Radical Scavenging Activity in *Pongamia pinnata* leaves

Concentration (µg/ml)	Ascorbic acid (% scavenging activity)	*P. pinnata* (% scavenging activity)
0	0	0
250	85.1±0.012	40.57 ± 0.021
500	87.4±0.019	55.20 ± 0.023
1000	88.1±0.018	61.45 ± 0.025
2000	89.7±0.021	69.54 ± 0.021

Values are mean ± SEM of three separate experiments; Statistical comparison has been done by student‘s t- test

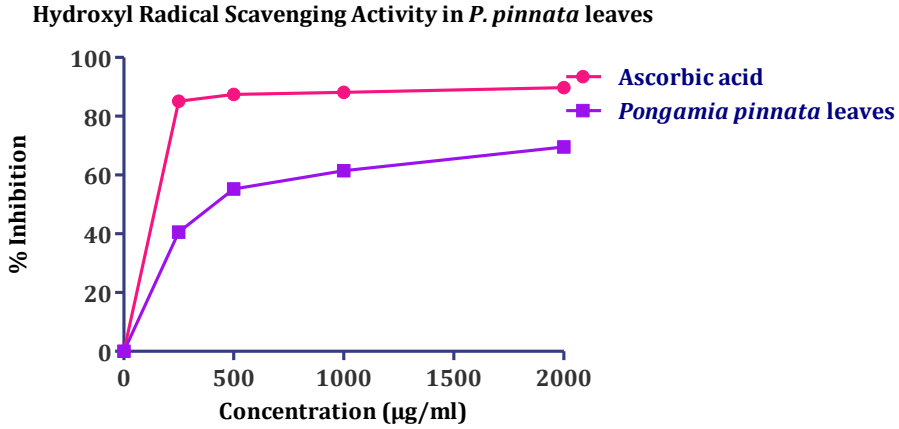


Figure 3:- Study on Hydroxyl Radical Scavenging Activity in *Pongamia pinnata* leaves at 532 nm

Kidney Function Study

Animals subjected to renal ischemia exhibited significant increases in serum urea, creatinine cystatin C and lactate dehydrogenase levels compared with the naive, sham and PP treated groups, suggesting a significant decrease in glomerular function due to renal I/R injury (P

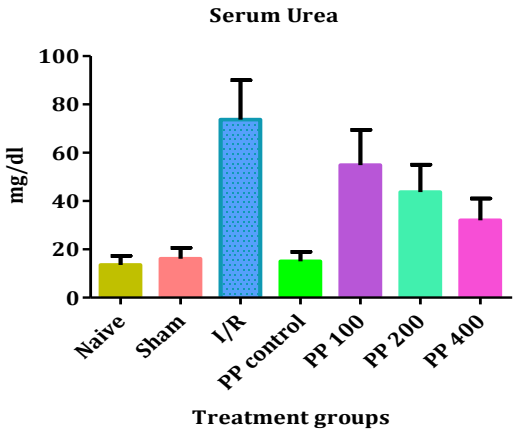
< 0.01). However, the rats treated with PP before I/R had significantly lower levels of serum urea, creatinine, cystatin C and lactate dehydrogenase compared with the I/R group (P<0.05) (Table 4)

Table 4:-Level of BUN, Creatinine, Cystatin C and LDH in Serum

Group	Treatment	BUN (mg/dl) (Mean ± SEM)	Creatinine (mg/dl) (Mean ± SEM)	Cystatin C (mg/dL) (Mean ± SEM)	LDH ((U/l) (Mean± SEM)
Group I	Naive	13.56±3.69	0.58±0.09	0.13±0.02	358.05±5.04
Group II	Sham -operated control	16.12±4.51	0.64±0.10	0.15±0.03	356.03±4.56
Group III	Ischemia/reperfusion (I/R)	73.67±16.4[a]	1.89±0.41[a]	0.38±0.11[a]	586.27±6.31[a]
Group IV	PP control	15.05±3.92	0.59±0.14	0.16±0.06	352.14±3.64
Group V	PP 100mg/kg + I/R	54.87±14.56[b]	1.48±0.39[b]	0.30±0.11[b]	436.13±2.54[b]
Group VI	PP 200mg/kg + I/R	43.73±11.28	1.21±0.32	0.25±0.12	408.96±2.35
Group VII	PP 400mg/kg + I/R	32.07±8.94	0.98±0.17	0.19±0.17	367.62±2.85

[a] P < 0.01 vs. naive, sham and PP groups.
[b] P < 0.05 vs. I/R
I/R, ischemia/reperfusion; PP, *Pongamia pinnata;* BUN, Blood Urea Nitrogen; LDH, Lactate dehydrogenase

Renal I/R-induced oxidative stress was associated with impaired kidney function, leading to a marked increase in serum creatinine, urea, cystatin C and LDH levels. Increased serum LDH concentration is usually a good marker of necrosis in tissues and is regarded as an index of generalized tissue damage. In the present study, serum creatinine, Cystatin C, BUN, and serum LDH levels were significantly increased with the application of I/R to the kidney. However, pretreatment with PP resulted in a decrease in serum creatinine, Cystatin C, BUN, and LDH levels was reduced by Pongamia pinnata treatment (p<0.001) most likely as a result of Reno protective effect by inhibition of ROS (Figure 4).



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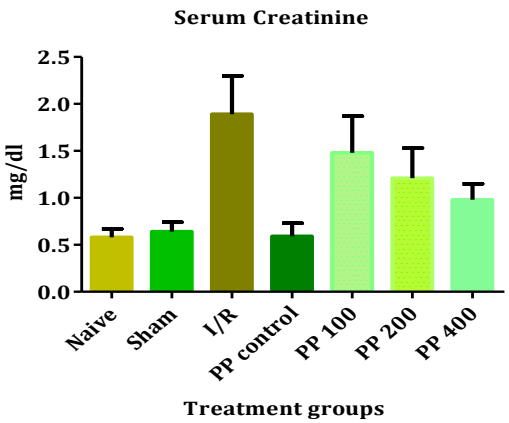

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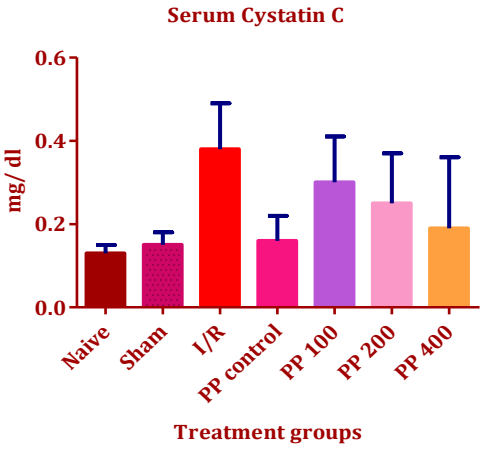
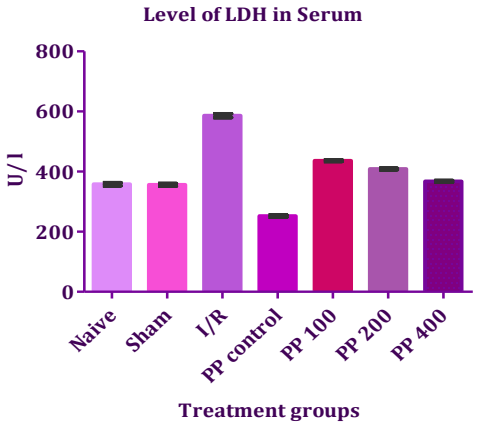
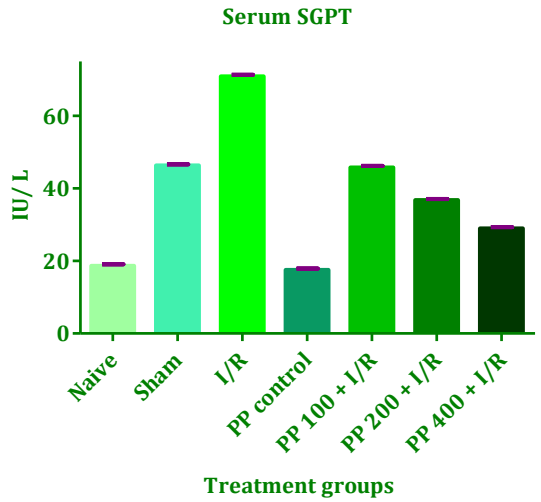


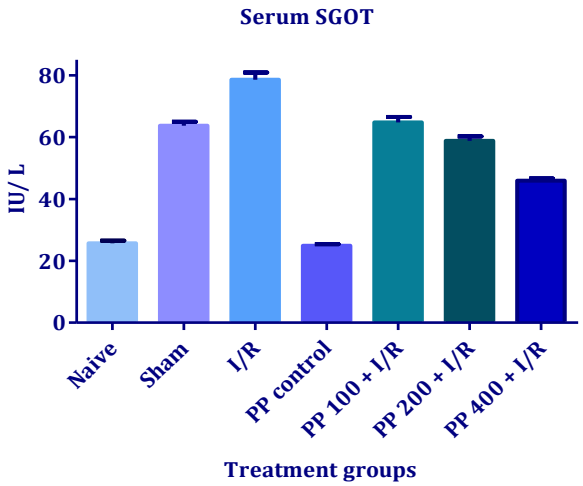


4 (C) 4 (D)

Figure 4:- Level of BUN, Creatinine, Cystatin C and LDH in Serum

Increased serum levels of SGPT and SGOT confirmed renal reperfusion injury (Figure 5). The SGPT and SGOT are not only specific to the liver but also found in other organs such as kidney and smooth muscle. These enzymes are especially elevated after renal tubular injury in rat [43]. Because SGPT and SGOT are present within the proximal tubulus and are regarded as a nonspecific marker of extensive cellular damage [44], we have used serum SGPT and SGOT in this study as markers of reperfusion injury (Table-5). Furthermore, evidence of tubular injury was supported by the histopathological scoring of renal injury as there was marked tubular injury (Table-9)





5 (A) **5 (B)**

Figure 5:- Level of serum pyruvate transaminase and serum oxaloacetate transaminase

Table 5:-Level of serum pyruvate transaminase and serum oxaloacetate transaminase

Group	Treatment	SGPT (IU/L) (Mean ± SEM)	SGOT (IU/L) (Mean ± SEM)
Group I	Naive	18.66±0.454	25.67±0.865
Group II	Sham	46.35±0.287	63.74±1.255
Group III	Ischemia/reperfusion (I/R)	70.92±0.410[a]	78.62±2.342[a]
Group IV	PP control	17.56±0.355	24.92±0.458
Group V	PP 100mg/kg + I/R	45.78±0.464[b]	64.78±1.746[b]
Group VI	PP 200mg/kg + I/R	36.75±0.328	58.83±0.448
Group VII	PP 400mg/kg + I/R	28.94±0.419	45.97±0.655

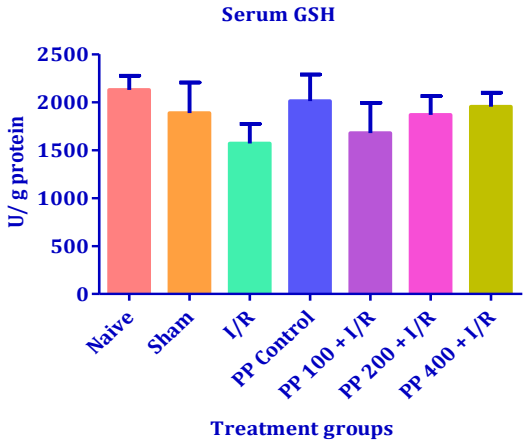
[a]P < 0.01 vs. naive, sham and PP groups.
[b]P < 0.05 vs. I/R + PP.
SGPT, Serum pyruvate transaminase; SGOT, Serum oxaloacetate transaminase

After renal I/R, the serum enzymatic activities of SOD and GSH significantly decreased in the I/R group. This reduction was significantly improved by treatment with PP in the I/R. PP group (P<0.01 and P< 0.05, respectively) (Table 6).

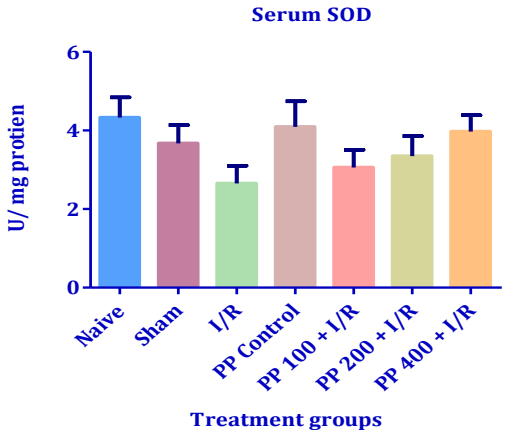
Table 6:- Serum SOD and GSH of the groups

Group	Treatment	GSH (U/g protein) (Mean ± SEM)	SOD (U/mg protein) (Mean ± SEM)
Group I	Naive	2128±147	4.33±0.51
Group II	Sham -operated control	1886±319	3.67±0.47
Group III	Ischemia/reperfusion (I/R)	1569±206[a, b]	2.65±0.45[a,b]
Group IV	PP control	2012±276	4.09±0.65
Group V	PP 100mg/kg + I/R	1678±315	3.06±0.45
Group VI	PP 200mg/kg + I/R	1867±195	3.35±0.51
Group VII	PP 400mg/kg + I/R	1953±147	3.97±0.42

a P < 0.01 vs. naive, sham and PP groups.
b P < 0.05 vs. I/R + PP
GSH, Reduced Glutathione; SOD, Super Oxide Dismutase



6 (A)



6 (B)

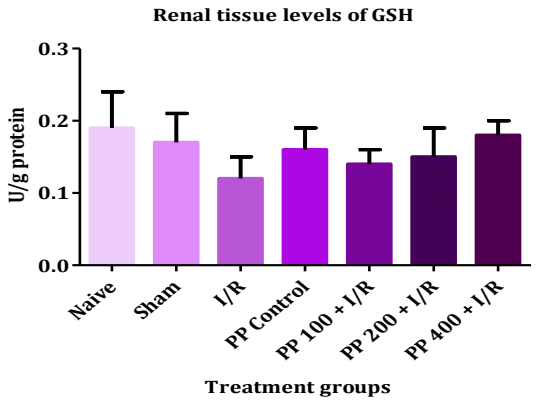
Figure 6:- Serum GSH and SOD

***Pongamia pinnata* (PP)-Induced Changes in the Antioxidant and Oxidant Pool in Rat Kidney Tissue**

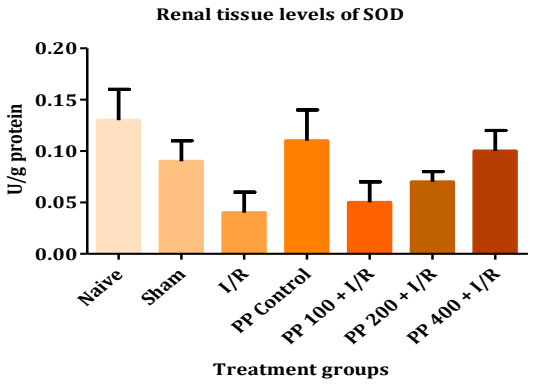
Oxidative stress can result from increased ROS production and/or from decreased ROS scavenging capability. The cells natural protective system against the devastating actions of ROS includes protective enzymes, including SOD, CAT and GSH. SOD, CAT, GSH and other antioxidants are believed to play important roles in reversing the pathological damage caused by I/R injury [42].

More studies have now established the ability of *Pongamia pinnata* to inhibit free radical generation and act as free radical scavengers and antioxidants [45].

The I/R process also resulted in significant decreases in tissue enzymatic activity of CAT, when compared with rats treated with PP before the process ($P<0.01$). Additionally, tissue SOD and GSH activities decreased after I/R and GSH activity improved with PP treatment; however, the difference in SOD was not statistically significant between the I/R and I/R+PP groups (Table 7).



7 (A)



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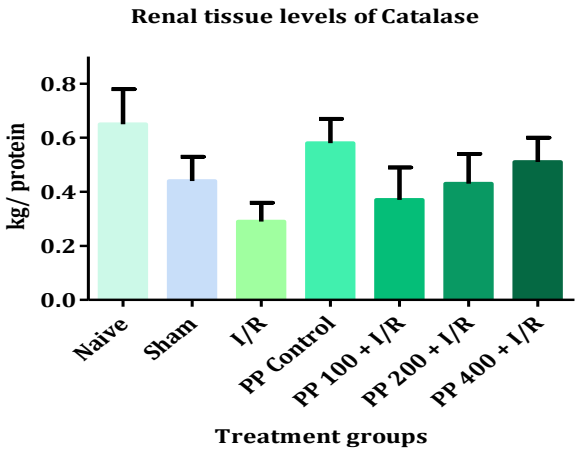

7 (C)

Figure 7:- The renal tissue oxidant and antioxidant enzyme levels of the groups

Table 7:- The renal tissue oxidant and antioxidant enzyme levels of the groups

Group	Treatment	GSH (U/g protein) (Mean ± SEM)	SOD (U/g protein) (Mean ± SEM)	CATALASE (kg/ protein) (Mean ± SEM)
Group I	Naive	0.19±0.05	0.13±0.03	0.65±0.13
Group II	Sham -operated control	0.17±0.04	0.09±0.02	0.44±0.09
Group III	Ischemia/reperfusion (I/R)	0.12±0.03 [a, b]	0.04±0.02[a,b]	0.29±0.07[a, b]
Group IV	PP control	0.16±0.03	0.11±0.03	0.58±0.09
Group V	PP 100mg/kg + I/R	0.14±0.02	0.05±0.02	0.37±0.12
Group VI	PP 200mg/kg + I/R	0.15±0.04	0.07±0.01	0.43±0.11
Group VII	PP 400mg/kg + I/R	0.18±0.02	0.10±0.02	0.51±0.09

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

In addition, renal ischemia and reperfusion produced a significant increase in MDA in serum and tissue compared with the naive, sham-operated and PP groups. Treatment with PP resulted in a significant reduction in MDA (P<0.05 for tissue and serum levels) (Table 8).

Table 8:- Lipid Peroxidation Activity

Group	Treatment	MDA *(In Serum)* (Mean± SEM)	MDA *(In Renal tissue)* (Mean± SEM)
Group I	Naive	0.21±0.03	2.58±0.47
Group II	Sham -operated control	0.33±0.07	3.46±0.60
Group III	Ischemia/reperfusion (I/R)	0.54±0.11[a,b]	4.79±0.72[a,b]
Group IV	PP control	0.28±0.07	3.17±0.53
Group V	PP 100mg/kg + I/R	0.46±0.08	4.04±0.67
Group VI	PP 200mg/kg + I/R	0.35±0.06	3.68±0.53
Group VII	PP 400mg/kg + I/R	0.31±0.04	3.24±0.56

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

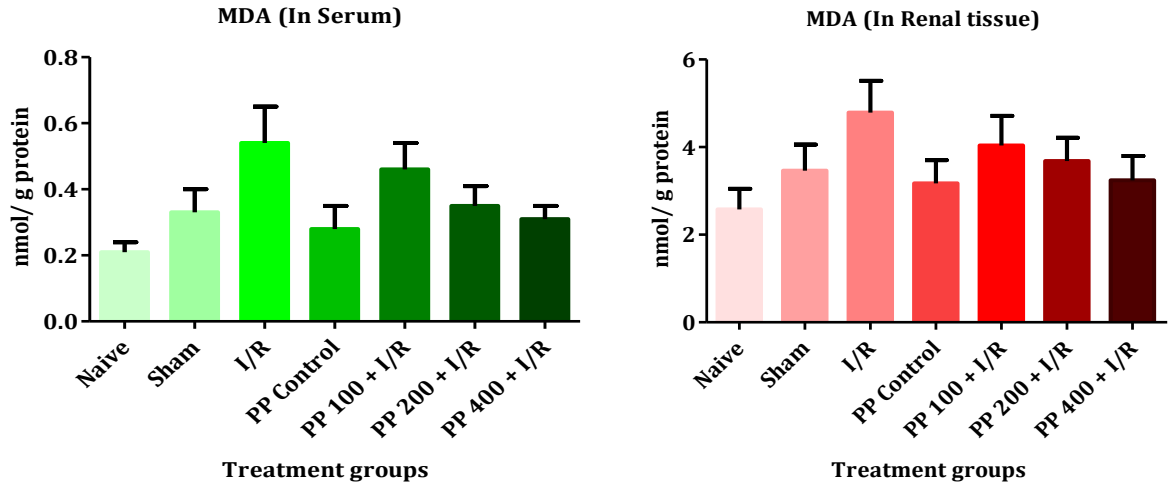

Figure 8:- Lipid Peroxidation Activity

Although the activity of antioxidant enzymes (CAT, SOD and GSH) decreased in the I/R group compared with the naive, sham and PP groups, pretreatment with PP improved the levels of CAT, SOD and GSH compared with the I/R group. There were statistically significant increases in renal tissue MDA levels in the I/R group compared with the naive, sham and PP groups. Pretreatment with PP significantly improved these levels compared with the I/R group

In our study, animals subjected to renal I/R demonstrated an increase in the renal MDA and attenuated the antioxidant enzymes pool (Figures 8, 6 and 7). Lipid peroxidation and antioxidant enzymes are important indexes of oxidant injury [46]. This study shows that pretreatment with PP protects rat kidneys against I/R injury, as demonstrated by improved renal function, normalized renal histopathology, improvement in antioxidant enzyme status (increased levels of GSH and activity of SOD and CAT), reduced oxidation products (reduced MDA levels). Our study also showed that tissue SOD and GSH activities and serum CAT, SOD and GSH levels were significantly decreased in the I/R group, when compared with the naive group (Figures 6). Furthermore, PP prevented depletion of SOD, GSH and CAT activity after I/R. Demonstrations of lipid peroxidation as index for oxidative damage may help us better understand the effects of ROS on the cellular components [47]

Histopathological Analysis

The histopathological changes were graded and summarized in the Table 9.

Table: - 9 Morphological changes assessed by Histopathological examination of kidneys of Rats Exposed to Ischemia/Reperfusion (I/R) Injury With and Without Preceded Treatment with *Pongamia pinnata* (PP) and Sham Operation

Rat group	Tubular cell swelling	Interstitial edema	Tubular dilatation	Necrosis of Epithelium
Naive	-	-	-	-
Sham	-	-	-	-
IR	+	+	+	+
IR + PP 100mg/kg	+	+	-	+
IR + PP 200mg/kg	+	+	-	-
IR + PP 400mg/kg	-	-	-	-

*The minus sign indicates no morphological change and plus sign indicates some change

The sham control group of rats did not show any morphological changes. By contrast, the kidneys of the rats with I/R only showed tubular cell swelling, interstitial edema, tubular dilatation, and moderate to severe necrosis, whereas, *Pongamia pinnata* preserved the normal morphology of the kidney (Figure 9).

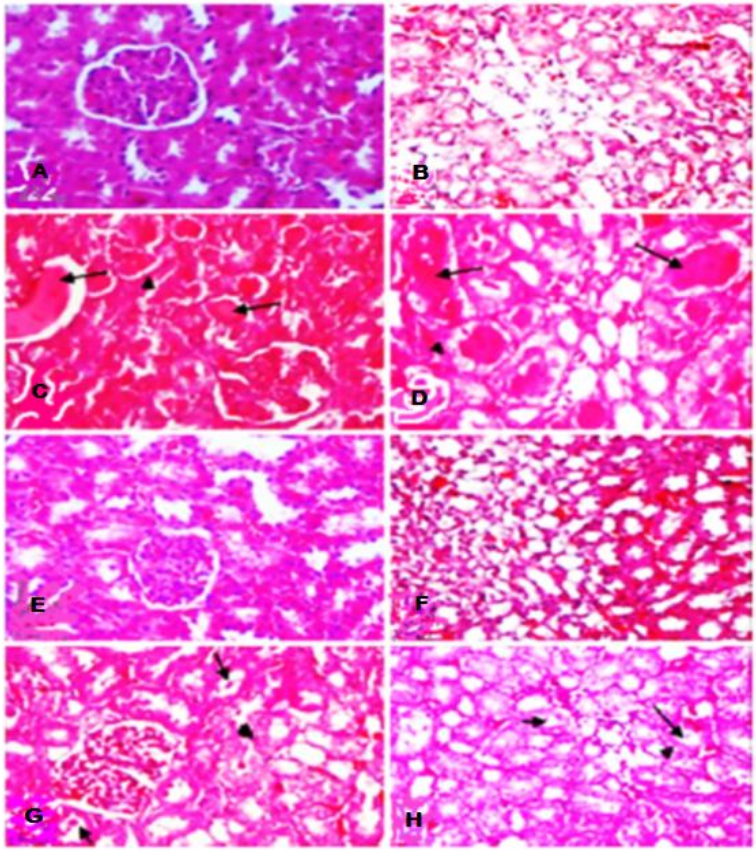

Figure 9:- Light photomicrograph of rat's kidneys (hematoxylin-eosin stained sections × 400)

A- Kidney sections from naive

B - Sham-operated group showing normal corpuscles and tubules

C, D - Ischemia-reperfusion group showing marked tubular epithelial degeneration (arrowhead) and intraluminal tubular eosinophilic casts (thin arrows)

E - Group received *Pongamia pinnata*, showing normal renal corpuscles and tubular cells

F, G & H - Ischemia-reperfusion group treated with *Pongamia pinnata* (100, 200 and 400 mg/kg body wt. respectively) showing marked improvement of tubular epithelium (arrowheads) and decline of luminal casts (arrows)

We studied the effects of PP on ischemia-induced tissue damage. Representative examples of these experiments are presented in Figure 9. I/R injury resulted in severe tissue damage in the S3 segment of the proximal tubules, and the outer medullary stripe exhibited loss of the brush border and detachment of epithelial cells from the basement membrane. This effect resulted in naked basement membranes (Figure 9C and Figure 9D) and tubular obstruction. In the naive group, renal tissue sections had a normal morphology (Figure 9A). No significant morphological

damage was observed in the PP-only and sham operated groups; most tubules were intact and demonstrated normal brush borders (Figures 9B and 9E). Renal sections obtained from rats treated with PP before the I/R process demonstrated a marked reduction in the histological features of renal injury, mainly consisting of focal and mild tubular necrosis (Figures 9F, 9G and 9H).

CONCLUSON

The results of our study allow us to conclude that PP protects kidneys against I/R injury. Administration of *Pongamia pinnata* hydro-alcoholic leaf extract attenuates the increase in markers of renal injury and oxidative damage. These findings provide a basis for the development of novel therapeutic strategies. *Pongamia pinnata* leaves have antioxidant properties and may be used in human studies in the future.

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