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Hepatoprotective Effects of Crude Extracts of *Pongamia pinnata* in Alloxan Induced Diabetic Albino Wistar Rats

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ABSTRACT

Diabetes mellitus is one of the most common endocrine disorders accompanied with many metabolic syndromes. Use of herbal medicines has always been an option to treat a great number of diseases such as cancer diabetes and its complications. The aim of the present study is to explore the hepatoprotective effects of *Pongamia pinnata* stem extracts in alloxan induced diabetic wistar albino rats. Thirty six Swiss wistar albino male rats were divided in 6 groups (6 per group weighing 250-300 g) such as NC, DC, TD1, TD2, SD and VC. Diabetes was induced in rats by injecting intraperitoneally alloxan monohydrate at a dose of 150 mg kg^{-1} b.wt. in five groups except Normal Control (NC). Aqueous and alcoholic extracts of *Pongamia pinnata* stem at a dose of 28 mg kg^{-1} b.wt. were given orally in diabetic rats, daily for three months (TD1, TD2). In diabetic rats (DC), the serum glucose, SGOT, SGPT and bilirubin levels were significantly increased whereas total protein and albumin levels were decreased in comparison with the control group. Significantly recovery was observed in all the parameters with aqueous (TD1) and alcoholic (TD2) extract of *Pongamia pinnata*. Histopathological observations were also in coordination with these results. The results are obtained after analyzing the observations of three such separate experiments. There was no change in Vehicle Control (VC) rats. The results suggested that crude extracts of *Pongamia pinnata* stem possesses antihyperglycemic and hepatoprotective activity in alloxan induced diabetic rats. Alcoholic extract proved to be more effective than aqueous extract.

Key words: *Pongamia pinnata*, diabetes mellitus, SGOT, SGPT, total protein, albumin, bilirubin

INTRODUCTION

Diabetes Mellitus (DM) is a chronic disease caused by inherited or acquired deficiency in insulin secretion and by decreased responsiveness of the organs to secreted insulin (Matsui *et al*., 2007). The liver plays a major role in the regulation of carbohydrate metabolism, as it uses glucose as a fuel, it has the capability to store glucose as glycogen and also synthesize glucose from non-carbohydrate sources. This key function of liver makes it vulnerable to diseases in subjects with metabolic disorders, particularly diabetes (Rigobelo, 2011). Nearly 70-80% of the diabetic subjects have been reported to have hepatic fat accumulation, referred to as is nonalcoholic fatty liver (NAFL) (Gupte *et al*., 2004). NAFL leads to nonalcoholic steatohepatitis (NASH), a progressive fibrotic disease which can result in cirrhosis or liver related death (Wong *et al*., 2004; McCullough,

2004). As diabetes aggravates and β-cell function deteriorates the insulin level begins to fall below the body's requirements and causes prolonged and more severe hyperglycemia (Gerich, 2003). Furthermore proof for the association of liver disease with diabetes comes from the Insulin Resistance Atherosclerosis Study (IRAS) which showed that liver function markers like the Serum Glutamate Oxaloacetate Transaminase (SGOT) and Serum Glutamate Pyruvate Transaminase (SGPT) are predictors of incident diabetes (Hanley *et al*., 2004).

Though different types of oral hypoglycemic agents are available for the treatment of diabetes mellitus, there is increasing demand by patients to use antidiabetic natural products because of the undesirable side effects of the existing drugs (Zhou *et al*., 2012) after all, many of the currently available drugs have been derived directly or indirectly from plants (Patel *et al*., 2012). In addition, herbal remedies continue to be more accessible and affordable than conventional drugs and represent the first line of treatment available for many of the world's population (Okpara *et al*., 2007). The attributed antihyperglycemic effects of these plants is due to their ability to restore the function of pancreatic tissues by causing an increase in insulin output or inhibit the intestinal absorption of glucose or to the facilitation of metabolites in insulin dependent processes. Hence, treatment with herbal drugs has an effect on protecting β-cells and smoothing out fluctuation in glucose levels. Most of these plants have been found to contain substances like glycosides, alkaloids, terpenoids and flavonoids etc. that are frequently implicated as having antidiabetic effects (Loew and Kaszkin, 2002).

Animal research regarding diabetes has made significant progress since the late 1800s until present, with patients benefiting from the original discovery of insulin in dogs and the continuous assessment of the efficacy and safety of other therapies. The experience gained by the study of animal models has been enormous not only in the field of preclinical testing prior to clinical trials but also in that of genes predisposing to diabetes (Potenza *et al*., 2011). As diabetes in humans remains a disease with increased morbidity and mortality in conjunction with its multi-organ complications, its investigation using animal models remains a foremost area of research. Several reports directly or indirectly indicate that alloxan affects the membrane potential and ion channels in β-cells (Sharma *et al*., 2013a). Presently there are not much therapeutic options for nonalcoholic fatty liver except correction of obesity with hypocaloric diets and physical exercise and controlling hyperglycemia with diet, insulin or oral hypoglycemic agents (Medina *et al*., 2004). Any therapeutic intervention which can target fat accumulation and ameliorate hepatic histology, would be of great benefit. *Pongamia pinnata* has prominent free radical scavenging property so it may prove as a very good medicinal plant. In the current literature there is paucity of data concerning the effect of *Pongamia pinnata* on the lipid metabolism and hepatic enzymes which are severely altered in Diabetes. The aim of the present study is to investigate the hepatoprotective effect of Pongamia pinnata stem aqueous and alcoholic extract using various biochemical parameters from serum and liver histopathology compared with standard drug metformin.

MATERIALS AND METHODS

Animals: Healthy male albino wistar rats, weighing approximately 250-300 g were used in the pharmacological studies. Before and during the experiment the animals were maintained in well-ventilated room at room temperature with natural day-night cycle in polypropylene cages lined with husk in standard environmental conditions (temperature 22±2°C, relative humidity 55±10% and 12:12 (light: dark cycle)). The rats were feed on a standard pellet diet *ad labium* and had free access to water. The experiments were performed after approval of the protocol by the (CPCSEA Regd. No. 704).

Preparation of plant material: The stems of *Pongamia pinnata* were collected from local place (Mulund, Mumbai). The plant was authenticated by (Blatter herbarium, St. Xavier's College, Mumbai). The stems were washed with distilled water, dried completely under the mild sun and crushed with electrical grinder in to coarse powder. At a time 50 g of powder with 250 mL ethanol or distilled water were used to prepare extracts using Soxhlet apparatus (6-7 cycles). Each time the extracts was dried with vacuum evaporator and stored at 4°C to for further use. The yield was 3.7% for alcoholic extract and 4.6% for aqueous extract.

Chemicals: All chemicals were obtained from the following sources: Alloxan was purchased from the sigma chemical (Batch no-G204207), Mumbai. Commercially available kits of Aggape were used for chemical analyses such as glucose, SGOT, SGPT and bilirubin. Analytical grade ethanol was purchased from Merck Company (India).

Induction of hyperglycemia with alloxan: The selected rats were weighed, marked for individual identification and made to fast overnight. The diabetes was induced by a single intraperitoneal injection of 150 mg kg^{-1} monohydrated Alloxan dissolved in 0.9% sterile saline in all the experimental rats except in NC group (Szkudelski, 2001). Blood glucose level of these mice were estimated 72 h after Alloxan administration, diabetes was confirmed by blood samples collected from the retro orbital sinus puncture method using a blood glucometer (Sugar scan). Animals with blood glucose level equal or more than 250 mg dL^{-1} were declared diabetic and were used in the entire experimental group (Lenzen, 2008).

Experimental design: Rats were randomized into six groups, consisting of six specimens in each group and treated as follows:

- **Group I:** Normal control (NC) and distilled water
- **Group II:** Disease control (DC) and distilled water
- **Group III:** Test drug 1 (TD1) and aqueous extract 28 mg kg^{-1} b.wt.
- **Group IV:** Test drug 2 (TD2) and alcoholic extract 28 mg kg^{-1} b.wt.
- **Group V:** Standard drug (SD) and metformin 80 mg kg^{-1} b.wt.
- **Group VI:** Vehicle control (VC) and DMSO 0.1 mL mL^{-1} DW

All the rats were fed respective doses daily for 90 days with Gavages.

Animal sacrifice and sample collection: After the last dose, animals were fasted for 12 h blood samples were collected by orbital sinus puncture method (Sharma *et al*., 2013a). Then animals were scarified by cervical dislocation to collect the liver for histopathology. Serum was prepared by following procedure. Briefly, blood samples were withdrawn from orbital sinus using heparinized capillary tubes, collected in dried appendrop tubes and allowed to clot. Serum was separated from the clot and centrifuged at 3000 rpm for 15 min at room temperature. The serum was collected carefully and used for biochemical analysis (Sharma *et al*., 2013b). Serum Glutamate Pyruvate Transaminase (SGPT) and Serum Glutamate Oxaloacetate Transaminase (SGOT) activities were measured according to the method described by Reitmann and Frankel (Samudram *et al*., 2009) while bilirubin activity was measured by dimethylsulfoxide method.

Histopathological studies: A portion of the liver was cut into two to three pieces of approximately 5-6 mm^{-3} sizes and fixed in 10% formaldehyde solution. After embedding in paraffin wax, thin sections of 5 μm thickness of liver tissue were cut and stained with haematoxylin-eosin. The thin sections of liver were made into permanent slides and examined under high resolution light microscope (Sharma *et al*., 2013b).

Statistical analysis: Results were presented as Mean±SD and total variation present in a set of data was analyzed through one-way analysis of variance (ANOVA) at $p<0.05$ and $p<0.01$.

RESULTS

To evaluate the effect of the alcoholic and aqueous extracts of *Pongamia pinnata in vivo*, long term treatment of the extracts and standard drug (90 days) was given to the alloxan induced diabetic wistar rats. The quantification of the fasting blood sugar (BSF) after 90 days of treatment in different experimental groups is indicated in Fig. 1. It is evident from the Fig. 1 that the BSF level is significantly ($p<0.5$, $p<0.01$) reduced with TD1 and TD2 extracts with the dose of 28 mg kg^{-1} where as standard drug Metformin created the same effect with a higher dose of 80 mg kg^{-1} b.wt. The observed differences between the TD1, TD2 and the SD as well as NC are not statistically significant. VC group shows no significant variation than that of DC indicating that vehicle DMSO is neutral and does not have antidiabetic property. PPAqExt inhibited diabetes by 8% PPAlcExt by 14% whereas SD shows 15% which is not statistically significant then extract.

Diabetes not only affect the endocrine function of pancreas but also affects the metabolic functions of all the vital organs like liver, kidney, eyes, heart etc. hence the main objective of the present investigation was to focus on the damaged liver histopathology and its function.

Figure 1 show that the Blood Sugar Fasting (BSF) values of NC group were 72.55±18.77. After inducing diabetes with alloxan the values reach their peaks and they were 339.81±8.59. In DC BSF level remained on higher side (more than 300 mg dL^{-1}). Treatment with PPAqExt and PPAlcExt produced reduction in the blood glucose level and the values were 218.11±24.85 for TD1 and

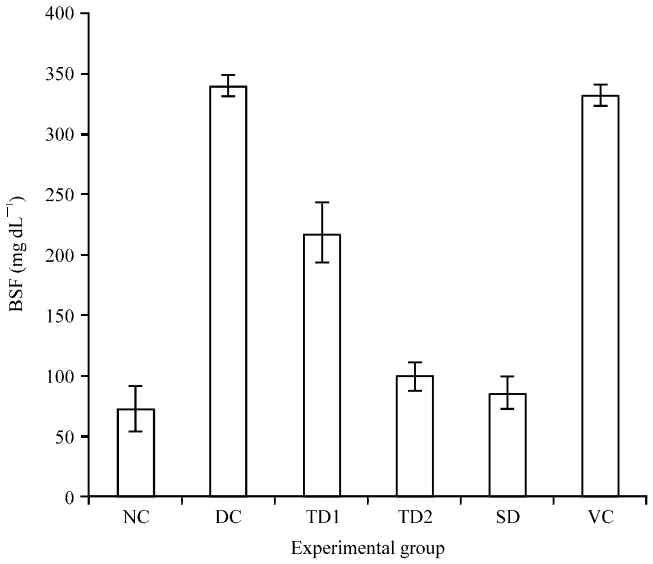

Fig. 1: Comparative BSF activities of each group for three months

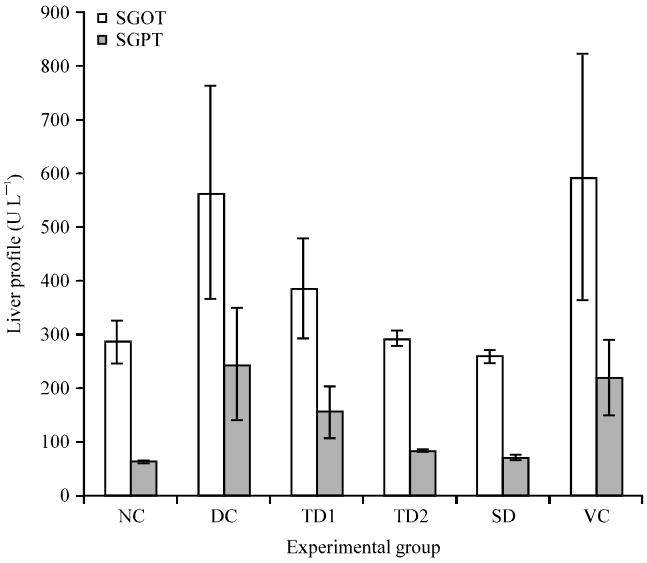

Fig. 2: Comparative SGOT, SGPT activities of each group for three months

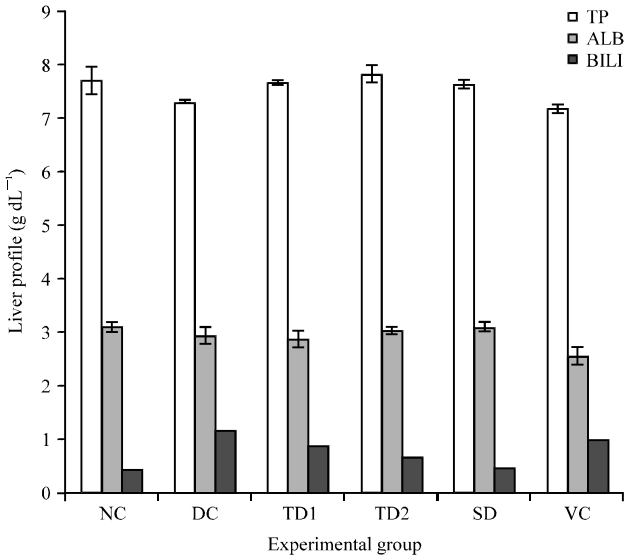

Fig. 3: Comparative TP, ALB and BILI activities of each group for three months

99.41±12.03 for TD2, respectively. Highly significant reduction was achieved with the alcoholic extract ($p<0.005$). Whereas for SD the BSF values were 86.37±13.49. The reduction in the BSF level by PPExts and standard drug Metformin were 121.7, 240.4 and 253.44 mg dL^{-1} for BSF.

Figure 2 and 3 shows that the significant reduction was achieved by PPExts and SD. The reductions were 180, 271, 306 U L^{-1} for AST and 89.21, 161.51, 175.96 U L^{-1} for ALT. Significant recovery like 0.35, 0.52, 0.33 g dL^{-1} for TPRO and 0.1, 0.9, 0.2 g dL^{-1} for ALB. Reductions observed were 0.25, 0.5, 0.67 g dL^{-1} for T Bili. The average value of AST in diabetic control group were 564.09 U L^{-1} which is reduced in PPExts treated group to 384.56 and 293.28 U L^{-1}. ALT levels

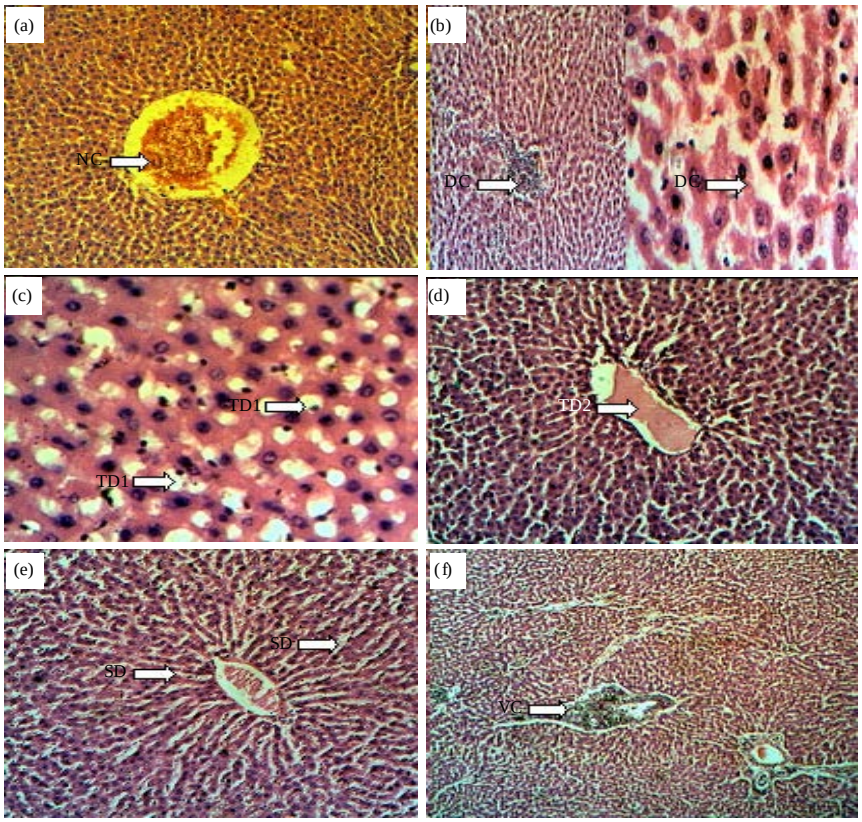

Fig. 4(a-f): Comparative histopathology of liver of experimental animals where arrows showing, (a) NC: Normal histological appearance of central vein and hepatic cords, (b) DC: Multifocal mild MNC infiltration, Diffuse mild SCN, (c) TD1: Multifocal mild fatty changes in hepatocytes, (d) TD2: Focal minimal dilated sinusoids with normal central vein, (e) SD: Focal dilated sinusoids with normal liver histology and (f) VC: Multifocal increased SCN with MNC infiltration in portal triad

were reduced from 244.72 to 155.51, 83.21 U L^{-1}. TPRO levels were increased from 7.31 up to 7.66 and 7.83 g dL^{-1}. ALB levels were increased from 2.9 up to 2.8, 3.0 g dL^{-1}. The BILI levels reduced from 1.15 to 0.9, 0.6.

Histopathology of liver: Investigation of the histology of the hepatocytes as examined in this study revealed multifocal mononuclear cell (MNC) infiltration, diffuse moderate, mild Single Cell Necrosis (SCN) moreover the sinusoids were non radiating tend to be wider and interrupted in the interrupted Alloxan diabetic rats. Also the hepatocytes were degenerated and number of nuclei reduced in DC (Fig. 4b) on the other hand NC (Fig. 4a) group liver histology showed normal histology of portal triad, sinusoids spaces and distinct lobulation with a central vein. Sinusoids radiate out from the central vain, hepatocytes were distinct single or polynuclei. Treatment with PPAqExt caused partial reversal in the lesions observed with Alloxan treatment where multifocal

mild central vein congestion with mild fatty changes in hepatocyte was observed. Also mild focal sinusoidal congestion with multifocal Mono nuclear cells infiltration was observed in TD1 (Fig. 4c) There was better improvement with PPAlcExt. The extract showed normal hepatocytes with central vein similar to normal control group histology in TD2 (Fig. 4d). Few areas showing mild mononuclear cell infiltration was observed. Treatment with standard drug metformin i.e., in SD group (Fig. 4e) rats showed normal liver histology were cellular architecture and integrity of the hepatocytes was normal along with distinct cell nuclei and sinusoids were non radiating, spaces between the sinusoids were normal, wider as similar as normal control. Where as in case of vehicle control VC (Fig. 4f) degenerative changes of hepatocytes was observed along with multifocal single cell necrosis. The extent of reversal and recovery was partial with PPAqExt and was distinct with PPAlcExt extract at a lower concentration of 28 mg kg^{-1} was compared to metformin 80 mg kg^{-1} b.wt.

DISCUSSION

We investigated at the biochemical and histological levels functioning of the antidiabetic aqueous and alcoholic extracts of *Pongamia pinnata* against the SD. The diabetic disorder being chronic in nature needs long term treatment to prevent the complications arising due to persistent high blood glucose level. Pharmacotherapy available for the treatment of diabetes in modern healthcare system includes insulin and 16 oral hypoglycemic drugs (Tripathi, 2003). However due to economic constraints; it is not possible for majority of the diabetic patients in developing countries like India to use these drugs on regular basis. Moreover these synthetic antidiabetic drugs are associated with large number of adverse effects. Hence, there is increase in the trend to use traditional indigenous plants widely available in India for the treatment of diabetes mellitus. Over 150 plant extracts and some of their active principles including flavonoids, tannins, alkaloids etc are used for the treatment of diabetes (Rahman *et al*., 2013).

Alloxan is relatively toxic to insulin producing pancreatic β-cells because it preferentially accumulates in β-cells through uptake via the GLUT-2 glucose transporter. This cytotoxic action is mediated by ROS source of generation of ROS is dialuric acid, a reduction product of alloxan. These radicals undergo dismutation to H_2O_2. The action of ROS with a simultaneous massive increase in cytosolic calcium concentration causes rapid destruction of beta cells, thereby decreasing the secretion of insulin which in turn increases the blood glucose level. Another result of alloxan, a β-cytotoxin, was preferred to produce the diabetic state in mice as it induces diabetes in a wide variety of animal species by damaging the insulin secreting pancreatic beta cell resulting in a decrease in endogenous insulin release which paves the ways for the decreased utilization of glucose by the tissues (Sharma *et al*., 2013c). During the present investigation, alloxan (150 mg kg^{-1} i.p) was used to induce diabetes in rats and their serum glucose levels were found to be significantly elevated as compared to normal rats. The increased levels of serum glucose may be due to the partial damage of the pancreatic β-cells. Alloxan, a β-cytotoxin, induces "Chemical diabetes" in a wide variety of animal species including rats by damaging the insulin secreting β-cells (Jelodar *et al*., 2007). Similar results reported by Vuksan and Sievenpiper (2005), show that the administration of alloxan significantly increases the level of glucose when compared to control which might account for the cytotoxic effect of alloxan on beta cells. On the other hand, with the treatment of extract (28 mg kg^{-1} b.wt.) for 90 days, the elevated level of serum glucose was significantly decreased. Present results are similar to previous reports (Ayyanar *et al*., 2013; Grover *et al*., 2002). The antidiabetic activity of aqueous and Alcoholic extract of *Pongamia pinnata* may be promoting insulin secretion by closure of K^+-ATP channels, membrane depolarization and

stimulation of calcium influx, an initial key step in insulin secretion. In this context, numbers of other plants have also been reported to have antidiabetic and insulin stimulatory effects (Latha and Pari, 2003). Flavonoids sterols, triterpenoids, alkaloids and phenolics are known to be bioactive antidiabetic principles (Shyam and Kadalmani, 2014). Flavonoids are known to regenerate the damaged beta cells in the alloxan induced diabetic rats (Bussa and Pinnapareddy, 2010). Phenolics are found to be effective antihyperglycemic agents. On this basis we have selected the glucose induced hyperglycemic model to screen the anti-hyperglycemic activity of the plant extracts.

Liver is an important insulin-dependent tissue which plays a pivotal role in glucose and lipid homeostasis and is severely affected during diabetes (Kumar *et al*., 2010). Liver is an important site for insulin clearance and fabrication of inflammatory cytokines play an important role in maintaining normal glucose concentration in fasting and post prandial states. Elevated levels of serum enzymes, such as ALT, AST and ALP are well known markers of hepatic damage; these enzymes are believed to leak from the cytosol into the bloodstream as a consequence of damage to hepatic tissue (Anandakirouchenane *et al*., 2013). Other researchers have reported an association between elevated ALT activity and fatty liver in obesity, insulin resistance and type 2 diabetes. On the contrary in the present study the treatment of the diabetic rats with *Pongamia pinnata* extracts caused highly significant ($p \leq 0.001$) reduction in the activity of these enzymes in serum compared to the values of diabetic group. The reduction noted is 32, 48 and 54% of TD1, TD2 and SD for AST whereas 35, 66 and 72% in TD1, TD2 and SD for ALT. Since raised levels of AST and ALT enzymes indicate the incidence of heart and liver diseases, these liver marker enzyme levels have significantly reduced to normally compared to the diabetic condition. Decrease in their levels in treated groups suggest that risk of liver and heart diseases can be reduced in diabetic patients. Results of our investigation are more significant than Prashant Kumar rai (Rai *et al*., 2010). Where reduction was only 31% for AST and 30% for ALT.

Proteins form the major portion of dissolved substances in the plasma. They form the basic structural components of the body. They constitute the enzymes present in our body and also act as secondary source of energy. Increased levels are found in dehydration and myeloma. Decreased levels are found in liver disorders and Nephrotic syndrome (Dhinaa and Palanisamy, 2010). In the present investigation decrease in the levels of proteins which was 0.39 g dL^{-1} (5%) as compared to NC was observed. After treatment with PPExt the recovery was 0.35, 0.52, 0.33 g dL^{-1} for TD1, TD2, SD, respectively. Similar results were observed by Sutar *et al*. (2009). Albumin which is synthesized in the liver constitutes a major part of the total proteins in the body. Albumin helps in distribution of extracellular fluids, regulation of osmotic pressure, act as a transport agent for a wide variety of substances such as hormones lipids, vitamins etc. Decreased levels are observed in liver disease (Hepatitis, Cirrhosis), malnutrition, kidney disorders, increased fluid loss during extensive burns and malabsorption (Kiple, 2003). Due to diabetic condition there is partial loss of proteins in urine which was clear indication from our findings that serum albumin levels observed in DC rats were decreased by 0.2 g dL^{-1} which is 6% when compared with NC. After treatment significant recovery was observed which is 4.7, 7.11 and 4.51% for TD1, TD2 and SD, respectively. Bilirubin is formed by the breakdown of RBC's in the spleen, liver and bone marrow. Small amount of bilirubin circulates in the plasma loosely bound to albumin which is not water soluble. This is referred to as indirect or unconjugated bilirubin. In the liver bilirubin is conjugated with glucuronic acid which forms a soluble compound. This is referred to a direct bilirubin. Elevated levels are found in hepatitis, cirrhosis, haemolytic jaundice, obstruction of biliary tract and drug induced reactions (Kremer *et al*., 2011). Bilirubin is excreted by the liver, therefore, interference with the normal liver

function affects its rate of conjugation and excretion. Thus a high level of bilirubin is used as indices for liver function and bile excretion status (Usha *et al.*, 2007). The present study showed a significant increase ($p<0.05$) in total bilirubin which was 0.73 g dL^{-1} in diabetic untreated control. These levels are, however, reduced in the extract treated groups and they were brought down by 0.25, 0.5, 0.67 g dL^{-1} in TD1, TD2 and SD rats suggesting the enhancement of liver functions by the extracts as well as standard drug Metformin. These accords with similar findings on this liver parameter by Maruthappan and Shree (2010) in his 28th day study.

The liver is one of the tissues that bear the brunch of chronic hyperglycemia, since glucose is freely permeable to its cells (Mayes, 2000). This unrestricted entry, in the presence of excess and sustained glucose in blood, is bound to cause metabolic derangements which would express themselves on the gross architecture of the tissue. In the present study, untreated diabetic rats presented with a sequestered and disoriented cellular architecture in accordance with other authors observation (Atangwho *et al.*, 2007). They reported that the altered architecture of the hepatocytes allows for leakage of liver enzymes (AST, ALT) into serum and hence their increased activities in serum. In normal control rats normal histological appearance of central vein and hepatic cords was observed whereas Multifocal mild Mononuclear cell (MNC) infiltration with diffuse mild Single Cell Necrosis (SCN) was observed in diabetic rats which are in accordance with the previous findings (Badole and Bodhankar, 2011). Administration of PPExt illustrate that hyperglycemia is ameliorated or reversed, normal metabolism gradually becomes reestablished, so also the gross architecture of the tissues.

CONCLUSION

In conclusion, the present study demonstrated a comprehensive and detail picture of effects of diabetic state on the hepatocytes of albino wistar rats. Specifically, we have reported that at later stage of diabetes fatty liver is seen in disease control group. The treatment of diabetic rats with *Pongamia pinnata* has exerted a considerable hypoglycemic effect along with hepatoprotective effect. In addition, study results suggest that *Pongamia pinnata* protects and improves liver function in diabetic rats.

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