Effect of N-Trisaccharide on liver and kidney: Toxicity evaluation studies

Introduction

Phytomedicines are in great demand in the world market, yet there are still concerns associated not only with their use but also their safety. The products are not truly standardized to known active components and quality control measures are not strictly followed (Kennedy and Wightman, 2011). Very little is known about the active and/or toxic constituents present in majority of these plant products. These phytomedicines are not subjected to the same regulatory standards in terms of efficacy and safety as orthodox drugs. This raises concern on their safety and implications for medicinal use. The risks that may be associated with their use can be revealed only by conducting toxicity tests thereby overcoming the potential harmful effects.

The toxicological assessment of phytomedicine allows identifying adverse effects and determining limits of exposure level at which such effects occur. Testing of toxicity reveals the risks associated with the use especially in sensitive populations. The detection of toxic plant extracts or active compounds derived in the pre-clinical and clinical stages of drug discovery remains equally important objective of toxicity testing. The identification of toxicants which can be either discarded or modified during processing facilitates to create an opportunity for extensive evaluation of safer and promising alternatives (Gamaniel, 2000). Certain compounds tolerability can be improved by modifications such as dosage adjustment, chemical grouping or structural adjustments.

In vertebrate body system, Liver performs detoxification of drugs but chemical agents and routine use of drugs cause cellular as well as metabolic liver injury. It has been considered as a major target of injury in patients with type 2 diabetes or the metabolic syndrome. Several biochemical tests have been considered as standard safety tests which include the measurement of serum glutamate oxaloacetate transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT) and alkaline phosphatase
(ALP) level, that have proven to be very good indicators of liver toxicity (Friedman et al., 1996). Serum creatinine and urea reflects the functioning of kidneys (Smith et al., 2006). Hence, measurement of these parameters enables study of the toxic effects of the drugs on the liver and kidney.

Alanine transaminase or ALT also called as serum glutamic-pyruvic transaminase (SGPT) is a transaminase enzyme (EC 2.6.1.2), found in serum and most commonly associated with the liver, but present in smaller amounts in muscles, heart, pancreas and kidneys. The enzyme level is measured for the diagnostic evaluation of liver injury. A minor elevation is a good predictor of mortality from liver disease and other medical problems such as viral hepatitis, liver damage, diabetes, bile duct problems, congestive heart failure, infectious mononucleosis, or myopathy. Also, elevated levels of ALT due to liver-cell damage can be distinguished from biliary duct problems by measuring alkaline phosphatase.

Aspartate transaminase (AST) also known as serum glutamic oxaloacetic transaminase (SGOT) is a pyridoxal phosphate (PLP)-dependent transaminase enzyme (EC 2.6.1.1), found in liver, brain, skeletal muscle, heart, kidneys, and red blood cells. AST is similar to ALT and are associated with liver parenchymal cells. The difference is that ALT is found predominantly in the liver, with clinically negligible quantities in heart skeletal muscle and kidney, while AST is normally found in red blood cells, liver, heart, muscle tissue, pancreas and kidneys. AST levels get elevated in diseases affecting organs, such as acute pancreatitis, musculoskeletal diseases, acute renal disease, myocardial infarction, acute hemolytic anemia, severe burns and trauma.

Selective pancreatic islet β-cell cytotoxicity by streptozotocin is well known and resembles human hyperglycemia non-ketotic diabetes mellitus in many animal species (Weir et al., 1981). Streptozotocin (STZ) induced rats display features relating to human subjects with uncontrolled DM, which may contribute to microvascular complications such as retinopathy, neuropathy and nephropathy are invaluable (Obrosova et al., 2005). The functioning of pancreas, liver and kidney may be affected due to decreased levels of insulin and as a result of hyperglycemic consequences. In the present investigation, the
histological changes in these tissues of diabetic rats and the effect of N-Trisaccharide of
*C. prophetarum* on these were studied.

**Histopathological examination**

Liver is considered to be consisting of large number of hexagonal lobules. Each lobule is delineated by a connective tissue and consists of a central vein, from which cords or rows of liver cells radiate (Chaudhari, 1998). Microscopic examination of normal liver shows hepatocytes consisting of glycogen granules as reddish purplish material with periodic acid-Schif (PAS) staining (Mitra *et al*., 1996). Histology of liver during diabetes shows structural alterations due to lack of insulin and decreased deposits of glycogen granules. In liver cells, the sinusoid spaces and the vein lumen get enlarged. The major alterations in diabetic state are wall thickening of the blood vessels and capillaries. The distortion in the usual arrangement of the hepatic cells could be brought about by the increase in the lumen of the veins which might have pushed the surrounding cells (Anil and Paulose, 1995; Anil *et al*., 1996). The fibrosis in diabetic liver shows extensive damage which is replaced by the fibrous tissue (Balazs and Halmos, 1985).

Kidney is covered by a capsule and below lies cortex and inner to it is medulla. Cortex occupies upper 4/5th region of the kidney which consists of circular structures called renal capsules, surrounding which are tubules in various shapes. The dark rounded thick walled tubules are part of the proximal convoluted tubule. The light thin wall tubule with lumen (small and indistinct) is called distal convoluted tubule (Singh, 1992). Enlarged kidney in alloxan or streptozotocin induced diabetic rats is reported (Seyer, 1983) which shows thickening on the walls of nephron filled with lumen along with glomerulopathy. Structural changes with glomerulus are due to diuresis which is a common feature associated with diabetes (Anil and Paulose, 1995; Anil *et al*., 1996). Also, hyalin thickening of afferent glomerular arterioles and deposition of hyaline in the mesangium of glomerular lobules with associated thickening of the glomerular capillary basement membrane is the feature of diabetic kidney. Renal failure is an important complication in diabetes as it causes more than 10% of the death in all diabetics and in over 50% of those who develop diabetes during their childhood (Anderson, 1985).
Pancreas is a compound tubular alveolar, partly exocrine which is in the form of serous acini, secreting the secretions into intralobular duct and partly endocrine which is in the form of numerous rounded collections of cells known as islets of Langerhans, embedded within the exocrine part that is separated by the surrounding alveoli with a thin layer of reticular tissue. The average islet in rats is 150 µm in diameter and contains about 45 ng of insulin. In mammalian islet, four major endocrine cell types exist; insulin producing β-cells, glucagon producing α-cells, somatostatin producing δ-cells and pancreatic polypeptide producing pp-cells. β-Cells are polyhedral and are usually well granulated with secretory granules of 250-300 nm in diameter. Microscopic examination shows abundant patches of β-cells in the pancreas of normal rats that contains granules and moderately dense immature granules (Bonner and Smith, 1994), which are absent in diabetic pancreas (Anil et al., 1996). Small shrunken islets and destruction of β-cells are observed under the diabetic condition (Mitra et al., 1996). Selective destruction of β-cells could be observed in alloxan or streptozotocin induced diabetic rats with lytic and vascular changes of cellular components. Insulitis with heavy lymphocytic infiltration in and around the islets may be present.

**Materials and Methods**

Diabetes was induced as described in the Chapter II, rats with the fasting blood glucose levels of ≥ 250mg/dL were used in the experiment. The rats were divided into 6 groups each having 6 rats.

- **Group 1**: normal untreated rats
- **Group 2**: normal rats treated with 50 mg N-Trisaccharide/kg.b.w/day
- **Group 3**: diabetic untreated rats
- **Group 4**: diabetic rats treated with 25 mg N-Trisaccharide/kg.b.w/day
- **Group 5**: diabetic rats treated with 50 mg N-Trisaccharide/kg.b.w/day
- **Group 6**: diabetic rats treated with 25 mg glibenclamide/kg.b.w/day

The animals in Group 2 and 5 were given daily oral dose of 50 mg N-Trisaccharide per kg body weight, while those rats in Group 1 and 3 were given water alone and Group 4 and 6 rats were treated with N-Trisaccharide and glibenclamide at a
dose of 25 mg/kg.b.w at morning time for a period of 28 days. All the 6 groups were
sacrificed on the last day of treatment by administering anesthesia and then blood was
drawn from heart, pancreas, liver and kidney were collected and stored in 10% formalin
after washing 3 times with normal saline. Serum was separated immediately and then
stored for further biochemical investigations. The activities of SGOT, SGPT and ALP
and the levels of urea and creatinine were estimated in serum by the methods as described
in Chapter II.

**DNA fragmentation analysis**

DNA fragmentation was assessed by the method of Wang et al. (2007) with slight
modifications. Briefly, the liver tissue was homogenized in lysis buffer (pH 8.0)
containing 100 mM Tris, 20 mM EDTA and 0.8% SDS. Proteinase K (0.4 μg/mL) was
added and incubated at 50°C for 3 h. Then added to the mixture of phenol: chloroform:
isoamyl alcohol (25:24:1) and centrifuged at 13000 rpm for 10 min. The resulting
aqueous phase was added 2 volumes of ice-cold absolute ethanol, 1/10th volume of 3 M
sodium acetate and incubated for 30 min on ice. DNA was pelleted by centrifuging at
13000 rpm for 10 min at 4°C and after separating supernatant, pellet was washed with 1
mL of 70% ethanol. The extracted DNA was re-suspended in Tris-EDTA buffer,
electrophoretically separated on 1.5% agarose gel and stained with ethidium bromide.
The DNA pattern was examined by ultraviolet transillumination.

**Histological studies**

The preparation of tissue sections in paraffin wax using microtone is discussed in
Chapter II.

**Results**

**DNA fragmentation analysis**

The effect of N-Trisaccharide on DNA fragmentation was examined in the control
and experimental groups (Fig. 6.1). Diabetic rats treated with N-Trisaccharide showed
significant decrease in DNA fragmentation compared to untreated diabetic rats, whereas
there was no difference in its laddering pattern in the normal rats treated with N-
Trisaccharide as compared to control. These observations suggest that N-Trisaccharide
treatment significantly suppressed the progression of apoptosis in the liver and kidney of diabetic rats.

**Effect of N-Trisaccharide on hepatic and renal functional markers**

Fig. 6.2 and Table 6.1 show the levels of hepatic (SGOT, SGPT and ALP) and renal function markers (urea and creatinine) respectively in different experimental groups. The activities of SGOT, SGPT and ALP increased significantly in diabetic untreated group. Renal function markers in serum also increased in diabetic rats when compared to normal rats. Administration of N-Trisaccharide significantly ($p \leq 0.05$) decreased the levels of SGOT, SGPT, ALP, and urea and creatinine in treated rats compared to untreated rats. There were no significant changes in the levels of hepatic and renal function markers in the normal treated rats.

**Histopathology**

Histological changes in liver and kidney in normal rats, diabetic rats and rats treated with N-Trisaccharide are presented below.

**Liver**

Histopathological results supported the biochemical findings. Histology of liver section of one animal from each group is presented in Fig. 6.3. Liver section of normal control rat and normal treated rats exhibited distinct hepatic cells, the central vein is free from visible lesion, sinusoids are normal and the epithelium lining is normal (Fig. 6.3.a and 6.3.b). In Fig. 6.3.c, diabetic liver shows total loss of hepatic architecture, necrosis, lymphocyte infiltration, loss of cellular boundaries, and joining together of nucleus. In addition, congestion of sinusoids, Kupffer cell hyperplasia, crowding of central vein and apoptosis are also evident. Rats treated with glibenclamide (Fig. 6.3.f) show less necrosis and almost normal liver architecture with no obvious necrosis as compared to diabetic rats. Sections of Fig 6.3.d and Fig 6.3.e treated groups with N-Trisaccharide (25 and 50 mg/kg) show gradual recovery of hepatic architecture. The central vein is seen and the sinusoids are having hepatitis alteration. Slight lymphocyte infiltration is observed in Fig. 6.3.d. These sections are nearly comparable to the glibenclamide treated group.
**Pancreas**

Histological changes in pancreas have been discussed in Chapter 4.

**Kidney**

Fig 6.4.a and 6.4.b are the sections of the kidney of normal and normal treated rats showing normal architecture of kidney with normal glomeruli and normal tubular epithelial cells. Fig 6.4.c shows atrophy of the glomeruli, necrotic tubular epithelial cells, infiltration and dark pyknotic nuclei typical of diabetic untreated rats. Fig. 6.4.d and 6.4.e are the sections of diabetic rats treated with N-Trisaccharide, which show normal glomeruli, normal intertubular vessels and tubular epithelial cells indicating restorative changes. Similar observations were made in rats treated with glibenclamide as evident from Fig. 6.4.f.
Fig. 6.1: Effects of N-Trisaccharide treatment on STZ-NA induced liver DNA fragmentation. Gel represent as lane I & II showing intact DNA (Normal and Normal treated with N-Trisaccharide respectively), lane III showing fragmented DNA (STZ-NA induced diabetic rat), Lane IV (N-Trisaccharide treated diabetic rats at 25 mg/kg.b.wt) partially reverted to normal and lane V & VI (Diabetic rats treated with N-Trisaccharide at 50 mg/kg.b.wt and glibenclamide at 25 mg/kg.b.wt respectively) showing fragmentation reverted near to normal rat liver
**Fig. 6.2:** Effect of N-Trisaccharide treatment on serum SGOT, SGPT and ALP in normal and experimental animals. Values are mean ± SD ($n = 6$)
Table 6.1: Effect of N-Trisaccharide treatment on serum urea and creatinine in normal and experimental diabetic animals

<table>
<thead>
<tr>
<th>Groups</th>
<th>Urea</th>
<th>Creatinine</th>
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<tr>
<td>Normal</td>
<td>41.22± 2.27&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.61 ± 0.036&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>Normal + N-Trisaccharide (50 mg/kg)</td>
<td>41.54 ± 1.92&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.61 ± 0.03&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Diabetic</td>
<td>68.33 ± 1.21</td>
<td>0.71 ± 0.036</td>
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<tr>
<td>Diabetic + N-Trisaccharide (25 mg/kg)</td>
<td>51.18 ± 1.59&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>0.62 ± 0.057&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Diabetic + N-Trisaccharide (50 mg/kg)</td>
<td>58.83 ± 2.22&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>0.60 ± 0.028&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Diabetic + Glibenclamide (25 mg/kg)</td>
<td>62.79 ± 3.07&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>0.61 ± 0.043&lt;sup&gt;b&lt;/sup&gt;</td>
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</table>

Values are mean ± SD (n = 6).
Values in the same column not sharing common superscript differ significantly at p ≤ 0.05.
Fig. 6.3: Photomicrographs showing the effect of N-Trisaccharide and glibenclamide treatment on liver histopathology of STZ treated rats. Plate-a: Liver section of normal group; Plate-b: normal saline + treated with N-Trisaccharide (50 mg/kg); Plate-c: diabetic control group; Plate-d: diabetic + N-Trisaccharide (25 mg/kg); Plate-e: diabetic + N-Trisaccharide (50 mg/kg); Plate-f: diabetic + glibenclamide (25 mg/kg) (Magnification 10X)
Fig. 6.4: Photomicrographs showing the effect of N-Trisaccharide and glibenclamide treatment on kidney histopathology of STZ treated rats. Plate-a: Kidney section of normal group; Plate-b: normal saline + treated with N-Trisaccharide (50 mg/kg); Plate-c: diabetic control group; Plate-d: diabetic + N-Trisaccharide (25 mg/kg); Plate-e: diabetic + N Trisaccharide (50 mg/kg); Plate-f: diabetic + glibenclamide (25 mg/kg) (Magnification 10X)
Discussion

DNA fragmentation occurs massively due to oxidative stress and increased fragmentation may enhance apoptotic cell death in liver and kidney. Significant DNA strand breaks and mutations were earlier reported in STZ induced diabetic rats and mice by Imaeda et al. (2002) and Schmezer et al. (1994), respectively. In the present study, increased DNA fragmentation in liver of STZ-NA induced diabetic rats was observed. Treatment with either N-Trisaccharide or glibenclamide significantly reduced DNA fragmentation in liver of diabetic rats. Antioxidant activity of N-Trisaccharide may be attributed to the reduction in DNA fragmentation.

Measurement of hepatic function markers (SGOT, SGPT and ALP) is of clinical and toxicological importance as changes in their levels are indicative of tissue damage in disease conditions or hepatic dysfunction. Higher secretion of these enzymes from the cells is the indication for cellular leakage and loss of functional integrity of the cell membrane in liver (Drotman and Lawhorn, 1978) and this could be as a result of hepatocyte necrosis or abnormal membrane permeability. SGOT is a sensitive indicator of acute liver damage and elevated level in non-hepatic diseases is unusual. SGPT is more selectively a liver parenchymal enzyme when compared to SGOT (Shah et al., 2002). The rise in the activity of SGPT in hepatocellular damage is usually accompanied by a rise in SGOT level (Rao et al., 1989). SGOT is similar to SGPT in that it is another enzyme associated with liver parenchymal cells. Its level is raised in acute liver damage, but it is also present in red blood cells, cardiac and skeletal muscle and hence not specific to liver alone. The ratio of SGOT to SGPT is sometimes useful in differentiating between causes of liver damage. ALP level in plasma will rise with large bile duct obstruction, intrahepatic cholestasis or infiltrative diseases of the liver (Gautam et al., 2004).

In the present study, significant increase in serum AST, ALT and ALP levels were observed in diabetic untreated rats compared to normal control rats. Significant increase in the levels of SGOT and SGPT in STZ-diabetic rats were reported by Yanardag et al. (2005) and Rajasekaran et al. (2006). The elevated levels of these marker enzymes in the present study were observed in diabetic rats. However, during the investigation, reduction of SGOT, SGPT and SALP concentrations were observed due to
the influence of N-Trisaccharide. Present findings are in agreement with those of Prakasam et al. (2004), N-Trisaccharide treatment of normal rats for 28 days did not produce any hepatotoxicity and showed protective effect against liver toxicity caused by STZ.

Diabetes mellitus causes renal damage due to elevated glucose, abnormal regulation, glycosylated protein tissue levels, increased oxidative stress and hemodynamic changes within the kidney tissue. STZ-induced diabetic rats exhibited significant increase in the serum urea and creatinine levels, which are considered as markers of renal dysfunction (Bethesda, 2001). However, administration of N-Trisaccharide significantly reduced the levels of serum urea and creatinine in treated rats. Kaleem et al. (2008) reported that oral administration of Annona squamosa L. extract significantly lowered both hepatic and renal markers in STZ-induced diabetic rats. Das and Sharma (2009) reported the hepatoprotective activity of Eugenia jambolana in albino rats which significantly restored the level of hepatic markers.

**Histopathological studies**

The histological sections of the tissues were observed to know any protective or harmful effect of N-Trisaccharide in non-diabetic and diabetic rats.

The decrease in cellularity within islets of Langerhans reflected the cytotoxicity of STZ (Szkudelski, 2001). STZ possesses diabetogenic effect mediated through pancreatic β-cell destruction and it appears to cause cytotoxicity through several mechanisms. The entry of STZ into the β-cells via glucose transporter leads to its spontaneous decomposition into isocyanate and methylidazohydroxide which cause intramolecular carboxylation and DNA damage by alkylation of carbonium ion respectively (Varva et al., 1960). This DNA damage induces activation of poly ADP-ribosylation that leads to depletion of cellular NAD and ATP, a process that is more important for the diabetogenicity of STZ than just DNA damage itself. Enhanced ATP dephosphorylation supplies a substrate for xanthine oxidase resulting in the formation of superoxide radicals along with generation of hydrogen peroxide and hydroxyl radicals after STZ treatment. Further, STZ liberates toxic amounts of nitric oxide that inhibits
aconitase activity and participates in DNA damage. As a result, necrosis of $\beta$-cells occurs. STZ selectively destroys the pancreatic $\beta$-cells that secrete insulin, leaving less active cells and this result in a diabetic state (Szkudelski, 2001).

In the present study, sinusoidal haemorrhages, vasculations in the hepatocytes (fatty changes), granular appearance of the hepatocytes (degenerative changes) and cloudy swelling (hazy nucleus) and inflammation were noticed in the liver of diabetic rats. These changes were reduced in N-Trisaccharide treated rats. This effect could be attributable to the antioxidant activity of N-Trisaccharide, which attenuated the oxidative threat and restored normal physiological functions. The histological findings from the present study are in agreement with the degenerative structural changes reported in liver tissues as a result of insulin depletion (Can et al., 2004) in type 2 diabetic models. Also, the authors observed an increase in degeneration in central vein to portal veins, excess vacuolization, granular appearance in the cytoplasm, dilations in the sinusoids and moderate hyperemia.

Kidney has the main function to excrete waste products of metabolism and to regulate water and salt concentration. Histologically, multifocal clarifications and vacuolations in the kidneys of STZ-NA diabetic rats were observed. Floretto et al. (1998) reported that, STZ does not possess any significant nephrotoxic potential. All structural changes in kidneys can thus be attributed to altered metabolism in diabetes (Rasch, 1980). In a study reported by Bolkent et al. (2004) in the neonatal STZ (100 mg/kg STZ)-induced type 2 diabetes, alteration in the structural integrity of the apical membrane of proximal tubules of the kidney tissue occurs in the neonatal STZ induced type 2 diabetic rats. Normoglycemia in diabetic rats with N-Trisaccharide treatment as observed in this study could ameliorate the glomerular and tubular lesions that characterize diabetic nephropathy. The improvement of renal morphology and function of STZ-NA diabetic rats after treatment with N-Trisaccharide could be attributed to its anti-diabetic action resulting in alleviation of altered metabolic status in animals. However, the recovery of renal function expected with treatment of N-Trisaccharide can be explained by the regenerative capability of the renal tubules (Kissane, 1985). Kidney sections of the
healthy rats treated with N-Trisaccharide showed no pathological changes and were comparable to those of normal control rats.

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