Results
&
Discussions
General observations

Food intake, body weight and plasma glucose:

During the eight weeks of experimental period control rats showed no significant change in blood glucose level (64.14 ± 1.8 mg/dl) with gradual increase in body weight. PNAEt treated normal rats also showed the same trend as control rats regarding plasma glucose (66.46 ± 4.36 mg/dl) and gain in body weight during experimental period. No visible side effects of PNAEt treatment were observed in this group of rats. Where as STZ-induced diabetic rats (Diabetic untreated rats) showed a characteristic features of diabetes such as increase food and water intake, polyuria, failure to gain weight and significant elevation in fasting plasma glucose concentration (437.39 ± 4.4 mg/dl). In spite of increased food intake, the body weights of diabetic untreated group were found to be decreased when compared with control group.

Antihyperglycemic effect of PNAEt was revealed in PNAEt treated diabetic rats, form the 3rd week of treatment reaching near normal levels of plasma glucose (93.9 ± 2.9) in 8 weeks of treatment. In addition, the decreased in body weight due to hyperglycemia and symptoms of diabetes like polyphagia, polyuria, polydipsia ameliorated by PNAEt treatment.

A significant increase in kidney size and weights (6.68% KW/BW) were observed in STZ-induced diabetic rats compared to normal rats. Where as normal rats treated with PNAEt and diabetic rats treated with PNAEt did not show significant alterations in size and weights of kidney when compared to normal rats.

Oxidative stress:

Lipid peroxidation and protein oxidation

The oxidative stress and resultant tissue damage are hallmark of chronic diseases and cell death, and diabetes is not an exception. Hyperglycemia, advanced glycation end products, autooxidation of glucose, polyol pathway, intracellular accumulation of lipids and metabolic alterations all lead to the increased
formation of oxygen derived ROS which make the membrane vulnerable to oxidative
damage (Rosen et al., 2001).

Hyperglycemia the main symptom of diabetes not only increases the
production of ROS but also affect antioxidant reactions catalyzed by ROS
scavenging enzymes (Wohieb and Godin, 1987). Hence, therapeutic agents with
both antihyperglycemic and antioxidant properties would be useful as antidiabetic
agents. It has been recently suggested that diabetic subjects with complications may
have defective cellular antioxidant response against the oxidative stress generated
by hyperglycemia, which can predispose the patient to organ damage (Ceriello et al.,
2000).

The present study was undertaken to examine the possible antioxidant
activity of PNAEt in the kidney of STZ-induced diabetic rats and the protection action
of PNAEt against oxidative damage of rat lymphocyte DNA under in vivo and in vitro
conditions.

The diabetogenic agent, STZ is a pancreatic β-cell selective toxin that has
been extensively used to probe the mechanisms underlying oxygen-mediated
damage to rodent β-cells. Although STZ is rapidly cleared from the body (Adolphe et
al., 1975) the β-cell damage by STZ leads to hyperglycemia which in turn generates
ROS. ROS production and oxidative damage may contribute to the onset,
progression and pathological consequences in diabetes (Katerine et al., 2004). NO°
permeates cell membrane, contains an unpaired electron, and readily oxidizes
ferrous atom into ferrite, or forms secondary radicals with O_2° radical (Moncada et
al., 1991). Too much NO° in cells inactivates iron-containing enzymes and the
decomposition products of secondary radical induced peroxidation of lipids, oxidize
methionine and ~SH residues in proteins, deplete antioxidants and cause DNA
damage (Beckman et al., 1994).
Table 1  Effect of PNAEt treatment on lipid peroxidation, and protein oxidation in STZ–induced diabetic rats

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Normal</th>
<th>Normal treated</th>
<th>Diabetic Untreated</th>
<th>Diabetic treated</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Lipid peroxidation</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(nmoles of MDA formed/mg protein)</td>
<td>15.378a</td>
<td>14.833a</td>
<td>29.800c</td>
<td>20.666d#</td>
</tr>
<tr>
<td></td>
<td>(±0.314)</td>
<td>(±0.401)</td>
<td>(± 0.400)</td>
<td>(± 0.247)</td>
</tr>
<tr>
<td><strong>Protein oxidation</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(µM of protein carbonyl content / mg protein)</td>
<td>4.058a</td>
<td>4.00a</td>
<td>4.941c</td>
<td>4.215d#</td>
</tr>
<tr>
<td></td>
<td>(± .058)</td>
<td>(± .068)</td>
<td>(± 0.032)</td>
<td>(± 0.048)</td>
</tr>
</tbody>
</table>

- Values are given as mean ±SE from eight rats in each group. Values not sharing a common superscript letter differ significantly at P<0.05(Duncan's multiple range test).
- # indicates significance between diabetic and diabetic treated group
STZ rapidly decomposes in aqueous solution to a highly reactive carbonium ion that produces cytotoxicity by alkylation of DNA, and oxidation of proteins, and membrane lipids (Wilson et al., 1984). It can be stated that potent alkylating properties of STZ are the main reason of its toxicity. However, the synergistic action of both NO and ROS may also contribute to DNA fragmentation and other deleterious changes caused by STZ. NO° and ROS can act separately or form the highly toxic peroxynitrite (ONOO'). Therefore, intracellular antioxidants or NO scavengers substantially attenuate STZ toxicity.

Oxidative stress is associated with the peroxidation of cellular lipids, which is determined by measurement of TBA-reactive substances. The concentration of lipid peroxidation products may reflect the degree of oxidative stress in diabetes (Baynes, 1991; Kakkar et al., 1995).

Recently, free radical induced LPO has gained much importance because of its involvement in several pathologies such as aging, atherosclerosis (Rowley & Halliwell, 1983), diabetes (Rosen et al., 2001), wound healing, oxygen toxicity, liver disorder, inflammation etc (Salin and McCord, 1975). Protection of the cell membrane from the LPO could prevent, cure or delay the aforesaid pathologies.

The levels of renal LPO in four groups of experimental animals are represented in table (1).

Normal rats treated with PNAEt showed a no significant change in extent of LPO when compared to normal rats. Where as DUT group and DT group showed significant increase in extent of LPO (93.8 and 34.4% respectively) when compared with normal group. However DT group showed a significant decrease (30.4%) in LPO when compared to DUT rats.
The enhanced LPO of diabetic untreated (DUT) rats compared to normal rats indicates the presence of oxidative stress in DUT rats. Low levels of lipoxigenase peroxides stimulates the secretion of insulin, but when concentration of endogenous peroxides increase it may initiate uncontrolled LPO leading to cellular infiltration and islet cell damage (Metz, 1984). The observed slight and non significant decrease in LPO of PNAEt treated normal rats indicates the nontoxic effect of PNAEt treatment.

The increased LPO impairs membrane function by decreasing membrane fluidity and changing the activity of membrane-bound proteins and receptors (Soon and Tan, 2002). The enhanced LPO of diabetic rats in present study agrees with results of several other studies conducted in diabetic animals and humans (Bynes, 1991; Prince and Menon, 2000; Stanely et al., 2001).

The significant decrease in the LPO of DT rats compared to DUT rats indicates protective effect of PNAEt against STZ induced diabetic oxidative stress. This protection did not restore to normal or basal levels of malondialdehyde (MDA) indicating a partial restoration against oxidative stress by PNAEt treatment.

Oxidative stress has been suggested extensively as a potential mechanism for diabetic kidney diseases because oxidative stress promotes the formation of AGE as well as PKC-MAPK activation (Ha and Kim, 1999; Brownlee, 2001). Indeed, involvement of oxidative stress has been indicated by the presence of LPO products and 8-hydroxyguanosine in the kidney from STZ induced diabetic rats (Ha et al., 1994; Horie et al., 1997).

LPO may also play a potential role in diabetic glomerulosclerosis. The initial hyperfiltering phase of diabetic kidney disease, heralded by the appearance of microalbuminuria, is followed by gross proteinuria, progressive reduction in glomerular filtration rate and excessive accumulation of extracellular matrix proteins in the glomerular capillaries and mesangium. The latter process leads the way to eventual glomerulosclerosis and renal failure (Salahudeen, et al., 1997).
Studies of Houglum et al., (1991) on fibroblast cell culture, revealed the protection of the ascorbic acid induced LPO-mediated activation of collagen genes at the transcription level by vit E. Vit E inhibited high glucose induced excessive collagen production by mesangial cells in culture (Trachtman, 1994). Further in vivo studies of Wang and Sallahudeen (1995) on renal fibrosis indicated that antioxidant therapy had inhibited the cyclosporine A induced renal LPO and renal dysfunction – providing the potential link between LPO and renal fibrosis.

Protein oxidation:
All biomacromolecules are faced with oxidative stress, including proteins. ROS are responsible for peptide bond cleavage during protein oxidation. Gallagher et al, (1998) reported that cleavage sites could be anywhere around the peptide, and result in various products. Many of these oxidative products have newly formed carbonyl groups. It has been established that protein oxidation is associated with aging, oxidative stress and number of diseases. Oxidation of protein molecules not only inactivate them but also introduces a tag for in vivo protein degradation by proteosome system (Tsu-Chung et al., 2000)

The oxidative damage of proteins in the lens is an early event in myopic and diabetic patients, occurring prematurely compared with the subjects of same age (Vendemiale et al., 1996; Altomare et al., 1997; Grattagliano et al., 1998) Kyselova et al., (2004) also demonstrated that a quantitative relationship between the degree of protein oxidation and the rate of advanced cataract development in STZ induced diabetic rats. These studies provided a relationship between extent of protein oxidation and long term complications of diabetes.

The extent of protein oxidation in the four groups of experimental animals is represented in table (1). There is no significant difference in the extent of kidney carbonyl content of normal PNAEt treated group compared to normal rats. Where as DUT and DT groups showed a significant increase in extent of protein oxidation when compared to normal group with 21.8 and 3.9% enhancement respectively.
However, DT rats showed a significant decrease (14.7%) in protein oxidation when compared to DUT group. The enhanced protein carbonyl content of DUT rats compared to normal rats clearly indicates the existence of oxidative stress in STZ induced diabetic rats.

Kidney protein carbonyl content of 4 experimental group of present study indicates PNAEt treatment does not show any toxic effect in normal rats and a protective effect against enhanced protein oxidation as observed in DT group compared to DUT rats.

Oxidation of biomolecules always impairs its original physiological function, which means that it is not useful any more and should be removed, otherwise, the cell gets older or even dies (apoptosis) (Tsu Chung et al., 2000). Enhanced protein oxidation is usually associated with carcinogenesis, probably through the modification of key transducers e.g. p53 which control cell proliferation (Sun and Oberiey, 1996; Cimino et al., 1997).

Thus present study indicates a protective effect of PNAEt treatment against renal tissue damage caused by enhanced LPO and protein oxidation in STZ diabetic rats.

Antioxidant system:

Besides all the deteriorative effects of ROS, the cell possesses some innate mechanism by which it tries to combat oxidative insult by increasing its reserves of antioxidants. The cellular free radical scavengers and antioxidant enzymes normally protects the cell from toxic effect of ROS which include glutathione (GSH) and GSH dependant antioxidant scavenging systems viz., glutathione reductase (GR), glutathione peroxidase (GPx), glutathione-s-transferase (GST) and GSH independent antioxidant enzymes viz., superoxide dismutase (SOD) and catalase (CAT) (Simmons, 1984; Halliwell et al., 1984). Oxidative stress occurs when there is imbalance between free radical production reactions and scavenging capacity of
antioxidative defense mechanism of the organisms. Enzymatic antioxidative SOD, CAT, GR, GPx and GST functions by direct or sequential removal of ROS thereby terminating their activities (Sies, 1999).

\[ 2O_2^- + 2H^+ \xrightarrow{\text{SOD}} H_2O_2 + O_2 \]

\[ 2H_2O_2 \xrightarrow{\text{CAT}} 2H_2O + O_2 \]

\[ 2GSH + H_2O_2 \xrightarrow{\text{GPX}} GSSG + 2H_2O \]

\[ GSSG + NADPH + H^+ \xrightarrow{\text{GR}} 2GSH + NADP^+ \]

\[ X + GSH \xrightarrow{\text{GST}} X-S-G \]

Glutathione (GSH):

GSH comprising a major portion of cellular non-protein thiols, plays central role in diverse groups of cell metabolic functions; the transport, cellular protection and detoxification of the exogenous as well as endogenous toxins. Thus GSH is an important constituent of cell and considered to be vital component in the cell. It provides a protection to cell nonenzymatically by acting as a free radical scavenger, a nucleophilic shield and as a reductant. Enzymatically GSH participates in detoxification of \( H_2O_2 \) and lipid hydroperoxides through mediation of GPx and of electrophilic xenobiotics (X) through the reaction by GST by conjugation of electrophilic xenobiotics to GSH forming thiol esters.

Thus GSH is an important antioxidant that functions directly in elimination of toxic peroxides and aldehydes and indirectly in maintaining vitamin C and vitamin E and SH dependent enzymes in their reduced and functional forms. Hence, the measurement of cellular GSH provides the information about GSH associated
<table>
<thead>
<tr>
<th>Parameters</th>
<th>Normal</th>
<th>Normal treated</th>
<th>Diabetic Untreated</th>
<th>Diabetic treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutathione (µg of GSH / mg protein)</td>
<td>1.03 (±0.01)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.12 (±.02)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.74 (± 0.01)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.98 (± .05)&lt;sup&gt;d#&lt;/sup&gt;</td>
</tr>
<tr>
<td>Glutathione Peroxidase (µg of GSH consumed/min/mg protein)</td>
<td>1.42 (±0.01)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.48 (±0.01)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.14 (±0.02)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.35(±0.01)&lt;sup&gt;d#&lt;/sup&gt;</td>
</tr>
<tr>
<td>Glutathione reductase (mmoles of NADPH oxidized/min/mg of protein)</td>
<td>40.93 (±0.60)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>44.63 (±0.33)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>29.26 (±0.46)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>41.92 (±0.44)&lt;sup&gt;d#&lt;/sup&gt;</td>
</tr>
<tr>
<td>Glutathione –S- transferase (µmoles of GSH – CDNB conjugate formed/min/mg protein)</td>
<td>20.57(±0.39)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>24.03(±0.37)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>17.85(±0.26)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>43.75(±0.57)&lt;sup&gt;d#&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

- Values are given as mean ±SE from eight rats in each group. Values not sharing a common superscript letter differ significantly at P<0.05(Duncan’s multiple range test).
- # indicates significance between diabetic and diabetic treated group.
scavenging system against free radicals induced LPO in the metabolic disease conditions and aging. Resistance of many cells against oxidative stress in associated with high intracellular levels of GSH (Meister, 1991; Estrela et al., 1995). GSH and thiol redox status regulates expression of genes involved in the pathogenesis of different diseases, including cancer, diabetes (Sen and packer, 1996).

The levels of renal GSH in four groups of experimental animals are represented in table (2). Normal rats treated with PNAEt showed a significant increase in levels of GSH (8.24%) when compared to normal rats. Where as DUT group and DT group showed significant decrease level of GSH (38.04 and 4.95% respectively) when compared with normal group. However DT group showed an increase in GSH levels (32.07%) when compared with DUT group.

The enhanced renal GSH content of normal rats treated with PNAEt compared to normal rats indicates increased reserves of free radical scavengers by PNAEt treatment. The significant decrease in GSH content of DUT rats represents increased utilization of GSH due to oxidative stress (Anuradha and Selvam, 1993). Our reports of decreased GSH concentration in diabetic rats was supported by earlier studies (Lee et al., 2001; Obrosava et al., 2003; Ngozi et al., 2003), suggesting that the decreased GSH concentration plays a role in development of diabetic complications. The observed significant elevation of kidney GSH content of DT rats compared to DUT rats indicates that the PNAEt treatment can either increase the biosynthesis of GSH or reduce the oxidative stress leading to less degradation of GSH or have both effects. The increased GSH content in kidney of the rats treated with PNAEt may be one of the factor responsible for inhibition of LPO and protein carbonyl content in these group of animals.
Similar to the PNAEt treatment other plant extrat treatments viz., Phaseolus vulgaris (Venkateswaran and Pari, 2002) Gongronema latifolium (Ngozi et al., 2003) Morinda officinalis (Soon and Tan, 2002) of STZ-induced diabetic rats showed an enhancement in renal GSH content.

**Glutathione reductase, Glutathione peroxidase and Glutathione-S-transferase**

*Glutathione reductase*

Besides transport and de novo synthesis, levels of GSH is also regulated by GSH redox cycle composed of GPx, GR and G6PDH. GR catalyses the reduction of GSSG to GSH in the presence of NADPH which is generated by a reduction of NADP⁺ via the HMP shunt pathway.

Under normal conditions the balance of the equation is far in the direction of maintaining cellular glutathione in its reduced state. The significance of these enzymes lies in its ability to keep high levels of GSH. In order to understand the region for alterations in renal GSH levels in four experimental groups the activity of GR in renal tissue has been estimated and represented in table 2.

*Glutathione peroxidase*

Peroxides produced in a cell can be detoxified by the action of GPx and CAT. GPx has a complementary catalytic activity with catalase. The Km value for H₂O₂ of GPx (0.25 mmol/l) is lower than that of CAT (25 mmol/l), providing a preferential pathway for the degradation of low concentration of H₂O₂ present in intact cell (Wohaieb and Godin, 1987). GPx catalyses the reduction of peroxides with GSH to form GSSG and the reduction product of H₂O₂ (Chance et al., 1979). This enzyme is specific for its hydrogen donor GSH, and non-specific for the hydroperoxides ranging from H₂O₂ to organic peroxides (Freeman and Crapo, 1982) thus offering a major defending role in cells against peroxidative damage of complex biochemical compounds such as lipids and nucleic acids (Meister and Anderson, 1983; Uaybandyopadhyay et al., 1999).
Glutathione-S-transferase

GST is multifunctional proteins found in many tissues. They show a broad specificity for organic hydroperoxides but not for \( \text{H}_2\text{O}_2 \) (Bruce et al., 1982). GST catalyses conjugation between GSH with a very wide range of secondary substrates and a large number of xenobiotics with electrophilic center. Thus it plays an important role in detoxification of xenobiotic compounds there by protecting the cell from peroxidative damage (Deneke and Fanburge, 1989).

The data represented in table (2) indicates the effect of PNAEt treatment on the activities of GSH dependent antioxidant enzymes of kidney in normal and STZ diabetic rats. In the present study the activities of renal GR GPx, GST are found to be significantly increased in PNAEt treated normal rats compared to normal rats (15.05, 0.22 and 16.86% respectively). Where as, the activities of these enzymes are significantly reduced in DUT group (16.71, 19.78 and 13.23% respectively) when compared to normal animals. The depletion in the activities of these enzymes in DUT rats may results in deleterious oxidative stress due to accumulation of toxic products. These results are in consistence with earlier reports (Anuradha and Selvam, 1993; Stanely et al., 2001; Venkateswarum and Pari, 2002).

However, in the published literature the antioxidant enzymes response to diabetics has been unclear. Diabetes has been reported to be associated with either increased (Ndahinana et al., 1996) decreased (Uzel et al., 1987) or unchanged (Faure et al., 1995) activities of anti oxidant enzymes in various tissues.

The decreased activity of GR in diabetic rats may lead to decreased GSH content. The decreased activity of GR further correlates with the levels of GSH and G6PDH. Thus the decreased activity of G6PDH (Gupta et al., 1996; Jain, 1998; Sailaja et al., 2003) also resulted in the decreased production of NADPH, a necessary co-factor for GR activity. In addition to this the enhanced operation of polyol pathway under diabetic conditions (Peterson et al., 1990) might have resulted
Results & Discussion

in decreased availability of NADPH for GR activity in DUT rats which ultimately resulted in decreased regeneration of GSH.

Flohe (1971) reported that the kinetics of GPx are in first order in respect to GSH. Thus the decreased levels of renal GSH in DUT rats may be one of the factor for decreased activity of GPx. Thus the decreased renal GPx activity of DUT rats may be responsible for elevated renal LPO observed in this group of animals.

The decrease activity of GPx in DUT indicates higher accumulation of peroxides. The increased generation of reactive oxygen radicals such as superoxide, hydrogen peroxide and hydroxyl radicals further may alter various functions of biomolecules, indicating decreased detoxification capacity of DUT rats. Decreased GST levels of renal tissue in DUT group may also be due to the decreased levels of renal GSH in these rats.

As the alterations produced in the antioxidant activities indicate the involvement of deleterious oxidative changes, increased activities of the components of this defense system would therefore be important in protection against radical damage.

Administration of PNAEt to diabetic rats resulted in increased activities of renal GR, GPx and GST (15.65, 18.07 and 145.13% respectively) compared to DUT rats, indicates the protective effect of PNAEt treatment against oxidative damage observed in STZ-induced diabetes.

SOD and CAT:

SOD scavenges the superoxide ions produced as cellular byproducts. SOD is a major defense for aerobic cells in combating the toxic effect of superoxide radicals (McCord et al., 1976). SOD has been touted as one of the most important enzyme in the enzymatic antioxidant defense system. The superoxide anion has known to inactivate CAT, which is involved in the detoxification of hydrogen peroxide. SOD
Table 3  Effect of PNAEt on SOD and CAT activity in STZ-induced diabetic rats

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Normal</th>
<th>Normal treated</th>
<th>Diabetic Untreated</th>
<th>Diabetic treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Superoxide dismutase (U/min/mg protein)</td>
<td>42.071(±0.302)a</td>
<td>44.636(±0.515)b</td>
<td>29.268(±0.747)c</td>
<td>41.923(±0.628)a#</td>
</tr>
<tr>
<td>Catalase</td>
<td>46.773(±0.939)a</td>
<td>50.671(±0.555)b</td>
<td>33.888(±0.780)c</td>
<td>45.395(±0.795)a#</td>
</tr>
</tbody>
</table>

- Values are given as mean ±SE from eight rats in each group. Values not sharing a common superscript letter differ significantly at P<0.05(Duncan’s multiple range test).
- # indicates significance between diabetic and diabetic treated group
scavenges the superoxide anion to form hydrogen peroxide, hence, diminishing the toxic effects caused by this radical. Wohaieb and Godin, (1987) had suggested that the reactive oxygen free radicals could inactivate and reduce the hepatic SOD and CAT activities.

CAT is a hemoprotein, this antioxidant enzyme is widely distributed in all animal tissues. It reduces H$_2$O$_2$, produced by dismutation reaction and prevents generation of hydroxyl radical, thereby protecting the cellular constituents from oxidative damage. Studies have shown that the administration of catalase results in protection against H$_2$O$_2$ mediated LPO (Corroch et al., 1986). H$_2$O$_2$ is considered a key metabolite because of its relative stability, its diffusion and its involvement in cell signaling cascade (Fridovich, 1995; Khan and Wilson, 1995; Pantopoulso et al., 1997; Sujuki et al., 1997). Since, the first description of glutathione peroxidase in 1957, an intense debate was created on weather CAT or GPx was the primary enzyme in the removal of H$_2$O$_2$ (Scott et al., 1991).

A major role of GPx in decomposing H$_2$O$_2$ especially derived from studies on erythrocytes with a reduced capacity to generate NADPH (Cohen and Hochstein, 1963). Experiments with acatalasic cells provide more evidence that CAT is a first line of defense against H$_2$O$_2$ (Jacob et al., 1965). CAT decomposes H$_2$O$_2$ without generation of free radicals by minimizing one electron transfers (Sebastain et al., 1997).

The activities of renal SOD and CAT of four experimental groups of rats are shown in table (3). The activities of SOD and CAT enzymes are found to be significantly decreased in DUT rats compared with their levels in normal rats (30.4, and 27.6%). Previous studies have reported on the reduction of renal SOD and CAT activities in STZ induced diabetic rats when compared with normal rats (Loven et al., 1986; Godin et al., 1988; Anuradha and Selvam, 1993; Sivaprakasam et al., 1996; Stanely et al., 2001; Venkateswaran and Pari, 2002; Ananthan et al., 2004). Reduced activity of SOD and CAT in kidney has been observed during diabetes, and
Results & Discussion

this activity may result in a number of deleterious effects due to accumulation of superoxide radicals and hydrogen peroxide (Searle and Wilson, 1980).

PNAEt treated diabetic rats showed a significant enhancement of these enzyme activities when compared with DUT group (43.23 and 34.03%), thus these altered enzyme levels in diabetic rats were normalize by PNAEt treatment, and this may be due to its protective action on kidney against ROS or to suppression of the production of free radical production.

PNAEt treated normal rats showed a significant enhancement in the activities of these enzymes when compared with that of the normal rats, indicating enhanced antioxidant potential of PNAEt.

In conclusion altered (decreased) tissue defense systems in kidney of diabetic rats suggest an increased oxidative stress in kidney of STZ induced diabetic rats indicating the degenerative status in diabetes. The current findings suggest that the enhanced oxidative stress in the kidney of STZ induced diabetic rats was effectively reduced and controlled via the administration of PNAEt by enhancing the activities of antioxidant enzymes in DT rats. The over expression of antioxidant enzymes in kidney of DT rats and normal treated rats implies that, this potential oxidant defense is reactivated by active principles of PNAEt. This results in an increase in the capacity of detoxification through enhanced scavenging of oxy radicals.

DNA damage studies:

In conclusion PNAEt treatment resulted in decreased oxidative stress in kidney of STZ induced diabetic rats (reflected by decreased protein carbonyl content and LPO) by enhancing the activity of scavenging enzymes and antioxidant levels. Further in order to understand its protective action against DNA damage caused by ROS, studies were extended on rat lymphocytes.
Free radicals and other species are constantly generated \textit{in vivo} cause oxidative damage to biomolecules, a process held in check only by the existence of multiple antioxidant and repair systems as well as the replacement of damaged lipids and proteins (Grune and Davies, 1997). DNA is probably the most biologically significant target of oxidative attack, and it is widely thought that continuous oxidative damage to DNA is significant contributor to the age-related development of the major cancers (Halliwell, 2000).

Strand scission, destruction and fragmentation of bases and deoxyribose sugars have all been reported to occur following free radicals (mainly hydroxyl radical) attack on DNA. The resulting cytotoxicity, mutations and potential for malignant change occur as a result of induced chromosomal aberrations (Sinclair et al., 1991).

The role of hyperglycemia-induced oxidative stress in producing DNA damage is also supported by recent findings of that increased amounts of 8-hydroxyguanosine and 8-hydroxydeoxyguanosine (markers of oxidative damage to DNA) found in both the plasma and tissues of STZ induced diabetic rats (Park et al., 2001). Base modifications and DNA strand breaks have been demonstrated in diabetic patients as well (Lorenzi et al., 1987; Anderson et al., 1998; Astely et al., 1999; Sardas et al., 2001; Dince et al., 2003).

The antioxidant power of the individual compound depends on their chemical structure, which is also responsible for the stability of the reactive flavonoid radicals (Rice-Evans et al., 1996). The less stable radicals, formed during the redox-cycle reaction, can propagate the harmful events through the radical attack (Cao et al., 1997; Arora et al., 1998). Thus, plant extracts can act as an antioxidant or prooxidant, depending on the structure and composition of different classes of polyphenols (Bors et al., 1990; Cao et al., 1997; Catapano, 1997). The interaction between individual polyphenols may decide about the final outcome. Because of
Table 4 Genotoxicity assessment of PNAEt by Comet Assay

<table>
<thead>
<tr>
<th>Contents</th>
<th>Rat lymphocytes</th>
<th>Lymphocytes+ 0.4 mg/ml PNAEt</th>
<th>Lymphocytes+ 0.8 mg/ml PNAEt</th>
<th>Lymphocytes+ 1.2 mg/ml PNAEt</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cells scored from quadruplicate gels</td>
<td>400</td>
<td>400</td>
<td>400</td>
<td>400</td>
</tr>
<tr>
<td>Percent of cells showing comets</td>
<td>1.50 (±0.00)</td>
<td>1.50 (±0.280)</td>
<td>1.50 (±0.288)</td>
<td>1.45 (±0.37)</td>
</tr>
<tr>
<td>Comet tail length (µM) mean ± SE</td>
<td>0.80 (±0.081)</td>
<td>0.77 (±0.121)</td>
<td>0.86 (±0.054)</td>
<td>0.73 (±0.104)</td>
</tr>
</tbody>
</table>

Values are mean ± SE of 4 gels. P<0.05
Figure 5: Images of comets obtained by Single-cell gel electrophoresis of rat lymphocytes at various concentrations of H$_2$O$_2$ fig.a) Normal b) 100µM c) 200 µM d) 500µM e) PNAEt rat lymphocytes +500 µM.
Figure 2: Images of Single-cell gel electrophoresis at various doses of Phyllanthus niruri aqueous extract—fig. a) Untreated b) 0.4 mg/ml c) 0.8 mg/ml d) 1.2 mg/ml.
**Figure 3**: Images of comets obtained by Single-cell gel electrophoresis of rat lymphocytes fig.a) Normal  b) STZ - treated  c) PNAEt pretreated + STZ.

**Figure 4**: Images of comets obtained by Single-cell gel electrophoresis of rat lymphocytes fig.a) Normal  b) NO - treated  c) PNAEt pretreated + NO.
that, the results obtained when the crude plant extracts are used have to be considered with special precaution.

The first screening of any compound, drugs or potential nutraceuticals starts with the genotoxicity test. The comet assay, broadly used in recent years. We also applied in our study to analyze the effect of plant extracts on the DNA level (da Costa Lopes et al., 2000; O'Brine et al., 2000; Yen et al., 2001; Kassie et al., 2002)

The comet assay is a useful technique for studying DNA damage and repair with manifold applications. Cells with increased DNA damage display increased migration of chromosomal DNA on electrophoresis from the nucleus towards the anode, which resembles the shape of comet. It is alkaline version, which is mainly used, DNA strand breaks and alkali-labile sites become apparent, and the extent of DNA migration correlates with the amount of DNA damage in the cell. The comet assay combines the simplicity of biochemical technique for detecting DNA single strand breaks or alkali-labile sites with the single cell approach typical of cytogenetic assay. The advantages of the comet assay include its simple and rapid performance, its sensitivity for detecting DNA damage. The comet assay has already been used in many studies to assess DNA damage and repair include by various agents in a variety of cells in vitro and in vivo (Fairbairn et al., 1995; Tice, 1995). The test has wide spread applications in a variety of cells in genotoxicity testing in vitro and in vivo (Tice et al., 2000; Hartmann et al., 2003), DNA damage and repair studies (Hartmann et al., 2003; Collins, 2004), environmental biomonitiring (Cotelle and Ferard, 1999; Lee and Steinert, 2003) and human population monitoring (Moller et al., 2000).

Lymphocytes of normal rats subjected to SCGE showed no significant damage (0.5% comet cells with slight DNA migration lengths). SCGE of rat lymphocytes treated with PNAEt (0.2, 0.4 and 0.6 mg/ml) showed a no significant DNA damage when compared to basal level indicating that, this extract is devoid of genotoxicity or prooxidant property table (4) and fig.(2)
Table 5 The protective ability of PNAEt against STZ and NO radical induced DNA

<table>
<thead>
<tr>
<th>Contents</th>
<th>Rat lymphocytes</th>
<th>Rat lymphocytes +STZ (100μg/ml)</th>
<th>PNAEt pretreated lymphocytes +STZ (100μg/ml)</th>
<th>Lymphocytes +Nitric oxide generating system</th>
<th>PNAEt pretreated lymphocytes +Nitric oxide generating system</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cells scored from quadruplicate gels</td>
<td>400</td>
<td>400</td>
<td>400</td>
<td>400</td>
<td>400</td>
</tr>
<tr>
<td>Percent of cells showing comets</td>
<td>0.50(±0.00)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>97.50(±0.64)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>10.50(±0.64)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>98.0(±0.40)&lt;sup&gt;d&lt;/sup&gt;</td>
<td>8.25(±0.04)&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>Comet tail length (μm) mean±SE</td>
<td>0.56(±0.10)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.40(±0.07)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.76(±0.54)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>5.68(±0.10)&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.89(±0.04)&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values are mean ± SE of 4 gels. P<0.05
The aim of this study was to establish whether *phyllanthus niruri* aqueous extract (PNAEt) has the protective ability of against the oxidative stress induced by the $H_2O_2$, NO and STZ in rat lymphocytes.

In addition to STZ-induced cytotoxicity through DNA alkylation, recent studies have suggested that ROS, including superoxide ($O_2^{•−}$), hydrogen peroxide ($H_2O_2$), hydroxyl radical ($OH^{•}$) and nitric oxide ($NO^{•}$) play a central role in the mechanism of DNA damage and cytotoxicity of STZ (Bolzan and Bianchi, 2002).

Streptozotocin-induced hyperglycemia in mice was prevented by L-N-monomethyl arginine (L-NMMA), an inhibitor of inducible nitric oxide synthase (iNOS); thus, suggesting a role for NO$^{•}$ in STZ-induced diabetes mellitus (Lukic et al., 1991). More recent studies contradict the source of STZ stimulated NO$^{•}$, suggesting that STZ spontaneously produces nitrite, which is oxidized to NO$^{•}$ (Tanaka et al., 1995). These results seem rational due to the fact that STZ does contain a nitrosourea moiety. Further studies have demonstrated that NO$^{•}$ is generated during the cellular metabolism of STZ and not only by iNOS, as the formation of NO$^{•}$ in hepatocytes in the presence of STZ was not blocked by iNOS inhibitors (Kroncke et al., 1995). However, the STZ-mediated DNA damage was significantly attenuated by the presence of an intracellular NO$^{•}$ scavenger. Reactive oxygen species can cause DNA damage by targeting the base or sugar. DNA single strand breaks are often the result of free radicals targeting the sugar resulting in damage to the phosphate backbone (Bolzan and Bianchi, 2002).

PNAEt protection against STZ-induced DNA damage of rat lymphocytes was presented in table (5) and fig (3). The study indicate the DNA damage was observed when lymphocytes with STZ and pre treatment of lymphocytes with PNAEt (0.4 mg/ml) showed a protection against STZ induced DNA damage. Frequency of cells showing migration when exposed to STZ was significantly higher compared to controls with greater lengths of migration. Where as PNAEt pre treated lymphocytes
Table 6  H₂O₂ induced DNA damage of lymphocytes from normal and PNAEt rats

<table>
<thead>
<tr>
<th>Contents</th>
<th>Lymphocytes</th>
<th>Lymphocytes +H₂O₂ (100µmol)</th>
<th>Lymphocytes +H₂O₂ (200µmol)</th>
<th>Lymphocytes +H₂O₂ (500µmol)</th>
<th>PNAEt treated rat lymphocytes + H₂O₂ (100µmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cells scored from quadruplicate gels</td>
<td>400</td>
<td>400</td>
<td>400</td>
<td>400</td>
<td>400</td>
</tr>
<tr>
<td>Per cent of cells showing comets</td>
<td>1.50(±0.00)³</td>
<td>29.50(±0.64)²</td>
<td>57.75(±1.93)³</td>
<td>99.12(±0.71)³</td>
<td>7.8(±0.86)⁵</td>
</tr>
<tr>
<td>Comet tail length (µm) mean ± SE</td>
<td>0.50(±0.08)²</td>
<td>1.695(±0.20)²</td>
<td>2.05(±0.19)³</td>
<td>5.21(±0.12)³</td>
<td>0.729(±0.05)⁵</td>
</tr>
</tbody>
</table>

Values are mean ± SE of 4 gells. P<0.05
exposed to STZ showed a significant decrease in frequency of comet cells and almost similar to basal levels with decreased lengths of DNA migration.

Similar to that of STZ, NO generating system also caused significant DNA damage by showing increased per cent of comets. Further PNAEt pre treatment showed protection against NO°.

Earlier reports of Bolzan and Bianchi, (2002) indicate STZ stimulate HO° and H2O2 generation lead to DNA fragmentation in isolated rat pancreatic islet cells. H2O2 induced lymphocyte DNA damage was quantified at the different concentrations of H2O2 (100, 200 and 500 μmols) by observing the cells as relative tail length. Table (6) and Fig (4) revealed that DNA damage in lymphocytes when exposed to H2O2 and this damage was increased with increased H2O2 concentration. Complete or 100% cells showed comets with greater tail length (Table. 6) at 500 μmol concentration of H2O2.

In order to assess the protective action of PNAEt against H2O2 caused DNA damage, lymphocytes of PNAEt treated rats were used for comet assay in the presence of 500 μmol concentration of H2O2.

The data presented in table (6) and fig (4) indicates that the PNAEt treated rat lymphocytes showed an excellent protection against H2O2 induced DNA damage.

Thus the protective effects of PNAEt against STZ, NO, H2O2 induced DNA damage under in vitro and in vivo condition, clearly indicates its ROS scavenging capacity. Some of the phytochemicals of PNAEt may be responsible for ROS removal activity.

Kapiszeska et al., (2005) reported that quercetin shows the dose-dependent DNA protection against the oxidative DNA damage induced by H2O2. Quercetin is the plant flavonoid, with known antioxidant property. Phyllanthus niruri also reported
to have a quercetin flavonoids. Thus quercetin and/or other constitutes of *Phyllanthus niruri* may be responsible for its protective role against ROS induced DNA damage by scavenge these ROS.

In conclusion these observations show that the PNAEt possess antioxidant activity, which may exert a beneficial action against pathological alterations caused by the presence of ROS in STZ-induced diabetic rats and may help to prevent or delay the onset of diabetic complications. The present study shows that the aqueous extract of *Phyllanthus niruri* not only possess antihyperglycemic property but also reduces oxidative stress in diabetic rats.