ABSTRACT

Diabetes mellitus has been associated with oxidative stress. The present study was performed to investigate the pancreas protective activities of *Phyllanthus amarus* aqueous extract (PAAEt) in streptozotocin (STZ) induced diabetic rats. Oral administration of PAAEt (200mg/kg bw) to diabetic rats for 60 days significantly (p<0.05) decreased antioxidant enzymes such as glutathione peroxidase, glutathione-s-transferase, glutathione reductase, superoxide dismutase, catalase and depletion of reduced glutathione in pancreas which illustrates that increased lipid peroxidation (107 %) was restored to control levels. Co-treatment with PAAEt revealed the therapeutic efficacy against oxidative stress in pancreatic tissue. Hence extract of this plant could be used as an adjuvant therapy for the prevention and/or management of diabetes.


INTRODUCTION

In diabetes mellitus, chronic hyperglycemia produces multiple biochemical sequelae and diabetes-induced oxidative stress that play an important role in the symptoms and progression of the disease (Chis et al., 2009). There is increasing evidence that complications (macro and micro vascular) related to diabetes are associated with oxidative stress (Jakus, 2000). Improved antioxidant status helps to minimize the oxidative damage, and thus can delay or decrease the risk of developing many chronic age related, free radical induced diseases. Supplementation of therapeutics with antioxidants may have a chemo protective role in diabetes (Logani and Davis, 1979). Currently available synthetic antidiabetic agents besides being expensive produce serious side effects. Apart from currently available therapeutic options, many herbal medicines have been recommended for the treatment of diabetes mellitus, medicinal plants have the advantage...
of having no side effects (Ayodhya et al., 2010).

*Phyllanthus amarus* (Euphorbiaceae) can be found in all tropical regions of the world including Southern India and China. *P. amarus* is an erect annual herb of not more than one and half feet tall and has small leaves and yellow flowers. All parts of the plant are used in ayurvedhic medicines because of their medicinal properties. Leaves of this plant are reported to contain lignans, alkaloids, flavonoids, galloantnoids and glycosides (Dhalwal et al., 2006). In folk medicine, *P. amarus* has allegedly been used to treat jaundice, diabetes, gonorrhea, irregular menstruation, tachycardia, dysentery, spasmodic cough, itchiness, arthritis, otitis, swelling, skin ulcer and weakness of maleorgan (Calixto et al., 1998). Methanol extract of *P. amarus* was reported to have hypoglycemic effect on alloxan induced diabetes mellitus (Raphael et al., 2002). Earlier work in our laboratory has shown that plasma and hepatic tissue antioxidant properties of aqueous extract of *P. amarus* in rats (Karuna et al., 2009 and Vijayabharathi et al., 2014). Therefore, the present investigation was undertaken to study the protective role of *Phyllanthus amarus* aqueous extract on oxidative stress in pancreas of STZ induced diabetic Wistar rats.

**MATERIALS AND METHODS**

**Chemicals**

The chemicals used in the current study were procured from Sigma Chemical Company (St. Louis, MO, USA), and SISCO Research Laboratory Pvt. Ltd, Mumbai, India).

**Plant material**

*Phyllanthus amarus* aqueous extract (PAAE) whole herb was purchased from Chemiloids (manufacturer and exporter of herbal extract, Vijayawada, A.P, India). Prior to preparation of the plant extract, the plant was authenticated by Dr. Narasimha Reddy, Taxonomist, Laila Impex R&D Centre, Vijayawada. The extract was stored in an air-tight container and dissolved in water just before use. Herb to product ratio was 10:1.

**Induction of diabetes mellitus**

Male Wistar rats of 2-3 month old weighing (125-150 g) were procured from Sri Venkateswara Enterprises (Bangalore, India). They were acclimatized for 7 days two to three animals were housed per cage sized (41x28x14 cm) in our animal house (Regd. No.470/01/a /CPCSEA, dated 24th August, 2001) and maintained under standard conditions of temperature and relative humidity with 12 h light/dark cycles. Diabetes was induced in overnight fasted rats by a single intraperitoneal injection of freshly prepared STZ (55 mg/kg body weight) in 0.05 M ice-cold citrate buffer (pH 4.5) in a volume of 0.1 ml per rat. After 72 hours of STZ administration, the plasma glucose level of each rat was determined for confirmation of diabetic induction. Rats with plasma glucose level above 230 mg/dL were considered as diabetic and used subsequently.

**Experimental design**

In the present experiment, a total of 40 rats (20 diabetic rats; 20 normal rats) were used. The rats were divided into following four groups of 10 each: Control (C); Control rats treated with *P. amarus* (C+PA); Diabetic (D); and Diabetic rats treated with *P. amarus* (D+PA). Diabetic treated group and Control treated group rats received an aqueous extract of the *P. amarus* in 3 ml of distilled water per rat at once by orogastric tube at a dose of 200 mg/kg body weight for 60 days, whereas distilled water was administered to control and diabetic rats. The dose was fixed based on the results from preliminary experiments.
Animal sacrifice and organ collection
After the experimental period of 60days, the animals from each experimental group were starved for 12 h and sacrificed by cervical dislocation and immediately the whole pancreas was dissected out and washed with ice-cold saline and used for analysis. Ten percent homogenate of pancreas was prepared by using Potter-Elvehjem homogenizer with Teflon pestle at a 4°C in 0.15 M KCl. The whole homogenate was used for estimation of reduced glutathione (GSH) and lipid peroxidation (LPO). The homogenate was centrifuged in cooling centrifuge (12000 rpm for 45 min at 0-4°C) to remove the debris and supernatant was used for enzyme assays. The total protein content of the pancreas in the whole homogenate was estimated by the method of Lowry et al. (1951).

Biochemical investigations
Lipid peroxidation, enzymatic antioxidant enzymes: glutathione peroxidase, glutathione-s-transferase, glutathione reductase, superoxide dismutase, catalase and nonenzymatic antioxidant enzyme: reduced glutathione of pancreatic homogenate were assayed.

Estimation of lipid peroxidation
Lipid peroxidation in pancreas was estimated colorimetric ally by measuring the thiobarbituric acid reactive substances (TBARS) by the method of Utley et al. (1967). To 0.1 ml of 10% tissue homogenate, 2 ml of 10% TCA and 4 ml of 0.67%TBA were added and heated in a water bath for 30 min and cooled. Absorbance of the supernatant was read at 535 nm. The extent of LPO was expressed as nmol of malondialdehyde (MDA) formed/g tissue, using a molar extinction coefficient of MDA as $1.56 \times 10^5 \text{M}^{-1} \text{cm}^{-1}$.

Estimation of reduced glutathione
Reduced glutathione was estimated in pancreas homogenate (10%) using a disulfide compound, 5,5'-dithio-bis-nitrobenzoic acid (DTNB), which readily gets reduced by sulfhydryl compounds forming a highly colored anion having maximum absorbance at 412 nm as outlined by the method of Ellman (1959).

Assay of enzymes in pancreas homogenate
Catalase
Catalase (CAT) activity was assayed by the method of Beers and Sizer (1952). The assay mixture consists of 1.9 ml of sodium phosphate buffer (0.05 M, pH 7.0), 1.0 ml of H$_2$O$_2$ (0.059 M) and 0.1 ml of pancreas homogenate. The activity was expressed as moles of H$_2$O$_2$ decomposed/min/mg protein.

Superoxide dismutase
Superoxide dismutase (SOD) activity in pancreas was determined according to a modified procedure adopted by Soon and Tan (2000). Superoxide dismutase activity was measured based on the ability of the enzyme to inhibit the oxidation of pyrogallol. The assay mixture consists of 0.5 ml of phosphate buffer (50 mM, pH 7.8 containing 1 mM EDTA), 0.02 ml of pancreas homogenate and 0.86 ml of distilled water. The reaction was initiated by the addition of 0.02 ml of pyrogallol (10 mM, in 0.01N HCl). One unit of SOD was defined as the amount of enzyme required to inhibit the oxidation of pyrogallol by 50% in standard assay system of 3 ml. The activity was expressed as Units/min/mg of protein.

Glutathione reductase
Glutathione reductase (GR) catalyzes the reduction of oxidized glutathione (GSSG) by NADPH to GSH. The activity of GR in pancreas was measured by the method of Pinto and Bartley (1969). In brief, the assay mixture
consists of 0.5 ml of phosphate buffer (0.25 M, pH 7.4), 1.0 ml of EDTA (25 mM), 0.1 ml of NADPH (1 mM), 0.96 ml of distilled water and 0.1 ml of pancreas homogenate. The reaction was initiated by the addition of 0.024 ml of GSSG (50 mM). The activity was expressed as μmoles of NADPH oxidized/min/mg protein by using milli molar extinction coefficient of NADPH as 6.22.

**Glutathione peroxidase**

Glutathione peroxidase (GPx) in pancreas was measured by the method of Rotruck et al. (1973). Briefly, the reaction mixture consists of 0.5 ml of phosphate buffer (0.2 M, pH 7.0 containing 0.4 mM EDTA, and 10 mM sodium azide), 0.2 ml of pancreas homogenate, 0.2 ml of GSH (2 mM) and 0.1 ml of H2O2 (0.2 mM) and incubated for 10 min at room temperature along with blank containing all reagents expect tissue homogenate. The reaction was arrested by the addition of 0.5 ml of TCA (10%), centrifuged at 4000 rpm for 5 min and GSH content in 0.5 ml of supernatant was estimated by following the method of Ellman (1959). The activity was expressed as μg of GSH consumed/min/mg protein.

**Glutathione-S-transferase**

Glutathione-S-transferase (GST) in pancreas was measured by the method of Habig et al. (1974). The assay mixture consists of 1.7 ml of sodium phosphate buffer (0.14 M, pH 6.5), 0.2 ml of GSH (30 mM) and 0.04 ml of homogenate. The reaction was initiated by addition of 0.06 ml of 1-chloro-2, 4-dinitrobenzene (CDNB) (0.01 M dissolved in 50% ethanol). The activity was calculated by using milli molar extinction coefficient of CDNB-GSH conjugate as 9.6. The activity was expressed as μmoles of CDNB-GSH conjugate formed/min/mg protein.

**Statistical analysis**

Data were expressed as the mean ± SE for the number, n = 10 of animals in the group as indicated in (Tables 1–2). The data were subjected to statistical analysis by Duncan’s Multiple Range (DMR) (Duncan, 1955) test. Values of p < 0.05 were considered statistically significant

**RESULTS and DISCUSSION**

**General observation and characteristics**

No visible side effects and variations in animal behaviour (respiratory distress, abnormal locomotion and cathelepsy) were observed in C+PAAE group of rats, indicating the non-toxic nature of P. amarus administration.

**Effect of PAAE on oxidative stress markers and antioxidant enzymes**

Table 1 & 2 illustrate that significant decreased in the activities of pancreatic oxidative stress markers viz. GSH and LPO and antioxidant enzymes glutathione dependent enzymes (GPX, GST, and GRx), anti lipidperoxidative enzymes (SOD and CAT) in STZ induced diabetic rats was observed in comparison to normal control rats. This study represents decreased levels of tissue antioxidant status and increased lipid peroxidation, reduced glutathione in STZ induced diabetic rats. Co-administration with PAAE significantly (p<0.05) restore the adverse changes in pancreatic tissue and maintained near to normal levels.
Table 1: Effect of *P. amarus* treatment on Pancreas oxidative stress markers in STZ induced diabetic rats.

<table>
<thead>
<tr>
<th>Experimental groups</th>
<th>C</th>
<th>C+PA</th>
<th>D</th>
<th>D+PA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipid peroxidation (nmol of MDA/mg protein)</td>
<td>4.404±0.110^b</td>
<td>3.109±0.020^a</td>
<td>21.863±0.075^d</td>
<td>10.229±0.055^c</td>
</tr>
<tr>
<td>Reduced glutathione (µg of GSH/mg protein)</td>
<td>0.832±0.010^b</td>
<td>0.929±0.013^c</td>
<td>0.409±0.009^a</td>
<td>0.783±0.037^b</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SEM (n=10) Values with different superscripts across the row are significantly different at P<0.05 (Duncan’s multiple range test). C: control, C+PA: control treated with *P. amarus*, D: diabetic, D+PA: diabetic treated with *P. amarus*, PAAEt: *Phyllanthus amarus* aqueous extract.

Table 2: Effect of *P. amarus* treatment on Pancreas antioxidant enzymes in STZ induced diabetic rats.

<table>
<thead>
<tr>
<th>Experimental groups</th>
<th>C</th>
<th>C+PA</th>
<th>D</th>
<th>D+PA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Catalase (mmol of H$_2$O$_2$ decomposed/min/mg protein)</td>
<td>1.890±0.007^b</td>
<td>2.956±1.104^d</td>
<td>1.104±0.008^a</td>
<td>2.006±0.009^c</td>
</tr>
<tr>
<td>Superoxide dismutase (U/min/mg protein)</td>
<td>30.841±0.191^b</td>
<td>34.395±0.109^c</td>
<td>24.963±0.168^a</td>
<td>40.054±0.197^d</td>
</tr>
<tr>
<td>Glutathione peroxidase (µg of GSH consumed/min/mg protein)</td>
<td>0.410±0.005^c</td>
<td>0.510±0.006^d</td>
<td>0.227±0.007^a</td>
<td>0.289±0.005^b</td>
</tr>
<tr>
<td>Glutathione reductase (µmols of NADPH oxidized/min/mg protein)</td>
<td>6.112±0.039^c</td>
<td>7.236±0.061^d</td>
<td>40246±0.049^a</td>
<td>5.874±0.044^b</td>
</tr>
<tr>
<td>Glutathione-S-transferase (µmols of GSH-CDNB conjugate formed/min/mg of protein)</td>
<td>0.060±0.002^b</td>
<td>0.087±0.102^c</td>
<td>0.410±0.001^a</td>
<td>0.061±0.001^b</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SEM (n=10) Values with different superscripts across the row are significantly different at P<0.05 (Duncan’s multiple range test). C: control, C+PA: control treated with *P. amarus*, D: diabetic, D+PA: diabetic treated with *P. amarus*, PAAEt: *Phyllanthus amarus* aqueous extract.
Oxidative stress is one of the most important mechanisms of diabetic complication genesis, and the imbalance between the production of active oxygen and its opposing protective mechanisms is known to be associated with even greater cellular damage, a phenomenon that is seen in diabetic patients. Furthermore, free radicals are reported to destroy beta cells that produce and secrete insulin (Laybutt et al., Adeghate and parvez, 2000). Low levels of lipid peroxides stimulate the secretion of insulin, but when the concentration of endogenous peroxides increases, it may initiate uncontrolled lipid peroxidation, thus leading to cellular infiltration and islet cell damage in type I diabetes (Metz, 1984). Glutathione-S-transferase is thought to be the fundamental antioxidant enzyme, because it is closely related to the direct elimination of reactive oxygen species. It catalyzes the conjugation of reduced glutathione, via the sulfhydryl group, to electrophilic centers on a wide variety of substrates. This activity is useful in the detoxification of endogenous compounds such as peroxidised lipids (Valavanidis et al., 2006). GSH is considered an important defense against lipid oxidative damage in the pancreas where as it has a direct antioxidant function by reacting with superoxide radicals followed by the formation of oxidized glutathione (GSSG) and other disulfides. Therefore, GSH metabolizing enzymes, GST and GPx, will be affected when GSH level is reduced in the cells (Khan and Ahmed, 2009). Consequently, the reduction in the activity of these enzymes may result in a number of deleterious effects due to the accumulation of superoxide radicals and hydrogen peroxide (Khan et al., 2012). SOD protects tissues against oxygen free radicals by catalyzing the removal of superoxide radical, converting it into H$_2$O$_2$ and molecular oxygen, which both damage the cell membrane and other biological structures (Arivazhagan et al., 2000). Catalase is a haem-protein, which is responsible for the detoxification of significant amounts of H$_2$O$_2$ (Cheng et al., 1981). Reduced activities of SOD and catalase in the pancreas and pancreas during diabetes were reported, resulting in a number of deleterious effects due to the accumulation of superoxide radicals and hydrogen peroxide (Searle and Wilson, 1981). When the balance between ROS production and antioxidant defense is lost oxidative stress results; which through a serious of events deregulates the cellular functions leading various pathological conditions (Blokhina et al., 2002).

It has been noticed that animal body had an effective mechanism to prevent the free radical induced tissue cell damage; this was accomplished by a set of endogenous antioxidant enzymes and protein such as GST, SOD, CAT, GPX, GRD and GSH. The significant increase in the activities of these antioxidant enzymes and reduced lipid peroxidation suggests a greater level of endogenous antioxidant associated with PAAEt co-administration resulting in an enhanced free radical scavenging activity. The antioxidant activity of phenols is due to their redox properties that allow them to act as reducing agents by donating hydrogen, quenching singlet oxygen or acting as metal chelators. Aqueous extract of P. amarus contained high content of phenolic compounds which were found to have strong and significant positive correlations to free-radical scavenging potential and inhibition of lipid peroxidation (Halliwell, 1994). Hence extract of this plant have wide variety of compounds like flavonoids and polyphenols. These compounds may be responsible for increasing the antioxidant status.
CONCLUSION

The present study revealed that PAAEt possess antioxidant properties in pancreas and protects against STZ induced Diabetes. The most important protective mechanism offered by PAAE is through its ability to decrease pancreatic lipid peroxidation and increased levels of antioxidant enzymes. Thus, PAAE exert a protective role in pancreas of streptozotocin induced diabetic oxidative stress wistar male rats.

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CONFLICT OF INTERESTS

The authors have declared no conflict of interests.

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Corresponding author:
Prof. Dr. D. Saralakumari
Dept. of Biochemistry, Anantapuramu
515003, Andhra Pradesh, India
Tel: +91 08554 255879
E-mail: desiredysaralakumari8@gmail.com