Hypoglycemic effect of methanol extract of *Phyllanthus amarus* Schum & Thonn on alloxan induced diabetes mellitus in rats and its relation with antioxidant potential

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Methanolic extract of *P. amarus* was found to have potential anti-oxidant activity as it could inhibit lipid peroxidation, and scavenge hydroxyl and superoxide radicals in *vitro*. The amount required for 50% inhibition of lipid peroxide formation was 104 μg/ml and the concentrations needed to scavenge hydroxyl and superoxide radicals were 117 and 19 μg/ml respectively. The extract was found to reduce the blood sugar in alloxan diabetic rats at 4th hr by 6% at a dose level of 200 mg/kg body wt and 18.7% at a concentration of 1000 mg/kg body wt. Continued administration of the extract for 15 days produced significant (*P < 0.001*) reduction in blood sugar. On 18th day after alloxan administration values were almost similar to normal in the group taking 1000 mg/kg body wt.

**Materials and Methods**

*Plant and chemicals*—Leaves and stems of *Phyllanthus amarus* (Hindi-Bhonyaabali, Malayalam-Kizhkkayinelli) were collected from Thrissur district of Kerala and dried at 50°C. Voucher specimen of the plant was identified and has been kept at Amala Ayurvedic Hospital and Research Center. Alloxan monohydrate was obtained from Sigma Chemicals, St. Louis, M.O. Nitroblue tetrazolium sodium salt (NBT) was purchased from SRL, Bombay and thio­barbutric acid (TBA) was purchased from BDH Laboratory, England. All other chemicals used were of Analytical reagent grade.

*Preparation of P. amarus extract*—Dried leaves and stems of *P. amarus* were powdered and this powder was extracted twice in 5 vol of 75% methanol at room temperature by stirring overnight and centrifuged. The supernatant was evaporated to dryness at 50°C. Complete extraction of the plant was identified and has been kept at Amala Ayurvedic Hospital and Research Center. Alloxan monohydrate was obtained from Sigma Chemicals, St. Louis, M.O. Nitroblue tetrazolium sodium salt (NBT) was purchased from SRL, Bombay and thio­barbutric acid (TBA) was purchased from BDH Laboratory, England. All other chemicals used were of Analytical reagent grade.

*Animals*—Male Wistar albino rats (200-300 g) were used for the study. They were fed with a standard pelleted diet (Sai Durga Feeds, Bangalore) and water *ad libitum*.

**a) Determination of superoxide radicals scavenging activity**

Superoxide scavenging was determined by the nitroblue tetrazolium reduction method. The reaction mixture contained EDTA (6 μM) containing NaCN (3 μg), riboflavin (2 μM), NBT (50 μM),...
various concentrations of the extracts (10-60 µg/ml) and phosphate buffer (67 mM, pH 7.8) in a final volume of 3 ml. The tubes were uniformly illuminated with an incandescent lamp for 15 min, and the optical density was measured at 530 nm before and after the illumination. The percentage inhibition of superoxide generation was evaluated by comparing the absorbency values of the control and experimental tubes.

b) Determination of hydroxyl radical scavenging activity

Hydroxyl radical scavenging was measured by studying the competition between deoxyribose and the test compounds for hydroxyl radicals generated from the Fe³⁺/ascorbate/EDTA/H₂O₂ system. The hydroxyl radical attacks deoxyribose, which eventually results in thiobarbituric acid reacting substance (TBARS) formation. The reaction mixture contained deoxyribose (2.8 mM), FeCl₃ (0.1 mM), EDTA (0.1 mM), H₂O₂ (1 mM), ascorbic acid (0.1 mM), KH₂PO₄-KOH buffer (20 mM, pH 7.4), and various concentrations of the extract (30-180 µg/ml) in a final volume of 1 ml. The reaction mixture was incubated for 1 hr at 37°C. Deoxyribose degradation was measured as TBARS and percentage inhibition was calculated.

c) Determination of lipid peroxidation activity

Lipid peroxidation activity was induced in rat liver homogenate by the method described by Bishayee and Balasubramanian. Reaction mixture (0.5 ml) containing rat liver homogenate (0.1 ml, 25% w/v) in tris-HCl buffer (40 mM, pH 7.0), KCl (30 mM), ferrous iron (0.16 mM) and ascorbic acid (0.06 mM) was incubated for 1 hr at 37°C in the presence and absence of the extract (40-240 µg/ml). The lipid peroxide formed was measured by TBARS formation. For this incubation mixture 0.4 ml was treated with sodium dodecyl sulphate (SDS-8.1%, 0.2 ml), thiobarbituric acid (TBA-0.8%, 1.5 ml) and acetic acid (20%, 1.5 ml, pH 3.5). The total volume was then made up to 4 ml by adding distilled water and kept in a water bath at 100°C for 1 hr. After cooling, 1 ml of distilled water and 5 ml of a mixture of n-butanol and pyridine (15:1 v/v) were added and shaken vigorously. After centrifugation, the absorbance of the organic layer was measured at 532 nm. The percentage inhibition of lipid peroxidation was determined by comparing the results of the test compounds with those of controls not treated with the extracts.

**Determination of antidiabetic activity**

a) Effect of *P. amarus* extract on blood glucose levels in alloxan-induced diabetic rats (single and multi dose study)

Diabetes was induced in male rats by injecting a single ip injection of 120 mg/kg body wt. of alloxan monohydrate. Serum glucose level was checked after 72 hr. Animals with serum glucose levels >250 mg/dl were considered diabetic and were used for the study. The diabetic rats were divided into three groups of 6 rats each. Control diabetic rats (group I) were given distilled water while those of group II and III were given aqueous suspension of the extract of *P. amarus* orally at a dose level of 200 and 1000 mg/kg body wt. respectively on 3rd day after alloxan treatment.

Fasting blood samples were collected from the tail vein on 3rd day of alloxan treatment prior to the administration of the drug and at 1, 2, 4 and 6 hr intervals. Serum was separated and glucose levels were estimated by enzymatic GOD/POD method. In multi dose study, same animals were continued with the same dose of the extract once daily for 15 days. Serum glucose levels in the blood collected at random were measured on 6, 9, 12, 15 and 18th day after alloxan treatment.

b) Determination of toxicity

Normal and diabetic animals were treated with the extract from day3-day18 (15 days) at dose levels of 200 and 1000 mg/kg body weight. They were sacrificed on day 19, blood was collected and serum was separated and glutamate pyruvate transaminase (Bergmeyer & Bernt method), blood urea nitrogen (diacetyl monoxime method) and creatinine(alkaline picrate method) were estimated. The organs like liver, kidney, spleen and thymus were collected and their weights determined. Biochemical changes in liver like glutamate pyruvate transaminase, alkaline phosphatase (King and Armstrong method) and protein(Lowry et al. method) were estimated. Total count and differential count were estimated using haemocytometer and recorded on various days after bleeding from the tail vein.

**Statistical analysis**

Statistical comparisons with animals of non treated group with *P. amarus* group were performed with Student's *t* test for paired observations, and data were expressed as mean ± standard error.
Results

The extract of Phyllanthus amarus was found to scavenge the superoxide generation by the method of photoreduction of riboflavin. The concentration needed for scavenging 50% of superoxides was 19 μg/ml. The concentration needed for 50% inhibition of hydroxyl radical generation was 117 μg/ml. Lipid peroxidation induced by Fe²⁺/ascorbate in rat liver homogenate was found to be inhibited and the concentration needed for 50% inhibition was 104 μg/ml (Fig. 1).

The effect of P. amarus extract in alloxan diabetic rat is shown in Tables 1 and 2. The single administration of the extract 200 mg/kg body wt on 3rd day after alloxan administration produced insignificant reduction in serum glucose level (6.07%) at 4th hr, and at 1000 mg/kg body wt dose level there was 18.7% reduction. Continuous administration of the extract (200 mg/kg body wt) showed significant reduction from 6th day onwards and produced 75.9% reduction in the elevated glucose level on 18th day. Similarly at dose level of 1000 mg/kg body wt, there was 81.2% reduction in blood glucose level on 18th day after alloxan treatment. In animals treated with 1000 mg/kg body wt blood sugar was almost similar to normal rats on 18th day of alloxan treatment. In the case of untreated control, percent reduction was only 44.4% on 18th day.

Normal animals treated with dose level of both 200 and 1000 mg/kg body wt did not produce any weight loss. A decrease in rat body weight (P < 0.005) was noted in alloxan induced diabetic rats. But, when the animals were treated with P. amarus extract, the decrease in body weight was almost completely suppressed (data not shown).

Serum GPT, blood urea nitrogen and creatinine were elevated in alloxan induced diabetes indicating that alloxan administration produced hepatic and renal damage. When treated with 200 and 1000 mg/kg body weight of P. amarus extract there was significant

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment (Dose/kg body wt)</th>
<th>Blood sugar in mg/dl (hr)</th>
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<tbody>
<tr>
<td>I</td>
<td>Normal</td>
<td>75.6 ± 2.8</td>
</tr>
<tr>
<td></td>
<td>Diabetic Control (alloxan)</td>
<td>479.2 ± 16.5</td>
</tr>
<tr>
<td>II</td>
<td>P. amarus (200 mg)</td>
<td>488.9 ± 27.4</td>
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<td>P. amarus (1000 mg)</td>
<td>501.4 ± 20.2</td>
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**P < 0.005, *P < 0.01 (compared to values at 0 hr of the same groups)

Values represent the fasting sugar level on 3rd day after alloxan administration in presence and absence of P. amarus extract

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</table>

**P < 0.001 (compared to values on 1st day's 0 hr of the same group)

Values represent random glucose level in alloxan treated animals with or without extract from 3rd day-18th day.
(P<0.001) reduction in the elevated levels of SGPT, blood urea nitrogen and creatinine. Liver GPT was found to be elevated from 528.1 to 869.1 U/mg protein in alloxan induced diabetes in rats and ALP was found to be elevated from 33.94 to 56.9 KA/dl 15 days after alloxan administration (Table 3). Animals treated with P. amarus extract 200 and 1000 mg/kg body wt along with alloxan had lowered liver GPT levels by 23.5% and 31.4% respectively. Similarly, elevated ALP levels in liver during alloxan induced diabetes were found to be significantly lowered by the extract (15.1% and 25.3%) respectively.

Total WBC was considerably reduced from 10366 to 6066, on 3rd day of the alloxan injection and gradually increased to 7800 on 18th day. Administration of P. amarus (1000 mg/kg body wt) prevented alloxan-induced white blood cellular damage as seen from the increased number of total WBC which was 9800 in the case of 200 mg/kg body wt and 10533 in the case of 1000 mg/kg body wt on 18th day. Administration of drug alone to normal animals did not alter the liver enzymes (GPT, ALP) or haematological values indicating that main action of the extract is to reduce the WBC damage induced by the free radicals generated by alloxan.

Discussion

In the present study we report the antidiabetic activity of P. amarus extract, which is very commonly used in Indian System of Medicine. P. amarus is mainly used in traditional medicines for liver problems including hepatitis. Unander et al.22, have shown that P. amarus could inhibit viral DNA polymerase and down regulate hepatitis B virus RNA transcription and replication23. Aqueous extract was found to inhibit the liver cancer induced by nitrosodiethylamine7. Hexane extract of P. amarus contained several hydrolysable tannins and lignans such as phyllanthin, hypophyllanthin; and some of the lignans such as niranthin, nirtetralin, and phyltetralin were reported to be strong inhibitors of protein kinase which may be responsible for its anti carcinogenic activity24.

**Table 3—Effect of methanolic extract of P. amarus on liver and serum biochemical parameters in normal and diabetic rats**

<table>
<thead>
<tr>
<th>Treatment (Dose/kg body wt)</th>
<th>Liver</th>
<th>Blood</th>
<th>Serum</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>GPT (U/mg Protein)</td>
<td>ALP (KA/dl)</td>
<td>BUN (mg/dl)</td>
</tr>
<tr>
<td>Normal</td>
<td>528.1±66.3</td>
<td>33.9±2.2</td>
<td>18.0±2.1</td>
</tr>
<tr>
<td>Diabetic</td>
<td>869.1±84.0</td>
<td>56.9±6.4</td>
<td>66.0±3.5</td>
</tr>
<tr>
<td>Control (Alloxan)</td>
<td>665.0±80.8</td>
<td>48.3±5.2</td>
<td>38.0±2.7*</td>
</tr>
<tr>
<td>P. amarus (200 mg)</td>
<td>596.0±56.0</td>
<td>42.5±13.8</td>
<td>27.0±3.8*</td>
</tr>
<tr>
<td>P. amarus (1000 mg)</td>
<td></td>
<td></td>
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</tbody>
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*P < 0.001 (compared to normal group)

Biochemical parameters were determined at the end of the experiment (day 18)
P. amarus extract was found to have strong antioxidant activity and was found to scavenge the superoxide, hydroxyl radical and inhibited the lipid peroxidation. Extract contains several polyphenols such as ellagic acid, flavonoids and lignans which are potent antioxidants and anticarcinogens. Alloxan produces oxygen radicals in the body, which cause pancreatic injury and could be responsible for increased blood sugar seen in the animals. However, it is found that action is not specific to pancreas as other organs such as liver, kidney and haemopoietic system also affected by alloxan administration as seen from the elevation of marker enzymes and reduction of haematological parameters. This was reversed by the continued administration of P. amarus extract. Present study indicates that in alloxan induced diabetes elevation of blood sugar was reversed by simultaneous administration of P. amarus. Decrease in blood sugar was seen within hours and upon continued administration the blood sugar value was found to be almost normal. Results shown in this study indicate that P. amarus extract reduces blood sugar in alloxan diabetes rats and the extract was found to scavenge oxygen free radicals in vitro. These results need to be confirmed by animal studies in vivo to find out whether P. amarus extract has any role in reducing oxidative stress in diabetes. P. amarus induced blood sugar reduction may be due to a possible inhibition of free radicals and subsequent inhibition of tissue damage induced by alloxan.

Acknowledgement

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References