Effect of potent ethyl acetate extract of *Amaranthus hybridus* indstreptozotocin-induced diabetic rats

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**ABSTRACT**

The present study was aimed to evaluate ethyl acetate extract of *Amaranthus hybridus* leaves for antihyperglycemic effect in streptozotocin (STZ)-induced diabetic rats by acute and sub-acute models. In this research, various partitioned extracts of crude ethanol extract from *Amaranthus hybridus* were prepared and their effects on blood glucose levels in STZ-induced diabetic rats were studied after a single oral administration (200 mg/kg). The STZ-induced diabetic rats were also treated orally with ethyl acetate extract of *Amaranthus hybridus* for 14 days once daily at the dose of 200 mg/kg. The parameters such as the fasting blood glucose and hepatic antioxidant levels were monitored. Effects of the extract on the pancreatic and hepatic histopathological profile in diabetic rats were also evaluated. The acute study investigation showed that the ethyl acetate fraction is the most potent in reducing the fasting serum glucose levels of the diabetic rats. Administration of the ethyl acetate fraction for 14 days significantly \((P < 0.001)\) reduced the fasting blood glucose and hepatic TBARS level and significantly \((P < 0.001)\) increased the liver superoxide dismutase and catalyses activities as well as reduced glutathione levels in diabetic rats. The histopathological study of liver and pancreas in drug treated rats shows significant protective effect against STZ oxidative stress. Our study concludes that the ethyl acetate fraction of *Amaranthus hybridus* possesses significant anti-hyperglycemic and antioxidant effects. Hence, ethyl acetate fraction could be considered as a potent phytochemical source for development as a safe and effective new antidiabetic drug.

**Keywords:** *Amaranthus hybridus, Ethyl acetate extract, Antihyperglycemic effect, Antioxidant*
INTRODUCTION

Diabetes mellitus, a metabolic disorder, is a multifactorial disease characterized by hyperglycemia with or without glycosuria, lipoprotein abnormalities, azoturia and some-times ketonemia, resulting from an absolute deficiency or predominantly insulin resistance with relative deficiency of insulin [1]. In the absence of reliable and effective modern antidiabetic drugs and available traditional medicines employed for the disease treatment, concerted efforts are currently channeled toward exploring complementary or alternative medicine from natural sources having potent antidiabetic activity with fewer side effects. Amaranthus hybridus L. (Amaranthaceae), commonly known as ‘Cheera’ in Malayalam, is an annual herb distributed throughout tropical and temperate regions of India as a common weed in the agricultural fields and wastelands. Traditionally, the plant has been used in treating dysentery, diarrhoea, ulcers and hemorrhage of the bowel due to its astringent property [2, 3].

In southern India, the leaves are used in folk medicine for the treatment of diabetes. Leaves possess antibacterial effect, and also help to reduce tissue swelling [2]. The Amaranthus species contain amaranthine, quercetin, and kaempferol glycosides [4]. Amaranthus hybridus (A. hybridus) leaves are used as an antidote for snake and scorpion bite [5]. A. hybridus has been used traditionally for the treatment of liver infections and knee pain and for its laxative, diuretic and cicatrisation properties [6].

Amaranthus species were of great importance in pre-Colombian American people’s diets and Amaranthuscruentus and Amaranthus hybridus have a high nutritional value [6, 7]. The consumption of Amaranthuscruentusproducts is advised for patients with celiac disease and, therefore, also for diabetic persons [8].

Furthermore, recent studies established the anti hyperglycemic activities of other species of Amaranthus genus as A. spinosus [9] and Amaranthus viridis [10, 11]. However, based on the literature survey, there is no scientific report proving the anti-hyperglycemic efficacy of ethyl acetate fraction this particular species. Therefore, the current study was designed to evaluate the antidiabetic activity of Amaranthus hybridus in STZ induced diabetic rats.

MATERIALS AND METHODS

Drugs and chemicals

Streptozotocin (STZ) was purchased from SISCO Research Laboratory, India. Glibenclamide was obtained from Prudence PharmaChem, Ankeshwar, Gujarat. All other chemicals including solvents used in the experiments were purchased locally (Merck and SD fine Chemicals) and were of analytical grade. Standard kits were obtained from Span Diagnostics, Mumbai, India.

Plant material

The leaves of Amaranthus hybridus L. (A. hybridus) were collected during the month June 2013 from agricultural and fallow fields of Kulukkallur, Palakkad district, Kerala. The plant was taxonomically identified and authenticated by Dr. Prabhu Kumar, Scientist, Plant Systematics and Genetic Resources Division, Centre for Medicinal Plant Research (CMPR), Department of AYUSH, Government of India, Kottakal, Kerala and a voucher specimen (ACPPCTB) has been preserved in our laboratory for further reference. The leaves of the plant were shade dried and powdered with a mechanical grinder. The powdered plant material was then passed through a 60-mesh sieve and stored in an air-tight container for future use.

Preparation of plant extracts

The shade dried coarse powdered leaves of A. hybridus (500 g) was packed in the soxhlet extraction apparatus and extracted with 1.5 L of 95% ethanol at temperature of 40–50°C for 72h. The extract was filtered and then concentrated to dryness in a rotary evaporator under reduced pressure at temperature of 40°C. Then the crude ethanolic extract of A. hybridus (AHELE)(100 g) was dissolved in distilled water (500 mL) and further partitioned with pet ether, chloroform and ethyl acetate.

The resultant black color residues were stored in a dessicator for use in subsequent experiments and considered as the crude ethanol extract and fractions. The yield of the crude ethanol extract, pet ether, chloroform, ethyl acetate and aqueous fractions were 24, 5, 4.16, 7.02 and 10.45% w/w respectively.
Weighed amount of fractions were suspended in 5% tween 80 in normal saline and used for the present study.

Qualitative phytochemical analysis

Preliminary phytochemical screening was performed for the ethyl acetate fraction of AHELE [11-13].

Experimental animals

Studies were conducted using Wistar albino rats of either sex weighing 150–200 g. They were purchased from, Small animal breeding station (SABS), Government Veterinary Medical College, Mannuthy, Thrissur (Dist), Kerala, India. The animals were randomly grouped (n = 6) and housed in polyacrylic cages (38 × 23 × 10 cm) and maintained under standard laboratory conditions (Temperature 25 ± 2 °C; relative humidity 55 ± 10%) with dark and light cycle (14/10 h). They were fed on a standard dry pellet diet (Small animal breeding unit, Government Veterinary College, Mannuthy, Thrissur District, India) and water ad libitum. The rats were acclimatized to laboratory condition for 1 week before commencement of experiment and were maintained in a well-ventilated animal house. Animals described as fasting had been deprived of food for at least 12 h but were allowed free access to drinking water. All procedures described were reviewed and approved by the AI Shifa College of pharmacy animal ethical committee (Reg. No: 1195/ac/08/CPCSEA 21 August 2013).

Acute oral toxicity study

An acute oral toxicity study was performed per Organization for Economic Co-operation and Development (OECD)-423 guidelines [14]. Wistar albino rats (150–200 g) of either sex were randomly distributed to twenty four groups of three each. The animals were fasted overnight and the extracts were administered orally at a dose of up to 2000 mg/kg body weight. Mortality and general behavior such as grooming, sedation, hyperactivity, loss of righting reflex, respiratory rate, and convulsions of the animals were observed individually after dosing at least once during the first 30 minutes, periodically for 24 h, special attention was provided during the first 4 h, and daily thereafter, for a total of 14 days.

Experimental design

Induction of experimental diabetes

Rats were fasted for 16 h before the induction of diabetes with STZ. A freshly prepared solution of STZ (50 mg/kg) followed by nicotinamide (120 mg/kg) in 0.1M cold citrate buffer, pH 4.5, were injected intraperitoneally in a volume of 1 mL/kg, and the control rats were injected with citrate buffer alone. In order to control the hypoglycemia during the first day after the STZ administration, diabetic rats were given 5% glucose solution orally. Hyperglycemia was confirmed by the elevated fasting glucose levels in blood, determined at 48 h and then on day 6 after injection. Rats with diabetes exhibiting fasting blood glucose levels in the range of 260–325 mg/100 mL were selected for the studies and blood glucose levels were measured by reflective glucometer (Accu-chek) using the glucose oxidase method.

Acute antihyperglycemic study

The rats were fasted for 16 h, divided into seven groups of six each, and treated as follows: Group I, nondiabetic control, was given 5% tween 80 in normal saline orally at a dose of 5 mL/kg. Group II, STZ-diabetic control, received 5% tween 80 in normal saline (5 mL/kg) orally. Groups III–VI, STZ-diabetic rats, were treated with pet ether, chloroform, ethyl acetate, and aqueous fractions (200 mg/kg, orally), respectively. Group VII, STZ-diabetic rats, was administered with standard drug Glibenclamide at a dose of 0.5 mg/kg orally. Blood samples were taken from the tail vein at 0, 0.5, 1, 2, 4, and 5 h after the oral administration and fasting blood glucose levels were determined [15].

Subacuteantihyperglycemic study (14 Days)

Rats were fasted for 16 h and divided into four groups of six each [16]. Group I, nondiabetic control, were given 5% tween 80 in normal saline orally at a dose of 5 mL/kg. Group II, STZ-diabetic control, received 5% tween 80 in normal saline (5 mL/kg) orally. Groups III–VI, STZ-diabetic rats, were treated with pet ether, chloroform, ethyl acetate, and aqueous fractions (200 mg/kg, orally), respectively. Group III, STZ-diabetic rats, was administered with standard drug Glibenclamide at a dose of 0.5 mg/kg orally. The treatment was continued once daily for 14 days. Fasting blood
glucose level of each animal was determined on days 1, 4, 7, 10, and 15 after the initiation of treatment. The body weights of animals were also monitored on the same days. On the 15th day, all the rats were sacrificed by euthanasia and the liver and pancreas were excised immediately and washed with ice cold saline solution.

**Effects on hepatic in vivo antioxidant activities**

A 10% w/v of liver homogenate was prepared in 0.15 M Tris-HCl buffer (pH: 7.4). The homogenate was centrifuged at 2000xg for 20 min at 4°C to remove the cell debris and then the supernatant was centrifuged (REMI C-24) at 12,000xg for 1 h at 4°C. The supernatant obtained were used for the determination of lipid peroxidation [17], reduced glutathione content [18], Superoxide Dismutase (SOD) [19] and Catalase (CAT) [20].

**Histopathological study**

The fragments from the pancreas and liver tissues were fixed in 10% neutral formalin solution, embedded in paraffin, and then, stained with Hematoxylin (H) and Eosin (E). The sections were examined microscopically for the evaluation of histopathological changes.

**Statistical analysis**

The experimental data were expressed as mean ± Standard Error Mean (SEM). The data were analyzed using one-way ANOVA and Dunnett’s test. The results were considered statistically significance if \( P < 0.05 \). All statistical analyses were performed using GRAPH PAD Software.

**RESULTS**

**Phytochemical screening**

The qualitative phytochemical screening of the ethyl acetate extract of AHELE revealed presence of flavonoids, glycosides, saponins, alkaloids and tannins.

**Acute oral toxicity study**

The pet ether, chloroform, ethyl acetate and aqueous fractions of ethanol extract of *A. hybridus* did not show any mortality and toxic manifestations upto the dose of 2000 mg/kg. Further dosing was not performed to estimate the LD\(_{50}\) (lethal dose) value. According to the OECD guidelines for the acute toxicity, an LD\(_{50}\) dose of 2000 mg/kg and above is categorized as unclassified and hence the drug is found to be safe. Based on the acute toxicity studies, the dose 200 mg/kg of the fractions has been selected as the therapeutic dose.

**Acute antihyperglycemic study**

The effects of the pet ether, chloroform, ethyl acetate, and aqueous fractions of AHELE on the blood glucose in STZ-induced diabetic rats results are shown in Table 1. The blood glucose levels were significantly \( (P < 0.001) \) elevated in diabetic control rats as compared to nondiabetic control rats. Oral acute administration of pet ether \( (P < 0.05, P < 0.001) \) and ethyl acetate \( (P < 0.001) \) fractions of EESS at the dose of 200 mg/kg significantly lowered the elevated blood glucose level in STZ-induced diabetic rats as compared to diabetic control rats, while chloroform and aqueous fractions were devoid of antihyperglycemic activity. The ethyl acetate fraction produced more potent effects than pet ether fraction in acute antihyperglycemic model.

**Subacute antihyperglycemic study**

**Effect on blood glucose levels**

The effect of the ethyl acetate fraction of *Amaranthus hybridus* on the fasting blood glucose levels in STZ-induced diabetic rats results are shown in Table 2. Repeated oral administration with a dose of 200 mg/kg of the ethyl acetate fraction to STZ-induced diabetic rats for 14 days significantly \( (P < 0.001) \) reduced the elevated 1, 4, 7, 10, and 15 after initiation of treatment, when compared to diabetic control rats. The effect of ethyl acetate fraction is comparable to that of glibenclamide.

**Effects on hepatic in vivo antioxidant activities**

The treatment of rats with ethyl acetate fraction (200 mg/kg) and glibenclamide resulted in a significant decrease in the concentration of MDA than in the diabetic control rats. There was a significant reduction in the activities of SOD, CAT and GSH levels in liver during diabetes. The hepatic SOD, CAT activities and GSH levels were significantly elevated in the diabetic rats treated with
ethyl acetate fraction of amaranthus (200 mg/kg) and Glibenclamide when compared with the diabetic control rats. The results are shown in Table 3.

**Histopathological studies of pancreas**

Histopathological section of nondiabetic control pancreas (Figure 1(a)) showing normal islets with clusters of purple-stained $\beta$-cells. Figure 2(b) presents the section of diabetic pancreas showing atrophy of $\beta$-cells and vacuolar degenerative changes in islets and mild infiltration of inflammatory cells. Figures 3(c) and 4(d), treatment with ethyl acetate fraction and glibenclamide, respectively, show maximum cellular regeneration of pancreatic $\beta$-cells with well-granulated and an increased number of islets.

**Histopathological studies of liver**

The section of a normal control rat liver showing normal cellular architecture with distinct hepatic cells, sinusoidal spaces and well brought out central vein, the result is shown in Figure 2(a). The section of STZ diabetic control liver showing disarrangement of normal hepatic cells with intense centrilobular necrosis and fatty degeneration, the result is shown in Figure 2(b). The section of the liver tissue of ethyl acetate extract of AHELE treated animals, showing moderate accumulation of fatty lobules and cellular necrosis, the result is shown in Figure 2(c). The section of the liver tissue of glibenclamide treated animals showing normal lobular pattern with a mild fatty change, feathery necrosis almost comparable to the normal, the result is shown in Figure 2(d).

**Table 1: Effect of various fractions of ethanol extract of Amaranthus hybridus in blood glucose level by acute treatment in STZ-induced diabetic rats.**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Blood glucose level (mg/dL) at different time intervals</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>0 min</td>
</tr>
<tr>
<td>Normal control (5% tween 80 in 0.9% NaCl, 5 mL/kg)</td>
<td>83.23 ± 2.34</td>
</tr>
<tr>
<td>DM control (5% tween 80 in 0.9% NaCl, 5 mL/kg)</td>
<td>293.46 ± 1.31**</td>
</tr>
<tr>
<td>DM + pet ether extract 200 mg/kg</td>
<td>289.30 ± 1.28</td>
</tr>
<tr>
<td>DM + chloroform extract 400 mg/kg</td>
<td>285.83 ± 2.37</td>
</tr>
<tr>
<td>DM + ethyl acetate extract 200 mg/kg</td>
<td>292.20 ± 1.44</td>
</tr>
<tr>
<td>DM + aqueous extract 200 mg/kg</td>
<td>287.50 ± 2.56</td>
</tr>
<tr>
<td>DM + glibenclamide 0.5 mg/kg</td>
<td>290.260 ± 1.16**</td>
</tr>
</tbody>
</table>

DM control: Diabetic mellitus rats.
Values are given as mean ± SEM, 6 rats in each group; *P <0.01 as compared to normal control group; **P<0.05, ***P<0.001 as compared to control group.
Table 2. Effect of ethyl acetate extract of *Amarantus hybridus* in blood glucose level by sub-acute treatment in STZ-induced diabetic rats.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Fasting blood glucose level (mg/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1&lt;sup&gt;st&lt;/sup&gt; day</td>
</tr>
<tr>
<td>Normal control (5% tween 80 in 0.9% NaCl, 5 mL/kg)</td>
<td>83.23 ± 2.34</td>
</tr>
<tr>
<td>DM control (5% tween 80 in 0.9% NaCl, 5 mL/kg)</td>
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<td>DM + glibenclamide 0.5 mg/kg</td>
<td>290.260 ± 1.16</td>
</tr>
</tbody>
</table>

DM control: Diabetic mellitus rats.
Values are given as mean ± SEM, 6 rats in each group; a,**P < 0.001 as compared to normal control group; b,**P < 0.001 as compared to control group.

Table 3. Effect of ethyl acetate extract of *Amarantus hybridus* on liver in vivo antioxidant system by sub-acute treatment in STZ-induced diabetic rats.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Lipid peroxidation (nmol of MDA/mg protein)</th>
<th>Glutathione (μM/gm protein)</th>
<th>Superoxide dismutase (IU/mg protein)</th>
<th>Catalase (nmol/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control (5% tween 80 in 0.9% NaCl, 5 mL/kg)</td>
<td>10.24 ± 0.51</td>
<td>61.28 ± 1.01</td>
<td>20.45 ± 0.48</td>
<td>47.44 ± 1.64</td>
</tr>
<tr>
<td>DM control (5% tween 80 in 0.9% NaCl, 5 mL/kg)</td>
<td>24.39 ± 0.7**</td>
<td>30.81 ± 0.79**</td>
<td>10.65 ± 0.27**</td>
<td>17.55 ± 0.59**</td>
</tr>
<tr>
<td>DM + ethyl acetate extract 200 mg/kg</td>
<td>11.69 ± 0.45**</td>
<td>57.08 ± 0.70**</td>
<td>18.45 ± 0.46**</td>
<td>43.69 ± 1.74**</td>
</tr>
<tr>
<td>DM + glibenclamide 0.5 mg/kg</td>
<td>20.86 ± 0.23b**</td>
<td>48.43 ± 1.26b**</td>
<td>17.93 ± 0.66b**</td>
<td>42.23 ± 1.54b**</td>
</tr>
</tbody>
</table>

DM control: Diabetic mellitus rats; MDA: Malondialdehyde.
Values are given as mean ± SEM, 6 rats in each group; a,**P < 0.001 as compared to normal control group; b,**P < 0.001 as compared to diabetic control group.
Figure legends

Figure 1(a-d). Photomicrographs of pancreatic sections of control and diabetic rats treated with ethyl acetate extract of *Amaranthus hybridus* leaf extract. (a) Normal control, (b) Diabetic control, (c) Diabetic rats treated with ethyl acetate extract of 200 mg/kg *Amaranthus hybridus* ethanol leaf extract, (d) Diabetic rats treated with 0.5 mg/kg glibenclamide.
DISCUSSION

The present study was performed to find out the potent antihyperglycemic fraction from ethanol extract of *Amaranthus hybridus* (AHELE). In this study, we induced an experimental diabetes mellitus in Wistar rats by Streptozotocin (STZ) injection. STZ when administered at a high single dose induces diabetes by the direct toxic effects on pancreatic β-islet cells [21].

The fundamental mechanism underlying hyperglycemia in diabetes mellitus involves the overproduction of glucose by excessive hepatic glycogenolysis and gluconeogenesis, decreased hepatic glycogenesis and decreased utilization of glucose by the tissue [22].

As a preliminary antihyperglycemic activity assessment as well as to isolate a potent fraction, the various fractions of AHELE were administrated to STZ-induced diabetic rats at a single dose level (200 mg/kg) to determine the acute effect on blood glucose concentration. Consequently, the pet ether and ethyl acetate fractions showed significant antihyperglycemic activity in STZ-induced diabetic rats, while no remarkable effect on blood glucose level was observed on the rats treated with chloroform and aqueous fractions. However, in comparison to pet ether fraction, ethyl acetate fraction has shown potential activity in decreasing the blood glucose level in STZ-induced diabetic rats.

In subacute study, daily administration of ethyl acetate fraction of AHELE for 14 days significantly decreased the blood glucose levels in STZ-induced diabetic rats, when compared to the diabetic control rats. The possible mechanism of the ethyl acetate fraction for its antihyperglycemic effect may be through potentiation of pancreatic secretion of insulin from remaining β-cells of islets and/or regenerated β-cells or due to enhanced transport of blood glucose to the peripheral tissues and/or the reduction of hepatic gluconeogenesis and glycogenolysis, and increased hepatic glyconeogenesis. The histopathological studies of pancreas also show that the ethyl acetate fraction regenerates the β-cells. The result finding suggests that the antihyperglycemic activity of ethyl acetate fraction of AHELE may be due to potentiation of insulin secretion by regeneration of β-cells.

We also examine antioxidant capacity of the plant extracts, since antioxidants have been reported to prevent oxidative stress and diabetic complications. In diabetes, hyperinsulinemia increases the activity of the enzyme, fatty acyl coenzyme A oxidase, which initiates β-oxidation of fatty acids, resulting in lipid peroxidation [23]. In the present study, the hepatic TBARS levels were significantly lower in the AHELE treated groups compared to the diabetic control rats. These findings support that the AHELE may exert antioxidant activities and protect the liver tissues from lipid peroxidation.
Glutathione (GSH), a major intracellular non-protein sulphydryl compound present in micromolar concentrations in all the cells, is an important antioxidant. SOD and CAT are enzymes that destroy the peroxides and play a significant role in providing antioxidant defenses to an organism [24]. Therefore, reduction in the activity of these enzymes may result in a number of deleterious effects due to the accumulation of superoxide anion radicals and hydrogen peroxide. In our study, it was observed that the ethyl acetate fraction of AHELE caused a significant increase in the hepatic SOD, CAT and GSH activities of the diabetic rats and indicating the free radical scavenging activity and their protective effect against cellular damage. The above in vivo antioxidant status reveals support to antidiabetic effect of ethyl acetate fraction. In the present study, histopathological findings also provided supportive evidence for the antioxidant potential of AHELE during diabetes. The significant antioxidant and antidiabetic potential of ethyl acetate extract in diabetic rats may be attributed to the presence of flavonoids, saponins and tannins in the plant *Amaranthus hybridus*.

**CONCLUSION**

The present research clearly indicates that the ethyl acetate fraction of ethanol extract of *Amaranthus hybridus* exhibits antihyperglycemic in addition to antioxidant effects in STZ-induced diabetic rats, thereby justifying its ethnomedicinal use. However, further studies are necessary to find out the active phytochemicals as well as the exact mechanisms of action involved in antidiabetic potential of this plant.

**REFERENCES**


