Background: Amaranthus hybridus is claimed to be useful in treating dysentery, diarrhoea, hemorrhage of the bowel, ulcers, liver infections and kidney pain in Indian traditional system of medicine, and in southern India, the leaves are used in folk medicine for the treatment of diabetes mellitus. The present research was conducted to evaluate the nephron protective effect of ethanol extract of Amaranthus hybridus leaves in Streptozotocin (STZ)-induced diabetic rats.

Materials and methods: Wister albino rats were induced diabetic by a single dose of STZ (50 mg/kg i.p.). The serum and urine renal function parameters-creatinine, urea, uric acid, albumin and total proteins were measured on 15th day after daily oral administration of Amaranthus hybridus ethanolic leaves extract (AHELE) for 14 days at doses of 200 and 400 mg/kg. The antioxidant potential of extract was also determined. Hence, the effects of the AHELE treatments on the kidney histological profile in STZ nephrotoxic rats were observed.

Results: The present study investigation showed that the AHELE significantly (P<0.001) attenuated elevations in the serum levels of creatinine, urea and uric acid, and urine levels of total proteins and albumin, in diabetic treated rats as compared with diabetic control rats. The extract also improved altered serum total protein associated with diabetes nephropathy. A significant decrease in TBARS (P<0.001), and significant increase in SOD (P<0.001), CAT (P<0.01) and reduced glutathione levels (P<0.001) were observed in the kidney of rats treated with AHELE. Furthermore, the histopathological study of kidney in drug treated rats shows significant protective effect against STZ oxidative stress.

Conclusions: Our results suggest that Amaranthus hybridus possesses significant nephronprotective effect against oxidative damage in diabetic rats.

Keywords: Amaranthus hybridus; Nephronprotective; Diabetic rats; Histopathology; Creatinine

Introduction

Diabetes Nephropathy, a chronic metabolic complication of diabetes mellitus, is characterized by elevated levels of serum glucose, creatinine, urea and uric acid in addition to abnormal histopathological changes in kidney. In the recent past, many antidiabetic agents are introduced; still the diabetes and the related nephropathy complication continue to be a major medical problem, not only in developed countries but also in developing countries. Not with standing much research work, the diabetic kidney damages are increasing rapidly and patients with diabetes kidney failure undergo either painful dialysis or kidney transplantation [1] which is both costly and harmful. More and more interest is now growing about plant use as an alternative therapy for protecting kidney damage in patients with diabetes mellitus. Reactive oxygen species (ROS) have been widely implicated in the pathogenicity of diabetes mellitus and its nephropathy. A number of clinical studies suggest that the antioxidants in medicinal plants are key factors in reducing the incidence of diabetic nephropathy. Traditional medicines and extracts from medicinal plants with antioxidant potential have been extensively used as alternative medicine for better control and management of diabetes nephropathy [2]. However, searching for new antidiabetic drugs with nephroprotective properties from natural plants is currently very important.

Amaranthus hybridus L. (Amaranthaceae) commonly known as ‘Cheera’ in Malayalam, is an erect branched annual herb distributed throughout tropical and temperate regions of India as a common weed in the agricultural fields and wastelands. In traditional medicinal system different parts of the plant Amaranthus hybridus (A. hybridus) have been mentioned to be useful in a variety of diseases. Traditionally, the plant has been used in treating dysentery, diarrhoea, ulcers and hemorrhage of the bowel due to its astringent property [3-5]. In southern India, the leaves are used in folk medicine for the treatment of diabetes. Leaves possess antibacterial effect, cleansing effect and also help to reduce tissue swelling [5]. In Nigeria, A. hybridus leaves combined with condiments are used to prepare soup [6-8]. In Congo, their leaves are eaten as spinach or green vegetables [6,9]. These leaves boiled and mixed with a groundnut sauce are eaten as salad in Mozambique and in West Africa [10,11]. The Amaranthus species contains amaranthine, quercetin, and kaempferol glycosides [12]. A. hybridus leaves are used as an antidote for snake and scorpion bite [13,14].

Amaranthus species were of great importance in pre-Colombian American people’s diets [15] and A. cruentus and A. hybridus have a high nutritional value [16] (Fernand et al.). The consumption of A. cruentus products is advised for patients with celiac disease and, therefore, also for diabetic persons [17]. A. hybridus has been used traditionally for the treatment of liver infections and knee pain and for its laxative, diuretic, and cicatrization properties [16].

Furthermore, recent studies established thioantihyperglycemic activities of other species of Amaranthus genus as A. spinosus [18] and A. viridis [19,20]. However, based on the literature survey, there

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is no scientific report proving the anti-hyperglycemic efficacy of this particular species. Therefore, the current study was designed to evaluate the nephroprotective activity of *Amaranthus hybridus* in STZ induced diabetic rats.

**Materials and Methods**

**Drugs and chemicals**

Streptozotocin (STZ), Trichloro Acetic Acid (TCA), Thiobarbituric Acid (TBA), Reduced Glutathione (GSH), Tris HCl, Sodium Dodecy Sulphate (SDS), Nitro Blue Tetrazolium (NBT), reduced Nicotinamide Adenine Dinucleotide Phosphate (NADH), Dimethylsulfoxide (DMSO) and Phenazinemethosulfate (PMS) were purchased from SISCO Research Laboratory, Mumbai, India. Gilbenclamide was obtained from Prudence Pharma Chem, Thiruvananthapuram, Kerala, India. The solvents and chemicals used were of analytical grade.

**Plant material**

The leaves of *A. hybridus* L. were collected during the month of 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, 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 extract**

The coarse powdered leaves of *A. hybridus* (500 g) were packed in the Soxhlet apparatus and extracted with 1.5 L of 95% ethanol at a temperature of 40°-50°C for 72 h. The solvent was completely removed in a rotary evaporator under reduced pressure at a temperature of 40°C and a semisolid mass was obtained (AHELE, yield 14% w/w). The dried AHELE was suspended in 5% tween 80 in normal saline and used for the present study.

**Preliminary phytochemical screening**

The extract was screened for the presence of various phyto constituents employing following standard screening tests [21-23].

- Test for Steroids: Libermann-Burchard Test, Salkowski Test
- Test for the Triterpenoids; Noller test
- Test for Alkaloids: Mayer’s test, Dragendorff’s test, Wagner’s test, Hager’s test.
- Test for Flavonoids: lead acetate test, Shinoda’s Test.
- Test for Glycosides: Legal’s Test, A. Tollen’s Test.
- Test for Tannins: Ferric chloride test.
- Test for Saponins: Foam test, Blood haemolysis test.
- Test for Proteins: Millon’s test, Ninhydrin test, Biurette test.
- Test for Carbohydrate: Molisch’s test, Fehling’s test, Benedict’s test.

**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 polycracyl 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 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 as per Organization for Economic Co-operation and Development (OECD) – 423 guidelines [24]. Wistar albino rats (150-200 g) were randomly distributed to six groups of three each. The animals were fasted overnight and AHELE was 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 periodically for 72 h. The animals were observed continuously for the initial 2 h and intermittently for the next 6 h and then again at 24, 48, and 72 h, following drug administration.

**Induction of experimental diabetes**

Rats were fasted for 16 h before the induction of diabetes with Streptozotocin (STZ). A freshly prepared solution of STZ (40 mg/kg body weight) in 0.1 M cold citrate buffer, pH 4.5, were injected intraperitoneally in a volume of 1 ml/kg [25] and the control rats were injected with the 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 moderate diabetes exhibiting fasting blood glucose levels in the range of 250-280 mg/100 ml were selected for the studies.

**Nephroprotective activity study in diabetic rats (14 days):** Rats were fasted for 16 h and classified into five groups of six each [26]. Group I, normal control, were given normal saline orally at a dose of 5 ml/kg. Group II, STZ-diabetic control, received normal saline at a dose of 5 ml/kg orally. Groups III and IV STZ-diabetic rats were treated with AHELE orally at a dose of 200 and 400 mg/kg, respectively. Group V, STZ-diabetic rats, were administered with glibenclamide at a dose of 0.5 mg/kg orally. The treatment was continued once daily for 14 days.

**Effect of ethanol extract of *Amaranthus hybridus* on serum and urine renal parameters:** On the 15th day, blood was collected from the overnight-fasted rats by retro-orbital bleeding, using micro capillary technique. Fasting blood glucose level of each animal was determined. Serum was separated and used for the determination of biochemical parameters, such as creatinine, urea, uric acid and total proteins (using Automated Span Diagnostic Reagents, Mumbai, India). Rats were accommodated in metabolic cages for urine collection for 2 days in order to become familiar with the environment of the cage. Twenty-four hour urine samples were collected from all groups to determine urine total protein and albumin. After urine collection, all the rats were sacrificed by euthanasia. Kidneys were excised immediately, rinsed in ice-cold normal saline (pH 7.4), blotted dry, and weighed.
Estimation of antioxidant assays: A 10% w/v of kidney homogenate was prepared in 0.15 M Tris-HCl buffers (pH: 7.4). The homogenate was centrifuged at 2000 x g for 20 min at 4°C to remove the cell debris and then the supernatant was centrifuged (REMI C-24) at 12,000 x g for 1 h at 4°C. The supernatant obtained were used for the determination of lipid peroxidation [27], reduced glutathione content [28], Superoxide Dismutase (SOD) [29] and Catalase (CAT) [30].

Histopathological study: The fragments from the kidney 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 ± SEM. The data were analyzed using ANOVA and Dunnett’s test. The results were considered statistically significant if P<0.05.

Results

Preliminary phytochemical analysis

The qualitative phytochemical screening of the AHELE has revealed presence of flavonoids, Glycosides, terpenoids, saponins, alkaloids, tannins and steroids (Table 1).

Acute oral toxicity study

Administration of *Amaranthus hybridus* ethanol leaf extract (AHELE) to Wistar rats did not show any mortality and gross behavioral changes up to 2000 mg/kg body weight. Further dosing was not performed to estimate the LD50 (lethal dose) value. According to the OECD 423 guidelines for the acute toxicity, an LD50 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 (low dose i.e., 10%) and 400 mg/kg (high dose i.e., 20%) of the AHELE was selected as the therapeutic dose.

Effect on serum and urine renal function parameters

Repeated oral administration of AHEL Eat a dose of 200 and 400 mg/kg in STZ induced diabetic rats for 14 days significantly (P<0.05, P<0.001) reduced the elevated fasting blood glucose levels when compared to diabetic control rats. In STZ induced diabetic control rats there was a significant (P<0.001) increase in serum creatinine, urea and uric acid, but were reduced by AHELE (200 and 400 mg/kg). In addition, there was a significant (p<0.001) decrease in serum total proteins levels in diabetic control rats compared with normal control. Oral administration of AHELE significantly (P<0.001) increased the serum total protein. Compared to the non-diabetic control rats, urine albumin and total proteins levels increased significantly (P<0.001), in STZ-induced diabetic control rats. Treatment of STZ-induced diabetic rats with AHEE resulted in marked decrease in urine albumin and total proteins (P<0.001) as compared to diabetic control rats (Table 2).

Effects on renal in vivo antioxidant activities

Lipid peroxidation: As depicted in Tables 1 and 2, the amount of MDA, an end product of lipid peroxidation, in the rats kidney tissues, significantly increased in STZ-induced diabetic control rats, compared to the non-diabetic control rats. The treatment of rats with AHELE (200 and 400 mg/kg) and Glibenclamide resulted in a significant decrease in the concentration of MDA than in the diabetic control rats (Table 3).

Reduced glutathione content superoxide dismutase and catalase: The total GSH content, SOD and catalase (CAT) activities in the STZ-induced diabetic rats were significantly (P<0.001) decreased in kidney. However, the renal SOD, CAT activities, and GSH levels were significantly elevated in the diabetic rats treated with AHELE (200 and 400 mg/kg) and Glibenclamide when compared with the diabetic control rats (Table 3).

Histopathological studies of kidney

Histopathological evaluation of non-diabetic control rats kidneys shown normal architecture (Figure 1). In STZ-induced diabetic control groups (Figure 2) resulted in glomerular hypertrophy (GL), mild thickening of basement membrane, increased Bowman’s space (BM), tubular dilation, intra-tubular hyaline casts and interstitial inflammatory cell infiltration. Treatment with AHELE (200 and 400 mg/kg reduced tubular necrosis, reduced cell infiltration, show normal Bowman’s space with glomerulus, and maintaining near normal kidney structure (Figures 3 and 4).

Discussion

Diabetes nephropathy is probably the fastest growing complex disorder in the world and as knowledge of the heterogeneous nature of the disease increases so does the need for more challenging and appropriate therapies. In people with diabetes mellitus, the diabetic nephropathy is the most important cause of death, of whom, 30-40% eventually develop end-stage renal failure [31]. As there is a growing trend towards using natural remedies adjunct to conventional therapy, traditionally used plants might provide a useful source of new anti-hyperglycemic compounds with nephroprotective effect. The present research provides an evidence for the beneficial effects of *Amaranthus hybridus* leaf extract on glucose, serum and urine renal function parameters and oxidative defense system in STZ induced diabetic rats.

In the present study, acute oral toxicity studies revealed that *Amaranthus hybridus* ethanolic leaf extract (AHELE) did not show any mortality and toxic signs up to 2000 mg/kg body weight and concludes the drug is safe. In this study, we induced a 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 [32].

The sub-acute anti hyperglycemic studies clearly demonstrated that the AHELE (200 and 400 mg/kg) significantly produced a dose-dependent anti hyperglycemic effect in the diabetic rats throughout the course of study.

Several studies reported that STZ administration elevated serum renal markers in rats [33,34] which is the indicator of diabetic nephropathy with altered glomerular filtration rate. The current study...
diabetic nephropathy. In diabetic nephropathy, oxidative stress has been found to be mainly due to an increased production of reactive oxygen species and a sharp reduction of antioxidant defenses [35]. 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 [36]. In the present study, the renal 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 renal tissues from lipid peroxidation. The possible mechanism by which AHELE may bring about its diabetic nephroprotective action in STZ-induced diabetic rats may be by inhibiting lipid peroxidation in kidney tissues.

Glutathione (GSH), a tripeptide present in all the cells is an important antioxidant system and glycogen by sub-acute treatment in STZ-induced diabetic rats.

In the present study, histopathological findings also provided supportive evidence for the antioxidant potential of AHELE during diabetes. Moreover, it has been reported that in STZ-induced diabetic rats, the renal undergo pathological changes [39,40] and the important pathologic features of diabetic nephropathy are glomerular hypertrophy, tubular dilation, interstitial inflammatory cell infiltration, mild thickening of basement membrane along with mild changes in the density of mesenchyme with increased Bowman’s space, and tubulointerstitial fibrosis [41]. These diabetic nephropathic changes were also observed in our study, in the STZ diabetic control rats. Treatment with ethyl acetate fractions (200 and 400 mg/kg and glibenclamide (0.5 m/kg) (Figures 3 and 4) reduced cell infiltration,
the presence of biologically active compounds flavonoids, saponins, tannins, and terpenoids in the plant Amaranthus hybridus.

According to these results, AHELE could be a supplement, as an antioxidant therapy, and may be beneficial for correcting the hyperglycaemia and preventing diabetic nephropathy due to lipid peroxidation and free radicals. As the present study is a preclinical one, further proceedings with human volunteers may pave a way for the usage of the drug in human beings.

Conclusion

Food and food supplements have increasingly become attractive alternatives to prevent or treat diabetes and its complications. The present investigation clearly indicates that Amaranthus hybridus exhibits nephroprotective effect in addition to antioxidant effects in STZ induced diabetic rats. 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.

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