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ENICOSTEMMA LITTORALE BLUME AQUEOUS EXTRACT IMPROVES THE ANTIOXIDANT STATUS IN ALLOXAN-INDUCED DIABETIC RAT TISSUES

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Abstract: This study investigates the effect of oral administration of an aqueous *Enicostemma littorale* whole plant extract on antioxidant defense in alloxan-induced diabetes in rats. A significant increase in blood glucose and increased concentration of thiobarbituric acid reactive substances (TBARS) and hydroperoxides (HP) in liver, kidney and pancreas were observed in alloxan diabetic rats. Decreased concentration of reduced glutathione (GSH) and decreased activities of superoxide dismutase (SOD), catalase and glutathione peroxidase (GPx) were also observed in these tissues of diabetic rats. Oral administration of aqueous *E. littorale* whole plant extract (1 and 2 g/kg) to diabetic rats daily for 45 days significantly decreased blood glucose, TBARS, HP and increased GSH, SOD, catalase and GPx. *E. littorale* extract at the dose of 2 g/kg was more effective than 1 g/kg. Insulin (6 units/kg) administration to diabetic rats for 45 days brought back all the parameters to near normal status.

Keywords: *Enicostemma littorale*, whole plant, alloxan diabetes, antioxidants

Enicostemma littorale Blume is a glabrous perennial herb belonging to the family Gentianaceae. It is a 2-5 inches tall herb, growing throughout India. It is more common in the plains and near the sea. In Indian Ayurvedic medicine, *E. littorale* is being used for the treatment of diabetes mellitus (1). Reports have shown the antidiabetic effect of *E. littorale* in alloxanized rabbits (2), alloxanized rats (3, 4) and streptozotocin treated rats (5). The methanol extract of *E. littorale* lowered blood glucose and lipid peroxides in alloxan-induced diabetic rats (6). The efficacy of *E. littorale* in type 2 diabetic patients was reported by Umesh et al. (7).

Enicostemma littorale Blume is a plant with a number of antioxidative phytochemicals, which include alkaloids, catechins, saponins, sterols, triterpenoids, phenolic acids, flavonoids and xanthones. It also contains minerals like iron, potassium, sodium, calcium, magnesium, silica, phosphate, chloride, sulphate and carbonate (5). In our lab, we have reported he effect of aqueous *E. littorale* Blume extract on key carbohydrate metabolic enzymes, lipid peroxides and antioxidants in alloxan-induced diabetic rats (8). There are no available reports pertaining to the antioxidant potential of aqueous extract of *E. littorale* in diabetic liver, kidney and pancreas. This study attempts to evaluate its effect on tissue antioxidants in alloxan diabetic rats.

EXPERIMENTAL

Enicostemma littorale growing at various places of Cuddalore, Tamil Nadu, India were collected. The collection was made by uprooting method and thereby taking care to collect the roots as well. The plant was identified at the Herbarium of Botany Directorate in Annamalai University, Annamalai Nagar, Tamil Nadu. A voucher specimen (No. 641) was also deposited there. The plant was cleaned and dried in the shade and packed into loose masses in plastic bags.

The aqueous extract of *E. littorale* whole plant was prepared as described by Murali et al. (5). A total of 3 kg of the shade-dried herb containing all vegetative and reproductive parts of *E. littorale* were mixed with 24 L of water and heated till it was reduced to half (12 L). The whole mass was then filtered and concentrated to further half twice (6 L) by heating. The marc obtained from this was again mixed with fresh 12 L of water and heated till approximately 6 L of water left behind. The whole mass was filtered again and the filtrate was concentrated to 3 L by heating. This time, the marc remaining was discarded. Both the filtrates were mixed and further concentrated at low temperature. The concentration of dried *E. littorale* was 2 g/ml in the final extract. A 2% sodium benzoate solution was added

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as a preservative. The extract was stored at temperature of 15-20°C in well-closed glass containers (5).

Female albino Wistar rats (180-210 g) raised in the Central Animal House of the Faculty of Medicine, Rajah Muthiah Medical College, Annamalai University were used for the study. Rats were housed in polypropylene cages (47 × 34 × 18 cm) lined with husk. They were fed with standard laboratory diet (Pranav Agro Industries Limited, Maharashtra, India) and given tap water *ad libitum*. The Ethical Committee of Annamalai University has approved this study.

The rats were injected with 150 mg/kg body weight of alloxan in saline (0.9% NaCl) intraperitoneally to induce diabetes. Control rats were injected with saline only. Prior to this, the rats were fasted for 12 h. The rats with high urine sugar and blood glucose (230-280 mg/dL) were selected for the diabetic group.

In our study, 36 rats were used. The rats were divided into 6 groups and each group contained 6 rats. Group I: normal untreated rats given saline orally using an intragastric tube daily for 45 days (8); Group II: normal rats orally administered with *E. littorale* extract (2 g/kg) daily for 45 days using an intragastric tube (8); Group III: alloxan (150 mg/kg) induced diabetic rats given saline orally for 45 days using an intragastric tube; Groups IV & V: diabetic rats treated orally with *E. littorale* extract (1 and 2 g/kg, respectively) daily for 45 days using an intragastric tube (8); Group VI: diabetic rats administered protamine zinc insulin (6 units/kg) intraperitoneally for 45 days (8).

After 45 days of treatment, all the rats were decapitated after fasting overnight. Blood was collected in potassium oxalate and sodium fluoride containing tubes for the estimation of fasting blood glucose. Liver, kidney and pancreas from the rats were excised immediately, rinsed in ice-chilled normal saline and stored for further biochemical estimations.

Blood glucose was estimated by the method of Sasaki et al. (9). 0.1 mL of freshly drawn blood was immediately mixed with 1.9 mL of 10% TCA solution to precipitate proteins and then centrifuged at 3000 rpm. 1.0 mL of the supernatant was mixed with 4.0 ml of *o*-toluidine reagent and kept in a boiling water bath for 15 min. The green colour developed was measured at 620 nm. Values are expressed as mg/dL.

Tissue TBARS were estimated y the method of Fraga et at. (10). To 0.5 mL of the tissue homogenate, 0.5 mL of saline and 1.0 mL of 10% TCA were added, mixed well and centrifuged at 3,000 rpm for 20 min. To 1.0 mL of the protein free filtrate, 0.25 mL of thiobarbituric acid reagent was added, the contents were mixed well and boiled for 1 h at 95°C. The tubes were then cooled to room temperature under running water and the absorption was measured at 535 nm. Values are expressed as mM/100 g wet tissue.

Lipid hydroperoxides were estimated by the method of Jiang et al. (11). 1.8 mL of the Fox reagent was mixed with 0.2 mL of the tissue homogenate and incubated for 30 minutes at room temperature and read at 560 nm. Values are expressed as mM/100 g wet tissue.

Reduced glutathione (GSH) in tissues was determined by the method of Beutler and Kelly (12). The reaction mixture contained 0.2 mL of tissue homogenate, 1.8 ml of EDTA solution, 3.0 mL of precipitating reagent (1.67 g of metaphosphoric acid, 0.2 g of EDTA disodium salt, 30 g sodium chloride in 1 L of distilled water). The contents were mixed thoroughly and kept for 5 min and then centrifuged. To 2.0 mL of the filtrate, 4.0 mL of 0.3 M disodium hydrogen phosphate solution and 1.0 mL of DTNB (5,5-dithiobis-2-nitro benzoic acid) reagent were added and the absorbance was read at 412 nm. Values are expressed as mM/g wet tissue.

Glutathione peroxidase was assayed by the method of Rotruck et al. (13). To 0.2 mL of Tris buffer, 0.2 ml of EDTA, 0.1 mL of sodium azide and 0.5 mL of tissue homogenate were added. To this mixture, 0.2 mL of glutathione followed by 0.1 mL of hydrogen peroxide were added. The contents were mixed well and incubated at 37°C for 10 minutes along with a tube containing all the reagents except sample. After 10 minutes the reaction was stopped by the addition of 0.5 mL of 10% TCA, centrifuged and the supernatant was estimated for glutathione by the method of Beutler and Kelly (12). Values are expressed as µg of GSH consumed/min/mg protein.

Superoxide dismutase (SOD) was assayed according to the method of Kakkar et al. (14). Tissues were extracted with 0.25 mol/L sucrose and an initial purification was performed using ammonium sulphate. The ammonium sulphate-fractioned superoxide dismutase preparation was dialysed overnight against 0.0025 mol/L Tris hydrochloride buffer (pH 7.4) before being used for enzyme assay. The assay mixture contained 1.2 mL of sodium pyrophosphate buffer (pH 8.3, 0.025 mol/L), 0.1 mL of 186 µmol/L phenazine methosulphate, 0.3 mL of 300 µmol/L nitroblue tetrazolium, 0.2 mL of 780 µmol/L NADH and approximately dilutedenzyme preparation and water in a total volume of 3.0 mL. After incubation at 30°C for 90 s, the reaction was

terminated by the addition of 1.0 mL of glacial acetic acid. The reaction mixture was stirred vigorously and shaken with 4.0 mL of *n*-butanol. The colour intensity of the chromogen in the butanol layer was measured at 560 nm against *n*-butanol. Values are expressed as units/mg protein. One unit of enzyme activity is defined as the enzyme concentration required to inhibit the OD at 560 nm of chromogen production by 50% in 1 min.

Assay of catalase was carried out according to the method of Maehly and Chance (15). The assay was done spectrophotometrically following the decrease in the absorbance at 230 nm. The reaction mixture contained 0.01 M phosphate buffer (pH 7.0), 2 mM H_2O_2 and the enzyme extract. Values are expressed as µmoles of H_2O_2 consumed/min/mg protein.

The protein content of tissue homogenates was estimated by the method of Lowry et al. (16). 0.5 mL of tissue homogenate was mixed with 0.5 mL of 10% TCA and centrifuged for 10 min. The precipitate obtained was dissolved in 1.0 mL of 0.1 M NaOH. From this, an aliquot was mixed with 5.0 mL of alkaline copper reagent and allowed to stand at room temperature for 10 min. 0.5 mL of Folin's phenol reagent was added and the blue colour developed was read after 20 min at 640 nm. Values are expressed as mg protein.

Statistical analysis was performed using SPSS package version 6.0 one way analysis of variance (ANOVA) followed by Duncan's Multiple Range Test (DMRT). All the results were expressed as mean ± S.D. for 6 rats in each group; p values < 0.05 were considered as significant.

RESULTS

Table 1 shows the level of blood glucose in normal and diabetic rats. A significant increase in blood glucose was observed in diabetic rats as compared to normal rats. Oral administration of *E. littorale* at doses of 1 and 2 g/kg for a period of 45 days to diabetic rats decreased significantly the levels of blood glucose.

The concentration of tissue TBARS and HP in normal and diabetic rats is presented in Table 2. The concentration of TBARS and HP increased in the liver, kidney and pancreas of diabetic rats as compared to normal rats. Treatment with *E. littorale* (1 and 2 g/kg) significantly decreased TBARS and HP concentration in these tissues of diabetic rats.

Table 3, shows the activity of GPx and the concentration of GSH in liver, kidney and pancreas of normal and diabetic rats. The activity of GPx and concentration of GSH significantly decreased in liver and pancreas of diabetic rats as compared to normal rats. GSH and GPx in kidney did not show significant effect in diabetic rats. Diabetic rats, when orally administered with *E. littorale* (1 and 2 g/kg) significantly increased the activity of GPx and the concentration of GSH in the liver and kidney.

In Table 4, the activities of SOD and catalase in the tissues of normal and diabetic rats are presented. The activities of these enzymes significantly decreased in the liver, kidney and pancreas of diabetic rats as compared to normal rats. Diabetic rats, when orally administered with *E. littorale* (1 and 2 g/kg) significantly increased the activities of these antioxidant enzymes in liver, kidney and pancreas.

Enicostemma littorale (2 g/kg) administration to normal rats did not show any significant effect on all the parameters studied. The dose of 2 g/kg was found to be more effective than that of 1 g/kg. Intraperitoneal administration of insulin (6 units/kg) to diabetic rats for a period of 45 days normalized all the biochemical parameters studied.

DISCUSSION

Free radicals and other reactive species are involved in many human diseases including diabetes and the increased formation of free radicals accompanies tissue injury. These free radicals make a significant contribution to the progression of diabetes mellitus and its complications (17). An increase in the concentration of TBARS has been observed in diabetic tissues. The increased concentration of TBARS in diabetic tissues indicates the

Table 1. Effect of *E. Littorale* on blood glucose (mg/dL) in normal and diabetic rats.

Groups	Blood Glucose (mg/dL)	
	Initial[1]	Final[2]
I	75.3 ± 3.2	79.7 ± 3.3[a]
II	77.0 ± 4.8	74.0 ± 3.0[a]
III	272.8 ± 11.6	337.6 ± 18.1[b]
IV	260.0 ± 20.0	204.1 ± 13.6[c]
V	277.8 ± 11.4	118.5 ± 7.8[d]
VI	258.4 ± 12.7	91.7 ± 3.9[a]

Each value is mean ± SD for six rats in each group.
Values not sharing a common superscript differ significantly at $p < 0.05$ (DMRT).
[1] Initial values prior to drug administration
[2] Final values at the end of the treatment period (45 days)

Table 2. Effect of *E. Littorale* on tissue TBARS and HP in normal and diabetic rats.

Groups		I	II	III	IV	V	VI
TBARS (mM/100 g wet tissue)	Liver	0.90 ± 0.03^{a}	0.90 ± 0.04^{a}	1.57 ± 0.08^{b}	1.44 ± 0.06^{bc}	1.08 ± 0.05^{d}	0.96 ± 0.02^{ad}
	Kidney	2.02 ± 0.11^{a}	2.0 ± 0.12^{a}	2.69 ± 0.23^{b}	2.61 ± 0.20^{c}	2.30 ± 0.17^{d}	2.14 ± 0.18^{a}
	Pancreas	29.6 ± 3.1^{a}	39.3 ± 2.0^{a}	67.7 ± 4.5^{b}	52.0 ± 3.0^{c}	42.2 ± 1.9^{d}	40.8 ± 1.8^{a}
HP (mM/100 g wet tissue)	Liver	50.2 ± 1.6^{a}	49.3 ± 2.0^{a}	89.8 ± 3.7^{b}	76.4 ± 4.0^{c}	61.5 ± 3.1^{d}	52.0 ± 2.3^{a}
	Kidney	33.1 ± 2.1^{a}	31.1 ± 1.8^{a}	76.5 ± 4.0^{b}	62.0 ± 3.0^{c}	40.94 ± 2.2^{d}	34.2 ± 2.1^{a}
	Pancreas	10.8 ± 0.8^{a}	10.4 ± 0.4^{a}	16.09 ± 1.8^{b}	24.7 ± 1.5^{bc}	13.2 ± 1.0^{d}	11.1 ± 0.7^{a}

Each value is mean ± SD for six rats in each group.
Values not sharing a common superscript differ significantly at $p < 0.05$ (DMRT).

Table 3. Effect of *E. Littorale* on the concentration of GSH and activity of GPx in normal and diabetic rats.

Groups		I	II	III	IV	V	VI
GSH (mM/g Wet tissue)	Liver	22.6 ± 1.3^{a}	22.7 ± 1.0^{a}	15.2 ± 1.3^{b}	17.8 ± 1.0^{bc}	21.1 ± 1.4^{d}	22.0 ± 0.8^{a}
	Kidney	17.5 ± 0.61^{a}	17.7 ± 0.4^{a}	16.9 ± 1.0^{a}	17.1 ± 0.8^{a}	11.3 ± 1.1^{a}	17.4 ± 0.8^{a}
	Pancreas	26.4 ± 1.2^{a}	26.5 ± 1.8^{a}	13.8 ± 1.0^{b}	18.0 ± 1.03^{c}	25.1 ± 1.7^{d}	25.9 ± 1.9^{a}
GPx (µg of GSH consumed/ min/mg protein)	Liver	6.3 ± 0.4^{a}	6.3 ± 0.3^{a}	4.0 ± 0.3^{b}	4.8 ± 0.3^{bc}	5.7 ± 0.31^{d}	6.1 ± 0.3^{a}
	Kidney	6.8 ± 0.2^{a}	6.91 ± 0.3^{a}	6.6 ± 0.4^{b}	6.6 ± 0.4^{a}	6.6 ± 0.5^{a}	6.7 ± 0.4^{a}
	Pancreas	27.9 ± 1.0^{a}	28.0 ± 1.1^{a}	15.2 ± 1.3^{a}	19.8 ± 1.8^{c}	25.6 ± 1.9^{d}	27.4 ± 1.6^{a}

Each value is mean ± SD for six rats in each group.
Values not sharing a common superscript differ significantly at $p < 0.05$ (DMRT).

Table 4. Effect of *E. Littorale* on the activities of SOD and catalase in normal and diabetic rats.

Groups		I	II	III	IV	V	VI
SOD (U^{a}/mg Protein)	Liver	11.3 ± 0.8^{a}	11.3 ± 0.6^{a}	5.55 ± 0.3^{b}	8.8 ± 0.4^{b}	10.0 ± 0.8^{c}	11.1 ± 0.6^{a}
	Kidney	12.0 ± 0.4^{a}	12.2 ± 0.7^{a}	7.4 ± 0.5^{b}	8.6 ± 0.5^{c}	10.7 ± 0.6^{d}	11.7 ± 1.0^{a}
	Pancreas	3.18 ± 0.14^{a}	3.19 ± 0.17^{a}	2.36 ± 0.12^{b}	2.57 ± 0.1^{c}	2.88 ± 0.10^{d}	3.15 ± 0.17^{a}
Catalase (µ moles of H_2O_2 consumed/mi	Liver	73.7 ± 3.0^{a}	74.0 ± 4.38^{a}	59.0 ± 6.0^{b}	64.5 ± 3.6^{b}	70.5 ± 5.8^{d}	73.2 ± 5.0^{a}
	Kidney	36.0 ± 2.1^{a}	36.8 ± 1.7^{a}	17.5 ± 1.2^{b}	21.3 ± 1.9^{c}	32.1 ± 2.0^{d}	35.4 ± 2.6^{a}
	Pancreas	13.492 ± 0.71^{a}	13.54 ± 0.4^{a}	7.05 ± 0.63^{b}	8.30 ± 0.41^{c}	11.08 ± 0.66^{d}	13.21 ± 0.88^{a}

U^{a} – enzyme concentration required to inhibit the OD at 560 nm of chromogen production by 50% in 1 min.
Each value is mean ± SD for six rats in each group.
Values not sharing a common superscript differ significantly at $p < 0.05$ (DMRT).

activation of lipid peroxidation system. Kamalakannan and Stanley Mainzen Prince (2004) have reported that increased concentration of lipid peroxides in the liver can result in decreased activity of cytochrome P_{450} and cytochrome b_5 and this may affect the drug metabolizing activity in the chronic diabetes (18).

Hydroperoxides are molecules with high toxicity and a high potential for destroying enzymatic proteins and cell membranes. An increase in HP in liver, kidney and pancreas have been observed in diabetic rats. These results are also consistent with other reports on an increased HP in diabetic rats (19, 20). The increase in HP is possibly caused by the decreased activity of antioxidant enzymes, which is also a favorable factor for uncontrolled generation of free radicals and subsequent generation of lipid hydroperoxides (19).

A decrease in GSH and GPx activity has been registered in diabetic tissues. Depletion of tissue glutathione is one of the primary factors that permit lipid peroxidation. Decreased concentration of GSH in the diabetic tissues might be due to its increased utilization or due to its utilization by glutathione dependent antioxidant process. The decreased activity of GPx might be due to increased concentration of HP or due to decreased concentration of glutathione in the diabetic rats tissues (18). It has been proposed that antioxidants, which maintain the concentration of GSH may restore the cellular defence mechanism, block lipid peroxidation and protect the tissues against oxidative damage (21).

SOD is an antioxidant enzyme, which reduced superoxide radicals to H_2O_2 and oxygen. Catalase is a hemoprotein, which catalyzes the dismutation of H_2O_2. A reduction in the actvities of these enzymes may result in a number of deleterious effects due to the accumulation of superoxide and hydroxy radicals. These radicals induce various injuries to the organs and play a vital role in clinical disorders. Other studies have shown that reactive oxygen free radicals could inactivate and reduce the activities of SOD and catalase (22). The diminished activities of these antioxidant enzymes in diabetic tissues observed indicate an imbalance between excessive generation and insufficient elimination of free radicals in experimental diabetes (23). Hence, removal of superoxide and hydroxyl radicals is the most effective defense of a body against diseases.

Enicostemma littorale administration to diabetic rats, decreased the concentration of TBARS and HP and increased the concentration of GSH and the activities of GPx, SOD and catalase in liver, kidney and pancreas. This indicates the antioxidant potential of the aqueous extract of *E. littorale* Blume. Studies have shown that alkaloids, sterols, catechins and flavonoids exhibit antioxidant effect. Thus the improved levels of antioxidants in diabetc rats might be due to the presence of these constituents in aqueous extract of *E. littorale*. The exact mechanism of action is yet to be known and further research is underway to identify the constituents that are responsible for the antioxidant activity.

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