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BIOCHEMICAL EVALUATION OF HYPOGLYCEMIC AND ANTIOXIDANT POTENTIALS OF *ENICOSTEMMA LITTORALE* LEAVES STUDIED IN ALLOXAN INDUCED DIABETIC RATS

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ABSTRACT

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Diabetes is a metabolic syndrome characterized by chronic hyperglycemia resulting in development of oxidative stress which contributes to the micro and macrovascular complications. Traditional antidiabetic plants provide a useful source of new oral hypoglycemic compounds for development as pharmaceutical entities or as simple dietary adjuncts to existing therapies. *Enicostemma littorale* (Gentianaceae), perennial herb that belongs to the family *Gentianaceae* has been reported for its wide array of pharmacological properties. The present study was aimed to evaluate the antidiabetic and antioxidant potentials of *Enicostemma littorale* leaves extract in alloxan-induced experimental diabetes in rats. Oral administration of *E. littorale* leaves extract (300 mg/kg b.w./rat/day) for a period of 30 days indicated the hypoglycemic nature of the extract. Determination of the lipid peroxides, hydroperoxides, enzymatic and nonenzymatic antioxidants evidenced the antioxidant potential of the leaves extract. The observed hypoglycemic and antioxidant potentials might be due to the presence of biologically active ingredients present in the leaves extract.

INTRODUCTION

Diabetes mellitus is a metabolic syndrome characterized by chronic hyperglycaemia associated with absolute or relative deficiency in insulin secretion and/or action¹. Diabetes is rapidly emerging at an alarming rate and is considered to be one of the biggest health catastrophes in the world², causing significant health and economic burdens on patients and communities³. The International Diabetes Federation estimates direct costs of diabetes of economically developed countries to be approximately 6% of the total health budget⁴.

Insulin therapy and oral hypoglycaemic agents offer effective glycaemic control, but insulin therapy has shortcomings such as ineffectiveness following oral administration, short shelf life, of the need for constant refrigeration, and fatal hypoglycaemia, in the event of excess dosage⁵. The oral hypoglycemic agents that are capable of reducing blood sugar level belong to two chemical classes - sulfonylureas and biguanides. However, the use of oral antidiabetics is limited due to their adverse side effects including hematological, cutaneous and gastrointestinal reactions, hypoglycaemic coma and disturbances of liver and kidney functions⁶. The situation necessitates the active search for the novel therapeutic agents preferably from natural sources. Plants are reputed in the indigenous systems of medicine for their hypoglycemic activities. More than 1200 plants worldwide have been documented as beneficial in the treatment of diabetes⁷.

Enicostemma littorale (Gentianaceae) is a perennial herb that belongs to the family Gentianaceae⁸. It grows throughout India up to 1.5 feet height and more frequently near the sea. It is called as Chota chirayata in Hindi, and Vellarugu or Vallari in Tamil. The herb has already been reported for its anti-inflammatory⁹ and anticancer property¹⁰. The plant was also used in an antidiabetic herbomineral preparation¹¹. The aerial part of *Enicostemma littorale* was reported to show hypolipidaemic effect in p-dimethylaminobenzene (p-DAB) induced hepatotoxic animals¹². In the absence of systematic literature, the present study was designed to evaluate the antidiabetic and antioxidant effect of *Enicostemma littorale* leaves extract in alloxan induced diabetic rats.

MATERIALS AND METHODS

Plant material

Fresh, mature *Enicostemma littorale* Linn plants were collected from Marthandam, Tamil Nadu, India. The plants were identified and authenticated and a voucher specimen was deposited at the Department of Botany, University of Madras, Chennai.

Preparation of plant extract

The *Enicostemma littorale* leaves were shadow dried for 5 days for complete removal of moisture content. After drying, the leaves were powdered in an electric grinder separately and stored at 5°

C until further use. The powdered leaves were delipidated with petroleum ether (60 - 80° C) for overnight. It was then filtered and Soxhalation was performed with 95% Ethanol. Ethanol was evaporated in a rotary evaporator at 40 – 50° C under reduced pressure.

Preliminary phytochemical screening

The ethanolic extract of *Enicostemma littorale* leaves were subjected to preliminary phytochemical screening of various plant constituents^{13, 14}.

Experimental animals

Male albino Wistar rats (150-180 g) were purchased from TANUVAS, Madavaram, Chennai. The rats were housed in polypropylene cages lined with husk and kept in Animal house, Department of Biochemistry. It was renewed every 24 hours. The rats were fed with commercial pelltated rats chow (VRK Nutritional Solutions, Maharashtra, India) and had free access to water. The experimental rats were maintained in a controlled environment (12:12 hours light/dark cycle) and temperature (30 ± 2° C). The experiments were designed and conducted in accordance with the ethical norms approved by Ministry of Social Justices and Empowerment, Government of India and Institutional Animal Ethics Commity Guidelines (IAEC No. 01/057/09) for the investigation of experimental pain in conscious rats. The rats were acclimatized for one week before starting the experiments.

Induction of Diabetes Mellitus

Experimental diabetes was induced in overnight fasted rats by single intraperitoneal injection of alloxan monohydrate dissolved in sterile normal saline at a dose 120 mg/Kg body weight. After 1 hour alloxan administration, the animals were fed with standard pellets and water *ad libitum*. Rats were supplied with 5% glucose solution for 48 hours after alloxan injection in order to prevent severe hypoglycaemia¹⁵. After 1 week time, the rats having persistant glycosuria and hyperglycemia (Blood glucose range of above 250 mg/dL) were considered as diabetic rats and used for the experiment. The treatment was started on the eighth day after alloxan injection and this was considered as first day of treatment.

Experimental Design

The rats were grouped into 4 groups, comprising of 6 rats in each group as follows:

Group I : Control rats received saline alone

Group II : Alloxan induced diabetic rats

Group III : Diabetic rats treated with *Enicostemma littorale* leaves extract (300 mg/Kg body weight/rat/day) in aqueous solution orally for 30 days.

Group IV : Diabetic rats treated with gliclazide (5mg/Kg body weight/rat/day) in aqueous solution orally for 30 days.

At the end of the experimental period, the rats were fasted over night, anaesthetized, and sacrificed by cervical decapitation. The blood was collected with and without anticoagulant for plasma and serum separation respectively.

Biochemical parameters

Blood glucose level was estimated by the method of glucose oxidase/peroxidase as described by Trinder¹⁶, urea by Natelson et al.,¹⁷. Levels of hemoglobin and glycosylated hemoglobin were estimated according to methods of Drabkin and Austin¹⁸ and Nayak and Pattabiraman¹⁹, respectively. Plasma was used for protein assay²⁰ and serum for determination of creatinine²¹.

Preparation of tissue homogenate

Liver tissue was excised, washed in ice-cold saline, and then homogenized in Tris-HCl buffer (pH 7.4) using a Teflon homogenizer. The liver homogenate was then centrifuged at 5,000 g to remove cellular debris and supernatant was used for determination of enzymatic antioxidants. Lipid peroxides were determined using thiobarbituric acid reactive substances by the method of Ohkawa et al.²². Levels of vitamin C, vitamin E, ceruloplasmin and glutathione (GSH) were determined by the methods of Omaye et al.²³, Desai²⁴, Ravin²⁵, Sedlak and Lindsay²⁶, respectively. Enzymatic antioxidants such as superoxide dismutase²⁷, catalase²⁸, glutathione peroxidase²⁹ were assayed.

Statistical analysis

All the grouped data were statistically evaluated with SPSS 13.00 software. Hypothesis testing methods included one-way analysis of variance followed by least significant difference (LSD) test. $p < 0.05$ was considered to indicate statistical significance. All results are expressed as mean \pm standard deviation (SD) for six rats in each group.

RESULTS

Table 1 shows the qualitative analysis of phytochemical in the ethanolic extract of *Enicostemma littorale* leaves. Phytochemical evaluation indicated the presence of alkaloids, flavonoids, proteins, saponins, tannins, triterpenoids and phenols.

Table 1: Phytochemical screening of the ethanolic extract of *Enicostemma littorale* leaves extract

PHYTOCONSTITUENTS	INFERENCE
Alkaloids	+
Flavonoids	+
Carbohydrates	+
Saponins	+
Tannins	+
Phytosterol	+
Triterpenoids	+
Proteins	+
Anthraquinones	-
Phenols	+

Table 2: Effect of *Enicostemma littorale* leaves extract on the levels of biochemical parameters in the experimental groups of rats.

Groups	Control	Alloxan-Diabetes	Diabetic+ <i>E.littorale</i>	Diabetic + gliclazide
Blood glucose (mg/dl)	84.23 ± 7.71	307.97 ± 44.17*	93.06 ± 9.15 [@]	90.61 ± 6.84 [@]
Total protein(g/dl)	9.14 ± 0.62	4.27 ± 0.86*	8.63 ± 0.62 [@]	8.71 ± 0.55
Serum creatinine (mg/dl)	0.71 ± 0.04	1.94 ± 0.23*	0.87 ± 0.05 [@]	0.85 ± 0.06 [@]
Blood urea(mg/dl)	21.64 ± 2.51	40.13 ± 3.42*	26.81 ± 1.99 [@]	25.37 ± 1.86 [@]
Urine sugar	Nil	+++	Nil	Nil

+++ indicates more than 2% sugar. Values are given as mean ± SD for groups of six rats in each. Values are statistically significant at $p < 0.05$. Statistical significance was compared within the groups as follows:

*compared with control, [@] compared with diabetic rats.

The effect of oral administration of *Enicostemma littorale* leaves extract on the levels of blood glucose, total protein, serum creatinine and blood urea and urine sugar in the control and experimental groups of rats were depicted in Table 2. The levels of blood glucose, urea, creatinine were significantly increased in the diabetic group of rats. Oral administration of *Enicostemma littorale* leaves extract at a dose of 300mg/Kg b.w. reverted back the levels to near normalcy. The levels of total protein were decreased significantly compared to control. Treatment with the leaves extract increased the levels to near normal. Urine sugar which is present in the diabetic group of rats was absent in *Enicostemma littorale* leaves extract as well as gliclazide treated diabetic group of rats.

Table 3: Levels of total hemoglobin and glycosylated hemoglobin in control and experimental group of rats

Groups	Hemoglobin (g/dl)	Glycosylated hemoglobin (% Hb)
Control	13.81 ± 4.71	6.05 ± 1.65
Alloxan-Diabetes	6.84 ± 2.35*	15.32 ± 4.46*
Diabetic + <i>E.littorale</i>	11.28 ± 2.74 [@]	8.19 ± 1.36 [@]
Diabetic + Glyclazide	11.94 ± 2.64 [@]	7.92 ± 0.97 [@]

Values are expressed as mean ± S.D. [n=6]. One-way ANOVA followed by post hoc test LSD.* $p < 0.05$.

The results were compared with * control; [@]Diabetic control.

The effect of oral administration of *Enicostemma littorale* leaves extract on the levels of hemoglobin and glycosylated hemoglobin are presented in Table 3. The levels of hemoglobin was significantly decreased and the glycosylated hemoglobin was increased in alloxan induced diabetic rats. The altered levels of these parameters were reverted back to near normalcy upon the treatment with the leaves extract.

Table 4: Effect of *E.littorale* extract on the level of lipid peroxidative markers in plasma of experimental groups of rats

Groups	TBARS	Hydroperoxides
Control	3.72 ± 0.83	8.18 ± 1.47
Alloxan-Diabetes	9.37 ± 0.71*	14.64 ± 2.75*
Diabetic + <i>E.littorale</i>	4.13 ± 0.42 [@]	9.53 ± 0.84 [@]
Diabetic + gliclazide	3.94 ± 0.53 [@]	9.32 ± 1.02 [@]

Units: mM/100 g in tissues; nM of TBA reactants/ml in plasma.

Values are given as mean ± SD for groups of six rats in each. Values are statistically significant at $p < 0.05$. Statistical significance was compared within the groups as follows: *compared with control, [@] compared with diabetic rats.

Table 4 represents the effect of *Enicostemma littorale* leaves extract on the levels of lipid peroxides in the plasma of experimental groups of rats. The levels of lipid peroxides were significantly ($p < 0.05$) elevated in the diabetic group of rats. Upon oral administration of *Enicostemma littorale* leaves extract as well as gliclazide to diabetic group of rats were significantly ($p < 0.05$) reverted to normal levels when compared to control group of rats.

Table 5: Effect of *E.littorale* extract on the levels of non enzymatic antioxidants in plasma of experimental groups of rats

Groups (Plasma)	Vitamin C	Vitamin E	Ceruloplasmin	GSH
Control	1.81 ± 0.32	1.93 ± 0.44	15.83 ± 1.29	30.43 ± 4.34
Alloxan-Diabetes	0.73 ± 0.19*	4.36 ± 0.79*	28.31 ± 2.46*	14.63 ± 2.53*
Diabetic + <i>E.littorale</i>	1.31 ± 0.37 [@]	2.11 ± 0.53 [@]	21.17 ± 2.41 [@]	25.13 ± 5.11 [@]
Diabetic + gliclazide	1.69 ± 0.16 [@]	2.29 ± 0.74 [@]	22.17 ± 3.13 [@]	26.31 ± 4.94 [@]

Units: mg/dl. Values are given as mean ± SD for groups of six rats in each. Values are statistically significant at $p < 0.05$. Statistical significance was compared within the groups as follows: *compared with control, [@] compared with diabetic rats.

The effect of *Enicostemma littorale* leaves extract on the plasma levels of non-enzymatic antioxidants such as vitamin C, vitamin E, reduced glutathione and ceruloplasmin in experimental groups of rats are shown in table 5. The diminished levels of non-enzymatic antioxidants in the diabetic group of rats were significantly ($p < 0.05$) improved to near normal values by the oral administration of *Enicostemma littorale* leaves extract as well as glyclazide, after 30 days of treatment.

Table 6: Effect of *E.littorale* extract on the activity of enzymatic antioxidants in liver tissues of experimental groups of rats

Groups	SOD	Catalase	Glutathione peroxidase	Glutathione S transferase
Control	14.21 ± 1.32	82.77 ± 5.64	9.07 ± 1.24	8.27 ± 1.61
Alloxan-Diabetes	$6.37 \pm 0.65^*$	$30.73 \pm 6.12^*$	$5.03 \pm 0.63^*$	$4.32 \pm 1.67^*$
Diabetic + <i>E.littorale</i>	$10.78 \pm 1.29^@$	$77.53 \pm 5.08^@$	$7.29 \pm 1.51^@$	$7.15 \pm 1.63^@$
Diabetic + gliclazide	$10.25 \pm 0.28^@$	$78.13 \pm 4.92^@$	$8.89 \pm 1.18^@$	$7.16 \pm 1.48^@$

Activity is expressed as: 50% of inhibition of epinephrine autoxidation/min for SOD; μM of hydrogen peroxide decomposed/min/mg of protein for catalase; μM of glutathione oxidized/min/mg of protein for GPx. GSH expressed as mg/100g of tissue. Values are given as mean \pm SD for groups of six rats in each.

Values are statistically significant at $p < 0.05$. Statistical significance was compared within the groups as follows: *compared with control, @ compared with diabetic rats.

Table 6 depicts the activities of enzymatic antioxidants such as superoxide dismutase, catalase, glutathione peroxidase and glutathione-S-transferase in the liver tissues of experimental groups of rats. The decreased activity of enzymatic antioxidants observed in the diabetic group of rats were significantly ($p < 0.05$) elevated to near normal levels after treatment with *Enicostemma littorale* leaves extract as well as gliclazide.

DISCUSSION

Alloxan and streptozotocin are the widely used diabetogens to induce experimental diabetes in animals. Alloxan (2, 4, 5, 6-tetraoxypyrimidine; 5,6-dioxyuracil) and the product of its reduction, dialuric acid, establish a redox cycle with the formation of superoxide radicals. These radicals undergo dismutation to hydrogen peroxide. Thereafter highly reactive hydroxyl radicals are formed by the Fenton reaction. The action of reactive oxygen species with a simultaneous massive increase in cytosolic calcium concentration causes rapid destruction of B cells³⁰.

Hyperglycemia is a significant factor in the development and progression of the complications of diabetes mellitus³¹. Experimentally induced diabetes leads to pancreatic beta cell necrosis that is caused due to diabetic oxidative stress. As a result of pancreatic beta cell necrosis insulin deficiency predominates resulting in repression of glycolytic enzyme and expression of gluconeogenic enzyme which promotes gluconeogenesis in liver, and decreased utilization of glucose by the peripheral tissues contributes to hyperglycemia³². The elevated blood glucose level observed in the diabetic rats was significantly decreased in extract treated rats suggesting insulin secretory effect of *E.littorale* extract from the remnant β cells. Earlier studies had confirmed hypoglycemic potential of the plant in alloxan induced diabetic rats^{33,34}.

Persistent hyperglycemia ultimately results in glycation of hemoglobin leading to the formation of glycosylated hemoglobin³⁵. Glycosylated hemoglobin (HbA1c) is a standard biochemical marker in assessment of diabetes. The HbA1c concentration reflects the patient's average plasma glucose over the previous several weeks making it useful in assessing diabetic control³⁶. A high glucose concentration has been found to lead to the glycosylation of amino groups of lysine residue in proteins. Nonenzymatic glycosylation of protein occurs by direct reaction between reducing sugars and aminogroups in protein. This condition favors reduction in the level of total hemoglobin and elevation in glycosylated hemoglobin, which is directly proportional to blood glucose³⁷. Diabetic rats showed higher levels of glycated hemoglobin indicating their poor glycemic control. Oral administration of *E. littorale* leaves extract to diabetic rats decreased the HbA1C level with a concomitant rise in hemoglobin level indicating the improved glycemic control.

Creatinine is a byproduct of the breakdown of creatine and phosphocreatine, which are considered as an energy storage compounds in muscle. The serum creatinine concentration may vary based on a number of factors including diet composition, muscle mass and gender. Serum creatinine values also depend on the

ability of the kidney to excrete creatinine. An elevation in creatinine usually occurs simultaneously with an increase in blood urea nitrogen. In the present study, the oral treatment with the leaves extract for 30 days significantly reduced the serum creatinine level indicating an improved renal function.

Urea is the main end product of protein catabolism in the body. Accumulation of urea nitrogen in experimental diabetes may be due to the enhanced breakdown of both liver and plasma proteins³⁸. Alterations in nitrogen homeostasis may lead to increased hepatic elimination of urea nitrogen and increased peripheral release of nitrogenous substances. Thus, the observed negative nitrogen balance may be partly because of changes occurring within the hepatocytes. Oral administration of *E.littorale* leaves extract to diabetic rats significantly decreased the altered levels of blood urea.

Experimentally induced diabetic model indicates several alterations of amino acid metabolism, which may be attributed to increased muscle proteolysis, reduced protein synthesis, an energy-dependent process in the liver, and stimulated hepatic gluconeogenesis utilizing gluconeogenic amino acids³⁹. This readily accounts for observed decrease in the total protein content in diabetic rats. Administration of *E. littorale* leaves extract to diabetic rats significantly inhibits proteolysis caused by insulin deficiency and improves total protein level.

Oxidative stress is an imbalance between the production of reactive oxygen species and antioxidant defense. Diabetic oxidative stress coexists with a reduction in the antioxidant status, which can further increase the deleterious effects of free radicals. The role of oxidative stress in the pathogenesis and complications of diabetes mellitus is well recognized⁴⁰. Animal models of diabetes exhibit chronic oxidative stress due to persistent hyperglycemia, which depletes the activity of free radical scavenging enzymes and thus promotes free radicals generation⁴¹. Oxidative stress has lately been reported to be responsible, to a certain extent, for the β -cell dysfunction caused by glucose toxicity⁴². Antioxidant treatment could be a potential therapeutic procedure for diabetic complications⁴³.

The high level of lipid peroxidation marker, TBARS, in the diabetic rats is a reflection of insufficiency of antioxidant defenses in combating ROS-mediated damage. The maintenance of persistent normoglycemia by the administration of *E. littorale* leaves extract may attenuate lipid peroxidation in tissues and thus prevent tissues from hyperglycemia mediated oxidative stress.

The activities of enzymatic antioxidants and the levels of non-enzymatic antioxidants in diabetic group of rats were found to be decreased. Enzymatic antioxidants are involved in detoxification of free radicals and

peroxides formed during oxidative stress, including diabetes. Enzymatic antioxidants such as SOD, CAT, GPx, and GST are crucial cellular components of antioxidant defense system in the body, thus playing a crucial role in the maintenance of a balanced redox status⁴⁴. The enzymatic antioxidants were found to be decreased in diabetic rats due to hyperglycemia mediated oxidative stress⁴⁵. However, oral treatment with *E. littorale* leaves extract to diabetic rats resulted in increased activities of antioxidant enzymes which may be attributed to the free radical scavenging of *E. littorale* leaves.

The non-enzymatic antioxidants such as Vitamin C, E, ceruloplasmin and reduced glutathione are found to be decreased in diabetic state due to their free-radical scavenging property⁴⁶. The declined levels of the non enzymatic antioxidants due to the increased production of free radicals⁴⁷ during hyperglycemia mediated oxidative stress. However, oral administration of *E. littorale* leaves extract to diabetic group of rats showed improved status of non-enzymatic antioxidants suggesting the free radical scavenging potential of *E. littorale* leaves extract.

Phytochemical evaluation indicated the presence of alkaloids, flavonoids, proteins, saponins, tannins, triterpenoids and phenols. Phytochemicals include compounds with various biological properties (i.e. antioxidant, antiproliferative, DNA repair) which allow plants to cope up with environmental challenges including exposure to radiation and toxins⁴⁸. Most plants with antidiabetic properties have been found to contain secondary metabolites such as glycosides, alkaloids and flavonoids⁴⁹. It has been shown that many plants exhibit efficient antioxidant properties owing to their phenolic constituents. Flavonoids are the polyphenolic compounds that act as primary antioxidants or free radical scavengers⁵⁰. Plant alkaloids have the tendency to release insulin from pancreatic beta cells and also have the potential to protect it from alloxan induced pancreatic damage in experimental animals⁵¹. Similarly, terpenoids as vitamins acts as the regulators of metabolism and play a protective role as antioxidants⁵².

The plant *E.littorale* is rich in phytoconstituents such as alkaloids, flavonoids, proteins, saponins, tannins, triterpenoids and phenols. The hypoglycemic and antioxidant nature of the leaves extract is due to the presence of the biologically active ingredients of known pharmacological action.

CONCLUSION

The results of the present study, supports the use of *E.littorale* leaves extract for the treatment of diabetes mellitus. In conclusion, *E.littorale* leaves extract possess potent antidiabetic and antioxidant properties. The extract exhibited anti-hyperglycemic activity comparable to that of a standard anti-diabetic drug,

gliclazide. The observed hypoglycemic and antioxidant nature of the leaves extract is due to the synergistic action of biologically active ingredients of known pharmacological action. Further studies are in progress to isolate and characterize the active principle responsible for its pharmacological action.

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