

Protective Effect of *Enicostemma littorale* Blume on Rat Model of Diabetic Neuropathy

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Abstract: Problem statement: Poor glycemic control and oxidative stress is implicated as a common pathway in the development of diabetic neuropathy. **Approach:** In the present study, we investigated the protective effects of *Enicostemma littorale* Blume (EL) (2.5 g kg⁻¹), a hypoglycemic and antioxidant herbal medicine in alloxan-induced diabetic neuropathy in male Charles foster rats. **Results:** Tail flick latency in hot immersion test and the formalin test were used to evaluate nociceptive responses in diabetic rats compared to non-diabetic rats. Preventive treatment of EL (for 45 days) and standard drug glibenclamide (Glib) significantly improved nociception in diabetic rats. The changes in lipid peroxidation status and anti-oxidant enzymes (super-oxide dismutase, glutathione peroxidase and catalase) levels observed in diabetic rats, were significantly restored by EL and glib treatment. Decrease in Na-K⁺ ATPase activity was also significantly restored by EL. Glibenclamide was used as standard drug in present study. **Conclusion:** This study provided experimental evidence to preventive effect of EL on nerve function and oxidative stress in animal model of diabetic neuropathy. Hence EL may be tried clinically for the treatment of diabetic neuropathy since it was used as folk medicine in diabetic patients.

Key words: Diabetes, neuropathy, *Enicostemma littorale*, glibenclamide

INTRODUCTION

Diabetic neuropathy occurs in 50% of diabetic patients. These patients are suffering from sever and unremitting pain. Diabetic neuropathic patient generally complain about persistent burning or tingling sensation, usually in the legs and feet. Other symptoms includes an inability to detect heat and cold, cutaneous hyperaesthesia, loss of vibration sensation and paradoxically, the loss of pain perception. The pathophysiology of the condition remains unclear, although it has been associated with peripheral demyelination, a decrease in peripheral nerve conduction and degeneration of myelinated and unmyelinated sensory fibers^[11].

Hyperglycemia can induce oxidative stress via glucose autooxidation and the subsequent formation of advanced glycation end products, disruption of the polyol pathway, altered eicosanoid metabolism and decreased antioxidant defenses^[14,8].

To combat oxidative stress in diabetic neuropathy antioxidants treatment has been tried in both animals and diabetic patients. Administration of the antioxidants vitamin C or α -lipoic acid, as well as free amino acids,

also improves responses to insulin and thus can provide additional benefit to the proposed reduction of oxidative stress in tissues^[15,16,27,34]. Vitamin E decreases blood glucose in type 1 diabetic rats through an unknown mechanism^[42].

In traditional practice, medicinal plants are used in many countries to control DM. The National Center for Complementary and Alternative Medicine, established in 1998 by the United States Government where development of herbal medicine is one of the important subjects of study^[47].

Enicostemma littorale Blume, a small herb of family Gentianaceae is commonly used as an antidiabetic agent by rural folks of Gujarat, India. It is cited in ancient literature as an antimalarial, antipyretic and as a laxative^[41]. Our earlier studies had confirmed its hypoglycemic potential in alloxan-induced diabetic rats^[24-26,40] and also reported it's hypoglycemic, antioxidant and hypolipidaemic potential in newly-diagnosed NIDDM patients^[39]. It is also used as an antidiabetic herbomineral preparation^[43]. This herb is also known for its anti-inflammatory^[45] and anticancer property^[40]. Recently aerial part of *Enicostemma littorale* was reported to show hypolipidaemic effect in p-

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Dimethylaminobenzene (p-DAB) induced hepatotoxic animals^[44].

MATERIALS AND METHODS

Extract preparation: Authentic plant material was purchased from local market and identified at the Botany Department, Maharaja Sayajirao University, Baroda, Voucher specimen [Oza 51,51 (a)] is present at the Herbarium of Botany Department, Maharaja Sayajirao University, Baroda. Whole dried plant was powdered in grinding meal. The powder was extracted as per our earlier report^[40]. Methanolic extract thus obtained, was further used in the experiment.

Animals and treatment: Male Charles Foster rats (body weight 200-250 g) were used for the study. They were allowed ad libitum access to water and food. Diabetes was induced chemically by alloxan (120 mg kg⁻¹ body weight, i.p.). After the stabilization period of 15 days, animals showing blood glucose levels more than 200 mg dL⁻¹ were considered as diabetic. Rats were divided into four groups of six rats each as follows: Group I: Control (C), treated intragastrically (i.g) with 1% Carboxymethylcellulose (CMC). Group II: Alloxan treated (ALX- Treated) 1% CMC by i.g., route. Group III: ALX + EL. EL was given by i.g route at a dose of 2.5 g of methanol extract kg⁻¹ body wt day⁻¹^[40]. Group IV: ALX + Glibenclamide (Glib). Glib was given by i.g., route at a dose of 2.5 mg kg⁻¹ b.wt day⁻¹. All the groups were treated for 45 days and following parameters were measured.

Blood glucose level measurement of nociceptive threshold: Before sacrificing the animals, blood was collected for the determination of the blood glucose and testosterone levels. The plasma was obtained after centrifugation (3000 g for 10 min, 4°C). Blood glucose was estimated by GOD-POD kit method^[46] (Monozyme, India).

Measurement of nociceptive threshold: All groups of animals were experimented for this test. Animals are placed into individual restraining cages leaving the tail hanging out freely. The animals are allowed to adapt to the cages for 30 min before testing. The lower 5 cm portion of the tail is marked. This part of the tail is immersed in a cup of freshly filled water of exactly 55°C. Within a few seconds the rat reacts by withdrawing the tail. The reaction time is recorded in 0.5 sec units by a stopwatch. After each determination the tail is carefully dried. The cut off time of the

immersion is 15 sec. The withdrawal time of untreated animals is between 1 and 5.5 sec. A withdrawal time of more than 6 sec therefore is regarded as a positive response.

All groups of animals were administered 0.05 mL of 10% formalin into the dorsal portion of the front paw. Each individual rat is placed into a clear plastic cage for observation. Readings are taken at 30 and 60 min and scored according to a pain scale. Pain responses are indicated by elevation or favoring of the paw or excessive licking and biting of the paw. Analgesic response or protection is indicated if both paws are resting on the floor with no obvious favoring of the injected paw.

Determination of aldose reductase activity from sciatic nerve and Na-K ATPase activity: Tissue was homogenized in 10 mM sodium phosphate buffer containing 5 mM 2-mercaptoethanol (pH 7.0) at 0°C. A cell-free extract is obtained by centrifuging the total homogenate at 17300 g for 10 min. The enzyme reaction mixture (final volume 1 mL) contains 0.4 M (NH₄)₂SO₄, 0.1 M HEPES buffer adjusted to pH 7.0 with sodium hydroxide, 10 mM DL-glyceraldehyde and 0.12 mM NADPH. A 20 µL aliquot of supernatant is added to initiate the reaction. Decrease in absorbance at 340 nm is followed spectrophotometrically. Enzymatic activity is expressed as nM of NADPH oxidized min⁻¹^[49]. Na-K ATPase activity was measured by^[48].

Antioxidant parameters: The tissues were minced and washed repeatedly with the sucrose buffer pH 7.4 to remove adhering blood and 10% (w/v) homogenates were prepared using a Potter-Elvehjem type glass-Teflon homogenizer. Then the mitochondrial and post-mitochondrial fractions were prepared^[17].

Lipid peroxides reacts with TBA (thiobarbituric acid) and forms TBARS (thiobarbituric acid reacting substances) giving a characteristic pink color which can be measured colorimetrically at 532 nm indicating the levels of LPO^[4]. GSH levels were estimated by monitoring the reduction of DTNB (dithiobis-2-nitrobenzoic acid) forming a yellow colored anion at 412 nm^[5]. Measurements were carried out to determine the activities of Superoxide Dismutase (SOD)^[1,23,34], catalase^[1] Glutathione Peroxidase (GPox)^[32].

Protein estimation: Protein estimation was according to the method of Lowry *et al.*^[21] using bovine serum albumin as the standard.

Statistical analyses: Statistical evaluation of analytical data was done by Student's t-test using the statistical

software-GraphPad Prism 3.0. In all the comparisons a two tailed p-value ≤ 0.05 was considered statistically significant. The values expressed as mean \pm SE.

RESULTS

Blood glucose level: Diabetic rats were having hyperglycemic condition. Rats treated with EL for 45 days showed significant reduction in blood glucose levels by 54% (Fig. 1).

Nociceptive threshold: In the present study, thermal hypoalgesia occurred in alloxan-induced diabetic rats. Response to a thermal noxious stimulus of diabetic rats was prolonged significantly compared to normal rats (Fig. 2). Administration of EL prevented this elevation of tail-flick threshold by 56%.

Injection of 5% formalin into the hind-paw of control rats evoked a series of flinching responses of the afflicted paw. In diabetic animals it has been observed that number of flinches increased significantly as compared to control animals. Upon EL treatment noxious stimulus was reduced to 52% as reflected in decrease in number of flinches in 30 min. (Fig. 3).

Aldose reductase and Na-K ATPase activity from sciatic nerve: There was significant increase in aldose reductase enzyme activity in sciatic nerve of diabetic animals as compared to normal animals. Diabetic rats treated with EL showed decrease in aldose reductase activity by 64% (Fig. 4). Sciatic nerve Na-K ATPase activity (Fig. 5) was significantly reduced in diabetic rats as compared to normal control. This was largely corrected by EL treatment by 64%.

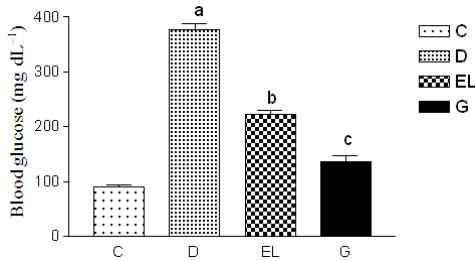


Fig. 1: Effect of EL on blood glucose levels of diabetic rats (a): $p < 0.001$ as compared to normal control; (b): $p < 0.001$ as compared to diabetic control; (c): $p < 0.001$ as compared to diabetic control

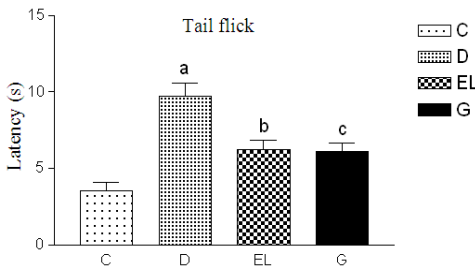


Fig. 2: Effect of EL on thermal nociception responses (a): $p < 0.001$ as compared to normal control; (b): $p < 0.01$ as compared to diabetic control; (c): $p < 0.01$ as compared to diabetic control

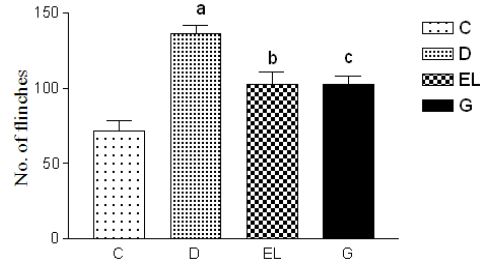


Fig. 3: Effect of EL on formalin induced flinches (a): $p < 0.001$ as compared to normal control; (b): $p < 0.01$ as compared to diabetic control; (c): $p < 0.01$ as compared to diabetic control

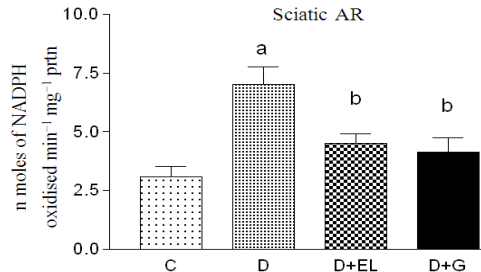


Fig. 4: Effect of EL on sciatic nerve AR activity (a): $p < 0.01$ as compared to normal control; (b): $p < 0.05$ as compared to diabetic control; (c): $p < 0.05$ as compared to diabetic control

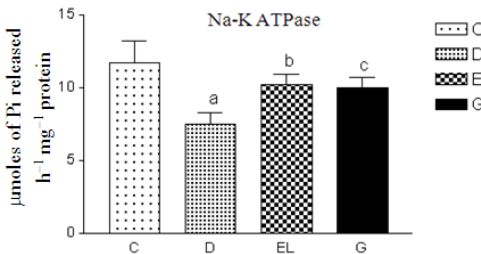


Fig. 5: Effect of EL on sciatic nerve Na-K-ATPase activities (a): $p < 0.05$ as compared to normal control; (b): $p < 0.05$ as compared to diabetic control; (c): $p < 0.05$ as compared to diabetic control

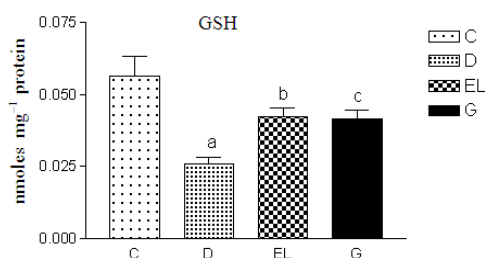


Fig. 6: Effect of EL sciatic nerve GSH content (a): $p < 0.05$ as compared to normal control; (b): $p < 0.05$ as compared to diabetic control; (c): $p < 0.05$ as compared to diabetic control

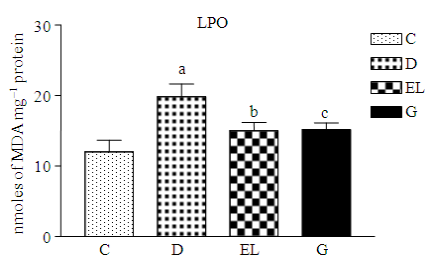


Fig. 7: Effect of EL on lipid peroxidation of sciatic nerve (a): $p < 0.01$ as compared to normal control; (b): $p < 0.05$ as compared to diabetic control; (c): $p < 0.05$ as compared to diabetic control

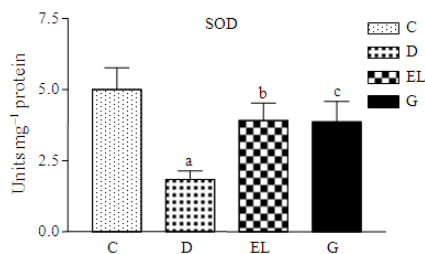


Fig. 8: Effect of EL on superoxide dismutase activity of sciatic nerve (a): $p < 0.01$ as compared to normal control; (b): $p < 0.05$ as compared to diabetic control; (c): $p < 0.05$ as compared to diabetic control

Antioxidant parameters: The GSH contents (Fig. 6) of sciatic nerve was reduced to 54% by diabetes. The GSH level was significantly improved to 55% by EL treatment to diabetic rats. MDA, lipid peroxidation product significantly increases in sciatic nerve of diabetic rats as compared to normal rats. Diabetic rats treated with EL showed significant decrease in MDA levels by 62% (Fig. 7).

Diabetic rats showed decrease in sciatic nerve SOD activity as compared to normal rats. EL treatment brings the activity of SOD to near normal by 66% (Fig. 8).

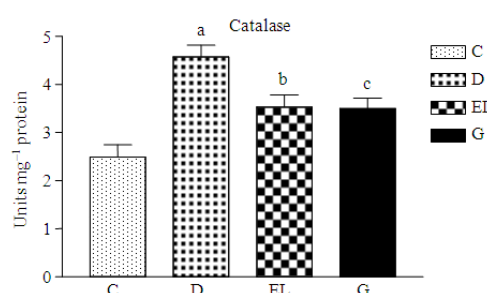


Fig. 9: Effect of EL on catalase activity of sciatic nerve (a): $p < 0.001$ as compared to normal control; (b): $p < 0.05$ as compared to diabetic control; (c): $p < 0.01$ as compared to diabetic control

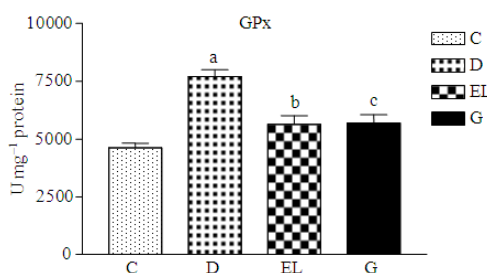


Fig. 10: Effect of EL on Glutathione peroxidase activity of sciatic nerve (a): $p < 0.001$ as compared to normal control; (b): $p < 0.01$ as compared to diabetic control; (c): $p < 0.01$ as compared to diabetic control

Similarly it has been observed that sciatic nerve Catalase (Fig. 9) and GPx (Fig. 10) activity increases significantly in diabetic rats as compared to normal control. Diabetic rats treated with EL showed decrease in these enzymes by 51 and 67% respectively.

DISCUSSION

Neuropathic pain is most common symptom associated with diabetic neuropathy, thus we evaluated the nociceptive response in diabetic rats. There are many studies of hyper and hypoalgesia in STZ-induced diabetic animal models. While thermal hypo-algesia is reported in diabetic rats using the tail-flick test or the hot-plate test^[2,3,6,10,19,20], others have found hyperalgesia in diabetic animals^[13,31,37,38]. In the present study, alloxan-induced diabetic rats showed hyperalgesia in the formalin test and showed thermal hypoalgesia.

In the present study we observed a significant reduction in nociception with EL treatment for 45 days. This could be due to hypoglycemic and antioxidant

activity of EL^[24]. Several reports have ascribed free radical induced oxidative stress under diabetic conditions to hyperglycemia^[7,9,29]. Oxidative stress causes vascular impairment leading to endoneurial hypoxia resulting in impaired neural function and reduced nerve conduction velocity^[35,43,44]. Hence, from the above reported results it is convincing to assume that the amelioration of oxidative stress using potent hypoglycemic and antioxidants can be beneficial in diabetic neuropathy.

In this study, we evaluated the effect of preventive effect of EL on the development of diabetic neuropathy. We observed effectiveness of preventive treatment of EL in diabetic neuropathy.

In diabetic rats, we observed a significant increase in MDA levels and reduction in antioxidant enzymes activity. Kishi *et al.*^[18] have reported that changes in antioxidant enzyme activity may be related to duration of diabetes^[18]. In our study, lipid peroxidation was significantly reduced by EL treatment. SOD activity was significantly improved following treatment with EL. Catalase activity in EL treated diabetic rats was improved as compared to control rats. Several antioxidant compounds (vitamin E, lipoic acid and taurine) have been investigated for their potential in the treatment of diabetic neuropathy^[28,30].

Na-K-ATPase activity in diabetic rats was significantly reduced down to about half level of the control rats. EL treatment restored the Na-K-ATPase activity. Na-K-ATPase activity was possibly improved by inhibition of oxidative stress^[36] and also by amelioration of vascular function^[22].

Thus, this study supports the potential of EL use in treatment of diabetic complications. Results of our study demonstrate the protective effect of EL, on diabetic neuropathy, which may be a consequence to improved glycemic control and in the antioxidant defense system and as well due to improvement in the Na-K ATPase activity. Since, EL is already being used as folk medicine by the diabetic patients it may be evaluated for preventive and curative therapy in diabetic patients at risk of developing neuropathy.

CONCLUSION

Present study indicates that hyperglycemia and oxidative stress is responsible for the development of diabetic neuropathy. Protective effect of EL against neuropathy could be due to controlling hyperglycemia and reducing oxidative stress. Thus our study suggests that EL can be used clinically for the management of diabetic neuropathy.

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