Indian Journal of Experimental Biology
Vol. 47, September 2009, pp. 737-742

Antinociceptive property of *Emblica officinalis* Gaertn *(Amla)* in high fat diet-fed/low dose streptozotocin induced diabetic neuropathy in rats

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Received 16 January 2009; revised 23 April 2009

Diabetic neuropathic pain is an important microvascular complication in diabetes mellitus and oxidative stress plays a vital role in associated neural and vascular complications. The present study investigated flavonoid rich fruit extract (ethyl acetate:methanol fraction) of *E.officinalis* (10 mg/kg), in type II diabetes (high fat diet fed/low dose streptozotocin) induced diabetic neuropathy in male Sprague-Dawley rats. Diabetic rats exhibited a significant hyperalgesia (nociception) as compared to control rats. Treatment with *E.officinalis* extract (EOE) and quercetin in diabetic rats showed significant increase in tail flick latency in hot immersion test and pain threshold level in hot plate test compared to control rats. The changes in lipid peroxidation status and anti-oxidant enzymes (superoxide dismutase and catalase) levels observed in diabetic rats were significantly restored by *E.officinalis* extract and quercetin treatment. Both, *E.officinalis* extract and quercetin attenuated diabetic induced axonal degeneration. The study provides experimental evidence of the preventive and curative effect of *E.officinalis* on nerve function and oxidative stress in animal model of diabetic neuropathy. Since, *E.officinalis* fruit is already in clinical use for diabetic patients it may be evaluated for preventive therapy in diabetic patients at risk of developing neuropathy.

Keywords: Antioxidant, Diabetic neuropathy, *Emblica officinalis,* Nociception, Oxidative stress

Diabetic neuropathic pain is one of the common complications of diabetes mellitus. The pathways contributing to the development of diabetic neuropathy include increased activation of polyol pathway, oxidative stress, advanced glycation end product formation, nerve hypoxia/ischemia, protein C and reduction of nerve growth factor support[1,2]. Oxidative stress plays a vital role in contributing to neural and vascular complications[3] because once the reactive oxygen species are formed they deplete antioxidant defenses (superoxide dismutase, catalase and glutathione peroxidase), rendering the affected cells and tissues more susceptible to oxidative damage. Increased lipid peroxidation and accelerated advanced lipoxidation endproducts (ALE) formation, possibly catalyzed by hyperglycemia and oxidative stress, may play a critical role in the development of neurovascular complications in diabetes[4].

Diabetic neuropathic pain is difficult to treat because it is resistant to analgesics like morphine[5]. Antidepressants, topical capsaicin, anticonvulsants[6] are therapeutically used in the management of diabetic neuropathy. However these are only partially effective[7], develop tolerance[8] and produce potential toxicity[9]. There are reports on the antinociceptive activity of certain flavonoids such as hydroxyethylrutoside[10]. Flavonoids are polyphenols found frequently in fruits, vegetables and grains[11]. Quercetin, a bioflavonoid is reported to attenuate thermal hyperalgesia in a mouse model of diabetic neuropathic pain[12]. *Emblica officinalis* Gaertn is reported to have hypolipidemic[13], antioxidant[14] and hypoglycemic activities[15]. *E. officinalis* is being used in several indigenous medical preparations against a variety of conditions such as atherosclerosis[13] and diabetes[16]. Based on these reports, the present study has been designed to evaluate the effect of flavonoid rich extract of *E.officinalis* fruit in high fat diet fed /low dose streptozotocin (HFD fed/low dose STZ) - induced diabetic neuropathic rats.

Materials and Methods

Chemicals and drugs — Streptozotocin was purchased from Sigma-Aldrich Chemical Company, St. Louis, MO, USA and quercetin from HiMedia laboratories, Bombay, India. The feed ingredients

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such as casein (Himedia laboratories, Mumbai, India), dl-methionine (Loba Chemie, Mumbai, India), vitamin, mineral mix (Sarabhai chemicals, Baroda, India) and lard were obtained from the commercial sources and were of analytical grades. Fructose was purchased from El-Nasr Chemical Co., Abou Zaabal, Cairo, Egypt.

Preparation of fructose diet — Fructose diet was prepared as per Sleder *et.al*[17] and consisted of 660 g fructose, 100 g protein, 80 g fat, 0.04 g zinc carbonate, 5 g vitamins mixture, 5 g mineral mixture and 150 g cellulose, all of commercial grade.

Extraction of flavonoids — Ground dried material of *E.officinalis* fruit was obtained locally. The plant material was identified and authenticated by Mrs. Shalini, Department of Pharmacognosy, Krupanidhi College of Pharmacy. The dried material of *E.officinalis* fruit was extracted thrice with hot 80% methanol[18]. The combined extract was evaporated to dryness and the residue was dissolved in water and extracted with ethyl acetate. The ethyl acetate extract was evaporated in vacuum. The residue obtained was adsorbed on silica gel and transferred to a column of silica gel equilibrated with hexane. Elution performed with ethyl acetate: methanol (50:50) fraction[19] gave fraction rich in polyphenols. This fraction was concentrated in vacuum and used in the experiment.

Animals — After obtaining the permission from institutional animal ethics committee for conducting the above study, male Sprague–Dawley (SD) rats (160–180 g) were housed in standard polypropylene cages (three rats/cage) and maintained under controlled room temperature (22°±2°C) and 55±5% RH with 12:12 h light and dark cycle. All the rats were provided with normal pellet diet (Amrut Diet, New Delhi) and water *ad libitum*, prior to the dietary manipulation.

Development of high fat diet -fed/low dose streptozotocin - treated type 2 diabetic rats[20] — The animals were fed high fat diet (HFD), once a day for 2 weeks followed by ip injection of streptozotocin (35 mg/kg) dissolved in 0.1 *M*/l citrate buffer (*p*H 4.4) after overnight fasting. The rats with the non-fasting plasma glucose level of ≥300 mg dl^{-1} were considered diabetic. Blood sample was collected from tail vein and glucose was measured by using glucose diagnostic kit (Accurex, India).

Treatment schedule — After a basal recording of nociceptive reaction, the control and diabetic rats were randomly selected such that their basal mean biochemical parameters were similar to each other. The control rats were divided into three groups (I-III) of 8-10 rats each and were treated with vehicle of quercetin (0.5% Na-CMC 2 ml kg^{-1}, po), quercetin (10 mg kg^{-1} once daily, po)[12] and *E.officinalis* extract (EOE) (10 mg kg^{-1}once daily, po)[19] respectively. The diabetic rats were divided into three groups (IV-VI) of 8-10 rats each and were treated with vehicle of quercetin (0.5% Na-CMC 2 ml kg^{-1}, po), quercetin (100 mg kg^{-1}once daily, po) and *E.officinalis* extract (EOE) (10 mg kg^{-1}once daily, po) respectively. Drug solution and extract were freshly prepared and administered for a period of 8 weeks and treatment schedule was started one day before the administration of STZ.

Effect of EOE and quercetin on blood glucose levels in normal and diabetic animals (single and multiple dose study) — Normal and diabetic rats were administered with a single dose of the EOE extract and the blood glucose level were estimated just prior to extract administration and at 1, 2 and 4 h intervals. Glucose levels were estimated using a glucose diagnostic kit. For multiple dose study, the same groups of normal animals were continued with the same dose level once daily, up to 11 days. The glucose levels of all the animals were measured on day 3, 5, 7, 9 and 11, respectively.

Oral glucose tolerance test — The rats were divided into two groups. The first group was treated with vehicle and the second group was treated with EOE extract. The animals were fasted for 12 h and then orally administered with 2.0 g/kg glucose. Blood glucose levels were measured at 0, 30, 60 and 120 min after glucose load.

Measurement of lipid peroxidation — Sciatic nerve was removed bilaterally from the inguinal ligament to its trifurcation. Nerve was then homogenized in 2.5% 50 m*M* phosphate buffer saline (PBS) buffer *p*H 7.0 using polytron homogenizer after incubation in triton X 100 for 20 min. Homogenate was used for the measurment of thiobarbituric acid reactive substance (TBARS) at absorbance of 535 nM according to Buege and Aust[21]. Amount of thiobarbituric acid reactive substance was calculated using an extinction coefficient of 156 mmol/cm and the values expressed as malondialdehyde levels in μM/mg protein.

Measurement of superoxide dismutase (SOD) and catalase activity — Sciatic nerve homogenate was centrifuged 17,500 *g* at 4°C, for 10 min. The supernatant was used for the measurement of SOD

activity by hematoxylin autooxidation method[22] and catalase activity by H_2O_2 degradation method[23].

Estimation of tissue protein — Protein concentrationwas estimated according to the method of Lowry *et.al*[24], using bovine serum albumin (BSA) as a standard.

Estimation of reduced glutathione — Reduced glutathione was measured according to the method of Ellman[25]. Equal quantity of sciatic nerve homogenate was mixed with 10% trichloroacetic acid and centrifuged to separate proteins. To 0.01 ml of this supernatant, 2 ml of phosphate buffer (*p*H 8.4), 0.5 ml of 5, 5' dithio, bis 2-nitrobenzoic acid and 0.4 ml double-distilled water was added. Mixture was vortexed and the absorbance was measured at 412 nm within 15 min. The concentration of reduced glutathione was expressed as µg/mg of protein.

Measurement of antinociceptive activity

Hot immersion test: Nociception was assessed by tail immersion test. The rat tail was immersed in warm (45º ± 1ºC) water and the tail flick latency (withdrawal response of tail) was recorded one week post STZ injection; 15 sec was considered as the cut of time.

Hot-plate test: The hyperalgesic response on the hot-plate is considered to result from a combination of central and peripheral mechanisms. In this test, animals were individually placed on a hot-plate (Eddy's hot-plate) with the temperature adjusted to 55°±1°C. The latency to the first sign of paw licking or jump response to avoid the heat was taken as an index of the pain threshold; the cut-off time was 15 sec in order to avoid damage to the paw. The responses were recorded one week post STZ injection.

Histopathological analysis — Samples of sciatic nerve were kept in the fixative solution (10% formalin) and cut into 4-µm thickness. Staining was done by using hematoxylin and eosin. Nerve sections were analyzed qualitatively under light microscope (400×) for axonal degeneration.

Gastric–ulcerogenic side effect — After the antinociceptive activity, rats were killed under deep ether anesthesia and stomachs were removed. Then the abdomen of each rat was opened through the greater curvature and examined under dissecting microscope for lesions or bleedings on the gastric mucosa.

Statistical analysis — All the results were expressed as mean±SE. Data of behavioural tests were statistically analyzed using two-way repeated ANOVA, while data of biochemical parameters were analyzed using one-way ANOVA. In both cases, Tukey's multiple range test was applied for *post hoc* analysis[26]. A value of $P < 0.05$ was considered to be statistically significant.

Results

Effect of EOE and quercetin on blood glucose levels in normal and diabetic animals (*single and multiple dose study*) — After the 2nd week of STZ injection, diabetic rats showed significant increase in blood glucose levels than control rats. Blood glucose level was unaffected in quercetin treated group. In normal rats the extract EOE showed decrease (30.88%) in blood glucose level until 2 hr when treated with single dose. Multiple dose study exhibited a significant reduction (20.8-26.22%) in blood glucose level between 9th and 11th day. In diabetic rats, single dose of the extract exhibited decrease (14.68%) in blood glucose level until 4 hrs and then tended to increase and multiple doses showed significant reduction (22.89-39.62 %) in blood glucose level between 7th and 11th day.

Effect of EOE on oral glucose tolerance test — In glucose fed rats (2 g/kg), administration of EOE extract exhibited a significant increase in glucose tolerance after 3 hrs of dosing because it produced a significant reduction (35.47%) in blood glucose level in comparison to normal control.

Effect of EOE and quercetin on sciatic nerve lipid peroxidation and glutathione — Sciatic nerve malondialdehyde (MDA) levels was significantly (P<0.05) high in diabetic rats as compared to age matched non-diabetic rats. There was significant reduction in the level of reduced glutathione. Eight weeks EOE and quercetin treatment significantly (P<0.05) reduced the sciatic nerve MDA content and raised the levels of reduced glutathione (Table 1). However, total protein levels were not significantly altered.

Effect of EOE and quercetin on sciatic nerve SOD and catalase activity — Sciatic nerve SOD activity was significantly (P<0.05) low in 10 weeks diabetic rats as compared to age matched non-diabetic rats. Eight weeks treatment with EOE and quercetin significantly (P<0.05) restored SOD activity to normal. There was a significant per se effect of EOE and quercetin in control rats after 8 weeks of treatment (Table 1). Sciatic nerve catalase activity was significantly (P<0.05) high in 10 weeks diabetic rats as compared to age matched non-diabetic rats. Eight weeks treatment with EOE and quercetin significantly (P<0.05)

reverted catalase activity to control values. A significant per se effect of EOE and quercetin was observed in normal control rats after 8 weeks of treatment (Table 1).

Measurement of antinociceptive activity—The nociceptive threshold was significantly lower in diabetic rats as compared with the basal value tested in both the tail-immersion (Table 2A) and hot-plate test (Table 2B). Hyperalgesia was evident in the tail-immersion and hot-plate after 1 week (P<0.05) and 2 weeks (P<0.01), respectively, and the maximum decrease in pain threshold was observed at 4 weeks after streptozotocin injection in rats as compared to non-diabetic control rats ($P < 0.001$) and the level of significance was maintained until 10 weeks. EOE extract and quercetin administration to diabetic rats produced a dose and time dependent increase in pain threshold level as compared to untreated diabetic rats. The maximum increase in pain threshold level was observed in a progressive manner in both tail immersion and hot plate test (Table 2). EOE produced a significant antinociceptive activity from the 4th week

Table 1—Effect of *E. officinalis* extract in diabetic-induced alterations in MDA, reduced GSH catalase and SOD activity

[Values are mean ± SE from 8 animals in each group]

Parameters	Normal Control	Normal +quercetin	Normal +EOE	Diabetic control	Diabetic +quercetin	Diabetic +EOE
MDA level (µM/mg protein)	1.65 ±0.05	1.34 ±0.07	1.42 ±0.04	4.76 ±0.71[a]	2.67 ±0.03[c]	2.59 ±0.04[c]
Catalase activity (U/mg protein)	0.26 ±0.02	0.49 ±0.05[a]	0.56 ±0.05[a]	0.41 ±0.03[a]	0.26 ±0.02[b]	0.29 ±0.01[c]
SOD activity (U/mg protein)	32.26 ±2.0	42.56 ±1.91[a]	46.23 ±2.10[a]	12.1 ±0.25[a]	18.77 ±0.51[c]	24.23 ±0.35[b]
Protein (mg/ml)	4.35 ±0.11	4.39 ±0.03	4.51 ±0.02	4.6 ±0.02	4.61 ±0.22	4.35 ±0.02
GSH (g/mg of protein)	73.34 ±2.54	65.5 ±2.65[d]	64.25 ±5.68[d]	49.25 ±3.4	58.0 ±4.32[e]	67.25 ±3.86[c]

P values < 0.001 [a]vs normal control; [b]vs diabetic control
[c]< 0.01 vs diabetic control
< 0.05 [d]vs normal control; [e]vs diabetic control

Table 2—Effect of quercetin (Q -10 mg/kg, po) and *E. officinalis* extract (EOE; 10 mg/kg, po) on (A) tail immersion and (B) hot-plate pain threshold in control and diabetic rats.

[Values are expressed as means ± SE from 8 animals in each group]

Treatment groups		Reaction time (sec)							
		Treatment (weeks)							
		3	4	5	6		8	9	10
Control	A	12.89±0.77	12.87±0.66	13.0±0.61	14.02±0.47	14.06±0.55	14.01±0.55	13.76±0.26	14.02±0.48
	B	14.01±0.48	12.68±0.75	13.5±0.5	13.0±0.63	14.5±0.43	13.5±0.38	14.75±0.38	13.75±0.5
Control +Q	A	12.38±0.85	12.75±0.96	12.69±0.91	13.88±0.35	13.63±0.54	13.51±0.25	14.0±0.47	13.63±0.54
	B	13.79±0.31	13.5±0.5	14.0±0.9	13.0±0.8	14.75±0.52	13.0±0.25	13.75±0.25	14.5±0.43
Control+EOE	A	13.0±0.89	13.38±0.88	13.5±1.02	13.29±1.15	13.6±0.61	13.35±0.15	13.23±0.19	13.35±0.15
	B	14.16±0.42	13.4±0.38	15.0±0.76	15.0±0.63	14.26±0.63	13.25±0.63	14.5±0.63	14.0±0.66
Diabetic control	A	11.26±1.65[c]	10.08±2.06[a]	9.5±2.04 [a]	7.13±2.2 [a]	7.36±1.83 [a]	7.05±2.16 [a]	6.98±2.24 [a]	6.8±2.51 [a]
	B	13.08±1.23[e]	11.56±1.26[e]	10.2±1.51[a]	9.5±1.26 [a]	8.67±1.76 [a]	7.76±2.18 [a]	7.24±2.18 [a]	6.4±3.04 [a]
Diabetic + Q	A	11.81±0.91	9.13±0.66	9.06±0.66	10.02±0.60 [b]	9.85±0.2 [b]	10.25±0.81 [b]	12.08±0.99 [b]	11.83±0.67 [b]
	B	12.71±0.86	11.68±0.63[f]	9.5±0.38	10.26±0.75	12.68±0.25 [b]	12.98±0.8 [b]	13.5±0.8 [b]	14.26±0.38 [b]
Diabetic + EOE	A	12.06±0.63	12.43±0.31[d]	12.13±0.52 [b]	10.81±0.43 [b]	10.36±0.87 [b]	10.24±0.38 [b]	12.44±0.85 [b]	12.2±0.66 [b]
	B	12.94±0.78	12.98±0.52[f]	11.5±0.66	12.62±0.5 [b]	13.34±0.38 [b]	13.56±0.29 [d]	13.95±0.29 [b]	14.35±0.52 [b]

P values:
< 0.001 [a]vs normal control; [b]vs diabetic control
< 0.01 [c]vs normal control; [d] vs diabetic control
< 0.05 [e]vs normal control; [f]vs diabetic control

onwards in diabetic rats but quercetin produced the significant effect only at 7th week. EOE produced a significant antinociceptive activity when compared to quercetin from 5th to 6th week of the treatment period.

Effect of E.officinalis on axonal degeneration—Histopathological changes were assessed by using a longitudinal section of sciatic nerve. In longitudinal sections, the fiber derangement and decrease in number of Schwann cells were noted in diabetic control. However, *Emblica officinalis* administration attenuated diabetic induced axonal degeneration and histopathological alterations.

Discussion

Metabolic syndrome is characterized by a cluster of pathological changes including obesity, hypertriglyceridemia, impaired glucose tolerance and insulin resistance. A modified diet (fructose diet) was adopted to induce insulin resistance because the role of fructose in the development of diabetic complications was well documented[20,27] and injection of a single dose of STZ induced a diabetic state similar to prediabetic, insulin resistant state in humans[28]. Peripheral nerve pathology in diabetic patients is characterized by progressive nerve fiber loss[29]. Distal fiber loss is typically found in the skin of calf in the subjects with impaired glucose tolerance[30] (IGT).

The early presence of neuropathy is supported by a survey of IGT patients who had reduced nerve conduction velocity[31] and glycemic control could prevent the development and progression of diabetic neuropathy[32]. Diabetic oxidative stress induced free radicals are involved in vascular endothelial damage of epineural arterioles of the sciatic nerve in diabetic rats[33]. Impaired blood flow contributes to noxious stimulus hypersensitivity and vasodilators have been demonstrated to reduce allodynia in diabetic rats[34]. Dietary antioxidants by scavenging reactive oxygen species has improved vascular resistance in diabetic rats[35-37].

Diabetic rats exhibited a significant increase in MDA levels, an index of lipid peroxidation and reduction in antioxidant enzyme activity. These parameters regained to normal levels when treated with EOE and quercetin. Quercetin has antioxidant- scavenging activity[38], delays lipid peroxidation of cell membranes[39], and reduces Cu^{2+}-induced LDL oxidation[40]. EOE significantly scavenges superoxide and as well as inhibits its generation[41] and aqueous EOE has been found to be potent antioxidants *in vitro*[42].

Quercetin and EOE has proven to protect against the development of diabetic neuropathy by inhibition of lipid peroxidation and restoration of antioxidant enzymes in diabetic rats. Thus reverses the oxidative stress induced changes in nerve physiology of diabetic rats as reported earlier[43,44].

Flavonoids are reported to have anti-inflammatory activity but do not produce any apparent acute toxicity or gastric damage. Therefore, it may be proposed that *E.officinalis* fruit extract -induced reduction in oxidative stress secondary to decrease in blood glucose level may be responsible for ameliorating axonal degeneration and neuropathy in sciatic nerve EOE has produced a pronounced antinociceptive activity from 3rd week onwards but not quercetin and thus it can prevent as well as cure diabetic neuropathy. The curative and preventive property of EOE in diabetic neuropathy may be due to its improvement in glucose intolerance and antioxidant property. Since, *Emblica officinalis* fruit is already in clinical use for diabetic patients it may be evaluated for preventive therapy in diabetic patients at risk of developing neuropathy.

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