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Research Article

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Neuroprotective effect of ethanolic root extract of Boerhaavia diffusa (Linn.) against Streptozotocin induced Diabetic neuropathy in animal model

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ABSTRACT

The present study was designed to screen the neuroprotective effect of Boerhaavia diffusa (Linn.) on Streptozotocine induced diabetic neuropathy in rats. Diabetes was induced in rats with a single intraperitoneal injection of streptozotocin (55 mg/kg b.w). The ethanol extract of B. diffusa at a dose of 100, 200 and 400 mg/kg of body weight were administered at single dose per day to diabetes induced rats for a period of 12 weeks. Neuropathic pain was assessed in diabetic rats with various painful procedures viz., hot and cold water tail immersion test, pinprick test, cold allodynia, hot plate test, photoactometer and rota-rod tests were performed to assess the degree of thermal, mechanical, cold hyperalgesia and locomotor activity as well as motor co-ordination. At the end of the study period experimental animals were scarified and biochemical parameters such as lipid peroxidation, superoxide dismutase, total protein, total calcium levels were evaluated in sciatic nerve tissue. Animals treated with Boerhaavia diffusa (100, 200, and 400 mg/kg p.o.) significantly alleviates hyperglycaemia induced mechanical, thermal hyperalgesia and cold allodynia and restored the reduced body weight and improved the biochemical parameters such as blood sugar levels, superoxide dismutase, and total protein and attenuated the calcium concentration, and lipid peroxidation in a dose dependent manner. Thus, from this study we conclude that Boerhaavia diffusa exhibits significant antidiabetic, antioxidant and neuroprotective activities against streptozotocin-induced diabetic neuropathy in rats.

Key words: Diabetic, hyperglycemic, Boerhaavia diffusa, streptozotocin.

INTRODUCTION

Diabetes mellitus is a metabolic disorder characterized by hyperglycemia resulting from defects in insulin secretion, insulin action or both [1]. Diabetes mellitus is a serious health problem in developing as well as developed countries. The world health organization stated it as an epidemic disease of the 21st century, due to its rising population of 382 million in 2013, and may projected to rise up to 592 million by the year 2035 [2]. Uncontrolled diabetes leads to microvascular and macrovascular complications [3]. The most common type of microvascular complication of diabetes is peripheral neuropathy [4,5].

The prevalence rate is 7% in one year of freshly diagnosed diabetes and 50% in long standing diabetes history patients aged more than 25 years [6]. In them 12% patients experience painful diabetic neuropathy [7] with generating the symptoms of allodynia-stimulus that normally doesn't provoke pain, hyperalgesia increased the response to a non painful stimulus [8]. Patients explain their symptoms as a sharp electric shock shooting their legs, and feeling of walking on broken glass [9].

Hyperglycemia induces fatal changes in nerve tissue, a verity classical metabolic pathways; polyol pathway resulting in accumulation of sorbitol and glucose, increased hexosamine shunt; excess/inappropriate activation of protein kinase C isoforms; resulting in accumulation of advanced glycation end products; weaken neurotrophic support and disrupt the repair mechanism; and stimulation of poly (ADP-ribose) polymerase (PARP); result in imbalanced nerve myo-inositol. Although all of these pathways fatal in the generation of reactive oxygen species through oxidation and reduction reactions in mitochondria lead to aggravation of neuropathy [10,11,12].

Several drugs such as tricyclic antidepressants and antionvulsant drugs are presently available to reduce the neuropathic pain. However, these drugs were reported to exhibit a wide spectrum of adverse effects in the management of painful neuropathy. Hence, there are a limited number of ideal medicines to treat diabetes neuropathy and its generating pain. Researchers, health care professionals and educated people acknowledge their interest towards new alternative medicines to treat diabetes associated neuropathy.

The present chosen trailing herb bearing a Latin name Boerhaavia diffusa Linn. (Family: Nyctaginaceae) is a creeping herb grown in tropical regions of South America, India and Africa [13]. Indian Ayurveda renowned it as 'Punarnava', rejuvenates itself from dried root in the rainy season as well as rejuvenates body [14]. Pharmacological studies on B. diffusa roots were proven its anticonvulsant [16], antioxidant, antidiabetic, antistress, hepatoprotective, antifibrinolytic and anti-inflammatory activities in experimental animals [15], due to presence of various phytochemicals like Punarnavine (alkaloid), boeravinone (rotenoid), flavonoids, amino acids, liriodendrons (lignans), β-sistosterol and stetracosanoic acid, ecosanoic, steroic and urosolic acid have proven capacity to cure and control disease prognosis [17]. Since B. diffusa were used principally in Ayurveda to give attractive results in curing and rejuvenating healthy life against diseases, and lack of scientific data on neuroprotective activity of Boerrhavia diffusa against diabetic neuropathy was gaining my attention towards this experiment.

EXPERIMENTAL SECTION

Plant materials: The roots of Boerhaavia diffusa was purchased from a supplier of medicinal herbs in Chennai, and it was authenticated by professor Dr. P. Jayaraman, Institute of Herbal Botany, Plant Anatomy Research Centre (PARC), Chennai, India. The voucher specimen (PARE/2013/2159) has been deposited in the Research laboratory, Department of Pharmacology for future reference.

Extract preparation: The roots of Boerhaavia diffusa were collected and dried under shade and grinded into powder. Ethanolic extract of Boerhaavia diffusa roots was done in the department of Pharmacology, Geetanjali Medical College, Udaipur using cold maceration.

Acute toxicity study: Acute toxicity study of ethanolic extract of the root of Boerhaavia diffusa was determined in wistar albino rats (150-180 gm) according to the OECD guidelines No.420 [18]. Based on performed toxicity tests the LD_{50} dose was selected in three doses of 100, 200, 400 mg/kg p.o.

Drugs and Chemicals: Streptozotocin was obtained from Sisco research laboratories Pvt. Ltd, Mumbai, India and Pregabalin was perchased from Swapnaroop drugs & pharmaceuticals, Aurangabad, Maharastra, India. All other chemicals and reagents used were of analytical grade.

Animals used:
Adult healthy Albino rats of Wistar strain of either sex weighing between 180-250 gm was gathered from the Central animal house, Geetanjali Medical College, Udaipur. The rats were housed in polypropylene cages under standard laboratory conditions 23±2^{o}C with 12hr light dark cycle and had free accesses to water with standard chow diet. Animal care should be taken as per guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA). Approval was taken from the Institutional Animal Ethics Committee for the study.

Experimental design:
In the present investigation, a total of 36 rats were taken and divided into six groups of 6 rats in each. Out of 6 groups, five were made diabetic with a single dose of prepared solution of Streptozotocin 55 mg/kg body weight in cold citrate buffer (P^{H} 4.5, 0.01 M) was administered intraperitoneally [19]. After 72 hrs blood glucose level of

surviving rats was measured and rats with fasting blood glucose levels above 250 mg/dl were used for further study [20].

The study of test compound and standard drugs were dissolved in distilled water and administered orally with the help of gastric oral tube. Rats were divided into the following groups;

Group-1: Normal control rats (Distilled water 5 ml/kg, p.o)
Group-2: Diabetic control rats (STZ 55 mg/kg, i.p)
Group-3: Diabetic rats served with Pregabalin (10 mg/kg, p.o)
Group-4: Diabetic rats served with B. diffusa ethanolic extract (100 mg/kg, p.o)
Group-5: Diabetic rats served with B. diffusa ethanolic extract (200 mg/kg, p.o)
Group-6: Diabetic rats served with B. diffusa ethanolic extract (400 mg/kg, p.o)

The study period was carried out for 12 weeks, behavioural parameters and fasting blood glucose levels as well as changes in the body weights of the animals were determined on 0, 4^{th}, 8^{th} and 12^{th} week respectively. At the end of the study period (i.e. 12^{th} week), rats were sacrificed by administering 50 mg/kg thiopental sodium intra peritoneally [21] and isolated sciatic nerves were analysed for lipid peroxidation, superoxide dismutase, total protein and total calcium levels.

EVALUATION OF BEHAVIOURAL ACTIVITY:

Assessment of hyperalgesia and Allodynia:

Cold water tail immersion test:

In cold water tail immersion test, distal 5 cm of tail was immersed in a cold water container by maintaining a constant temperature (10^{o} C). Duration of time taken for withdrawal of tail from cold water was noted. A cut-off time of 20 sec was maintained to prevent tissue injury. The procedure was repeated three times for each animal and the mean values are taken in consideration. The decrease in tail contact time with cold water was pointing towards nociception, whereas prolonged contact time was noted as anti-allodynic effect [22].

Hot water tail immersion test:

In hot water tail immersion test, heat hyperalgesia was measured by immersion of terminal part of the tail (1 cm) in warm water (52.5 ± 0.5°C). The duration of tail withdrawal reflex was recorded, as a response of heat thermal sensation and a cut-off time of 15 seconds was maintained. Shortening of tail withdrawal time is an indication for thermal hyperalgesia [23].

Paw heat- hyperalgesia test (Eddy's hot plate method):

The nociceptive threshold for heat was an index for thermal hyperalgesia. Eddy's hot plate, which is an instrument designed by Eddy and co-workers to assess thermal sensitivity. The plate was preheated and maintained at a temperature of 52.5±2.0°C. The rat was placed on the hot plate and nociceptive threshold, with respect to licking of the hind paw or jumping, was recorded in seconds. The cut-off time of 20 sec was maintained [24].

Mechanical hyperalgesia (Pin prick test):

According to Erichsen and Blackburn-Munro, the surface of the injured hind paw was touched with a point of the bent gauge needle at 90°, without piercing deep into tissue. The intensity was sufficient to produce a reflex withdrawal response in normal control animals. The duration of the paw withdrawal was recorded in seconds using 20 sec as cut-off time [25, 26]

Cold hyperalgesia (Acetone drop test):

Cold chemical thermal sensitivity was assessed using acetone drop method as described by Choi Y [27] with modification. Rats were placed in a metal mesh cage and allowed to habituate for approximately 20 minutes in order to acclimatise them for the new environment. Freshly dispensed acetone drop (50µL) was applied gently on to the mid plantar surface of the hind paw. It generates a cold chemical sensitive reaction i.e., paw licking, shaking or rubbing the hind paw with brisk foot withdrawal after application (2-5 sec) of acetone was considered as nociceptive response and absence or delay in these responses were considered as anti-nociceptive effect. Each test was repeated three times with 5 min interval between each application of acetone on both paws and mean was calculated.

Motor coordination:
The test was conducted with Rota rod apparatus (Scientec, India), by placing rats on 25 rpm rotating spindle. The falling time of each rat from rotating spindle was recorded during five minutes period [28].

Spontaneous Locomotor (Exploratory) Test:
Photoactometer test was engaged to assess the spontaneous motor (exploratory) behaviour of the rodents with actophotometer (Scientec, India). Each animal was observed for a period of 5 min in a square closed field area (30 × 30 × 30 cm) equipped with 6 photocells in the outer wall. Interruptions of photocell beam (locomotor/exploratory action) of rats were recorded by digital counter [29, 30].

ASSESSMENT OF BIOCHEMICAL PARAMETERS:
Determination of blood glucose levels: Determination of the blood glucose levels was done by the glucose-oxidase principle [31] at interval of 0, 4, 8, and 12 week by puncturing lateral vein of the rat's tail. Blood glucose was estimated by using glucometer (Accu-Chek sensor from Roche Diagnostic Corporation) and results were expressed as mg/dl [32].

At the end of the study duration rats were euthenized with 50 mg/kg thiopental sodium i.p injection [21]. The sciatic nerve was isolated and the nerve homogenate (10% w/v) was prepared by using 0.1 M Tris-Hcl buffer (P^H 7.4). The tubes with homogenate were kept in ice water for 30 minutes and centrifuged at 4^o C (2500 rpm, 10 min). The obtained supernatant employed to estimate lipid peroxidation (TBARS), SOD, total protein and total calcium levels.
The extent of lipid peroxidation was estimated by using the thiobarbituric acid method [33], Superoxide dismutase (SOD) activity by Paoletti F and Sapakal VD [34, 35], total protein by Bradford [36], total calcium by OCPC [37, 38] method.

Statistical analysis:
The results were expressed as Mean±SD. The intergroup variation was measured by using One way analysis of variance (ANOVA) followed by Bonferroni t-test. The statistical analysis was done by using the SigmaStat 3.5. Values of $P < 0.05$ were considered statistically significant.

RESULTS

Effect of B. diffusa on behavioural parameters:
Hot and cold water tail immersion test:
Streptozotocin (STZ) induced hyperglycaemia results in progressive heat hyperalgesia and cold allodynia, reflected as shortening of tail withdrawal latency in comparison with normal rats (group 1). B. diffusa treated rats were started to show early significant improvement in tail withdrawal latency at dose 400 mg/kg ($P<0.001$) from 4^{th} week and remaining doses 100, 200 mg/kg were showed the effect on 8^{th} week for hot water tail immersion test (Table-1). In cold water tail immersion all three doses of B. diffusa 100, 200 and 400 mg/kg were significantly improved tail withdrawal latency in a dose dependent manner. Whereas, pregabalin 10 mg/kg significantly ($P < 0.05$) improved the tail withdrawal latency when compared with group 2 rats (Table-2).

Paw heat- hyperalgesia test:
Diabetic rats showed significant reduction in paw withdrawal latency when compared with normal rats (group 1). Pregabalin 10 mg/kg significantly ($P<0.001$) improved the paw withdrawal latency from 4^{th} week. Whereas, B. diffusa 100, 200 and 400 mg/kg were significantly ($P<0.05$) improved the paw withdrawal latency from the 8^{th} week in comparison with disease control rats (Table-3).

Pinprick test:
In this test hyper responsiveness to a noxious stimulus was observed with significant rise in paw withdrawal latency in diabetic control as compared with normal control. Whereas, treatment with ethanolic extract of B. diffusa (100, 200 and 400mg/kg, p.o) caused significant reduction in paw withdrawal latency in a dose dependent manner. The anti-nococeptive effect of ethanol extract at was found similar effective to the reference standard, pregabalin (Table-4).

Table-1: Effect of ethanolic extract of Boerhaavia diffusa on rats subjected to hot water tail immersion test

S.No	Groups	Reaction time (Sec)			
		Week-0	4th week	8th week	12th week
1	Control	9.67±1.211	9.83±0.753	10.33±0.516	10.17±0.753
2	STZ control	5.67±1.366	4.83±1.169	4.67±1.211	5.17±0.753
3	Pregabalin (10 mg/kg)	5.33±1.211	8.50±1.049***	11.50±1.049***	13.67±1.211***
4	B. diffusa (100 mg/kg)	6.00±0.894	6.67±0.516	7.50±0.548**	8.10±0.753***
5	B. diffusa (200 mg/kg)	5.17±1.169	6.33±0.816	7.67±0.816***	8.83±0.753***
6	B. diffusa (400 mg/kg)	5.50±1.049	7.83±0.983***	9.83±0.753***	12.00±0.894***

All values are presented as Mean±SD, (n = 6), p<0.05, p<0.01**, p<0.001*** when compared to disease group.*

Table-2: Effect of ethanolic extract of Boerhaavia diffusa on rats subjected to cold water tail immersion test

S.No	Groups	Reaction time (Sec)			
		Week-0	4th week	8th week	12th week
1	Control	10.83±0.753	11.33±0.516	11.17±0.753	11.50±0.548
2	STZ control	6.50±0.548	5.33±0.516	4.83±0.753	4.33±0.516
3	Pregabalin (10 mg/kg)	6.17±0.983	9.50±1.049***	12.67±0.816***	14.33±0.816***
4	B. diffusa (100 mg/kg)	6.17±0.753	7.00±0.894*	7.83±1.169***	8.00±0.632***
5	B. diffusa (200 mg/kg)	6.33±0.816	7.33±0.516**	8.17±0.753***	9.33±0.516***
6	B. diffusa (400 mg/kg)	6.50±0.837	9.00±0.632***	11.00±0.894***	12.50±0.548***

All values are presented as Mean±SD, (n = 6), p<0.05, p<0.01**, p<0.001*** when compared to disease group.*

Table-3: Effect of ethanolic extract of Boerhaavia diffusa on rats subjected to paw heat- hyperalgesia test

S.No	Groups	Reaction time (Sec)			
		Week-0	4th week	8th week	12th week
1	Control	13.33±2.066	13.17±1.472	13.17±0.753	13.50±0.548
2	STZ control	6.50±1.049	5.67±0.816	4.83±0.753	5.00±0.632
3	Pregabalin (10 mg/kg)	5.83±0.983	8.50±1.049***	11.17±0.983***	13.33±1.033***
4	B. diffusa (100 mg/kg)	5.67±0.816	6.67±0.516	7.83±0.753***	8.17±0.753***
5	B. diffusa (200 mg/kg)	6.00±0.894	7.33±0.816	8.83±0.753***	10.17±0.753***
6	B. diffusa (400 mg/kg)	5.83±0.983	7.50±1.049	10.00±1.265***	11.67±1.366***

All values are presented as Mean±SD, (n = 6), p<0.05, p<0.01**, p<0.001*** when compared to disease group.*

Table-4: Effect of ethanolic extract of Boerhaavia diffusa on rats subjected to pinprick test

S.No	Groups	Reaction time (Sec)			
		Week-0	4th week	8th week	12th week
1	Control	1.83±0.983	2.00±0.894	2.17±0.753	2.00±0.894
2	STZ control	11.83±1.602	13.00±1.414	13.83±0.983	14.67±1.211
3	Pregabalin (10 mg/kg)	11.83±1.169	9.17±1.472***	6.83±1.472***	5.33±1.033***
4	B. diffusa (100 mg/kg)	11.50±1.049	10.33±1.033**	9.67±1.211***	8.83±0.753***
5	B. diffusa (200 mg/kg)	11.33±0.816	10.00±0.894**	8.50±1.049***	8.00±0.632***
6	B. diffusa (400 mg/kg)	11.67±1.211	9.83±0.983***	8.33±1.033***	6.83±0.753***

All values are presented as Mean±SD, (n = 6), p<0.05, p<0.01**, p<0.001*** when compared to disease group.*

Table-5: Effect of ethanolic extract of Boerhaavia diffusa on rats subjected to acetone drop test

S.No	Groups	Reaction time (Sec)			
		Week-0	4th week	8th week	12th week
1	Control	6.11±0.404	6.17±0.279	6.22±0.584	6.06±0.491
2	STZ control	28.06±2.471	31.78±3.507	34.17±3.189	32.44±3.089
3	Pregabalin (10 mg/kg)	27.83±5.853	20.33±3.783***	13.33±2.171***	11.17±2.030***
4	B. diffusa (100 mg/kg)	29.33±4.104	27.39±3.832	25.28±4.208***	23.44±3.331***
5	B. diffusa (200 mg/kg)	28.67±5.064	25.22±3.291	21.17±3.075***	18.17±2.614***
6	B. diffusa (400 mg/kg)	29.44±4.480	23.83±3.097**	18.94±2.551***	15.94±2.323***

All values are presented as Mean±SD, (n = 6), p<0.05, p<0.01**, p<0.001*** when compared to disease group.*

Acetone drop test:

In this test, applying acetone on plantar surface of hyperglycemic rats results cold allodynia, it is implicated as rised paw withdrawal duration than the normal control. Animals treated with B. diffusa at dose 400 mg/kg was given significant (P<0.05) results from 4th week, and remaining 100, 200 mg/kg were started to shown reduction in paw withdrawal latency from 8th week as compared to disease control (group 2). Whereas, pregabalin 10 mg/kg was significantly (P<0.001) decreased the paw withdrawal latency from the 4th week (Table-5).

Table-6: Effect of ethanolic extract of Boerhaavia diffusa on rats subjected to motor co-ordination test (Rota-rod)

S.No	Groups	Falling time (Sec)			
		Week-0	4th week	8th week	12th week
1	Control	134.00±7.772	137.00±7.668	136.00±8.462	136.67±6.713
2	STZ control	56.83±5.981	60.67±6.314	63.67±5.354	66.33±5.164
3	Pregabalin (10 mg/kg)	58.50±6.595	83.33±6.186***	101.83±8.377***	110.17±6.646***
4	B. diffusa (100 mg/kg)	60.50±4.037	65.50±3.674	72.00±3.742	75.83±4.167
5	B. diffusa (200 mg/kg)	58.67±6.250	67.83±5.492	76.83±5.529**	83.17±5.742***
6	B. diffusa (400 mg/kg)	57.00±6.229	74.50±4.930**	88.00±6.693***	94.50±6.775***

All values are presented as Mean±SD, (n = 6), p<0.05, p<0.01**, p<0.001*** when compared to disease group.*

Table-7: Effect of ethanolic extract of Boerhaavia diffusa on rats subjected to locomotor activity test (Actophotometer)

S.No	Groups	Reaction time (Sec)			
		Week-0	4th week	8th week	12th week
1	Control	121.50±10.877	123.50±11.692	121.83±8.565	120.33±9.501
2	STZ control	36.33±5.574	37.17±5.672	39.67±5.610	41.33±6.186
3	Pregabalin (10 mg/kg)	37.17±5.845	52.67±7.005***	64.83±8.377***	72.17±9.517***
4	B. diffusa (100 mg/kg)	38.17±5.776	42.17±4.535	45.67±5.046	48.50±5.357
5	B. diffusa (200 mg/kg)	38.17±6.494	45.33±7.967	49.83±8.796	53.17±9.786
6	B. diffusa (400 mg/kg)	35.67±7.202	46.33±5.428	56.17±6.210*	65.00±7.321***

All values are presented as Mean±SD, (n = 6), p<0.05, p<0.01**, p<0.001*** when compared to disease group.*

Table-8: Effect of ethanolic extract of Boerhavia diffusa on lipid peroxidation (TBARS), Superoxide dismutase (SOD) , total protein and total calcium levels in normal and diabetic rats nerve tissue

S.No	Groups	TBARS (nM/gm wet tissue)	SOD (U/gm wet tissue)	Total Protein (µg/mg tissue)	Total calcium (mg/dl)
1	Control	16.22±4.991	48.66±6.930	77.68±6.108	7.98±1.361
2	STZ control	61.50±11.738	10.09±5.579	30.65±4.247	31.42±3.031
3	Pregabalin (10 mg/kg)	36.22±4.763	32.14±4.913	62.48±7.360	16.99±3.340
4	B. diffusa (100 mg/kg)	55.11±5.314	16.22±4.771	35.77±4.273	26.16±3.100*
5	B. diffusa (200 mg/kg)	50.39±7.865	19.88±5.291*	38.28±5.496	24.09±2.432***
6	B. diffusa (400 mg/kg)	43.17±7.226***	25.02±3.608***	47.28±4.424**	20.88±2.749***

All values are presented as Mean±SD, (n = 6), p<0.05, p<0.01**, p<0.001*** when compared to disease group.*

Table-9: Effect of ethanolic extract of Boerhavia diffusa on fasting blood glucose levels

S.No	Groups	Day-0	Day-3	4th week	8th week	12th week
1	Control	84.33±6.91	86.5±7.71	84.5±7.91	85.16±6.67	87.833±5.45
2	STZ control	84.16±7.11	303.16±10.14	331.6±18.61	368.33±69.47	424.16±49.43
3	Pregabalin (10 mg/kg)	88.66±4.45	304±28.89	325.16±40.86	360.833±37.87	394±49.05
4	B. diffusa (100 mg/kg)	82±8.04	313.16±44.21	288.50±31.61*	265.5±17.47**	250.83±16.85***
5	B. diffusa (200 mg/kg)	85.66±8.52	302.33±49.15	278.33±43.55*	239.5±36.09**	212±14.66***
6	B. diffusa (400 mg/kg)	77.66±7.86	310.5±50.17	245.66±28.43***	192.66±21.91***	107±16.81***

All values are presented as Mean±SD, (n = 6), p<0.05, p<0.01**, p<0.001*** when compared to disease group.*

Table-10: Effect of ethanolic extract of Boerhavia diffusa on Body weights

S.No	Groups	Week-0	4th week	8th week	12th week
1	Control	207.33±10.94	218±4.19	226.5±4.50	230.66±7.76
2	STZ control	215±7.45	197.66±7.033	182±10.25	160±11.40
3	Pregabalin (10 mg/kg)	216.16±5.87	202.5±10.83	187.5±7.86	175±4.47*
4	B. diffusa (100 mg/kg)	210.83±8.28	196.16±12.25	190.16±7.46	186.83±9.28***
5	B. diffusa (200 mg/kg)	208.83±11.19	191.66±13.77	183.33±11.25	189±11.83**
6	B. diffusa (400 mg/kg)	210.66±6.91	195.83±7.02	187.66±10.01	192.16±9.13***

All values are presented as Mean±SD, (n = 6), p<0.05, p<0.01**, p<0.001*** when compared to disease group.*

Motor coordination and locomotor activity:

Rats treated with various doses of B. diffusa were shown significant improvement in muscle grip strength and locomotor activity as compared with disease control. Improvement in motor coordination activity was achieved with the dose of 200 and 400 mg/kg from 12th and 4th weeks respectively (Table-6). Whereas, B. duffusa 400 mg/kg and pregabalin 10 mg/kg were significantly improved the locomotor activity from 8th and 4th weeks respectively (Table-7).

Effect of B. diffusa on Biochemical Parameters:
During twelve weeks of the study, STZ induced diabetic rats showed elevated levels of lipid peroxidation, total calcium and decrease in SOD levels and total protein value significantly ($P<0.05$) in comparison with normal control (Table-8).

Lipid peroxidation (TBARS):
The rats treated with B. diffusa with 400 mg/kg and pregabalin 10 mg/kg p.o were significantly reduced the elevated lipid peroxidation levels.

Superoxide dismutase (SOD):
Pregabalin 10 mg/kg and B. diffusa 200, 400 mg/kg were showed significant improvement in SOD levels during 12 weeks of treatment as compared with group 2.

Total protein levels:
A significant ($P<0.05$) improvement in total protein levels was noted with the 400 mg/kg of ethanolic extract of B. diffusa roots (EEBD) and pregabalin 10 mg/kg only.

Total Calcium:
The raised total calcium levels has been significantly reduced by 100, 200 and 400 mg/kg of B. diffusa and pregabalin 10 mg/kg when compared with disease control rats.

Fasting blood glucose levels:
A marked rise in fasting blood glucose level was observed in STZ treated groups as compare to normal control. Whereas, treatment with EEBD (100, 200 and 400mg/kg, p.o) caused significant reduction in the levels of fasting blood glucose in a dose dependent manner. Whereas, standard reference pregabalin did not decrease the blood glucose levels in the entire study period (Table-9).

Body weight:
Normal rats gained weight significantly throughout the experimental period, while both the control diabetic and EEBD diabetic animals had significantly ($P < 0.001$) lower body weights when compared to normal animals. However, EEBD (100, 200 and 400 mg/kg) and pregabalin (10 mg/kg) treated diabetic rats were maintained their initial weights during the 12 weeks treatment period although at the end of the experiment their body weights were significantly less than those of normal rats. In contrast, the control diabetic rats showed significant weight loss when compared to both the normal rats and the EEBD treated diabetic rats at the end of the 12 weeks experiment (Table-10).

DISCUSSION

In this study, Boerhaavia diffusa extract was given for prevention as well as treatment of neuropathic pain in STZ induced diabetic rats. The development of neuropathy was observed at 0' week after STZ induction, which was consistent with previous reports [21]. Streptozotocin gain popularity to induce type-1 hyperglycaemia in rodents [39] which resembles to human insulin dependent diabetes mellitus [40]. Streptozotocin induced hyperglycaemia probably due to pancreatic DNA alkylation through GLUT2 transporter mechanism [41, 42], which intern triggers multiple biochemical pathways such as polyol pathway, hexosamine pathway, protein kinase C pathway (PKC), advance glycation end (AGE) product and poly adipose ribose polymerase (PARP) pathway all of these pathways contribute towards oxidative stress by generating ROS in a mitochondria results in nerve damage and neuropathy [43].

In this study diabetic rats showed a significant rise in blood glucose levels and decreased body weight than the normal rats. Hyperglycaemia in turn influence proteolysis in skeletal muscle and lipolysis in adipose tissues results in severe weight loss in the animal models.

B. diffusa Linn. (100, 200 and 400 mg/kg b.w) treated hyperglycaemic animals were shown a significant reduction in blood glucose levels throughout the experiment with significant improvement in body weight, it could be due to B. diffusa persisting insulin secretogoge action like sulfonylurias.

STZ induced diabetic animals are the models for chronic neuropathic pain with hyperalgesia and allodynia that reflect symptoms observed in diabetics [44,45]. Hence, the behavioural parameters such as thermal and cold hyperalgesia; and allodynia was assessed by using hotplate, pin prick, acetone drop test, and hot and cold water tail immersion tests along with motor coordination as well as locomotor activity.

In behavioural examination, diabetic rats were shown significant reduction in tail and paw withdrawal latency than the normal control rats, is an indication for decreased nociceptive threshold to heat resulting hyperalgesia and allodynia. Similar models of thermal hyperalgesia and tail flick latency have been reported previously in STZ induced diabetic animals. The delay in tail withdrawal response depicts the involvement of spinal reflex arc and delay in paw withdrawal latencies to noxious thermal stimuli depicts the involvement of supra spinal sensory pathways.

The hyperalgesic response to a noxious stimulus (pin prick) and development of cold chemical sensitivity in diabetic rats were shown significant rise in hind paw withdrawal latency than the normal rats. It has been reported that involvement of TRPA1 and ATP-gated purinergic ion-channel P2X3 may be responsible for mechanical hyperalgesia [46].

However, rats treated with 200 and 400 mg/kg were shown improvement in muscle grip strength at 12^{th} and 4^{th} weeks respectively as well as progress in locomotor activity was observed at 8^{th} week with 400 mg/kg.

B. diffusa alleviates hyperglycaemia induced mechanical, thermal hyperalgesia and cold allodynia. It might be due to straight glycaemic control reverses the hyperglycaemia induced generation of ROS, which intern involved in regulation of gene promoting inflammatory reaction results in neuronal dysfunction and generation of pain [43]. Moreover, it is well established that ROS are gravely involved in pain transmission [47]. The present study shows a significant rise in lipid peroxidation, and decreased nerve protein, superoxide dismutase levels are the indication of the involvement of oxidative stress in diabetes induced neuropathy.

Generation of peroxynitrite by a reaction between superoxide anions and nitric oxide results protein nitrosylation, lipid peroxidation, DNA damage and cell death shows direct toxic effects on nerve tissue [48]. Superoxide dismutase (SOD) protect biological tissues from highly reactive superoxide anions by converting them to hydrogen peroxide, this hydrogen peroxide intern converted to water with the help of reduced glutathione (GSH), hyperglycemia is known to involve in non-enzymatic glycosylation which results in reduced activity of SOD in sciatic nerve of animals [49]. Thus, the concurrent decrease in endogenous antioxidant defence system makes sciatic nerves more vulnerable to hyperglycemia-induced oxidative stress. Chronic treatment with B. diffusa significantly increases tissue SOD levels and reduces lipid peroxidation in diabetic animals.

The present study shows a significant rise in intracellular calcium concentration in neuropathic rats when comared with normal control rats. Excess calcium will participate in triggering a response of calpain and calmodulin and calcium dependent kinases leads to imbalanced homeostasis in the nervous system result in neuronal hyper excitation [50].

Chronic treatment with B. diffusa significantly blocks the calcium conduction in the nerve tissue and alleviates hyperglycaemia induced neuropathy. This could be due to presence of liriodendrin in the roots of B. diffusa has proven for its anticonvulsant activity [51] and calcium channel blocking property [52].

Our findings support the argument on generation of reactive oxygen species, calcium channel over activation in chronic hyperglycaemia may be major culprits involved in axonal degeneration and generation of neuropathic pain.

On the basis of data in hand and with support from the literature, it may be proposed that B. diffusa L., produced an ameliorative effect in STZ induced diabetic pain full peripheral neropathy which may be attributed to its multiple effects viz. anticonvulsant, antioxidant, antidiabetic, antistress, hepatoprotective, antifibrinolytic, anti inflammatory activity.

Pregabalin is a selective Ca 2.2 (a2- delta subunit) channel antagonist. It has potential actions like predecessor gabapentin, it is a structural analogue (but not functional) of the gamma aminobutyric acid. Pregabalin has analgesic, anti-convulsant and anxiolytic activities [53]. Preclinical trials have demonstrated an anti-hyperalgesic and anti-

allodynic effect of pregabalin in various animal models of neuropathic pain [54-56]. Data of our study also supports these reports.

Therefore, B. diffusa root extract was proposed that in addition to its anidiabetic, antioxidant, and voltage gated calcium channel blocking properties are the prominent features in attenuation of diabetes induced neuropathy and its generating pain. Nevertheless, further studies needed to substantiate these findings.

CONCLUSION

In conclusion, the present study has shown that, the ethanol extract of B. diffusa root have attenuated the STZ induced diabetic neuropathy in rats. These effects may be attributed to its potential antidiabetic, anti-oxidant and calcium channel blocking properties.

REFERENCES

[1] Amos AF, Mc Carty DJ and Zimmet P. *Diabet Med*., **1997**, 14, S1-85.
[2] IDF Diabetes Atlas, International Diabetes Federation, 6th ed, **2013**.
[3] Michael J. Fowler MD. *Clinical Diabetes*, **2008**, 26.
[4] Boulton AJ, Vinik Al, Arezzo JC. *Diabetes Care*, **2005**, 28, 956-62.
[5] Strotmeyer ES, deRekenerie N, Schwartz AV. *J Am Geriatr Soc.*, **2009**, 57, 2004-10.
[6] Pirart J. *Diabetes care*, **1978**, 1, 168-88.
[7] Said G. *Nat Clin Pract Neurol.*, **2007**, 3, 331-40.
[8] Baron R. *Clin J Pain*, **2000**, 16(2), S12-20.
[9] Qualttrini C, Tesfaye S. *Diabetes Metab Res Rev.*, **2003**, 19(1), S2-S8.
[10] Vinik AI, Maser RE, Mitchell BD, Freeman R. *Diabetes Care*, **2003**, 26, 1553-79.
[11] Kong MF, Horowitz M, Jones KL, Wishart JM. *Diabetes Care*, **1999**, 22, 503-7.
[12] Obrosova IG, Drel VR, Pacher P, Ilnytska O, Wang ZQ, Stevens MJ. *Diabetes*, **2005**, 54, 3435-41.
[13] Meena AK, Niranjan US, Yadav AK, Ajit K, Singh B and Rao MM. *International Journal of Pharmacognosy and Phytochemical Research*. **2010**, 2(1), 25-8.
[14] Ayurvedic Pharmacopoeia of India. MHFW, Deptt of Ayush, Govt. of India, Delhi. **2005**; 1(1), 40.
[15] Goyal GM, BansalP, Gupta V, Kumar S, Singh R, Maithani M. *IJPSDR*, **2010**, 2(1): 17-22.
[16] Adesina SK. *Quarterly Journal of Crude Drug Research*, **1979**, 17, 84-6.
[17] Ujowundu CO, Igwe CU, Enemor HA, Nwaogu LA, Okafor OE. *Pak J Nutr*., **2008**, 7(1), 90-2.
[18] Raghavan PV. *OECD guideline line*, **2000**, 420.
[19] Sharma SS, Kumar A, Kaundal R. *Life Sci.*, **2008**, 82, 570-6.
[20] Murti et al. *Pharmacologyonline*, **2011**, 1, 15-21.
[21] Ramdas, Pandhare B, Sangameswaran B, Popat, Mohite B, Shantaram, Khanage G. *RBFBJP*, **2012**, 22(2), 428-35.
[22] Saha L, Hota D, Chakrabarti A. *Pain Res Treat.*, **2012**, 43579.
[23] Necker R, Hellon RF. *Pain*, **1978**, 4, 231-42.
[24] Eddy NB. *J Pharmacol Exp Ther.*, **1950**, 98, 121-37.
[25] Erichsen HK, Blackburn-Munro G. Pain, **2002**, 98, 151-61.
[26] Jain V, Pareek A, Bhardwaj YR, Singh N. *BMC Complementary and Alternative Medicine*, **2013**, 13, 274.
[27] Choi Y, Yoon YW, Na HS, Kim SH, Chung JM. *Pain*. **1994**;59 (3):369-76.
[28] Carter RJ, Morton J, Dunnett SB. *Curr Protoc Neurosci.*, **2001**, 8, 8-12.
[29] Bushnell PJ. *Neurotoxicol Teratol.*, **1988**;10(6):569–77.
[30] Aswar M , Kute P, Mahajan S, Mahajan U, Nerurkar G. *Pharmacology, Biochemistry and Behavior*. **2014**, 124, 101-7.
[31] Beach, E.F. and J.J. Turner. *Clin.Chem.*,**1958**, 4, 462-75.
[32] Asha B, Krishnamurthy KH and Devaru S. *J. Chem. Pharm. Res*., **2011**, 3(1), 452-6.
[33] Ohkawa H, Ohishi N, and Yagi K. *Analytical Biochemistry*, **1979**, 95, 35l-8.
[34] Paoletti F, Aldinucci D, Mocali A, and Caparrini A. *Analytical Biochemistry*, **1986**, 153, 536-41.
[35] Sapakal VD, Shikalgar TS, Ghadge RV, Adnaik RS, Naikwade NS, and Magdum CS. *Journal of Herbal Medicine and Toxicology*, **2008**, 2(2), 1-8.
[36] Bradford MM. *Anal Biochem.*,**1976**, 72, 248–54.
[37] Moorehead, WR and Briggs Hc. *Clinical Chem.*, **1974**, 20, 1458.

[38] Tietz NW. Textbook of clinical Chemistry, W.B Saunders; 1350.
[39] Hayashi K, Kojima R, Ito M. *Biol pharmaceut Bull*, **2006**, 29, 1110-9.
[40] Bach JF. *Endocrine Revs.*, **1994**, 15, 516-42.
[41] Delaney CA, Dunger A, Di Matteo M, Cunningham JM, Green MH, Green IC. *Biochem Pharmacol.*, **1995**, 50, 2015-20.
[42] Elsner M, Guldbakke B, Tiedge M, Munday R, Lenzen S. *Diabetologia*., **2000**, 43, 1528-33.
[43] Vincent AM, Russell JW, Low P and Feldman EL. *Endocrin Rev.*, **2004**, 25, 612-28.
[44] Gul, Yildiz O, Dogrul A, Yesilyurt O and Isimer A. *Pain*, **2000**, 89, 39-45.
[45] Kamei J, Zushida K, Morita K, Sasaki M and Tanaka S. Eur. J. Pharmacol., **2001**, 422, 83-6.
[46] Kwan KY, Allchorne AJ, Vollrath MA, Christensen AP, Zhang, DS, Woolf CJ and Corey DP. *Neuron*, **2006**, 50, 277-89.
[47] Viggiano A, Monda M, Viggiano A, Viggiano D, Viggiano E, Chiefari M, Aurilio C, De Luca B. *Brain Res.*, **2005**, 1050, 72-78.
[48] Kim SY, Lee JH, Yang ES, Kil IS, Park JW. *Biochem Biophys Res Commun.*, **2003**, 301, 671-4.
[49] Halliwell, B. *Drugs*, **1991**, 42, 569-605.
[50] Young W. *J Neurotrauma*, **1992**, 9, 9-25.
[51] Kaur M and Goel RK. *Evidence-Based Complementary and Alternative Medicine*, **2011**, 4, 1-7.
[52] Lami N, Kadota S, Tezuka Y, and Kikuchi T. Chemical and Pharmaceutical Bulletin, **1990**, 38(6), 1558-62.
[53] Stump P. *Drugs Today (Barc).*, **2009**, 45, 19-27.
[54] Kumar N, Laferriere A, Yu JS, Leavitt A, Coderre TJ. *J Neurochem*., **2010**, 113, 552-61.
[55] Bender G, Florian JA Jr, Bramwell S, Field MJ, Tan KK, Marshall S, DeJongh Bies RR, Danhof M. *J Pharmacol Exp Ther*., **2010**, 334, 599-608.
[56] Park HJ, Joo HS, Chang HW, Lee JY, Hong SH, Lee Y, Moon DE. *Can J Anaesth*, **2010**, 57, 664-71.