Neuroprotective effect of *Canthium parviflorum* on streptozotocin induced diabetic neuropathy in rats

Neelakanth M. Jeedi* and Basavaraj C. Koti

Department of Pharmacology, K L E College of Pharmacy, Vidyanagar, Hubballi 580031, (A constituent unit of KLE Academy of Higher Education and Research, Belagavi), Karnataka, India

In traditional system to cure diabetes and related complications, *Canthium parviflorum* Lam plant leaves are used. Ethanolic extract of leaves were considered to investigate its neuroprotective property in streptozotocin elicited diabetic neuropathy rat model. Streptozotocin (60 mg/kg body weight) given by intraperitoneal injection to induce diabetes, with no treatment given, was intended to develop diabetic neuropathy. Fourteen days after induction, animals were given the extract (100 and 200 mg/kg) once daily for thirty five days. Hypersensitivity to temperature in diabetic neuropathy was assessed by tail immersion and eddy's hot plate tests. Nerve growth factor, blood glucose, and pro-inflammatory cytokines involved in pathological pain were measured in sciatic nerve. Neuropathy which is also associated with oxidative stress was assessed by measuring bio-markers in sciatic tissue. Diabetic induced neuropathic animals exhibited significant decrease in the paw and tail withdraw time thus signifying diabetic neuropathy. Extract treatment prevented diabetic neuropathy in animals thus showing normalized response to hot and cold. These actions in extract treatment groups are supported evidently by normal blood glucose, significant low levels of pro-inflammatory cytokines, oxidative stress bio-markers, and high nerve growth factor in sciatic nerve tissue. *C. parviflorum* leaves ethanolic extract display neuroprotective effect in STZ-induced diabetic neuropathy in rats.

Keywords: Antidiabetic, *Canthium parviflorum*, Diabetic neuropathy, Neuroprotective, Streptozotocin.

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Introduction

Diabetes Mellitus (DM) is one of the metabolic disorders that has burdened health and social wellbeing globally and has been a major contributor of death among the population[1]. Nearly 0.15 crore people die per year worldwide because of DM. According to World Health Organization (WHO), in 2000 nearly 31 million people suffered with DM and it may increase up to 79 million by 2030. Throughout the world in the year 2016, 422 million people suffered with DM, it may rise up to 642 million by 2040[2]. Diabetes is characterized by high blood glucose, abnormal lipid profile, and proteins due to altered metabolism of carbohydrates, proteins, and lipids metabolism. Also, defect in pancreas function with respect to insulin secretion, its action or tissue develops resistance to insulin[3]. If this abnormality is present for a long time, it causes various organs dysfunction or failure. The abnormality of lipids in

DM is one among risk factor for heart and organs failure due to coronary and peripheral artery blockages[4]. The dysfunction of various organs in diabetes is associated with pathological changes that eventually leads to diabetic complications[5]. Biological markers namely, high blood glucose levels, glycated hemoglobin, and oxidative stress are the key responsible factors that cause pathological changes in diabetes leading to neuropathy. Diabetic neuropathy (DN) is a chronic complication of diabetes, which is characterized by defect in afferent and efferent nerve functions[6,7]. Symptoms like less sensitivity to stimuli and tingling of upper and lower limbs, control on urinary bladder is lost, drooping of eyelid, weakness of muscle and defective speech etc.

First line therapy to treat diabetic neuropathy is to normalize the blood glucose level. Various drugs used are capsaicin, antidepressant drugs (amitriptyline, duloxetine, desipramine, etc), and anti-epileptic drugs (gabapentin, pregabalin, opioids, etc). The orally given hypoglycemic agents normally cause unwanted effects like disturbances of hepatic and renal functions[8]. Severe allergic reactions are associated

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*Correspondent author
Email: neelakant_jeedi@rediffmail.com
Mob.: 08951370708

with anti-epileptic drugs. Antidepressant drugs depress the central nervous system; constipation and dry mouth are also associated with these drugs. It is very difficult to prevent these unwanted effects and drugs are not available to permanently cure DN. But to some extent, we can prevent worsening of condition in DN by having control on blood glucose and making changes in life style. By knowing above mentioned, it is clear that a new drug without side effects is required to treat DN permanently. To fulfill these requirements, plant source drugs are considered because of little or no toxicities and low price. Various plant derived drugs are proven for their usefulness in illnesses including diabetes and related complications like DN[9].

Canthium parviflorum , a plant belonging to family Rubiaceae is a short tree, grown abundantly in South India. Different parts of this plant are used to treat various illnesses; some of them are proven scientifically but not all. Traditionally, *C. parviflorum* leaves are used to treat various ailments namely diabetes, hypertension and reduce pain in gastrointestinal tract. Stem bark of this plant has blood glucose lowering property[10,11]. Other parts of *C. parviflorum* are useful to treat high body temperature, piles, inflammation in glands, muscle, bones abnormalities and pain in the joints[12,13]. *C. parviflorum* is proven for its anti-inflammatory[14] and free radical scavenging properties[15]. But usefulness of *C. parviflorum* in diabetes and diabetic neuropathy are not supported with scientific study. The aim of this research work is to provide scientific evidence to this traditional claim. Therefore, this study was planned to assess the effects of the ethanolic extracts of *C. parviflorum* (EECP) leaves on diabetes and neuropathy in streptozotocin (STZ)-induced diabetes and neuropathy in experimental animals.

Materials and Methods

Plant material

The *C. parviflorum* leaves were collected in the month of June 2013, at surroundings of village Mudgal Taluk Lingsur, Raichur District, Karnataka. The leaves were identified by (Prof.) Dr. M Jayaraj, Botany Department, Karnatak University Dharwad. Authenticated (voucher specimen RMRC-966) by Dr. Harsha Hegde, Scientist, ICMR, Regional Medical Research Centre, Belagavi. The herbarium is kept at Regional Medical Research Centre, ICMR, Belagavi for further reference. Collected leaves were washed with normal-water followed by distilled water till leaves are free from the soil and other material. Then the leaves were shade dried and size reduced to get coarse and uniform size powder of size 40 mesh.

Preparation of plant extract

About 300 g of shade dried coarsely powdered leaves of *C. parviflorum* were extracted by using 500 mL of ethyl alcohol (70%) by Soxhletextractor[16]. After exhaustive extraction process nearly 80% volume of the ethanol solvent was removed by distillation process over boiling water bath at normal atmospheric pressure and remaining 20% volume of the ethanol solvent was removed under reduced pressure.

Chemicals

Streptozotocin was procured from Sisco Research Laboratories, Pvt. Ltd. Mumbai and diagnostic kits from Erba Diagnostic Pvt. Ltd. Other reagents and chemicals were purchased from the local suppliers of Hubballi, Karnataka, India.

Animals

The *Wistar albino* rats of either sex (150-200 g) were purchased from Sri Venkateshwara Enterprises, Bangalore, India. Animals were kept in controlled house (22±2 °C) with 12 hours shift of natural light-dark cycle. Water was given *ad libitum* and good quality chow was provided to animals. The 7 days acclimatized animals were divided into different groups. Research activities conducted as per guidelines of committee for the purpose of control and supervision of experiments on animal (CPCSEA). Protocol was approved by Institutional Animal Ethical committee of KLE College of Pharmacy, Hubballi, Karnataka, India (KLEU's-09-IAEC. HBL-31/Aug 2013).

Preparation of drug solutions

STZ was dissolved in cold citrate buffer (of pH 4.5). EECP was suspended in 1% Tween 80 solution prepared in distilled water. It was sonicated for 10 min to get desired concentrations.

Preliminary phytochemical test

The dried ethanolic extract of *C. parviflorum* leaves were investigated for the presence of various phytochemicals[16,17].

Acute toxicity study

The dose of EECP 100 and 200 mg/kg body weight was considered for the activity, based on previous study report[18].

Evaluation of effect of EECP on STZ- induced DN in rats

Antinociceptive activity of EECP was evaluated in STZ induced DN rat's model. Diabetes associated neuropathy was induced in animals by administering STZ (60 mg/kg). Two weeks later, animals were treated with extract subsequently for five weeks. Development of neuropathy and protective effect of the extract was assessed by studying response of the animals for noxious and non-noxious stimuli (Hot-plate and tail flick tests). The assessment was done on before (i.e., on zero day) and after induction (i.e., on 14th day) of neuropathy, and also assessed during the treatment period (i.e., on 26th, 38th, and 49th days)[19].

Experimental design

Normal control group (Group I) contained normoglycemic rats, no treatment only vehicle 1% Tween 80 was given. Diabetic neuropathy group (Group II) having blood glucose above 250 mg/dL received only vehicle (i.e., 1% Tween 80). Treatment groups **(**Group III and IV) were treated with EECP 100 and 200 mg/kg, respectively. The vehicle and treatment doses were given to the respective groups by oral gavages once daily for 5 weeks. DN was assessed by antinociceptive activity, measuring various biomarkers in the blood and sciatic tissue of animals[20-22].

Biochemical estimation

At the end of treatment period (of five weeks), fasted animals were sacrificed, blood and sciatic tissues were collected. The samples collected were used to measure diabetic and neuropathy bio-markers. Blood glucose was estimated by Trinder's method. Pro-inflammatory cytokines, tumour necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, and nerve growth factor (NGF) in sciatic nerve by enzyme-linked immunosorbent assay (ELISA) method using standard kits (R&D Systems, Inc.). Thiobarbituric acid reactive substances (TBARS) activity was determined spec-trophotometrically by calculating the amount of malondialdehyde (MDA), a product of lipid peroxidation (LPO). Reduced glutathione (GSH) was measured as explained by Sedlak and Lindsay[23]. Superoxide dismutase (SOD) activity was studied as described by Kono[24], catalase (CAT) level was measured as per Aebi *et al.*[25]. Glutathione peroxidase (GPx) and glutathione reductase (GR) were calculated by colourimetric method using kits.

Statistical analysis

The data was expressed in mean±standard error of mean (S.E.M). Each group contained six animals (n=6). Statistical analysis was done by one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison tests. The significance was expressed by *p* values, if *p* value was <0.05 results were considered statistically significant.

Results

Phytochemical constituents in EECP leaves

The preliminary phytochemical study confirmed the presence of phytoconstituents (carbohydrates, steroids, glycosides, triterpenoids, saponins, flavonoids, tannins, phenolic compounds and alkaloids) in EECP.

Effect of EECP on Hot-plate and Tail flick tests in STZ induced DN rats

Normal control group animals respond normally (no variation in response time) to the noxious and non-noxious stimuli. Diabetic rats became hypersensitive to both hot and cold stimuli, they exhibited decrease in paw withdraw latency and tail flick latency as compared to normal animals. That indicates diabetes had thermal hyperalgesia and cold allodynia. Treatment with ethanolic extracts of *C. parviflorum* after fourteen days of STZ induction showed improvement in response time of animals indicating that animals developed pain threshold. EECP 200 mg/kg reduced thermal hyperalgesia and cold allodynia (Table 1 & 2).

Effect of EECP on blood glucose, TNF α, IL 1β, IL 6 and NGF levels

Group I did not show changes in glucose levels, Proinflammatory, and nerve growth factors levels in their sciatic nerve during the study. Diabetic group animal showed a significant (*P* <0.001) increase in blood glucose TNF α, IL 1β, and IL 6 but significant (*P* <0.001) decrease in NGF levels during the study, compared to normal control. In the extract (low and high dose) treated groups, significant reduction of glucose, TNF α, IL 1β, and IL 6, but significant (*P* <0.001) increase in NGF levels as compared to diabetic control. It was a dose dependent activity (Table 3).

Effect of EECP on TBARS, GSH, and SOD levels

Group I animals did not demonstrate any significant changes in TBARS, GSH, and SOD levels in their sciatic nerve during study. However, Group II showed a significant (*P* <0.001) increase in TBARS

(P <0.001) and noticeable decrease in GSH and SOD levels compared to Group I animals. Treated Groups III (EECP 100 mg/kg) and IV (EECP 200 mg/kg) showed a significant (P <0.001) reduction of TBARS and significant (P <0.001) increase in GSH and SOD levels as compared to Group II. EECP at 200 mg/kg admirably reduced TBARS levels and improved GSH and SOD levels in neuropathic rats as compared to 100 mg/kg dose (Table 4).

Table 1 — Effect of EECP on paw withdraw latency (sec) in STZ induced DN rats.

Group/Duration	Normal control	DN	DN + EECP (100 mg/kg)	DN + EECP (200 mg/kg)
Before Induction Zero Day	7.43±0.70	7.39±0.56	6.48±0.67	5.51±0.18
After Induction 14th Day	7.60±0.73	4.56±0.5^c	4.00±0.43	2.88±0.12
26th Day	7.45±0.73	3.69±0.4^c	4.52±0.43	3.66±0.15
38th Day	7.67±0.66	3.03±0.3^c	5.04±0.39^x	4.54±0.13
49th Day	7.94±0.45	2.15±0.2^c	5.59±0.39^z	5.34±0.09^z

Values are Mean±SEM; n = 6 in each group; $^c P$ <0.05, compared to normal control;
$^x P$ <0.05, $^y P$ <0.01, $^z P$ <0.001, compared to diabetic neuropathy (DN); EECP–Ethanolic extract of *Canthium parviflorum*

Table 2 — Effect of EECP on tail flick latency (sec) in STZ induced DN rats

Group/Duration	Normal control	DN	DN + EECP (100 mg/kg)	DN + EECP (200 mg/kg)
Before Induction Zero Day	10.36±0.39	10.5±0.27	10.0±0.56	9.51±0.77
After Induction 14th Day	10.2±0.29	6.31±0.32^c	5.66±0.32	5.22±0.58
26th Day	10.25±0.19	5.22±0.31^c	6.57±0.32	6.56±0.56
38th Day	10.39±0.21	4.01±0.28^c	7.36±0.31^z	7.89±0.56^z
49th Day	10.46±0.26	2.82±0.25^c	8.25±0.32^z	9.23±0.56^z

Values are Mean±SEM; n = 6 in each group; $^c P$ <0.05, compared to normal control;
$^z P$ <0.05, compared to diabetic neuropathy (DN); EECP–Ethanolic extract of *Canthium parviflorum*

Table 3 — Effect of EECP on blood glucose, TNF-α, IL-1β, IL-6 and NGF levels in sciatic nerve of STZ induced DN rats

Group	Blood glucose (mg/dL)	TNF-α (pg/mg protein)	IL-1β (pg/mg protein)	IL-6 (pg/mg protein)	NGF (pg/mg protein)
Normal control	84.68±3.532	29.41±2.74	53.79±3.42	38.28±2.95	26.95±3.08
DN	376.5±5.86^c	87.39±4.58^c	106.28±5.31^c	93.51±4.63^c	12.48±1.73^c
DN + EECP (100 mg/kg)	148.2±3.176^z	54.32±2.65^z	74.86±3.63^z	69.47±3.65^z	17.84±2.37^z
DN + EECP (200 mg/kg)	113.7±4.152^z	31.96±2.34^z	58.68±2.51^z	47.58±3.86^z	23.64±2.76^z

Values are Mean±SEM; n = 6 in each group; $^c P$ <0.05, compared to normal control; $^z P$ <0.05, compared to diabetic neuropathy (DN); TNF-α - Tumor necrosis factor-α; IL-1β-Interleukin-1β; IL-6- Interleukin-6; NGF- Nerve growth factor; EECP–Ethanolic extract of *Canthium parviflorum*

Table 4 — Effect of EECP on TBARS, GSH and SOD levels in sciatic nerve of STZ induced DN rats

Group	TBARS (nM/mg protein)	GSH (nM/mg protein)	SOD (U/mg protein)
Normal control	13.87±1.86	12.48±1.74	02.65±0.43
DN	39.06±2.97^c	07.24±1.06^c	01.49±0.27^c
DN + EECP (100 mg/kg)	29.47±2.73^z	09.86±1.48^z	02.03±0.34^z
DN + EECP (200 mg/kg)	20.74±2.31^z	12.07±1.35^z	02.57±0.43^z

Values are Mean±SEM; n = 6 in each group; $^c P$ <0.05, compared to normal control; $^z P$ <0.05,compared to diabetic neuropathy (DN); TBARS - Thiobarbituric acid reactive substances; GSH- Reduced glutathione; SOD- Superoxide dismutase; EECP–Ethanolic extract of *Canthium parviflorum*

Table 5 — Effect of EECP on CAT, GPx and GR levels in sciatic nerve of STZ induced DN rats

Group	CAT (U/mg protein)	GPx (nM/mg protein)	GR (nM/mg protein)
Normal control	03.89±0.61	21.58±2.36	14.09±2.86
DN	02.16±0.42[c]	11.35±1.62[c]	06.87±1.38[c]
DN + EECP (100 mg/kg)	02.94±0.63[z]	16.24±1.95[z]	09.74±1.96[z]
DN + EECP (200 mg/kg)	03.46±0.75[z]	20.57±2.69[z]	13.58±1.63[z]

Values are Mean±SEM; n = 6 in each group; [c]P <0.05, compared to normal control; [z]P <0.05,compared to diabetic neuropathy (DN); CAT- Catalase; GPx- Glutathione peroxidase; GR- Glutathione reductase; EECP–Ethanolic extract of *Canthium parviflorum*

Effect of EECP on CAT, GPx, and GR levels

Sciatic nerves study of Group I did not exhibit any significant changes whereas, Group II animals showed a significant (P <0.001) decrease in CAT, GPx, and GR levels. Extract treated Groups III (EECP 100 mg/kg) and IV (EECP 200 mg/kg) showed a significant (P <0.001) increase in CAT, GPx, and GR levels as compared to diabetic control (Group II) animals. However, Group IV showed a prominent increase in CAT, GPx, and GR levels as compared to Group III (Table 5).

Discussion

Nature has been a major source of drugs in the form of plants. The use of medicinal plants for treating various illnesses is as old as mankind. In order to bring herbal source drug into the market it has to undergo number of test scientifically to fulfill the requirements. It should be safe, efficacious, and superior in all aspects to present available drugs in the market. The present research work was carried out to investigate the neuroprotective property of the ethanolic extract of *C. parviflorum* leaves in streptozotocin induced diabetic neuropathy in rat model. Streptozotocin is toxic or destructive and it suppresses pancreatic insulin secreting beta cells, leads to hyperglycemia, low insulin level, altered lipid metabolizing enzymes activity. The streptozotocin acts by a different mechanism, it reduces enzymes level and activity of those involved in synthesis of DNA[26,27]. Streptozotocin induces cell damage due to oxidative stress or generation of free radical thus leading to alteration of antioxidant defense property of pancreatic beta cells[28]. Animals in all the groups were normal and this was confirmed before the onset of experiment by measuring blood glucose levels. Diabetes was induced by intraperitoneal injection of STZ (60 mg/kg) and induction was confirmed after three days by checking glucose levels. The nitrosourea group of STZ alkylates and destructs the chromosomes of pancreatic beta cells selectively by releasing nitric oxide and free radicals. STZ also disrupts mitochondrial function by inhibiting aconitase enzyme. This enzyme is involved in Tricarboxylic Acid Cycle TCA cycle and protects the DNA by nucleoids reversible remodeling process. STZ also induces the activity of the enzyme xanthine oxidase, which is mainly responsible for free radical formation. All the above said biochemical changes are responsible for oxidative stress and pancreatic cells destruction by generation of more reactive free radicals like oxides, peroxynitrate, hydroxides, hydrogen peroxides etc. Because of all these circumstances, pancreatic beta cells lose their sensitivity to glucose thus leading to reduction in insulin secretion. This eventually causes hyperglycaemia and development of tissue resistance to insulin[29]. If this condition is left untreated, it becomes a focal reason for diabetic complications. These diabetic complications ultimately damage various organ systems namely cardiovascular, renal and nervous systems etc. Among all these, nervous system complication is DN, evidenced by altered structure and functioning of neurons. The mechanisms behind development of diabetic complications are activation of polyol pathway, aldose reductase, and sorbitol dehydrogenase mediated conversion of glucose to sorbitol. Due to neuronal membrane non permiability to sorbitol, it consequently accumulates in nerves thus causing reduction inability of nerve conduction[30]. Oxidative stress due to over activity of polyol pathway causes reduction in NADPH levels this ultimately leads to decreases in the level of glutathione[31-33].

All above mentioned biochemical, anatomical, and physiological changes brings about structural and functional abnormalities of nervous system[34]. The structural and functional abnormalities of nerves were induced in experimental animals by STZ induced diabetes and the complications were seen after two

weeks of induction. These complications were characterized by pain, burning sensation, tingling or numbness of feet, and thermal hypersensitivity. The complications were assessed in experimental animals by Eddy's hot plate and Tail immersion cold tests. As per the present study, results suggest that STZ induced untreated animals became thermal hypersensitive to both cold and hot. This is due to biochemical, anatomical, and physiological abnormalities in the nerves. This abnormality is evidently supported by high levels of TNF-α, interleukins, TBARS, and low levels of superoxide dismutase, reduced glutathione, catalase, glutathione reductase, glutathione peroxidase, and nerve growth factors. The *C. parviflorum* extract treated animals (Group III and IV) respond normally to the cold and hot stimuli because of normal neuronal structure and function. This is due to reversal of STZ induced biochemical, anatomical and physiological abnormalities in pancreas and nerves by EECP. These results are supported by low levels of proinflammatory cytokines, reactive oxygen species, and high levels of nerve growth factor in sciatic nerve tissue. The beneficial effect of extract is due to the phytoconstituents that have antioxidant properties and may suppress activities of free radical forming enzyme xanthine oxidase and hexosamine biosynthesis pathways. Extract may normalize aconitase enzyme activity in mitochondria of pancreatic beta cells. The development of neuronal complications may be prevented by the extract by suppression of aldose reductase enzyme activity involved in polyol pathway also by promoting the synthesis of functional proteins.

Phytochemicals are the active compounds present in the plants, they are responsible for pharmacological activity. Flavonoids protect the cells from highly reactive oxygen species[35]. Phenolic compounds enhance protective enzymes activity, also as antioxidant[36]. Steroids in *Elephantopus scaber*[37], glycosides of *Picralima nitida*[38], condensed tannins, tannic acid, and polyphenols of *Passiflora ligularis*[39-41] and alkaloids of *Aerva lanata*[42] have proven beneficial effects in diabetes and associated complications[43,44]. The phytochemicals in the leaf extracts of plant *C. parviflorum* are responsible for the results of this study. Neuroprotective property of extract is due to action of single, multiple or may be due to synergetic action of all the phytoconstituents. Based on the research work, it is confirmed that the ethanolic extract of plant *C. parviflorum* leaves possess neuroprotective property. Further detailed study is required to know which phytochemicals are responsible for these actions.

Conclusion

Ethanolic extract of *C. parviflorum* leaves possess a neuroprotective property in streptozotocin induced diabetic neuropathy in experimental rats. Extract prevents diabetic neuropathy by managing the parameters stated and assessed during the study procedure. This extract normalizes the physiology of the pancreatic and sciatic nerve tissue as per the present findings. A future study necessitates the fractionation of the ethanolic extract to explore the phytoconstituents that are responsible for the demonstrated neuroprotective activity.

Conflict of interest

The authors do not have any conflicts of interest in this research work.

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