

EFFECT OF ETHANOLIC FLOWER EXTRACT OF *SPATHODEA CAMPANULATA* ON STREPTOZOTOCIN INDUCED DIABETIC NEUROPATHY

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ABSTRACT

Objective: To evaluate the effect of ethanolic extract of the flower of *Spathodea campanulata* (EFESC) on streptozotocin-induced diabetic neuropathy.

Methods: Non-insulin dependent diabetes mellitus (NIDDM) was induced in overnight fasted adult wistar strain albino male rats weighing 160-200g by a single intraperitoneal injection (i. p) of streptozotocin (STZ-65 mg/kg). The rats were randomized into six groups, with six animals each, namely normal control (NC) (Treated with 1% carboxymethyl cellulose solution), diabetic control (DC) (65 mg/kg, i. p. STZ), test group treated at various doses of EFESC250, EFESC500, and EFESC750, standard control-glibenclamide 0.25 mg/kg b.w.(SCG) and the treatment has begun from the day of blood sugar level (BSL) detection after the STZ treatment. Body weight was checked daily and serum glucose levels were measured at 48 h, 15th and 28th d of study. Reaction time to thermal hyperalgesia and cold allodynia were measured after induction of diabetes. *In vitro*, aldose reductase inhibition assay was carried out.

Results: The preliminary phytochemical screening revealed the steroids, terpenoids, coumarins, carbohydrates, tannins, glycosides, and flavonoids in EFESC. DC group showed decreased in reaction time (hyperalgesia) compared to NC while a significant increase in reaction time was observed at various doses EFESC250, EFESC500, EFESC750 and SCG0.25. EFESC at various doses showed the significant reduction in BSL and body weight on 15th and 28th d in STZ diabetic rat at various dose levels. *In vitro*, aldose reductase inhibition was observed with an IC₅₀ at 131 µg/ml.

Conclusion: EFESC showed reduced in BSL and prevents hyperalgesia in experimental diabetic neuropathy. It also reduced aldose-reductase level that may play an important role in reducing the complication of diabetic neuropathy.

Keywords: *Spathodea campanulata*, Neuropathy, Hyperalgesia, Streptozotocin

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INTRODUCTION

Diabetes mellitus (DM) is a complex metabolic disorder characterized by hyperglycemia due to defects in insulin secretion, insulin action or both [1]. It affects the metabolism of carbohydrates, proteins and fats in the body. The long-term elevated glucose level in the blood is easy to cause a variety of diabetic complication such as a neuropathy [2], nephropathy [3], cardiopathy [4] and retinopathy [5]. As the prevalence of diabetes has risen to epidemic proportions worldwide, diabetic vascular complications have now become one of the most challenging health problems. Diabetic neuropathy is one of the most common complications of DM. Patients with this neuropathy experience symptoms such as spontaneous pain, allodynia, and hyperalgesia with an incidence rate of 10–20% [6-8].

Spathodea campanulata (SC) P. Beauv. (Bignoniaceae) is a plant species used in folkloric medicine in Ghana and several African countries. The generic name comes from the Ancient Greek word *Spathé*, in reference to the spadix-like calyx. In Ghana, it is a locally known medicinal plant used for the treatment of various disorders almost as a universal remedy. The bark has laxative and antiseptic properties, and the seeds, flowers and roots are used as medicines for several conditions [9, 10].

SC flowers and bark are used traditionally in the treatment of mental disorders, malaria, haemorrhoids, bacterial infections, HIV, poor blood circulation, gastrointestinal diseases, respiratory ailments, genital-urinary system disorders etc. [11-16]. The flowers of *Spathodea camanulata* possesses an anti-inflammatory and diuretic property. While its stem bark has anti-malarial, hypoglycemic, anti-complement, and anti-HIV properties [17, 18]. In Ghana decoction of the stem bark of SC exerted a hypoglycemic activity in STZ-induced rats [12, 18]. The flowers of SC reported that phytoconstituents responsible for its antidiabetic activity [19, 20]. On the basis

phytochemical and pharmacological evaluation the aim and objective of the present study to investigate the effect of EFESC of STZ-induced diabetic neuropathy in rats.

MATERIALS AND METHODS

Collection of plant material

The fresh flowers of plant materials of SC were collected from Pawan Nagar, New-Nashik (MS), India, during the mo of October 2014. The plant is authenticated and deposited at the Botanical Survey of India, Pune. A Voucher Specimen (RSB-1) was deposited at the Institute for further reference.

Chemicals, reagents and drugs

Petroleum ether (60-80°C), ethanol, (Loba Chemie-Mumbai), streptozotocin (Sigma Aldrich Chemicals) and glibenclamide (Cadila Pharmaceuticals Ltd.) were used as a standard control. Carboxymethylcellulose (CMC-Hi media Pvt. Ltd). All other chemicals, solvents and reagents used were of the highest analytical grade. A standardized EFESC was prepared in 1% CMC.

Extraction and phytochemical screening

The flowers of plant material of SC (2.0 kg) were dried at room temperature. It was ground to coarse powder. The powder materials were defatted three times with 15 l of petroleum ether (60-80°C) using soxhlet apparatus. The petroleum ether extracts were filtered and the solvent was removed under reduced pressure in a rota evaporator (Buchi Laboratories). The defatted materials were stored at-20°C. The defatted plant material of flowers of SC was obtained 0.9 g (45%) further flowers extract of SC extracted with 10 l of 90% ethanol at room temperature. EFESC concentrated under reduced pressure and dried in a freeze dryer. The practical yield of EFESC

was 7.0g (35%w/w). EFESC was further analyzed by various phytochemical tests for carbohydrates, proteins, amino acids, saponins glycosides, alkaloids, flavonoids, steroids, tannins and triterpenoids [21-23].

Animals

The study was carried out after the approval of the Institutional Animal Ethics Committee (Reg No. 1542/po/a/CPCSEA/2008). Adult male wistar albino rats, aged 10-12 w, weighing 160-200 g were obtained from Lacsmi Biofarm (Reg. No. 1277/CPCSEA/2000). They were acclimatised to room temperature with a relative humidity 55±10 % for 2 w during which time they were provided a standard feed (Amrut Feed) and filtered water ad libitum. They were housed in propylene cages.

Acute toxicity study

Acute toxicity assay was performed as per OECD guidelines 423(limit test). Six female wistar albino rats (three animals in each step) were randomly selected. The animals were kept fasting for overnight providing only water. The test drug was administered orally at one dose level of 2000 mg/kg b.w. The rats were observed continuously for the first 4 h and then periodically up to 24 h for toxic symptoms and mortality.

Experimental design

Induction of non-insulin dependent diabetes mellitus (NIDDM)

NIDDM was induced in overnight fasted adult wistar albino male rats weighing 160–200 g by a single intraperitoneal injection of 65 mg/kg streptozotocin (Sigma Aldrich, Germany). STZ was dissolved in citrate buffer (pH 4.5) as per international models of diabetes complications donsortium. Within 72h following STZ administration, blood glucose concentration was estimated by enzymatic GOD-POD (Glucose-oxidase-peroxidase) diagnostic kit method (Accurex). The rats with fasting blood sugar (FBS) more than 200 mg/dl means hyperglycemia was confirmed by the elevated glucose levels in plasma. The rats found with permanent NIDDM were used for the antidiabetic study. The EFESC was prepared in 1% CMC solution and given orally (p. o.) for the treatment group. Standard control-glibenclamide-0.25 (SCG-0.25) dissolved in 0.9% sodium chloride administered intraperitoneally. The rats were randomized into six groups, with a minimum of six animals each, and the treatment began from the day of blood sugar level (BSL) detection after the STZ treatment. Body weight was checked daily and serum glucose levels were measured after 48 h, 15th d and 28th d of study [24-25]

The animals were divided as per following groups,

I-normal control (NC) (Treated with 1 % CMC solution)

II-diabetic control-(DC)-(65 mg/kg, i. p. STZ)

III-EFESC 250 mg/kg, (p. o.)

IV-EFESC 500 mg/kg, (p. o.)

V-EFESC 750 mg/kg (p. o.)

VI-SCG-0.25 mg/kg b.w. (i. p)

Assesment of thermal hyperalgesia and cold allodynia

Tail immersion (warm water) test

The tail of rat was immersed in a warm water (47±1 °C) bath until tail withdrawal (flicking response) or signs of struggle were observed (cut-off 15s). Shortening of tail-withdrawal indicates hyperalgesia.

Tail immersion (cold water) test

The procedure is same as a warm water test but the temperature of the water was set at 10±0.5 °C, a temperature that is normally innocuous. The cut-off time was 15 s. the shorten duration of tail immersion indicates allodynia.

Hotplate test

In this test, animals were placed individually on a hot plate (Inco Digital) with temperature adjusted to 55±1 °C. The latency to the

first sign of paw licking or jump response to avoid heat pain was taken as an index of pain threshold. The cut-off time was kept 10 s so as to avoid damage to the paw. Both hyperalgesia and allodynia were assessed weekly till the end of the study [26-28].

In vitro aldose reductase inhibition assay

For the determination of the aldose reductase inhibitory activity of the different concentration of EFESC was selected, The incubation mixture, in a final volume of 250 µl, consisted of 67 mmol potassium phosphate buffer (pH-6.2), 0.4 M lithium sulphate, 150 µM NADPH, 300 µM DL-glyceraldehyde, enzyme (50 µl). Quercetin 0.5 µg/ml was used as standard inhibitor. Appropriate blanks were prepared for quercetin and the test samples without DL glyceraldehyde. The reaction was initiated by adding NADPH. The absorbance was read at 340 nm for 20 min using microplate reader (BMG Labtech) in a kinetic mode. The percent inhibition by test sample/quercetin was calculated by considering the control value as 100%, using the equation mentioned below. The median inhibitory concentration (IC₅₀) was calculated using Finney software. A similar procedure was carried out with EEFSC at strengths of 5, 10, 25, 50, 100 and 200 µg/ml [29]. The percentage inhibition was calculated with the following formula:

$$100 - \frac{(\Delta A340 \text{ sample} \times 100)}{\Delta A340 \text{ control}}$$

Where, Δ A340 = changes in absorbance at 340 nm,

Statistical analysis

Data were expressed as mean±SEM of the animal in each group. To determine statistical significance, ANOVA followed by Dunnett's test was used for post hoc analysis. P<0.05 was considered as statistically significant. IC₅₀ was calculated for the *in vitro* aldose inhibitory activity of EFESC.

RESULTS

Preliminary phytochemical screening of *Spathodea campanulata*

The petroleum ether extract of the flower of SC showed the presence of saponins, terpenoids, steroids, quinones, coumarins and proteins. The EFESC showed presence of flavonoids, saponins, terpenoids, proteins, carbohydrates, coumarins, quinones, glycosides and phenolic compounds.

Acute toxicity of EFESC

The acute oral toxicity study was carried out according to OECD guidelines No.423. The tested extract did not exhibit any toxicity symptoms and mortality in all groups when given orally at a dose 2000 mg/kg b.w. EFESC were safe up to the dose of 2000 mg/kg b.w. hence three different doses (250,500 and 750 mg/kg b.w.) were arbitrarily selected for the pharmacological study.

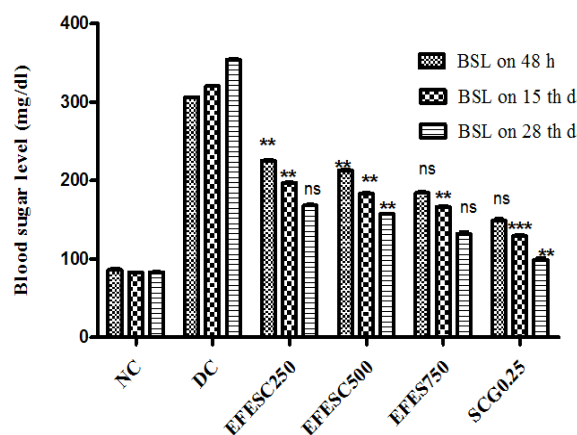


Fig. 1: Effect of EFESC on blood sugar level (BSL) in STZ induced diabetes in rats

Effect EFESC on blood sugar levels (BSL)

BSL of the NC group of animals did not alter throughout the experiment. In DC group BSL level increased steadily on 15th d (320±0.67) and on 28thd (354±1.1) after STZ treatment indicating the incidence of diabetes in the animals throughout the experiment (fig. 1). There were significant changes in BSL of SCG0.25 treated animals (149±2.00 mg/dl) 48 h post-STZ treatment as compared to DC group of animals (306±0.58 mg/dl) indicating the incidence of diabetes. There was a significant reduction in BSL of EFESC on 15th and 28th d in the STZ-induced diabetic rat at various dose levels.

Each Value represents as mean±SEM. (n = 6). The comparisons were made by one way ANOVA followed by Dunnett's test. ns=non-significant, STZ = streptozotocin. NC=normal control, DC=diabetic control, EFESC=ethanolic flower extract of *Spathodea campanulata*,

SCG0.25=standard control glibenclamide. *p<0.05, **p<0.01 and ***p<0.001 is considered as significant when compared to the control group.

Effect of EFESC on hyperalgesia produced by tail immersion (hot water) method

There was no change in tail flick latency (s) observed in NC group of the animal throughout the experiment. A gradual decline in the latency was observed in DC group of the animal from day 7th (8.4±0.10) onward which was observed minimum on day 28th (4.3±0.06), indicating neuropathic pain due to diabetes (table 1). At three different doses level of EFESC also showed a significant reduction in latency from 14thd to 28th d increase in pain threshold time, indicating an absence of algesia produced by tail immersion in hot water.

Table 1: Effect of EFESC on hyperalgesia produced by tail immersion (hot water) in STZ-induced diabetes in rats

Groups (n=6)	Treatment	Tail flick latency (s)			
		7 th d	14 th d	21 st d	28 th d
I	NC	13±0.07	11±0.09	12±0.12	11.0±0.14
II	DC	8.4±0.1	6.3±0.0	5.3±0.06	4.3±0.06
III	EFESC250	8.6±0.08	9±1.00**	9.4±0.03**	10.0±0.01**
IV	EFESC500	9.1±0.16	9.1±0.01**	10±0.07**	11.0±0.02**
V	EFESC750	9.1±0.01	9.9±0.02**	11±0.01*	11.0±0.02**
VI	SCG025	9.9±0.01***	10±0.01***	10±0.03***	11.0±0.05***

The data are expressed as mean±SEM. (n = number of animals in each group = 6). The comparisons were made by one way ANOVA followed by Dunnett's test. STZ=streptozotocin, NC=normal control, DC=diabetic control, EFESC=ethanolic flower extract of *Spathodea campanulata* SCG0.25=standard control glibenclamide.*p<0.05, **p<0.01 and ***p<0.001 is considered as significant when compared to the control group.

Effect of EFESC on allodynia produced by tail immersion (cold water) method

No significant change in latency was observed in NC of the animal throughout the study. For DC group of an animal, there was a gradual reduction in latency (s) was observed from day 7th

(10.0±0.01) till 28thd (4.3±0.06) indicating the presence of allodynia (table 2). In the treatment group of animals i.e. EFESC with increasing dose showed significant (**p<0.01) increased in latency time (s) at 14th and 28th d was exhibited which implies the protective action of drug treatment on allodynia produced by cold water.

Table 2: Effect of EFESC on allodynia produced by tail immersion (cold water) in STZ induced diabetes in rats

Groups (n=6)	Treatment	Tail flick latency (s)			
		7 th d	14 th d	21 st d	28 th d
I	NC	14.0±0.12	13.0±0.07	14.0±0.04	13.0±0.09
II	DC	10.0±0.01	7.3±0.05	5.4±0.08	4.3±0.06
III	EFESC250	12.0±0.06	12.1±0.05*	12.7±0.02**	13.1±0.04**
IV	EFESC 500	13.0±0.05	12.7±0.05 ^{ns}	13.5±0.02**	14.0±0.04**
V	EFESC750	13.0±0.02	12.9±0.03 ⁿ	14.0±0.56**	14.0±0.02**
VI	SCG0.25	13.66±0.04	13.80±0.02*	14.69±0.03***	14.30±0.03***

The data are expressed as mean±SEM. (n = number of animals in each group = 6). The comparisons were made by one way ANOVA followed by Dunnett's test. ns = non-significant, STZ = streptozotocin. NC =normal control, DC=diabetic control, EFESC=ethanolic flower extract of *Spathodea campanulata*, SCG0.25=standard control glibemclamide.*p<0.05, **p<0.01 and ***p<0.001 is considered as significant when compared to the control group.

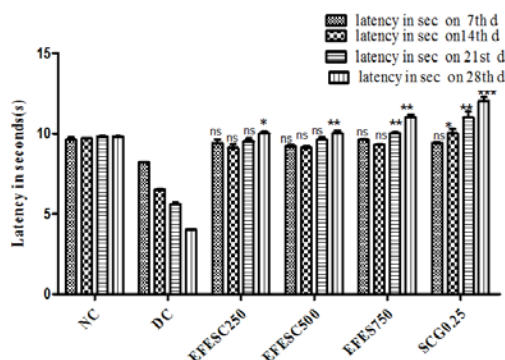


Fig. 2: Effect of EFESC on thermal hyperalgesia (hot plate) in STZ induced diabetes in rats

Effect of EFESC on thermal hyperalgesia by a hot plate method

No significant change in latency was observed in NC group of animals, throughout the study (fig. 2). For DC group of animals, there was a gradual reduction in latency (s) observed from day 7th d (8.20±0.01s) till 28thd (4.00±0.04s) where the pain was observed to be maximum, indicating the presence of algesia by heat. In the EFESC treated a group of animals, no significant lowering of pain latency was exhibited which implies the protective action of GLB on hyperalgesia produced in diabetic animals.

The data are expressed as mean±SEM. (n = number of animals in each group = 6). The comparisons were made by one way ANOVA followed by Dunnett's test. ns = non-significant, STZ = streptozotocin. NC =normal control, DC=diabetic control, EFESC=ethanolic flower extract of *Spathodea campanulata*, SCG0.25=standard control glibemclamide.*p<0.05, **p<0.01 and

*** $p < 0.001$ is considered as significant when compared to the control group.

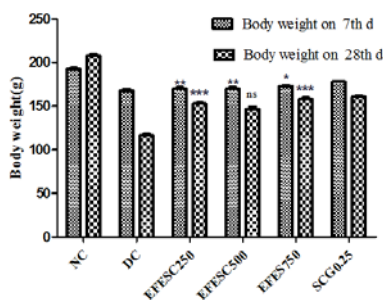


Fig. 3: Effect of EFESC on body weight

Effect of EFESC on body weight

There was a decrease in body weight in the DC group as compared to NC group which was not statistically significant.

The SCG0.25 group showed significantly increased in weight as compared to DC group (fig. 3). EFESC showed a significant reduction in body weight (g) compared to NC and DC value. There was no reduction in feed intake in any of the group.

The data are expressed as mean \pm SEM. (n = number of animals in each group = 6). The comparisons were made by one way ANOVA followed by Dunnett's test. ns = non-significant, STZ = streptozotocin.

NC =normal Control, DC=diabetic control, EFESC=ethanolic flower extract of *Spathodea campanulata*, SCG0.25=standard control glibemcamide.* $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ is considered as significant when compared to the control group.

Effect of EFESC on aldose enzyme reductase activity *in vitro*

Quercetin at a concentration of 0.5 $\mu\text{g/ml}$ exhibited 46.19 % inhibition of aldose reductase activity. Percent inhibition of aldose reductase enzyme by the EFESC samples at different concentrations were calculated and plotted on a graph. IC₅₀ of EFESC was 131 $\mu\text{g/ml}$ (table 3).

Table 3: Effect of EFESC on aldose enzyme reductase activity *in vitro*

Conc. tested ($\mu\text{g/ml}$)	(% Inhibition)
5	5.74
10	10.58
25	21.23
50	36.72
100	46.91
200	71.52
IC ₅₀ ($\mu\text{g/ml}$)	131

DISCUSSION

Pain associated with nerve damage from diabetes initially involves peripheral mechanisms causing sensory fibres hypersensitivity, which secondarily leads to central rearrangements responsible for central nociceptive system hyperexcitability. In this study the main peripheral and central mechanisms of diabetic neuropathic pain proposed by the work using the STZ-rat model. Peripheral diabetic neuropathy is a devastating complication of diabetes and a leading cause of foot amputation. Diabetic neuropathy is characterized by clinical features like allodynia, hyperalgesia due to elevated nociceptive response, neuronal hypoxia and reduced threshold to painful stimuli [30]. Similar symptoms are exhibited by STZ induced diabetic animals [31]. Hyperglycemia and inflammation unleash a cascade of events that affect cellular proteins, gene expression and cell surface receptor expression, ultimately resulting in progressive pathologic changes and subsequent diabetic complications [32]. The STZ induced diabetic rats are the most commonly employed animal model of painful diabetic neuropathy [33].

The model chosen in the present study was based on the principle that at an increased level of blood sugar in a diabetic rat, hyperalgesia due to the hyperactivity of C-nociceptive fibres will be observed. This can be detected as a reduced latent period (reaction time) for the tail flick in response to radiant heat by the analgesiometer [34]. EFESC would beneficial in DN will reverse the hyperalgesia.

The mechanisms of cold allodynia and hyperalgesia include peripheral and central sensitization, or central disinhibition, such as sensitization of C nociceptors or A δ fibres. Microneurographic recordings in a patient with small-fibre neuropathy and cold allodynia showed sensitization to cold and menthol responsiveness of subtypes of C nociceptors [35]. Blockade of A δ fibres during nerve compression [36,37] or disease [38] causes an increase in cold detection thresholds, a decrease in cold pain thresholds, and a change in the quality of cold sensation to icy, stinging, hot, and burning sensations. This is thought to result from disinhibition of C-polymodal nociceptive fibres (heat-pinch-cold fibres) by the loss of

A δ fibres [39] and could provide an explanation for cold allodynia in neuropathic pain patients.

Phytochemical studies were performed with different parts of SC, including stem barks, leaves, flowers and fruits. Spathodic acid, steroids, saponins, ursoilic acid, tomentosolic acid and phenolic substance were isolated from stem bark. The flowers showed the presence of anthocyanins, while the floral nectar contains the complex mixture of triterpenoids and steroids [40-41].

The studied EFESC are rich in flavonoids, steroids and anthraquinones like compound. The EFESC extract probably contains quercetin type of flavonoids like phytoconstituents. The effect of quercetin in DN could be mediated possibly through a peripheral mechanism. One of the possible mechanisms that could partially explain the beneficial analgesic property of quercetin may be attributed to its hypoglycemic and antioxidant effects. Since hyperglycemia in the diabetic state could induce some functional alterations in the nervous system [42], quercetin may have attenuated the hyperalgesia in diabetic rats through lowering blood glucose that may be the probable mechanism for the present study. The SC plant is also reported for its analgesic and anti-inflammatory activity. These phytoconstituent are a positive modulator for neuropathic pain and their antinociceptive effect.

The studied plant of SC ethanol leaf extract has reported for its anticonvulsant properties. SC used in traditional medicine to treat convulsion and epilepsy were studied in mice using pentylenetetrazole, picrotoxin, and electro-shock induced models in mice. On the basis of its biological activity, the plant might be responsible to treat diabetic neuropathy by an enhancement of GABA inhibitory action and/or inhibition of voltage-dependent sodium channels [43].

In the present study, STZ injected rats had significantly higher BSL level, which provokes diabetic neuropathy that was associated with the neuropathic pain. In addition, EFESC, attenuated STZ induced thermal hyperalgesia, mechanical hyperalgesia and tactile allodynia.

The nociceptive threshold was significantly lowered in DC rats compared to NC rats in tail immersion and hot plate technique. This indicates that diabetic animals exhibited thermal hyperalgesia, post development of diabetic neuropathy. Consequently, hyperalgesia and neuropathic pain caused further decrease in pain threshold on exposure to thermal heat in tail immersion and hot plate method in uncontrolled diabetic rats (DC rats). Contrary to that, administration EFESC in diabetic rats not only reduced the BSL level but also lead to maximum improvement in pain threshold in a progressive manner in tail immersion and hot plate tests, indicating the better control of BSL, reduction in neuropathic pain and thermal hyperalgesia. However, SC is scientifically reported for its analgesic effect [44], which indicates its possible role in relieving the pain of diabetic neuropathy.

A strong relationship exists between glycemia and diabetic microvascular complications in both type1 and type2 diabetes [45]. Generation of superoxide due to oxidative stress in diabetes may be responsible for vascular and neuronal complications of painful neuropathy [26]. Early in the course of diabetes, intracellular hyperglycemia causes abnormalities in blood flow and increased vascular permeability.

Hyperglycemia affects the pro-oxidant state of the tissue. Oxidative stress and neuroinflammation combined with the decline in antioxidative defence potential have been documented as the key pathophysiological mechanism, involved in various complications including diabetic neuropathy [46]. Hyperglycemia is also caused due to increased polyol pathway activity, oxidative stress, advanced glycation end product formation, increased activation of protein kinase C, nerve hypoxia/ischemia and impaired NGF support [32] and all these pathways contribute to the development of diabetic neuropathy. One of the important consequences of chronic hyperglycemia is the enhanced oxidative stress resulting from an imbalance between the production and neutralization of reactive oxygen species (ROS). Clinical trials with aldose reductase inhibitor, Sorbinil (Pfizer-CP45634) have demonstrated significant improvement in the pain relief, motor and sensory nerve conduction velocities with minimum toxicity in patients with DN. These observations suggest that aldose reductase inhibitors may be important in the treatment of symptomatic, somatic and autonomic neuropathies complicating diabetes [47]. Hence the *in vitro* effect of EFESC on aldose reductase inhibition was studied. It showed an inhibitory effect although the IC₅₀ was higher as compared to the standard (quercetin). Thus, the beneficial effect of SC on diabetic neuropathy appears to be due to its analgesic effect and unrelated to its anti-hyperglycemic effect. Aldose reductase inhibition may contribute to this action. An antioxidant role of SC cannot be ruled out as oxidative damage contributes to the causation of diabetic neuropathy and SC is proven to be an anti-oxidant in experimental models [48].

CONCLUSION

The data of the present study suggest a potential protective role of EFESC against STZ induced diabetes and diabetic neuropathy in rats. The onset of neuropathic complications could be prevented by early glycaemic controls. These results further validate the traditional use of SC against diabetes and its complications. In future experiments involving nerve conduction studies would be more efficacious for the claim against diabetic neuropathy.

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AUTHORS CONTRIBUTIONS

The experimental part of the work and writing of the manuscript was done by the first author Prof. Rishikesh Bachhav. The correction of the manuscript was done by the author Prof. Ravindranath Saudagar

CONFLICT OF INTERESTS

Declared none

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