# Antinociceptive Property of *TRIGONELLA FOENUM GRAECUM* (FENUGREEK SEEDS) in High Fat Diet-Fed/Low Dose Streptozotocin Induced Diabetic Neuropathy In Rats

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## Summary

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 the effect of fenugreek seed powder in type II diabetes (high fat diet fed/low dose streptozotocin) induced diabetic neuropathy in male Sprague-Dawley rats. Diabetic rats exhibited a significant hyperalgesic behavior to thermal & noxious stimuli (Formalin 0.5%) when compared to control group. Treatment with *Trigonella foenum graecum* seed and quercetin to diabetic rats showed significant increase in tail flick latency (hot immersion test) and a significant decrease in paw flinching response (Formalin test) compared to untreated diabetic control. *Trigonella foenum graecum* seed and quercetin attenuated diabetes induced axonal degeneration. The study provides experimental evidence of the preventive and curative effect of fenugreek seed powder on nerve function and oxidative stress in animal model of diabetic neuropathy. In conclusion, fenugreek seed powder may be evaluated for preventive therapy in diabetic patients at risk of developing neuropathy.

Keywords: Antioxidant, Diabetic neuropathy, *Trigonella foenum graecum*, Nociception, Oxidative stress

## Introduction

Diabetic neuropathic pain is one of the most important common complications of diabetes mellitus. The vital role contributing to neural and vascular complications is oxidative stress[1].

Oxidative stress is responsible in depleting antioxidant defenses rendering the affected cells and tissues more susceptible to oxidative damage. Hyperglycemia and oxidative stress catalyzes the increased lipid peroxidation and accelerated advanced lipoxidation endproducts (ALE) formation and may also play a critical role in the development of neurovascular complications in diabetes[2].

Narcotic analgesics, nonsteroidal anti-inflammatory drugs, antidepressants, topical capsaicin and anticonvulsants[3] are therapeutically used in the management of diabetic neuropathy but they are partially effective[4] and develop tolerance[5] with potential toxic effects[6]. Diabetic rats display exaggerated hyperalgesia behavior in response to noxious stimuli (0.5% formalin solution)[7]. Formalin injection produces spinal release of excitatory amino acids (EAA) and cyclooxygenase (COX) products. The antinociceptive doses of COX inhibitors, inhibits the spinal release of $PGE_2$ and its suppressive effect on EAA release indicates that prostaglandins may be involved in facilitation of afferent evoked EAA release in the spinal dorsal horn[8].

Many herbal medicines have been recommended for the treatment of diabetes[9]. Herbal drugs are frequently considered to be less toxic and lesser side effects than synthetic ones[10]. *Trigonella foenum graecum* seeds possess significant hypoglycemic[11], anti-inflammatory[12] and antinociceptive[13] activity. The hypoglycemic property of fenugreek was observed in alloxan-induced diabetic rats and diabetic patients[14-15]. A combination of *Trigonella foenum graecum* seed powder (TSP) and sodium ortho vanadate has been effectively used to control the long term complications of diabetes in tissues like peripheral nerve[16]. It has been reported that fenugreek seeds contains five different flavonoids, namely, vitexin, tricin, naringenin, quercetin, and tricin-7-O-β-D-glucopyranoside[17]. Polyphenolic flavonoids have been shown to protect various cell types from oxidative stress–mediated cell injury. Quercetin, a bioflavonoid was reported to attenuate thermal hyperalgesia in a mouse model of diabetic neuropathic pain[18]. It was also reported to have anti-inflammatory activity[19-20].

Based on these reports, the present study has been designed to evaluate the effect of *Trigonella foenum graecum* seed powder (TSP) in high fat diet fed /low dose streptozotocin (HFD fed/low dose STZ) -induced diabetic neuropathic rats.

## Methods

### Chemicals

Streptozotocin was purchased from Sigma-Aldrich Chemical Company, St. Louis, MO, USA. The feed ingredients such as casein (Himedia Laboratories, Mumbai, India), dl-methionine (Loba Chemie, Mumbai, India), vitamin and mineral mix (Sarabhai chemicals, Baroda, India) were procured from the commercial sources. Quercetin (HiMedia Laboratories, Bombay, India), lard and heparin were obtained from commercial sources. Quercetin was administered orally as suspension (0.5% Na-CMC 2 ml $kg^{-1}$, p.o). Trigonella foenum graecum seeds were obtained from the local market.

### Preparation of fructose diet

Fructose diet was prepared by the method reported elsewhere[21] 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 cellulose 150 g, all commercial grade.

### Experimental Animals

Male Sprague–Dawley rats (160–180 g) were housed in standard polypropylene cages (three rats/cage) and maintained under controlled room temperature (22°±2°C) and relative humidity (55±5%) with 12:12 h light and dark cycle. The rats were provided with normal pellet diet (Amrut Diet, New Delhi) and water *ad libitum*, prior to the dietary manipulation. Institutional Animal Ethics Committee approved the experimental protocol. Animals were maintained under standard conditions in an animal house approved by Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA).

### Development of HFD-fed / STZ-treated type 2 diabetic rats

The model of type 2-like diabetes was established according to the method reported earlier[22] with modification. 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 non-fasting PGL of ≥300 mg $dl^{-1}$ were considered diabetic and selected for further pharmacological studies.

### Experimental protocol

The control rats were divided into two groups of 8-10 rats each (I-II). Group I was treated with NPD (normal pellet diet) and group II (high fat-fed/low dose STZ diabetic rats) treated with vehicle [1% Na-CMC (2 ml $kg^{-1}$, p.o)] served as diabetic control. Groups III and IV were treated with quercetin (10 mg $kg^{-1}$ once daily, p.o)[18] and *Trigonella foenum graecum* seed powder (TSP) (8 g/kg, p.o)[23] respectively. All the above were administered intragastrically for 8 weeks and treatment schedule was started one day before the administration of STZ. TSP was mixed with the normal pellet diet. At the end of the treatment period, fifty µl of diluted formalin (0.5% for diabetic rats or 1% for non-diabetic rats) were injected subcutaneously into the dorsal surface of the right hind paw, to observe formalin induced flinching behavior[7].

### Experimental Procedure

### Effect of TSP and quercetin on blood glucose levels in normal and diabetic animals (single and multiple dose study)

A single dose of the TSP was administered to normal and diabetic rats and the blood glucose level were estimated just prior to TSP 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 14 days. The glucose levels of all the diabetic 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 TSP. After fasting for 12 h the animals were treated with TSP. Ninety minutes later; the rats were administered 2.0 g/kg glucose orally. Blood glucose levels were measured at 0, 30, 60 and 120 min after glucose load.

### 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. Cut off time was 15 sec.

### Behavioral assessment

Antinociception in non-diabetic and diabetic rats was assessed using the formalin test[24]. The rats were placed in open Plexiglas observation chambers for 30 min to allow them to acclimate to their surroundings; then they were removed for formalin administration. Fifty µl of diluted formalin (0.5% for diabetic rats or 1% for non-diabetic rats) were injected subcutaneously into the dorsal surface[25] of the right hind paw with a 30-gauge needle. The animals were returned to the chambers and nociceptive behavior was observed immediately after formalin injection. Mirrors were placed in each chamber to enable unhindered observation. Nociceptive behavior was quantified as the number of flinches of injected paw during 1-min periods every 5 min, up to 60 min after injection[26]. Flinching was readily discriminated and was characterized as rapid and brief withdrawal, or as flexing of the injected paw. Formalin induced flinching behavior was biphasic[24]. The initial acute phase (0–10 min) was followed by a relatively short quiescent period, which was then followed by a prolonged tonic response (15–60 min).

### 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 stomach 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±SEM. Where appropriate, between –group comparisons were made by unpaired t test or one-way ANOVA with the Student – Newman Keuls posthoc test. A value of $P<0.05$ was considered to be statistically significant.

## Results

### Effect of EOJ and quercetin on blood glucose levels in normal and diabetic animals (single and multiple dose study)

In our preliminary study, TSP (low dose, 2g/kg) did not show any significant decrease in blood glucose level until 2 hrs in normal rats. High dose of TSP (8g/kg) showed a significant reduction (13.93%) at 2 hrs and 28.76% reduction at 4hrs. Hence high dose of TSP was considered for the present study. After the 2nd week of STZ injection, diabetic rats showed significant increase in blood glucose levels than control rats. Blood glucose level was not significantly affected in quercetin treated group. In diabetic rats, multiple dose of TSP exhibited significant reduction (18.76-32.52 %) in blood glucose level between 7th and 14th day.

### Effect of EOE on oral glucose tolerance test

In glucose fed rats (2 g/kg), administration of TSP exhibited a significant increase in glucose tolerance after 2.5 hrs of dosing because it produced a significant reduction (44.53%) in blood glucose level in comparison to normal control.

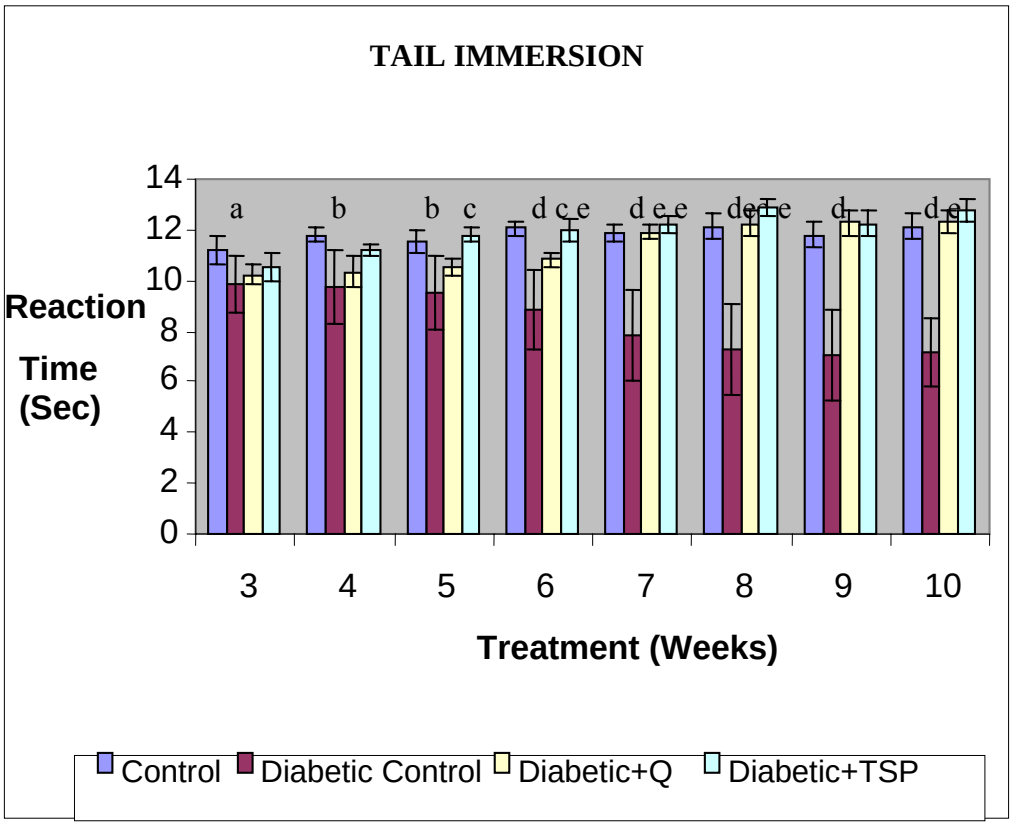
### Measurement of antinociceptive activity
### Tail immersion test

The rats exhibited hyperalgesia in the tail-immersion test and the decrease in pain threshold was observed until eight weeks after streptozotocin injection. The decrease in pain threshold was significant when compared to control rats ($P<0.001$). TSP and quercetin administration to diabetic rats produced a significant increase in pain threshold level as compared to untreated diabetic rats. The maximum increase in pain threshold level was observed in a progressive manner as shown in the Fig 1.

### Effect of TSP on formalin evoked behavior

Injection of 1.0% formalin into the hind paw produced a biphasic response in control rats with the active phases separated by a quiescent period of inactivity (sum flinches counted over the 60-min observation period, 91±8) (Fig 2). In diabetic rats, flinching was exaggerated within minutes of the injection of formalin, and this was maintained throughout the monitoring period (sum flinches counted over the 60-min observation period, 122±12; $P<0.01$ vs control by unpaired t test). Hyperalgesia in diabetic rats was significantlly attenuated by Quercetin ($P<0.01$) and TSP ($P<0.05$), as shown in the Fig 3, when compared to vehicle treated diabetic rats.

Fig 1: Effect of TSP (8G/kg, p.o.) and Quercetin (Q -10mg/kg, p.o.) on tail immersion pain threshold in control and diabetic rats. Values are expressed as means ± SEM (n=8 in each group).




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

Fig 2: Time course of formalin – evoked flinching in control and diabetic rats. Data are means ± SEM; n = 8/group.

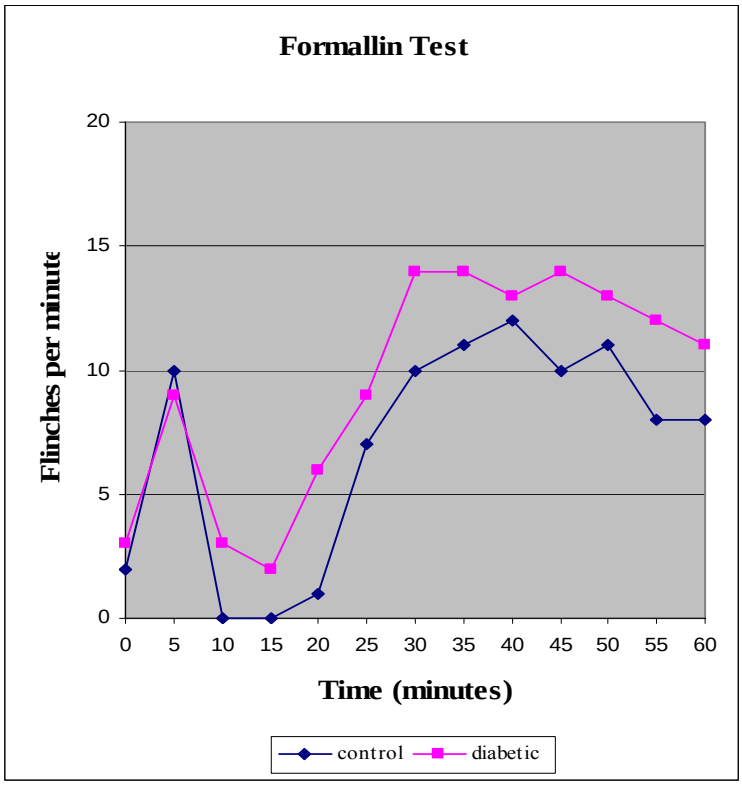
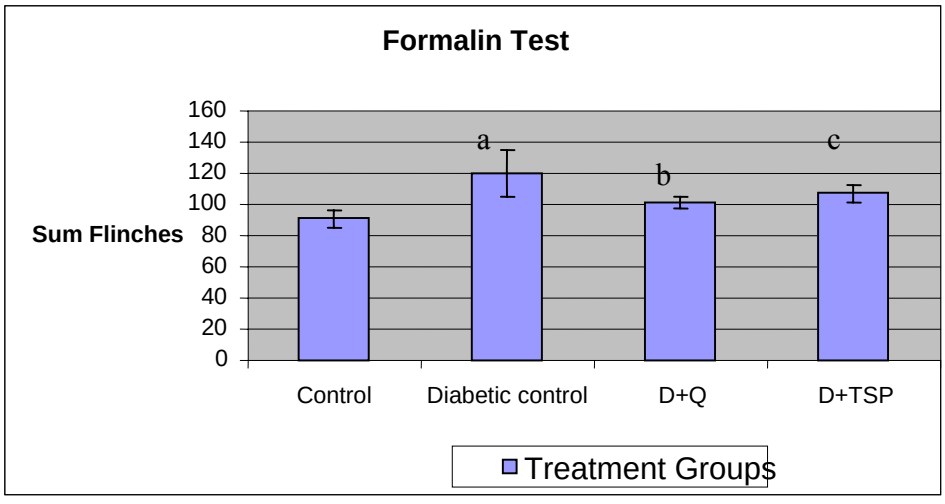



Fig 3: Sum of flinches counted in the 60-min period after paw formalin injection in control and diabetic rats that were untreated or treated with Quercetin (Q) or TSP. Data are means±SEM; n=8/group. Statistical comparison by ANOVA with Student-Newman-Keuls post hoc test.

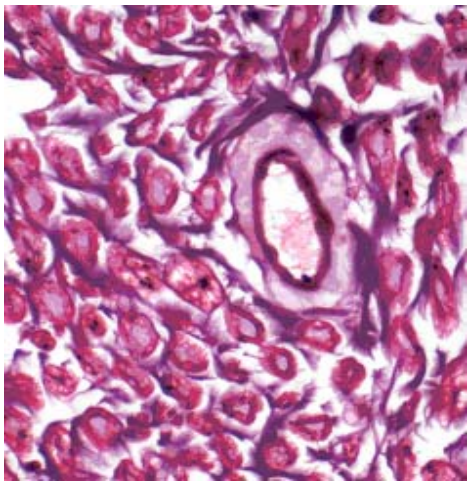



D +Q = Diabetic + Quercetin , D + TSP = Diabetic + TSP
$P < 0.01$ [a] vs Control; [b] vs Diabetic control
$P < 0.05$ [c] vs Diabetic control

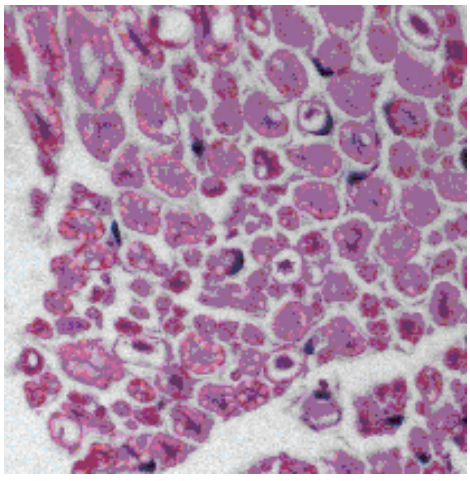
**Effect of TSP on axonal degeneration**

Histopathological changes were assessed by using a longitudinal section of sciatic nerve *Trigonella foenum graecum* seed powder and quercetin administration attenuated diabetic induced axonal degeneration and histopathological alterations.

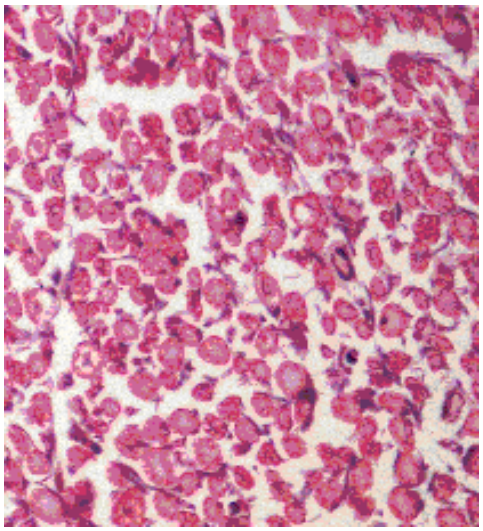
**Diabetic control (Fig 4A).:** Multifocal loss of both large and small myelinated fibers. Small myelinated fiber loss is more prominent than large diameter fiber loss. Thickened and hyalinised endoneurial vessel. (HE stain. Original magnification 320x)



**Quercetin (Fig 4B):** Fiber density is well preserved. There is mild loss in some funicles. The small myelinated fibers are relatively preserved (dotted large circle). Several regenerating clusters are seen (small solid lined circles). (HE stain. Original magnification 320x).



***Trigonella foenum graecum* (4 C):** Higher magnification showing well preserved fiber and axonal density. Endoneurial vessels are normal in morphology. (HE stain. Original magnification 320x).



## Discussion

Peripheral nerve pathology in diabetic patients is characterized by progressive nerve fiber loss[27]. Distal fiber loss is typically found in the skin of calf in the subjects with impaired glucose tolerance[28] (IGT). Fenugreek seeds possess significant hypoglycemic[29], antiathrosclerotic[30], anti-inflammatory[12], antinociceptive[31] and antiulcerogenic[32]activity. In agreement with the present results, the hypoglycemic effect of fenugreek seeds has been demonstrated in experimentally induced diabetic rats, dogs, mice, and healthy volunteers and insulin-dependent and non–insulin-dependent diabetic patients[33-35]. 4-Hydroxyisoleucine, a modified amino acid extracted and purified from fenugreek seed, also displayed an insulinotropic property *in vitro*, stimulated insulin secretion *in vivo*, and improved glucose tolerance in normal rats and dogs and in rat model of type 2 diabetes mellitus[36]. Previous study has reported the early presence of neuropathy in IGT patients who had reduced nerve conduction velocity. Glycemic control in these patients prevented the development and progression of diabetic neuropathy[37-38]. Fenugreek produces a significant antinociceptive activity from 4$^{th}$ week onwards. Fenugreek also prevented the development and progression of diabetic neuropathy due to its suppressive effect on blood glucose levels in normal, glucose fed hyperglycemia and type 2 diabetic rats. Thus fenugreek due to its improvement in glycemic index may be useful in preventing diabetic neuropathy. On the other hand quercetin did not have any significant effect on blood glucose level and showed significant antinociception only at 6$^{th}$ week. Thus quercetin may be used as curative agent.

Diabetic oxidative stress induced free radicals are involved in vascular endothelial damage of epineural arterioles of the sciatic nerve in diabetic rats[39]. Dietary antioxidants by scavenging reactive oxygen species has improved vascular resistance in diabetic rats[40]. Quercetin has antioxidant and free radical scavenging activity[41], delays lipid peroxidation of cell membranes[42], and reduces $Cu^{2+}$-induced LDL oxidation[43]. Administration of fenugreek seeds has been reported to reverse the disturbed antioxidant levels and peroxidative damage and therefore can be used to reverse the complications of diabetes[44]. Quercetin is reported to be present in fenugreek seeds[45]. Polyphenolic flavonoids have been shown to protect various cell types from oxidative stress–mediated cell injury. The present study also indicates the protective nature of quercetin and TSP in the development of diabetic neuropathy by reversing the oxidative stress induced changes in nerve physiology of diabetic rats as reported earlier[46-47].

Fenugreek seeds possess anti-inflammatory, antinociceptive and antiulcerogenic activity. The anti-inflammatory activity of quercetin and fenugreeks seeds have been useful in our study to attenuate hyperalgesia induced by formalin injection in diabetic rats because hyperalgesia behavior in diabetic rats is associated with both increased cyclooxygenase -2 protein and cyclooxygenase mediated $PGE_2$ release. Selective inhibitors of cyclooxygenase-2 or antagonists of prostaglandin receptors may have therapeutic potential for treating painful diabetic neuropathy[7]. Flavonoids are reported to have anti-inflammatory activity but do not produce any apparent acute toxicity or gastric damage. Fenugreek seeds, inspite of anti-inflammatory activity did not show any ulcerogenic effect.

The curative and preventive property of TSP in diabetic neuropathy may be due to improvement in glucose intolerance, anti-inflammatory and antioxidant property. Since, *Trigonella foenum graecum* seed 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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