PHYTOCHEMICAL SCREENING, HPTLC ANALYSIS AND ANTIDIABETIC POTENTIAL OF TRIGONELLA FOENUM GRAECUM ON ALLOXAN INDUCED DIABETIC RATS.

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Abstract

Seeds are now serving as one of important hub for isolation and extraction of secondary metabolites in pharmaceutical industry. Our present investigation was carried out to screen the phytochemical constituents and HPTLC analysis of seed extracts of Trigonella foenum-graecum and its antidiabetic potential in alloxan-induced diabetic rats. Different solvent extracts such as petroleum ether, ethyl acetate, chloroform, methanol and aqueous were subjected to phytochemical screening. Methanol extract was found to possess more amounts of secondary metabolites than other solvent extracts. In addition, HPTLC analysis of the plant sample revealed the presence of 35 peaks by using trigonellin as standard marker. Different biochemical parameters such as blood glucose, cholesterol, protein, urea, creatinine and triglycerides level were subjected for estimation by collecting the blood samples from the treated diabetic rats after 28 days. A sharp decline in blood glucose, cholesterol, triglycerides, creatinine and urea level was noticed when methanolic extracts of Trigonella foenum-graecum were given to experimental animals when compared with negative control. Increase in protein and weight of the animal was noticed when treated with methanolic extracts of Trigonella foenum-graecum. It can be concluded that antidiabetic potential of Trigonella foenum graecum can be attributed to wide range of active pool of secondary metabolites present in the seed part. Further, screening and isolation of secondary metabolites along with their mode of action is required for effective use of plant-based drugs as antihyperglycemic agent.

Key words: Seed, Trigonella foenum graecum, antidiabetic activity, Trigonellin, HPTLC analysis

Introduction

Plants are an excellent source of drugs, and many of the currently available drugs have been derived directly or indirectly from them (Arunugam et al., 2013). It is obvious due to the richness and complexity of the compounds in plants. A multiple targeting is a double-edged sword in diabetes therapies. The multiple targets associated with antidiabetic herbal medicine could play a beneficial role in the control of diabetics. Diabetes mellitus (DM) is a major chronic metabolic disorder and an extremely serious condition from both clinical and public health standpoints. It is recorded that every 5th Indian have diabetic by 2025, it may be assumed 40 million diabetics in India expected to be 70 million by 2025 (Aubert and Hilary, 2000). Diabetes mellitus can directly affect serum lipid levels causing diabetic dyslipidemia which is one of its complications (Wen et al., 2012). Among the available therapeutic agents, insulin, metformin, sulfonylureas (SU) and thiazolidinediones

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(TZDs) are mostly used for the control of diabetes (Stein et al., 2013).

*Trigonella foenum-graecum* belongs to the family Fabaceae which grows up to 30-45 cm height. The fenugreek plant can grow under a wide range of climatic conditions and has demon-strated to contain a substantial amount of beneficial constituents, including fiber, phospholipids, glycolipids, selected steroidal saponins, including diosgenin, yamogenin, tigogenin, and neotigogenin, selected amino acids, including L-trypto-phan, 4-hydroxyisoleucine and lysine, oleic acid, linolenic acid, linoleic acid, choline, vitamins A, B1, B2, and C, nicotinic acid, potassium, niacin, micronutrients, and many other functional ele-ments (Bahmani et al., 2013; Zameer et al., 2017). The major bioactive compounds in fenugreek seeds are believed to be polyphenol compounds, such as rhaponticin and isovitexin (He et al., 2014). The seeds of fenugreek contain lysine and L-tryptophan rich proteins, mucilaginous fiber and other rare chemical constituents such as saponins, coumarin, fenugreekine, nicotinic acid, sapogenins, phytic acid, scopoletin and trigonelline, which are thought to account for many of its presumed therapeutic effects, may inhibit cholesterol absorption and thought to help lower sugar levels (Billaud, 2001). Therefore, the present study was made to evaluate the antidiabetic potential of methanolic seeds of *Trigonella foenum graecum* on alloxan-induced diabetic rats and to identify the bioactive compounds using HPTLC analysis.

**Materials and Methods**

**Collection of Plant Materials:**

Seeds of *Trigonella foenum graecum* which were procured from Tamil Nadu Agricultural College and Research Institute (TNAU), Madurai, Tamil Nadu was identified and authenticated by an botanist (also an expert in plant taxonomist) from our institute by referring standard taxonomic characteristic features (Keys) according to Flora of Madras Presidency (Gamble, 1935) and the Flora of Tamil Nadu Carnatic (Mathew, 1991). The voucher samples (SN-9718) and photographs were deposited in the institute for future reference.

**Selection and maintenance of experimental animals:**

Albino Wister strain rats of both sex, six months old that weighed 180-220g with a mean weight of 200g was used in this study. The animals were allowed to acclimatize for two weeks in the animal house at the Department of Pharmacy in Ultra College of Pharmacy, Madurai. The rats were housed in polypropylene cages, maintained under standard laboratory conditions. Animals were fed with standard laboratory pellets diet and water. Animal studies were done at Department of Pharmacy in Ultra College of Pharmacy, Madurai and experimental protocols and procedures were approved by Institute of Animal Ethics Committee of Ultra College of Pharmacy Institute (Approval Number: MKU/IAEC/KMCP/88/ P9718 Ph.D./2013).

**Extraction of Trigonella foenum graecum**

Seeds of *Trigonella foenum graecum* were washed thoroughly with running tap water to remove dust particles, adhering epiphytes etc. They were air-dried and crushed into powder in a grinding machine. The powder (0.5 kg) was extracted in Erlenmeyer flasks with different solvents viz., 90% petroleum ether, ethyl acetate chloroform and methanol and aqueous at room temperature. The whole extract was combined, filtered (Whatman filter paper No. 1) and concentrated at 40°C in vacuum and finally, the extract was freeze-dried to get 50 gm of crude extract.

**Preparation of Aqueous extract:**

Fresh plant materials (0.5 kg) were harvested and surface sterilized with 0.1% (w/v) HgCl₂ solution for 5 min. The plant material was grounded with mortar and pestle and the tissue was centrifuged at 3500 rpm for 20 min. The supernatant alone was taken as aqueous extract. All the extracts were concentrated by distillation and evaporated to dryness using a flash evaporator. After evaporation, each of the solvent extracts was weighed and preserved at 5°C in airtight bottles.

**Phytochemical analyses of seeds of Trigonella foenum graecum.**

Phytochemical screening of crude solvent extracts were performed using the following reagents and chemicals: alkaloids with Wagner reagent, tannins with 5% ferric chloride, saponins with ability to produce foam by adding water and olive oil, carbohydrates with Molish reagents and concentrated sulfuric acid, glycosides with glacial acetic acid, ferric chloride and concentrated sulphuric acid, steroids with chloroform, acetic anhydride and concentrated sulphuric acid, terpenoids with chloroform and concentrated sulphuric acid and fixed oil using spot and oil staining methods (Trease and Evans, 1996; Sofowara, 1993).

**HPTLC analysis**

**Development of chromatogram**

Sample solutions were applied onto the plates with automated Camag HPTLC system comprising of Linomat V. as sample applicator (Camag, Muttenz, Switzerland) and TLC Scanner III controlled by win CATS Software 1.4.3 was used for quantitative evaluation (Sethi, 1996).
A TLC scanner III with win CATS software was used for scanning the TLC plates, and pre-coated silica gel aluminium plates 60 F 254 20 × 10 cm with 0.2 mm-μm thickness (Merck, Darmstadt, Germany) were used for all determinations. The plates were pre-washed with methanol and activated at 60°C for 5 min, prior to chromatography. Five different aliquots (2, 4, 8, 12 and 16 μl) of standard solution were applied in triplicates on 20 × 10 cm TLC plates for the preparation of calibration curve. Six such plates were prepared. A constant application rate of 0.1 μl s⁻¹ was employed with a bandwidth of 6 mm. Bandwidth was set at 20mm. The mobile phase (10ml) consisted of toluene, ethyl acetate, formic acid and methanol (3:6:1.6:0.4) (v/v). The chamber saturation time for mobile phase was 15 min (optimum) at relative humidity of 60% ± 5. Chromatogram run length was 8.0 cm. After development, chromatographic plates were dipped into derivatization reagent i.e. modified Dragendorff reagent and again dried for 10 min. using hair drier on hot mode. After drying, the plates were heated at 70°C for 15 min. in a pre-heated oven. The formation of orange coloured spots corresponding to various phyto compounds of plant extracts was observed. The plates were scanned within 10 minutes using densitometric scanner III in the remission mode at 254 nm. The spots and or peaks were detected and their Rf values and peak areas were calculated.

Identification of the optimum alloxan-monohydrate dose to induce diabetes

Intraperitoneal optimum dose of alloxan to induce diabetes was done by using a logarithmic scale with 5 dose levels (50, 100, 150, 200 and 250 mg/kg body weight) (Thomson, 1985). The doses were intraperitoneally administered in 0.1 ml physiological saline once for each level to five albinos Wister rats. The animals were frequently monitored for changes in blood sugar within 24 to 48 hours. After 48 hours, the diabetic animals were examined for suitability in the bioassays by measuring blood glucose levels after every 2 hours consistently for 24 hours. The observations were compared and the optimum dose was selected for further studies.

Induction of Hyperglycemia

Hyperglycemia was induced in albino Wister rats from both sex aged 6 months (180-220 g body weight) experimentally by intraperitoneal administration of a single dose of 150 mg/kg body weight (identified as optimum dose) of a freshly prepared 10% alloxan monohydrate (2, 4, 5, 6 tetraoxyypyrimidine; 5-6-dioxuyracil) obtained from Sigma (Steinhein, Switzerland). Rats with Fasting Blood Glucose ≥ 200 mg/dl were considered as diabetic. Before initiation of this experiment, the animals were fasted for 8-12 hours but providing water until the end of this experiment and maintained at room temperature in plastic cages (Rees and Alcolado, 2005).

Acute toxicity study:

Wistar albino rats weighing between 150 and 220g were used for the study. In the first phase, rats were distributed to four groups of four animals each with fourth group as control (1 ml saline). Methanolic extracts of Trigonella foenum graecum (250, 500, 1000, 1500, 2000 mg/kg) were administered. In the second phase, three higher doses of 1000, 1500 and 2000 mg/kg of the extract were administered to each rat from each group. The animals were monitored for 24 hours for behavior and mortality. Further, they were all placed for more observation for 72 hours (three days) for the signs of physical changes, lethality, toxic symptoms, behavioral changes or deaths (Lorke et al., 1983).

Experimental design:

After observing acute toxicity study, 500 mg of methanol extract of Trigonella foenum graecum were selected as dosage for this study. Doses were prepared in distilled water by dissolving 500 mg of methanol extract of Trigonella foenum graecum with 1% Tween-80 as a surfactant. The experimental animals with an average weight of 180-220 grams were randomly divided into five groups of five animals each. The extracts were administered orally for 28 days.

Group I consisted of normal rats orally administered with 1.0 ml physiological saline and fed with normal pellet diet as positive control.

Group II consisted of alloxan induced diabetic rats orally administered with 1.0 ml physiological saline, as diabetic control (negative control).

Group III consisted of alloxan induced diabetic rats orally administered with 1.8-2.2 mg of glibenclamide, standard commercial drug (10 mg/kg body weight) in 1.0 ml physiological saline.

Group IV consisted of alloxan induced diabetic rats orally administered with seed extract of Trigonella foenum graecum (500 mg/kg body weight) in 1.0 ml physiological saline.

Duration of treatment

The extracts/drug treatment was given orally for 28 days. Fasting blood glucose levels were estimated on 0, 7, 14, 21 and 28 days. Similarly, the blood samples were withdrawn from all animals at 7 days interval up to 28 days by retro-orbital plexus. Serum glucose (Trinder et al., 1969), serum protein (Rajagopal and Ramakrishnan,
1983), serum cholesterol (Zlatkis et al., 1953), serum triglycerides (Werner et al., 1981), serum creatinine (Owen et al., 1954) and serum urea (Varley, 1991) were estimated using standard procedures. The initial body weight and final body weight were also measured. Finally, euthanasia was performed by cervical dislocation under deep anesthesia with 10% isoflurane.

**Statistical Analysis**

The data were collected from five replicates of each experimental study. Values were expressed as mean of total number of replicates ± SE and compared with diabetic control.

**Results**

**Acute Toxicity Study**

During acute toxicity study, the extracts did not produce any drug-induced harmful physical signs and no mortality was detected. There was no lethality and no toxic reaction was observed at any of the doses selected till the end of the treatment. This designates that extracts as a safety product.

**Phytochemical screening:**

*Trigonella foenum graecum* when subjected to phytochemical screening exhibited different types of phytocompounds under different solvent extracts. Among various solvents used, positive response for alkaloids, carbohydrates, saponins, phenols, tannins, flavonoids, gums and mucilages were observed in methanolic and aqueous extracts. In addition, proteins, aminoacids, fixed oils and fat showed positive results in methanolic extract. The seed extract prepared using ethyl acetate exhibited the presence of proteins, amino acids, fats and fixed oils. Presence of saponins and steroids at moderate level were detected in petroleum ether extract. In chloroform extract, proteins and aminoacids were the only phytocompounds found. It was observed that triterpenoids showed negative results in all the solvent extracts. As per overall analysis, methanolic extract exemplified positive results to majority of phytochemical compounds followed by aqueous extract table 1.

**HPTLC Analysis:**

HPTLC analysis of methanolic solvent extracts of seeds of *Trigonella foenum-graecum* was scanned under UV 254 nm. The peaks and Rf values obtained were tabulated. Extracts of *Trigonella foenum-graecum* seeds exhibited 35 peaks with different Rf values in the chromatogram. The peaks appeared to show start Rf at 0.39 and end Rf at 0.51 in 4.0 µl concentration. The chromatogram showed start height at 0.5, maximum height at 368.9 and end height at 1.7. The 18th peak in the chromatogram of *Trigonella foenum-graecum* seeds appeared at same Rf value (0.46) as that of respective marker (standard trigonellin) with peak area 6893.2 Fig. 1.

![Fig.1 HPTLC chromatogram of methanolic extract of T.foenum-graecum](image1)

**Antidiabetic potential of fruit peel of *Trigonella foenum graecum* on alloxan-induced diabetic rats**

**a) Change in fasting blood glucose level**

Alloxan induced diabetic rats when treated with Glibenclamide and methanolic extracts of *Trigonella foenum-graecum* seeds showed a gradual decrease in fasting blood glucose level. As expected, the blood glucose level of untreated diabetic rats (negative control) continued to increase significantly on the following days when compared with initial values throughout the study. Treatment with glibenclamide was found to decrease the fasting blood glucose level (44%) when compared with untreated animals. Maximum reduction (50%) was found in diabetic rats which received methanolic extracts of *T. foenum- graecum* Fig. 2.

**b) Change in blood glucose level**

The diabetic animals treated with standard drug Glibenclamide recorded a reduction of 59% in blood glucose level when compared to other individual plant extracts Fig. 3. The animals which received methanolic extract of *Trigonella foenum-graecum* seeds reduced the blood glucose level by 60% which is found to be similar to standard drug glibenclamide (59%).

**c) Change in body weight**

A gradual increase in the body weight of both the normal control rats and treated diabetic rats was noticed throughout the study. Continuous oral treatment of diabetic rats with *T. foenum-graecum* seed extracts and glibenclamide enhanced the body weight by 16% and 14% respectively Fig. 4.
d) Change in serum protein level

There was a gradual reduction in serum protein level from day 1 to day 28 over control Fig. 5. Intraperitoneal administration of *T. foenum-graecum* seed extracts to alloxan-induced diabetic rats enhanced the protein level in serum by about 70% over negative control at day 28. Administration of standard drug Glibenclamide also increased the protein level to about 53% which is found to be lesser than *T. foenum-graecum* treatment.

e) Change in serum cholesterol level

Treatment of diabetic rats with *T. foenum-graecum* plant extracts and Glibenclamide for 28 days caused a marked reduction in cholesterol level by 57% and 60% respectively Fig. 6.

f) Change in serum triglycerides level

In case of serum triglycerides level, diabetic rats when administered with standard drug glibenclamide brought down the level triglycerides by 56%. But, intraperitoneal administration of *T. foenum-graecum* reduced the triglyceride level by 71% which was found to be higher than standard drug Fig. 7.

g) Change in serum creatinine level

At the end of 28 days treatment, decrease in serum creatinine level was observed in the diabetic rats when supplemented with *T. foenum-graecum* seed extract (48%). But, 69% of reduction in serum creatinine was observed when diabetic rats were fed with standard drug glibenclamide. It was clear that the plant extracts exhibited 10% less reduction efficiency as compared to glibenclamide Fig. 8.

h) Change in serum urea level

The result of our study indicated that urea content was found to be lesser in the diabetic rats which received *T. foenum-graecum* seed extracts by 40% followed by glibenclamide treated animals (41%) Fig. 9.

**Discussion**

Positive response for alkaloids, carbohydrates, saponins, phenols, tannins, flavanoids, gums and mucilages, fixed oils and fats were observed in methanolic extracts. Different groups of bioactive compounds, alkaloids (Zhuo et al., 2010), amino acids (Hilles and Mahmood, 2016), flavonoids (Huang and Liang, 2000; Han et al., 2001), saponins (Taylor et al., 1997), anthocyanins, fiber, lipids, vitamins and traces of inorganic elements have been reported in fenugreek plant. Sumayya et al., (2012) reported that the *Trigonella foenum-graecum* had considerable amount of carbohydrates, phenols, sterols, saponins, quinones, alkaloids, terpenoids and tannins but there was only slight presence of proteins, glycosides and flavonoids. The phytochemical analysis of aerial parts of *Trigonella foenum-graecum* indicated presence of tannins, alkaloids, saponins and flavonoids, proteins, starch, amino acids, fats and fixed oils, glycosides (Swati et al., 2014; Darshana et al., 2015). Flavonoids modulate glucose metabolism or insulin sensitivity at different levels, increasing glucose uptake and insulin secretion, and inhibiting glucose production (Alkhalidy et al., 2018). Additionally, some flavonoids and polyphenols, as well as sugar derivatives, are found to be effective due to some other extrapancreatic mechanisms (Jung et al., 2006). Further, fenugreek saponin was identified as diosgenin which improved glucose level, HbA1c level and antioxidant enzymes after 30 days of use in diabetic rats (Fuller et al., 2015).

Trigonelline is the major component of alkaloids in fenugreek. In our study, the 18th peak in the chromatogram of *Trigonella foenum-graecum* seeds appeared at same Rf value (0.46) as that of respective marker (standard trigonellin) with peak area 6893.2. Earlier reports indicate that trigonelline reduces blood glucose concentrations in rats (Moorthy et al., 2009) and in human (Olthof et al., 2011). Trigonelline protects β-cells of the pancreas and increases insulin sensitivity index as well as insulin content (Zhou et al., 2013). Similarly, Subramanian and Prasath (2014) reported that trigonelline supplementation attenuated the elevated levels of glucose, glycosylated hemoglobin, AST, ALT and ALP. The insulin level was improved with an improvement in hepatic and muscle glycogen content of insulin resistant diabetic rats and also

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**Table 1:** Phytochemical screening of extracts of seed of *Trigonella foenum-graecum*

<table>
<thead>
<tr>
<th>Extracts</th>
<th>Petroleum Ether</th>
<th>Chloroform</th>
<th>Ethyl Acetate</th>
<th>Methanol</th>
<th>Water</th>
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<tr>
<td>Alkaloid</td>
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<td>++</td>
<td>+</td>
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<tr>
<td>Carbohydrates</td>
<td>-</td>
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<td>++</td>
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<tr>
<td>Saponin</td>
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<td>Triterpenoids</td>
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<td>Phenolic Compounds</td>
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<td>&amp; Tannins</td>
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<td>Proteins &amp; Aminoacids</td>
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<td>Steroids &amp; Sterols</td>
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<td>Fixed Oil &amp; Fat</td>
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<td>Flavones &amp; Flavonoids</td>
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<td>Gums &amp; Mucilages</td>
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<td>+</td>
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</tbody>
</table>

+++ = Maximum  ++ = Moderate  + = Minimum  - = not detected
Values given above bar are percent decrease over diabetic control.

concluded that trigonelline exhibit significant insulin sensitization activity as well as improvement in the glucose homeostasis probably due to improved pancreatic -cell function which is evident from improved plasma insulin level. Earlier studies indicate that administration of trigonelline to diabetic rats restored b-cells mass and architecture in response to glucose stimulation. Administration of fenugreek alkaloids to diabetic rats reduced blood glucose and this was accompanied by an increase in plasma insulin concentration (Zhou et al., 2013). These findings are consistent with earlier reports that the antidiabetic effect of fenugreek seeds and the pure compounds trigonelline and diosgenin might be mediated through their ability to improve insulin sensitivity in T2DM models (Back et al., 2012).

In our study, T. foenum-graecum extract exhibited positive responses in all biochemical constituents except serum urea level. Moreover, potential of the plant extracts was found to be equal to the potential of standard drug glibenclamide. Our findings were found to be in accordance with Hasona et al., 2017; Shashikumar et al., 2019; Sree Sudha et al., 2019). Trigonella foenum-graecum seed powder solution taken by newly diagnosed type II diabetic patients produced a significant reduction in TC, TG, and LDL-C levels and increase in HDL-C level (Geberemeskel et al., 2019). Moreover, Trigonella foenum-graecum (2 gm/kg) showed significant reduction in blood glucose, increase in GIP levels, and showed reduction in glucagon levels in alloxan induced diabetic rats (Inbaraj and Muniappan, 2019). Significant decrease in blood glucose, cholesterol, triglycerides, LDL, VLDL
levels, SGOT, SGPT, urea, creatinine and increase in HDL levels and body weight was found in alloxan induced diabetic rats when *Trigonella foenum graecum* extracts was supplemented along with other plant extracts (Sree Sudha et al., 2019).

The hypoglycemic effect of the tested extracts may be due to the active principles present in these extracts such as polyphenols and flavonoids (Bahadoran et al., 2013) which possess the properties of regenerating pancreatic â-cell, increasing insulin secretion, enhancing glucose uptake by adipose or muscle tissues, inhibiting glucose absorption from intestine and glucose production from the liver and resolving the problem of insulin deficiency (Hasona et al., 2017). Fenugreek seed extracts have also been reported to exhibit antidiabetic potential by delaying both gastric emptying time and rate of glucose absorption. It reduced uptake of glucose in the small intestine mainly due to its high fiber content that slows the metabolism of carbohydrates and lowered blood glucose (Patel et al., 2012). It also restores the function of pancreatic tissues, protecting â cells, evaluating serum insulin level possibly through the regeneration of â cells or stimulation of insulin release by the existing â islet cells (Bera et al., 2013). 4-Hydroxyisoleucine, a novel amino acid from fenugreek seed, was reported to increase glucose-stimulated insulin release by isolated islet cells in rats, mice, and humans. A specific amino acid called 4-hydroxyisoleucine, which represents 80% of the free amino acid in fenugreek seeds, was reported to possess insulin-stimulating properties, and enhance insulin sensitivity and glucose uptake in peripheral tissues (Shashikumar et al., 2019). In our study, fenugreek seeds exhibited antidiabetic potential which might be due to attributed to the qualitative and quantitative occurrence of phychochemical compounds such as 4-hydroxyisoleucine, trigonelline etc., which could have regulated the carbohydrate metabolism and increasing the secretion of â cells in islets of the pancreas and thus exhibiting hypoglycemic potential.

**Conclusion**

Presence of secondary metabolites and other compounds in the plant extract could have been responsible for exhibiting hypoglycemic activity. It may be due to wide range of active bioactive compounds present in *Trigonella foenum-graecum* that could have played a vital role by increasing the insulin secretion, reducing hepatic glucose output, regulating certain enzymes that are involved in carbohydrate metabolism. Therefore, it is important to characterize the phytochemicals and also to expose their mode of action in controlling this multi factorial endocrine disorder. Thus, the use of individual plant extracts might provide a prelude that extracts could serve as natural remedy for diabetes overcoming the toxicity issues associated with synthetic drugs.

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