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## GERMINATION OF FENUGREEK SEEDS IMPROVES HYPOGLYCAEMIC EFFECTS AND NORMALIZES INSULIN SIGNALLING PATHWAY EFFICIENTLY IN DIABETES

Omi Laila<sup>1,2</sup>, Imtiyaz Murtaza\*<sup>1</sup>, M Z Abdin<sup>2</sup> and Sageera Showkat<sup>1</sup>

Biochemistry and Molecular Biotechnology Laboratory<sup>1</sup>, SKUAST-K, Shalimar Campus, Srinagar-191121, Jammu and Kashmir, India.

Centre for Transgenic Plant Development<sup>2</sup>, Department of Biotechnology, Jamia Hamdard, Hamdard Nagar, New Delhi-110062, India.

### Key words:

Fenugreek sprouts,  
Hypoglycemic activity,  
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### Correspondence to Author: Imtiyaz Murtaza

Biochemistry and Molecular  
Biotechnology Laboratory,  
Biochemistry Section, Division of Post-  
Harvest Technology, SKUAST-K,  
Shalimar Campuss, Srinagar-191121.  
Jammu and Kashmir, India.

**E mail:** imz\_murtaza@hotmail.com

**ABSTRACT:** Recent reports show that germination process increases the hypoglycemic activity of seeds many fold, and can act in a better way for diabetes management. Despite large use of fenugreek seeds than any part of the plant for the treatment of broad range of maladies including diabetes, the anti-diabetic effects of germinated seeds have not been assessed. In the present study, the effect of 21 days oral administration of germinated fenugreek seed extracts (100mg/kg bw, 200mg/kg bw, and 300mg/kg bw) on various biochemical markers like blood, glucose, blood insulin, hepatic glycogen content and key molecular markers of insulin signalling pathway in STZ-induced diabetic rats were assessed. Compared to its respective seed extract, the aqueous sprout extracts from IL8 seed genotype showed significantly higher hypoglycemic activity against the diabetic state and the best dose was established to be 300mg/kg bw. The antidiabetic effect of sprout extracts were quite comparable to standard antidiabetic drug, Voglibiose (1mg/kg bw). The germinated seed extract normalised significantly, various dysregulated biochemical and molecular markers of insulin signalling pathway than their seeds including Akt2 and GSK-3 $\beta$  and GS in the diabetic rats within 21 days of experimental period. To best of our knowledge, we report for the first time that germination of fenugreek seeds increases their antidiabetic activity many fold than their respective seeds. We also demonstrated a mechanistic rationale of germinated fenugreek seeds to be used alone or as an adjuvant to current therapies for better management of diabetes.

**INTRODUCTION:** At present, one of the global challenges the whole world is facing is the epidemics of type 2 diabetes that, in turn is stimulating the quest for new concepts and targets for better treatment of this incurable disease.

Although, medicinal plants have been used to treat diabetes, since ancient times as reported in the Ebers papyrus in Egypt in 1550 BC, but unfortunately, such an emerging potential of phytotherapy research is not yet, included in the evidence-based medicine, especially with respect to the diabetes therapy. To date, out of 400 traditional plant treatments reported for diabetes, only a small number of them have received scientific and medical evaluation to assess their efficacy<sup>1</sup>. In this regard, the World Health Organization expert committee on diabetes has recommended that

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traditional medicinal herbs should be further investigated, in order to make them realistic possibilities for the proper management of diabetes<sup>2</sup>. In glucose metabolism, deregulation of the various insulin associated targets like phosphoinositide-3-kinase (PI-3K)/v-akt murine thymoma viral oncogene homologue (Akt), mitogen-activated protein kinase (MAPK) and AMP-activated protein kinase (AMPK) pathways, which are essential for glucose homeostasis, often results in obesity and diabetes<sup>3</sup>. A large number of reports indicate that medicinal herbs or plant extracts have potential to exert their hypoglycemic effects through modulation of various components of insulin signalling pathway<sup>4</sup>.

Fenugreek is one such traditional herb that has been extensively used as a source of anti-diabetic compounds, from its seeds, leaves and extracts. The hypoglycemic effect of fenugreek seeds has been studied in many animal model systems, as well as in humans in both type 1 and type 2 diabetes patients<sup>5</sup>. The biological and pharmacological actions of fenugreek seeds is mostly attributed to the variety of phytochemicals namely, quercetin, diosgenin, trigonelline and free amino acids such as 4-hydroxyisoleucine, present in them<sup>6</sup>. These chemical components of fenugreek seeds have been reported to possess diverse activities and serve as raw materials for the manufacture of various hormonal and therapeutic drugs in pharmaceutical industry.

Seed germination has been found to improve outstanding sources of health maintaining nutrients like glucosinolates, phenolics, selenium-containing components and isoflavones in seeds<sup>7</sup>. One of the recent studies on phenolic enriched pea sprouts suggests them to possess much higher hypoglycemic activity than their seeds, in relation to diabetes management<sup>8</sup>. Till date the anti-hyperglycemic effect of ungerminated fenugreek seeds reported in various animals and human studies do not provide any concrete experimental evidence of direct relationship between glucose metabolism and any component of the insulin signalling pathway. Therefore, the prime contribution of current research study will be the first step towards understanding the role of

germinated fenugreek seeds against diabetes and determines the specific biochemical and molecular targets of such phytochemical rich sprout extracts, in response to its anti-diabetic effects under *in vivo* conditions. Such kind of study is of invaluable importance for the proper management of this disease and its associated complications with dietary based nontoxic sprout extract.

## **MATERIALS AND METHODS:**

### **Plant material:**

Ten Fenugreek seed genotypes (IL1-IL10) used in this study were obtained from a wide range of agro-climatic zones of India including six genotypes from Division of Vegetable Science, Sher-e-Kashmir University of Agricultural Sciences and Technology, Kashmir, and four genotypes from local markets of Delhi, Bhopal, Kerala and Punjab, respectively. All the genotypes were identified and validated by subject experts of University. The collected seeds were re-grown under identical environmental conditions in SKUAST-K and the seeds collected from their respective plants were then, used for further analysis.

### **Seed germination:**

The collected seeds were soaked in flasks containing distilled water and kept on orbital shaking incubator cum B.O.D. incubator (Tanco, India) at a speed of 120 rpm for 24 hours. After 24 hours, the seeds were transferred to glass jars and germination at 25°C for four days. The jars were covered with cheese cloth and the seeds germinated in dark. The germinating seeds were washed alternately with distilled water.

### **Preparation of fenugreek aqueous extract for *in vivo* studies:**

The fourth day fully germinated IL8 sprouts (showing maximum hypoglycemic activity under *in vitro* conditions- data not shown) as well as their respective seeds were washed with distilled water, surface sterilised by soaking in 0.1% sodium hypochlorite and 0.05% nonidet P-40 (NP-40) for 30 seconds and rinsed thoroughly with distilled water. The samples were oven-dried and crushed to a fine powder in a grinding machine. The powdered samples (500 g) were then extracted with double distilled water in Erlenmeyer flasks at room

temperature. The maceration was carried out five times, each in 24 hours with occasional shaking and stirring. The whole extract was combined, filtered (Whatman filter paper No.1) and concentrated at 40°C in vacuo. For lyophilization, the crude water extract was poured onto the freeze dry sample tray and loaded into the lyophilizer (Esquire Biotech, India). Finally, the extract was freeze-dried to get 40g of lyophilized extract powder. The lyophilized powdered extract was then re-dissolved in distilled water, aliquoted and stored at -80°C.

### **Experimental animals and their diets:**

The experiment was approved according to the rules of University of Kashmir Animal Ethics Committee, India (Reg. No. 801/03/CA/CPCSEA). A total of 40 male albino rats of Wistar strain (120-150g) obtained from the Animal House of Indian Institute of Integrative Medicine (IIIM), CSIR Laboratories, Jammu, India, were used in the study. The animals were acclimatized to standard animal house conditions, (temperature  $24 \pm 1^\circ\text{C}$ , relative humidity  $55 \pm 5\%$ ) for 12 hours photoperiod in suspended wire meshed galvanized cages (4-6 rats/cage) for one week's time in University of Kashmir before the commencement of the experiment. During the entire period of study, the rats were supplied with a semi-purified basal diet and water *ad libitum*.

### **Toxicity analysis:**

Acute toxicity tests of aqueous extract of IL8 sprouts were carried out in normal male albino rats. Rats were orally dosed with 100 mg/kg bw, 250 mg/kg bw, 500 mg/kg bw, 1000 mg/kg bw, 2000 mg/kg bw or 3000 mg/kg bw individually. Each treated groups was closely observed at different time intervals (one hour, four hour, and intermittently for next six hours, 24 hour and 48 hours<sup>9-10</sup>). The study was continued further for next 21 days in order to check any delayed toxic effects on gross behavioural activities<sup>11</sup>. Their food consumption and growth rate were also examined once daily up to 21 days.

### **Oral starch tolerance test (OSTT):**

The oral starch tolerance test was performed in overnight fasted (18 hour) normal rats. Rats were divided into five groups (n=6) and each group fed

with either drinking water or 1mg/kg bw voglibiose (Positive control) or with 250mg/kg bw, 500mg/kg bw, 1000mg/kg bw, aqueous extract of IL8 sprouts, respectively. Starch (3g/kg bw) was fed orally 10 minutes prior to extract administration and blood was withdrawn through tail snipping at 30, 60, 90 and 120 minutes interval. The glucose levels were measured using commercial electronic one-touch glucometer (Life Scan Europe, Switzerland).

### **Induction of diabetes:**

Diabetes in experimental animals was induced by intra-peritoneal injection of streptozotocin (STZ) (Sigma Aldrich, India) at a dose of 60mg/kg bw prepared in 0.1M cold citrate buffer of pH 4.5. The rats of control group were administered with citrate buffer (pH 4.5) only, in place of STZ solution. STZ-injected animals exhibited severe glycosuria and hyperglycemia within 3 days and rats were stabilized over a period of 7 days. The diabetic state was assessed by measuring the blood glucose levels after 48 hours of STZ administration. The rats with fasting blood glucose levels  $>300$  mg/dl were considered diabetic and were included in the current study.

### **Experimental design and treatment schedule:**

Out of total 36 rats, the effect of aqueous extract from IL8 sprouts was studied in thirty STZ induced-diabetic rats in parallel to six untreated non-diabetic normal rats (NC) during the 21 days of experimental period. The STZ induced diabetic rats were randomly divided into six experimental groups-group 2, group 3, group 4, group 5, group 6 and Group 7), with six rats in each group. The rats of group 1 representing the NC(Normal control) group along with Group 2 representing DC (Diabetic control) group, were orally treated with distilled water (1ml) daily whereas, group 7 acted as positive control group against extract treatments and was only treated with standard antidiabetic drug, voglibiose orally (1 mg/kg bw) throughout the experimental period. The group 3 was given orally aqueous extract of ungerminated IL8 seed extract (200mg/ kg bw while as 4, 5 and 6 diabetic groups were treated with aqueous extract of IL8 sprout extracts at a dosage of 100mg/kg bw, 200g/kg bw, and 300mg/kg bw, respectively on daily basis for a period of 21 days. Blood glucose levels, serum insulin levels and body weight of all the six groups

were measured on 0, 7, 14 and 21 day of treatment. The blood samples needed for routine blood glucose/serum insulin estimation were obtained through tail snipping. Finally, on 21st day of experiment, animals in all the six groups were deprived from food for 3 hours and sacrificed by prolonged ether anesthesia. All the blood samples collected by cardiac puncture were centrifuged for 10 minutes in a table-top clinical centrifuge at 4,000 rpm. The resulting serum samples obtained were stored at 0°C in a freezer for further biochemical analysis. Liver was dissected out rapidly, washed with cold saline, blotted dry with filter paper and weighed. Portions of liver (100 mg) were immediately digested in 30% KOH solution and used for determination of glycogen content.

#### **Estimation of serum insulin, serum triglycerides and serum cholesterol levels:**

Serum insulin levels of all the six experimental groups were estimated by Erba Lisa Scan II Touch screen Elisa reader (Erba Diagnostic Manheim, Germany) using rat insulin ELISA kit (Merck Millipore, India). The total cholesterol and triglyceride levels in serum samples were determined enzymatically by kits obtained from Agappe Diagnostic Ltd (India), using semi-autoanalyzer (RMS, India).

#### **Determination of liver glycogen content:**

Glycogen content was measured in the rat liver samples according to the method of Carroll *et al*<sup>12</sup> using anthrone reagent.

#### **Western blotting:**

Frozen liver tissue samples, weighing 0.2 to 0.5 g were cut into shivers with scissor and washed with cold saline to remove blood. Further, the liver cell lysates from the tissue samples were prepared in cold lysis buffer [0.05 mmol/l Tris-HCl, 0.15 mmol/l NaCl, 1 mol/l EGTA, 1 mol/l EDTA, 20 mmol/l NaF, 100 mmol/l Na<sub>3</sub>VO<sub>4</sub>, 0.5% NP-40, 1% Triton X-100, 1 mol/l phenyl methylsulfonyl fluoride (PMSF) (pH 7.4)] with freshly added protease inhibitor cocktail Set III (Sigma Aldrich, India). The lysates were collected and cleared by centrifugation, and the resulting supernatant aliquoted and stored at -80°C. The protein content in the lysates was measured by BCA protein assay kit (Genetix Biotech, India) as per vendor's

instructions, using Bovine Serum Albumin (BSA) as standard. For western blotting, 40 µg of protein from each sample was resolved over 12% Tris-glycine polyacrylamide gels (Novex, Life technologies, India) under non-reduced conditions. The resolved proteins were transferred onto polyvinyl difluoride membrane (0.45 µm, Immobilon-P Transfer Membrane, Merck Millipore, India), and subsequently incubated in blocking buffer (5% nonfat dry milk/1% Tween 20, in 20 mmol/L TBS, pH 7.6) for 2 hours.

The blots were next incubated with appropriate primary antibody [Phospho-GSK3β (ser 9), Phospho-Akt2(ser 474) and Phospho-GS(ser 641)], washed, and subsequently incubated with appropriate horseradish peroxidase (HRP)-conjugated goat-antirabbit IgG antibody (Cell Signaling Technology, India) at 4°C. The blots were detected with chemiluminescence (Signal Fire™ ECL kit, Cell Signalling Technologies, India) followed by autoradiography using Kodak XAR-5 film (Sigma Aldrich, India). Equal loading of protein was confirmed by stripping the blots and re-probing with β-actin (Santa Cruz Biotechnology, Inc., Santa Cruz, USA).

#### **Statistical analysis:**

The statistical analysis of data generated in the current study was determined by using one way analysis of variance (ANOVA) as well as correlation tests. In the current study, the comprehensive statistical package SPSS (Version 20) for windows was used.

## **RESULTS AND DISCUSSION:**

### **Germination of fenugreek seeds and Extract preparation:**

Numerous medicinal plants have been used, since ancient times in various traditional systems of medicine worldwide, especially for the management of diabetes<sup>13</sup>. Keeping the global epidemics of diabetes in mind, the current study was carried out to evaluate under *in vivo* conditions the antidiabetic potential of germinated fenugreek seeds (IL8) owing to their maximum hypoglycaemic activity under *in vitro* conditions (Data not shown), among the selected ten genotypes (IL1-IL10). Such plant based products being rich source of bioactive constituents are more

desired to act as potent and effective anti-diabetic agents, owing to their lesser side-effects and low cost<sup>14</sup>. However, before recommending any plant based agent for health purpose, it should be thoroughly evaluated for its toxicity, because some plants/plant products with pharmacological properties are found to be toxic even at lower doses<sup>15</sup>.

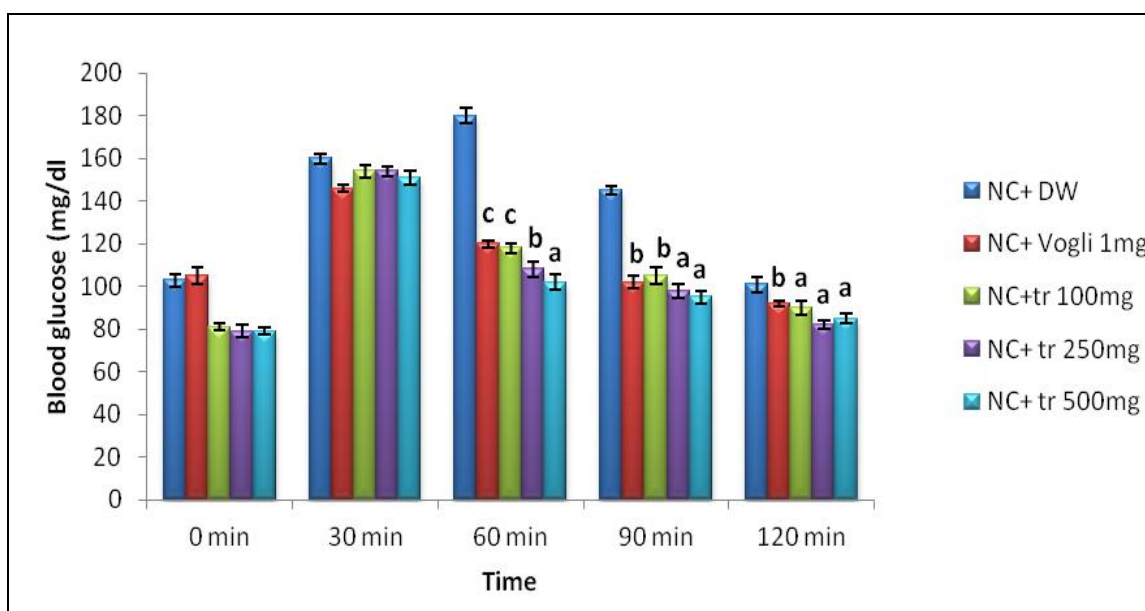
### Toxicity tests:

In order to assess any possible toxicity of fenugreek sprout extracts, the toxicity tests were carried out in normal wistar rats, using oral administration of different concentrations of IL8 sprout extract up to the highest dosage of 3000 mg/kg bw. Interestingly, no sign of toxicity or mortality was observed in any of the treated groups, even up to the highest dosage of 3000 mg of sprout extract/kg bw. The results revealed the non-toxic nature of fenugreek sprout extract as no lethality or any toxic reaction could be found with these selected doses until the end of the experimental period (21 days). The results obtained are not surprising because fenugreek is a nontoxic herb which is commonly used as a vegetable and spice throughout the world. Therefore, it became clear that the phytochemical rich fenugreek sprouts obtained through germination process are non-toxic, even if used at much higher doses and can be used as a potential antioxidant rich food against various oxidative

stress related degenerative diseases including diabetes.

### Oral starch tolerance test (OSTT) in normal wistar rats:

In order to evaluate the antidiabetic mode of action of specific plant extracts, some of the animal studies have focussed on analyzing the reduction in blood glucose levels after an oral starch tolerance test (OSTT), in which decline in starch digestion and absorption was expected immediately after extract ingestion<sup>16</sup>. OGTT, a normal routine blood test, in addition to OSTT, is used to assess metabolism of sugar in test subjects. Individuals are required to fast prior to consuming a fixed amount of glucose. Their blood sample is later analysed at designated time intervals in order to determine whether unusually high glucose levels will be reached in their blood<sup>17</sup>. Recently, radish sprout extracts have been reported to decrease blood glucose sugar levels within 120 minutes after extract ingestion, while performing OGTT test in diabetic rats<sup>18</sup>. Based on these findings, in the current study also, oral starch tolerance test was performed to identify the alteration of carbohydrate metabolism due to IL8 sprout extract treatment during post glucose administration. As shown in **Fig. 1**, the blood glucose levels of rats measured in between 0 to 120 minutes at different time intervals demonstrated a varied trend.



**FIG.1: EFFECT OF IL8 SPROUT EXTRACT ON ORAL GLUCOSE TOLERANCE TEST IN NORMAL SWISS ALBINO MALE WISTAR RATS**. Values are represented as Mean  $\pm$  SD; n = 6; cP < 0.05; bP < 0.01; aP < 0.001; P > 0.05 is considered as non-significant (ns). NC= Normal control; DW= Distilled water; tr = Extract treated; Vogli = Voglibiose; D= diabetes

The results suggest that starch loaded normal rats (control group) demonstrated an abrupt increase in blood glucose levels reaching to its maximum limit (42.77 % increase) of 180 mg/dl within 60 minutes after starch ingestion (3g/kg bw). However, in case of fenugreek sprout extract treated groups (100mg/kg bw, 250mg/kg bw and 500mg/kg bw), the blood glucose levels increased comparatively at much lower rate. In case of control group treated with distilled water, the blood glucose level were found to be higher (160mg/dl) within 30 minute after starch load as compared to extract (100mg/kg bw, 250mg/kg bw and 500mg/kg bw) treated groups i.e. 154mg/dl, 154mg/dl and 151 mg/dl, respectively.

STZ-induced diabetic rats have been reported to show regularly increased levels of blood glucose, and in order to decrease such glucose levels; various plant based regimes like fenugreek are being tested, owing to their lesser side-effects and low cost<sup>14</sup>.

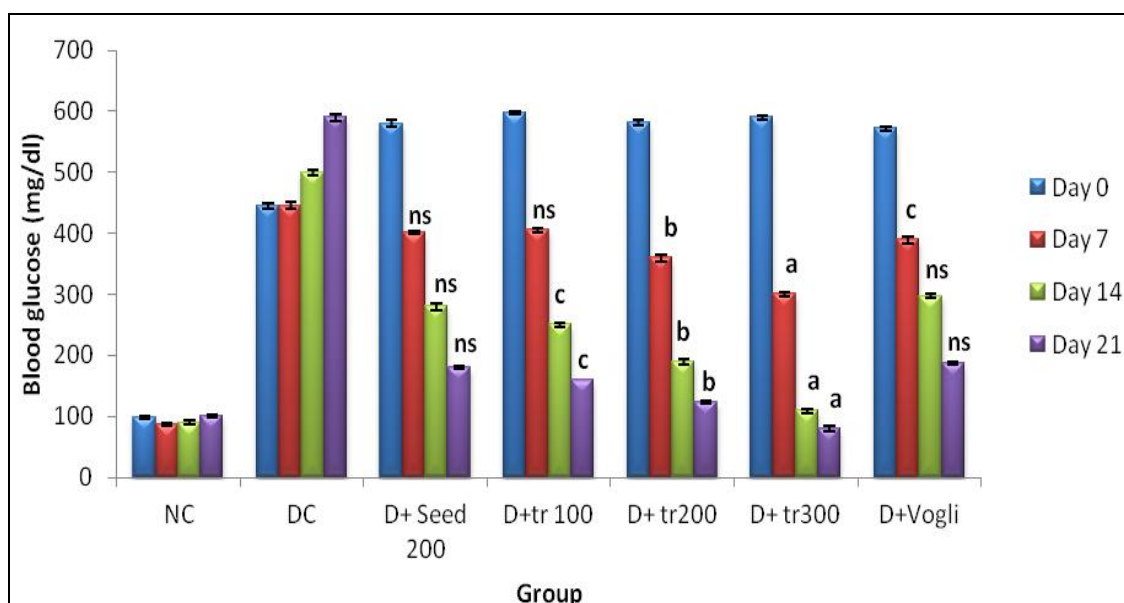
In the current study, the IL8 sprout extract treatment was observed to maximally delay the time of glucose absorption and reduce the area under the curve (AUC) of postprandial blood glucose levels, as evident from remarkable decrease in elevated blood glucose levels at 60 minute time interval after starch loading. It was found that compared to control group, the 100mg/kg bw sprout extract treated group showed 34.44% decrease in blood glucose levels within 60 minutes of starch load. Similarly, 250 mg/kg bw and 500 mg/kg bw caused 40% and 43.33% decrease in blood glucose levels, respectively at 60 minute interval as compared to 33.33% shown by a standard drug used against hyperglycemia i.e. voglibiose at a dose of 1 mg/kg bw. The blood glucose levels almost normalised within 90 minutes in extract treated group as compared to 120 minutes observed in distilled water treated group. In our study, the most potent dose of IL8 sprout extract causing maximum decrease in blood glucose levels in OSTT test was established to be 500 mg/kg bw. The determination of the mode of action of such food based medicines are thus need of hour and warrants scientific and systematic approach for their use as effective hypoglycemic agents.

### Effect of extract on overall blood glucose levels:

To carry out *in vivo* studies, in the present study; we preferred streptozotocin (STZ) to induce chemical diabetes in experimental animals (wistar albino rats) as compared to other diabetogenic agents. In mammals, this compound is toxic to insulin producing  $\beta$ -cells of pancreas. As a result of STZ,  $\beta$ -cells undergo destruction by necrosis leading to diabetes<sup>19</sup>. As evident from the **Fig. 2**, in all the six groups injected with a single intraperitoneal injection of STZ (60 mg/k.bw), in 95% cases a significant increase in the fasting blood glucose levels > 300mg/dl (Data not shown) were observed within 3 days<sup>4</sup>.

In the diabetic control group, the blood glucose level was constantly raised ( $\geq 590$  mg/dl,  $p < 0.001$ ) throughout the 21 days of study period. The increase was found to be almost more than 5 fold as compared to normal (101 mg/dl,  $p < 0.001$ ) control group. However, daily oral administration of IL8 sprout extract to diabetic rats caused significant decrease in elevated blood glucose levels throughout the 21 days experimental period. Interestingly, 100mg/kg bw of IL8 sprout extract demonstrated almost the same effects in fasting blood glucose levels as observed in diabetic group treated with 1 mg/kg bw standard drug voglibiose. However, the antihyperglycemic effects were more pronounced in 300mg/kg bw treated group followed by 200 mg/kg bw treated group. As compared to untreated diabetic control group, the 100mg/kg bw, 200mg/kg bw and 300mg/kg bw extract treated groups demonstrated encouraging results with 72.88 %, 78.98 %, and 86.44 % net reduction in blood glucose levels, respectively at the end of 21 days of experimental period.

The 1mg/kg bw voglibiose treatment, however, caused comparatively lesser (68.30%) effects. Interestingly, as evident from **Fig. 2**, IL8 seed extract treatment (200 mg/kg bw) caused comparatively much lesser glucose reducing effects than its germinated sprouts, and thus suggest fenugreek sprouts to be much more potent than their seeds in relation to hyperglycemia management. These results are in accordance to one of the recent studies indicating pea sprouts to possess higher hypoglycaemic activity than their seeds<sup>8</sup>.

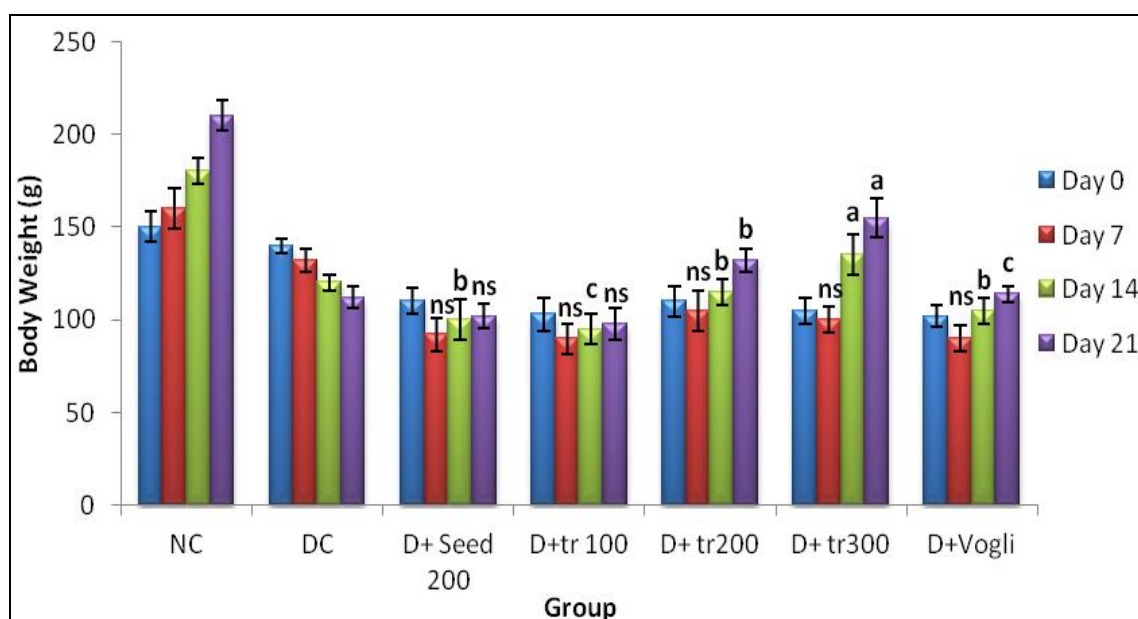


**FIG. 2: EFFECT OF IL8 SPROUT EXTRACT ON OVERALL BLOOD GLUCOSE LEVELS IN STREPTOZOTOCIN- INDUCED DIABETIC RATS.** Values are represented as Mean  $\pm$  SD; n = 6; cP < 0.05; bP < 0.01; aP < 0.001; P > 0.05 is considered as non-significant (ns). NC= Normal control; DC = Diabetic control; DW= Distilled water; tr = Extract treated; Vogli = Voglibiose

### Effect of extract on overall body weight:

Researchers have shown that hyperglycemia is intimately associated with decrease in body weight of diabetic animals and in STZ-induced diabetes, the characteristic loss of body weight occurs due to gluconeogenesis or catabolism of proteins and fats<sup>20</sup>. As expected in the current study also, STZ administration induced a drastic weight loss in the experimental animals compared to normal control group over a period of 21 days. In untreated diabetic control group, a significant decrease

corresponding to almost 20% reduction in total body weight was observed. However, as evident from **Fig.3**, in comparison to diabetic control group, a significant increase in net overall body weight of diabetic rats, corresponding to 16.66% and 32.25% was observed on treatment of 200 mg/kg bw and 300 mg/kg bw sprout extract, respectively. In voglibiose (1 mg/k.bw) treated diabetic group, an overall increase of only 10.52% was observed with respect to total body weight.



**FIG.3: EFFECT OF IL8 SPROUT EXTRACT ON OVERALL BODY WEIGHT IN STREPTOZOTOCIN- INDUCED DIABETIC RATS.** Values are represented as Mean  $\pm$  SD; n = 6; cP < 0.05; bP < 0.01; aP < 0.001; P > 0.05 is considered as non-significant (ns). NC= Normal control; DC = Diabetic control; DW= Distilled water; tr = Extract treated; Vog = Voglibiose

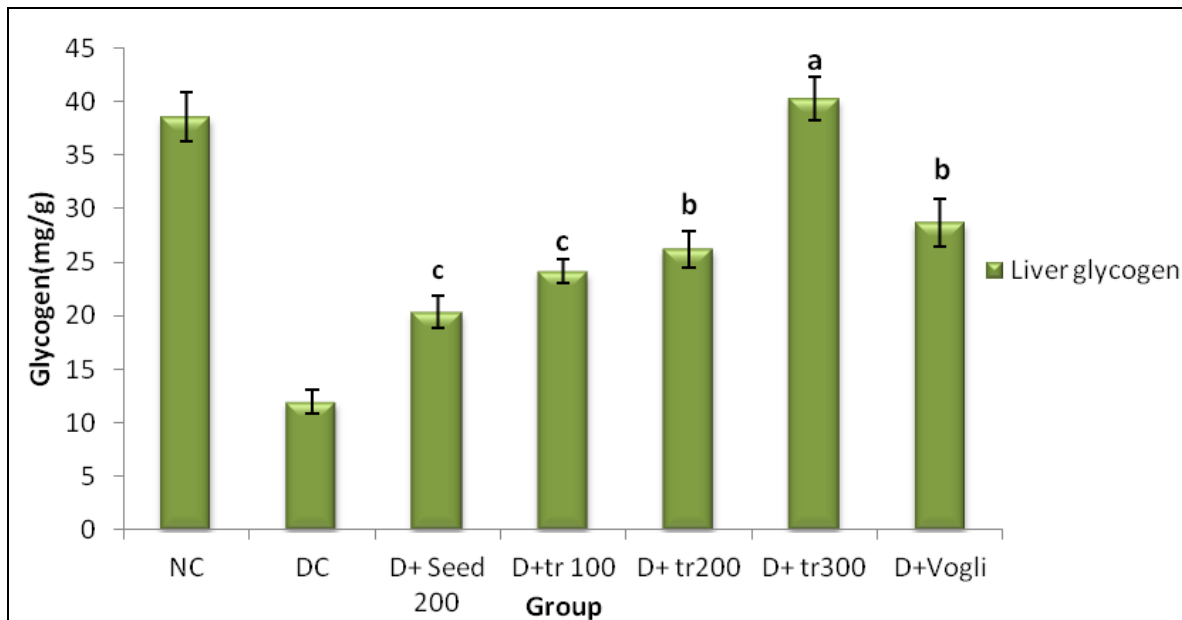
However, it was clear that 100 mg/kg bw dosage of IL8 sprout extract as well as dosage up to 200 mg/kg bw of IL8 seed extract treatment were unable to cause any significant change in overall body weight of STZ-induced diabetic rats. The potential mechanism of action involved by the fenugreek sprout extract may be most probably due to its protective effects against the muscle wasting i.e., reversal of gluconeogenesis<sup>21</sup>.

#### Effect of extract on liver glycogen content:

One of the major symptoms in diabetes, in addition to, defective glucose uptake of the cells, is the impairment of glucose storage capacity due to either poor pancreatic function and/or reduced glucose clearance<sup>22, 4</sup>. Liver (the master gland) is a major site for endogenous glucose

storage/production either by gluconeogenesis or glycogenolysis and plays an important role in the management of postprandial hyperglycemia. In light of these reports, in the current study, it was observed that the hepatic glycogen content showed a drastic decrease (71.85 %) in the liver samples of STZ- induced diabetic control group rats.

However, as shown in **Fig. 4**, IL8 sprout extract treatment for 21 days, significantly increased in a dose dependent manner the reduced liver glycogen content. Further, among the different concentrations used, it was found that the daily oral administration of 300 mg/kg bw of the extract almost normalized and restored the decreased glycogen levels within 21 days of experimental period.



**FIG. 4: EFFECT OF IL8 SPROUT EXTRACT ON SERUM INSULIN LEVELS IN STREPTOZOTOCIN- INDUCED DIABETIC RATS.** Values are represented as Mean  $\pm$  SD; n = 6; cP < 0.05; bP < 0.01; aP < 0.001; P > 0.05 is considered as non-significant (ns). NC= Normal control; DC = Diabetic control; DW= Distilled water; tr = Extract treated; Vogli = Voglibiose

The restored levels corresponding to 40.32 mg glycogen /g tissue were almost comparable to that of normal control group (38.59 mg glycogen/g tissue). The other two extract treatments (100 mg/kg bw and 200 mg/kgbw) also demonstrated encouraging results that almost paralleled with voglibiose treatment (28.72 mg glycogen/g tissue). However, the seed extract treatment (200mg/kgbw) demonstrated much lesser glycogen normalizing effects than its germinated sprout extract.

**Effect of extract on serum insulin levels:** Some research findings suggest that supplementation of

diet with fenugreek seeds in hyperglycemic rats prevent the increased levels of glucose by stimulating the process of glycolysis, inhibiting gluconeogenesis and subsequently increasing the secretion of insulin<sup>23</sup>.

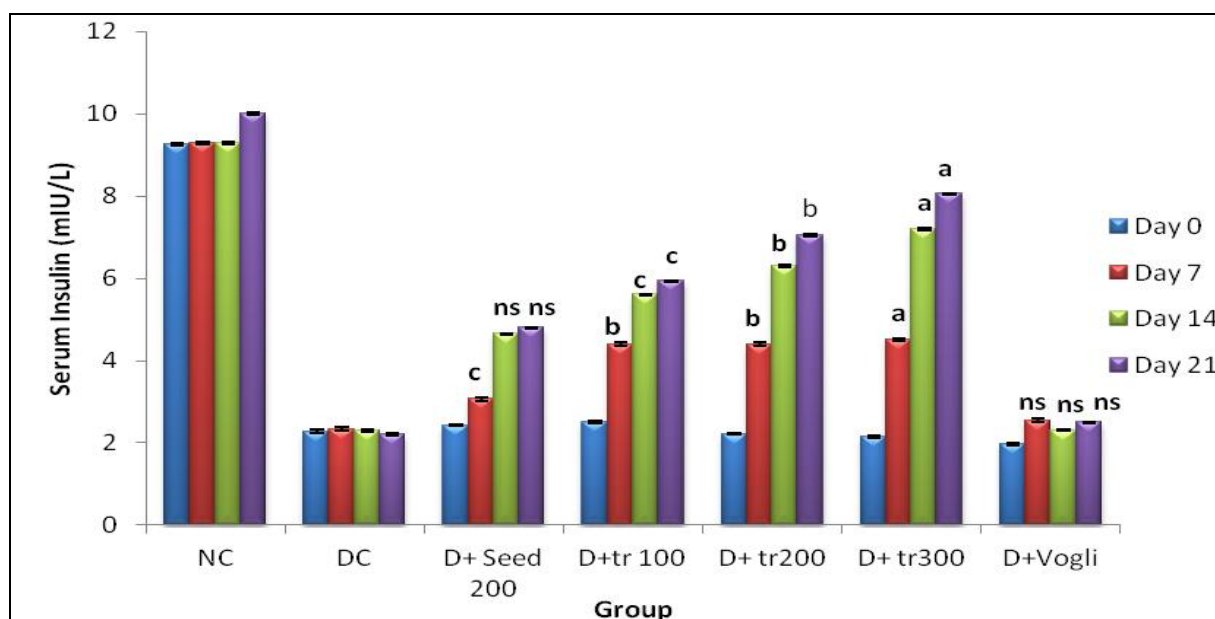
In the current study, as compared to normal control group, STZ administration was found to cause almost 70 fold reduction of serum insulin levels in diabetic rats after 21 days of experimental period. However, administration of three selected sprout extract doses (100mg/kg bw, 200mg/kg bw and 500mg/kg bw) efficiently reduced blood glucose



levels and increased insulin levels in STZ diabetic rats. From our investigation it was clear (Fig. 5) that oral administration of sprout extract i.e. 100 mg/kg bw, 200 mg/kg bw and 300 mg/kg bw, demonstrated insulinotropic property, as evidenced by significant increasing trend of serum insulin levels to 57.84%, 68.51%, and 73.29%, respectively compared to diabetic control group. These findings seem to be in accordance to the previous reports suggesting that hypoglycemic effects of natural plant extracts may be either due to their nature of inducing insulin secretion from pancreatic  $\beta$ -cells or possessing insulin like molecules or acting as insulin secretagogues<sup>18</sup>. Surprisingly, voglibiose was unable to cause any significant change in reduced serum insulin levels

in diabetic rats. Among the different doses used, 300mg/kg bw of IL8 sprout extract was found to be the best treatment that almost normalised the reduced serum insulin levels of diabetic rats within 21 days of experimental period.

Interestingly, in our study IL8 seed extract (200mg/kg bw) treatment demonstrated much lesser effects on serum insulin levels as comparison to their respective sprouted counterparts. Therefore, it becomes clear from our investigation that sprouts are better antidiabetic agents. Our argument is further strengthened by previous reports demonstrating that broccoli sprouts significantly improve insulin resistance among type 2 diabetic human subjects<sup>24</sup>.



**FIG.5: EFFECT OF IL8 SPROUT EXTRACT TREATMENT ON LIVER GLYCOGEN IN STREPTOZOTOCIN- INDUCED DIABETIC RATS.** Values are represented as Mean  $\pm$  SD; n = 6; cP < 0.05; bP < 0.01; aP < 0.001; P > 0.05 is considered as non-significant (ns). NC= Normal control; DC = Diabetic control; DW= Distilled water; tr = Extract treated; Vogli = Voglibiose

As clear from **Fig. 2** and **Fig. 4**, daily oral administration of IL8 sprout extracts for the 21 days of experimental period significantly improved plasma insulin levels and decreased blood glucose levels as compared to their seed extracts. In one of the recent studies, fenugreek seeds have been reported to significantly increase the reduced serum insulin levels of STZ-induced diabetic rats, via stimulation of insulin secretion and regeneration of the  $\beta$ -cells of the pancreas<sup>25</sup>. It is proposed that the possible antihyperglycemic mechanism involved by fenugreek sprout extracts may be either due to their insulin emission from the  $\beta$ -cells, followed by improved glucose transportation or consumption<sup>26</sup>.

#### Effect of extract on dysregulated insulin signalling pathway markers:

Insulin is a hormone released by pancreatic beta cells and triggers the uptake of glucose from blood into liver, adipose tissue and muscle and promotes its storage in the form of glycogen, lipids and proteins, respectively. Failure to uptake and store glucose from blood results in diabetes. Previous reports have shown the involvement of several mechanisms to explain the hypoglycemic action of fenugreek that includes modulation of insulin secretion and insulin-mimetic effects<sup>26</sup>. In Insulin signalling, among the key kinases, PI3-kinase leads to the phosphorylation of Akt, that triggers

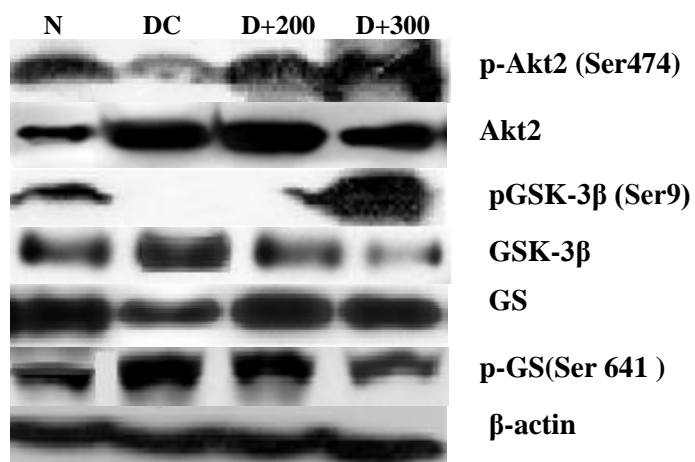
the phosphorylation of free cytoplasmic GSK3 $\beta$  and renders it inactive<sup>27-28</sup>. This in turn leads to the de-phosphorylation of downstream substrates such as glycogen synthase (GS) and thus eliciting an increase in glycogen synthesis. These are thus the possible candidate targets in diabetes therapy<sup>29-30</sup>.

Therefore, in the current study, an attempt was made to determine the putative antidiabetic action of fenugreek sprout extract on dysregulated Akt2, GSK3 $\beta$  and GS expression. As shown in **Fig. 6**, it is clear that IL8 treatment modulated the activation of these key chemotherapeutic targets of insulin signalling pathway. It was found that IL8 sprout extract treatment caused reasonable increase of phosphorylated levels of Akt2 in liver of diabetic rats, and finally leads to activation of glycogen synthase (GS) through its dephosphorylation via inactivation of GSK-3 $\beta$ . The activated glycogen synthase, then promotes glycogen synthesis, and, therefore, constitutes an important mechanism of glycemic control.

In our study, the liver samples obtained from diabetic rats orally fed with 300mg/kg bw of IL8 sprout extract daily for 21 days (**Fig. 6**) demonstrated the highest levels of phosphorylated GSK-3 $\beta$  (inactive form), in contrast to predominant unphosphorylated hepatic GSK-3 $\beta$  found in untreated diabetic rats.

It has been seen that this enzyme remains mostly active in the diabetic state and inactivates glycogen synthase via its phosphorylation and thus justifies the lowered levels of liver glycogen content seen in untreated diabetic control group. In accordance to previous reports, most probably the phytochemical rich IL8 sprout extract acts via phosphorylation of Ser-9/21 of GSK-3 $\beta$  and thus leading to inhibition of its kinase activity<sup>4</sup>.

In the present study, the liver samples obtained from diabetic rats treated orally with IL8 sprout extract (300mg/kg bw), daily for 21 days demonstrated the predominant levels of activated glycogen synthase (non-phosphorylated form), in contrast to maximum phosphorylated GS levels found in hepatic tissue of un-treated diabetic rats and thus corroborated well with previous reports on citrus fruits<sup>31</sup>.



**FIG. 6: WESTERN BLOT ANALYSIS DEMONSTRATING THE EFFECT OF IL8 SPROUT EXTRACT TREATMENT ON Akt, Gsk-3 $\beta$  AND GS EXPRESSION**

It is thus, quite clear that IL8 sprout treatment maintains higher levels of unphosphorylated form of glycogen synthase, a key player required in glycogen synthesis. Overall, the hepatic GSK-3 $\beta$  inhibition and activation of Akt as well as glycogen synthase in IL8 sprout extract treated diabetic rats may likely be due to potentiating of insulin production in the pancreas or its (sprout extract) mode of action mimics insulin like effects. The results clearly indicate that IL8 sprout extract treatment is capable of ameliorating the glucose metabolism by promoting glycogen synthesis and glycolysis in diabetic rats.

As shown in **Fig.6**, the western blot analysis clearly demonstrates that, in liver samples obtained from STZ-induced diabetic rats administered with aqueous extract of IL8 sprouts, the inactive (dephosphorylated) form of hepatic Akt2 was predominantly activated to phosphorylated (p-Akt2, ser-474) form, that in turn deactivated GSK-3 $\beta$  through conversion into its highly phosphorylated state (p-GSK3 $\beta$ , ser-9).

It was observed that hepatic Akt and GSK3 $\beta$  in non-treated diabetic rats (control) mostly remained in the non-phosphorylated state. The inactivation of GSK3 $\beta$  resulted in the increased conversion of glycogen synthase into its dephosphorylated state [p-GS (Ser 641) to GS] and activated it. The increased levels of non-phosphorylated form of GS thus, caused increased glycogen synthesis. Interestingly, treatment with 100 mg/kg bw of IL8 sprout extract couldn't cause any significant change, however, daily oral administration of 200 mg/kg bw and 300

mg/kg bw doses of IL8 sprout extract (Fig, 8) caused a significant difference. In diabetic rats, the dosage of 300 mg/kg bw almost restored these specific molecular markers within 21 days of treatment. Thus, at molecular level, it is clear that IL8 sprout extract exerts its hypoglycemic effects via restoration of insulin signal pathway through the modulation of Akt, GSK-3 $\beta$  and GS therapeutic markers.

**CONCLUSION:** It is concluded that under in vivo conditions, 4<sup>th</sup> day germinated IL8 sprout extract demonstrates the significant and beneficial glycemic control, has insulinotropic property, normalises liver glycogen content and normalises other related biochemical markers. It normalizes more efficiently various molecular markers of insulin signalling pathway than their seeds including Akt2, GSK-3 $\beta$  and GS in the diabetic rats, and can thus play a vital role in the treatment of diabetes as well as ameliorate liver and pancreatic damage at cellular level caused by diabetes. Although, through this investigation, it is clear that IL8 fenugreek sprout extract seems to be promising in the treatment of diabetes. It is, however, still early to recommend its use in humans. Before it can be considered as an important addition to the therapeutic armamentarium for treatment of diabetes, only thorough and full-fledged clinical studies can rationalise its use in humans.

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## REFERENCES:

1. Pandey A, Tripathi P, Pandey R, Srivastava R and Goswami S: Alternative therapies useful in the management of diabetes: a systemic review. *Journal of Pharmacy and Bioallied Science* 2011; 3(4): 504–512.
2. Murtaza I, Laila O, Abdin MZ, Parveen K, Raja T, Ali SA, and Sharma G: Maximum Phenylalanine Ammonium Lyase (PAL): Enzyme activity at Mid Stage of Growth Imparts Highest Hypoglycemic Property to Fenugreek. *Current Trends in Biotechnology and pharmacy* 2013; 7(4): 837-846
3. Stochmaova A, Sirotkin A, Kadasi A, Alexa R: Physiological and medical effects of plant flavonoid

- quercetin. *Journal of Microbiology, Biotechnology and Food Science* 2013; 2 (1): 1915-1926.
4. June CC, Wen LH, Sani HA, Latip J Gansau JA, Chin LP, Embi N, and Sidek HM: Hypoglycemic Effects of *Gynura procumbens* fractions on streptozotocin-induced diabetic rats involved phosphorylation of GSK-3 $\beta$  (Ser-9) in liver. *Sains Malaysiana* 2012; 41(8): 969–975.
5. Kulkarni CP, Bodhankar SL, Ghule AE, Mohan V, Prasad A, Thakurdesai PA: Antidiabetic activity of *Trigonella foenum graecum* L. seeds extract (IND01) in neonatal streptozotocin induced diabetic rats. *Diabetol Croat* 2012; 41(1): 29-40.
6. Mehrafarin A, Qaderi A, Rezazadeh SH, Naghdi-Badi H, Noormohammadi G, Zand E: Bioengineering of important secondary metabolites and metabolic pathways in fenugreek (*Trigonella foenumgraecum* L. *Journal of Medicinal Plants* 2010; 9(35): 1 – 18.
7. Khole S, Chatterjee S, Suchandra, Variyar P, Sharma A, Devasaayam TPA, Ghaskabdi S: Bioactive constituents of germinated fenugreek seeds with strong antioxidant potential. *Journal of Functional Foods* 2011; 6: 270-279.
8. Burguieres E, Mccue P, Kwon Y-I, Shetty K: Health-related functionality of phenolic-enriched pea sprouts in relation to diabetes and hypertension management. *Journal of Food Biochemistry* 2008; 32 (10): 3-14.
9. Kar A, Choudhary BK, Bandyopadhyay NG: Comparative evaluation of hypoglycaemic activity of some Indian medicinal plants in alloxan diabetic rats. *Journal of Ethnopharmacology* 2003; 84:105-108.
10. Han CC, Yuan JH, Wang YZ, Li LG: Hypoglycaemic activity of fermented mushroom of *Coprinus comatus* rich in vanadium. *Journal of Trace Elements and Medical Biology* 2006; 25: 191-196.
11. Mukund H, Rao CM, Srinivasan KK, Mamathadevi DS, Satish H: Comparison between the effects of alloxan and streptozotocin inducing diabetes in mice. *Pharmacognosy Magazine* 2008; 15: 819–824.
12. Carroll NV, Longley RW, Roe JH: The determination of glycogen in liver and muscle by the use of anthrone reagent. *The Journal of Biological Chemistry* 1956; 220: 586–593.
13. Pandhi L, Panda SK: Medicinal plants: Potential to fight superiority diseases. *Annals of Pharma Research* 2013;1 (1): 8-17.
14. Khan V, Najmi AK, Akthar M, Aqil M, Mujeeb M, Pillai KK: A pharmacological appraisal of medicinal plants with antidiabetic potential. *Journal of Pharmacy and Bioallied Science* 2012; 4 (1), 27–42.
15. Choudhury C, Bawar IM, Sharma GD: Evaluation of the harmful effect of a medicinal plant extract on mice brain. *Indian Journal of Applied Research* 2013; 3(6): 36-39.
16. Violet K, Fatima U and Imad H: Evaluation of the acute antihyperglycemic effects of four selected indigenous plants from Jordan used in traditional medicine. *Pharmaceutical Biology* 2011; 49(7): 687-695.
17. American Diabetes Association. Standards of medical care in Diabetes. *Diabetess Care- The Journal of Clinical and Applied Research and Education* 2015; 38(1).
18. Survay NS, Ko EY, Upadhyay CP, Mi J, Park SW: Hypoglycemic Effects of Fruits and Vegetables in Hyperglycemic Rats for Prevention of Type-2 Diabetes. *Korean Journal of Horticultural Science and Technology* 2010; 28(5): 1-10
19. Goyary D, Sharma R: Dietary restriction prevents diabetogenic effect of streptozotocin in mice. *Indian*

- Journal of Biochemistry and Biophysics 2010; 47: 254-256.
20. Zafar M, Naqvi SN: Effects of STZ-induced diabetes on the relative weights of kidney, liver and pancreas in albino rats: A comparative study. *International Journal of Morphology* 2010; 28(1): 135-142.
  21. Kumar V, Ahmed D, Anwar F, Ali M, Mujeeb M: Enhanced glycemic control, pancreas protective, antioxidant and hepatoprotective effects by umbelliferon- $\alpha$ -D-glucopyranosyl-(2I $\rightarrow$ 1II)- $\alpha$ -Dglucopyranoside in streptozotocin induced diabetic rats. *Springer plus* 2013; 2: 639-43.
  22. Soni B, Paramjit C, Rajbir S: Effect of sprouted fenugreek seeds on non insulin dependent diabetics. *Journal of Research* 2009; 46(1-2): 90-93.
  23. Kaur J, Singh H, and Khan MU: Multifarious therapeutic potential of fenugreek: A comprehensive review. *International Journal of Research and Pharmaceutical Biomedical Science* 2011; 2(3): 853-871.
  24. Bahadoran Z, Tohidi M, Nazeri P, Mehran M, Azizi F and Mirmiran P: Effect of broccoli sprouts on insulin resistance in type 2 diabetic patients: a randomized double-blind clinical trial. *International Journal of Food Science and Nutrition* 2012; 63 (7): 767-771.
  25. Kumar V, Anwar F, Ahmed D, Verma A, Ahmed A, Damanhoury ZA, Mishra V, Ramteke PW, Bhatt PC, and Mujeeb M: *Paederia foetida* Linn. leaf extract: An antihyperlipidemic, antihyperglycaemic and antioxidant activity. *BMC Complimentary and Alternative Medicine* 2014; 14: 76-81
  26. Santiago SI, Christudas S, Veeramuthu D and Savarimuthu I: Antidiabetic and antioxidant activities of *Toddalia Asiatica* (L.) Lam. Leaves in Streptozotocin induced diabetic rats. *Journal of Ethnopharmacology* 2012; 143: 515–523.
  27. Mackenzie RWA and Elliott BT: Akt/PKB activation and insulin signaling: a novel insulin signaling pathway in the treatment of type 2 diabetes. *Diabetes Metabolic Syndrome and Obesity* 2014; 7: 55–64.
  28. Maixner DW and Han-Rong Weng H-R: The Role of Glycogen Synthase Kinase 3 Beta in Neuroinflammation and Pain *Journal of Pharmacy and Pharmacology (Los Angel)* 2013; 1(1): 001.
  29. Nachar A, Vallerand D, Musallam L, Lavore L, Badawii A, Arnason J *et al.*: The action of antidiabetic plants of the canadian james Bay Cree traditional pharmacopeia on key enzymes of hepatic glucose homeostasis. *Evidence Based Complementary and Alternative Medicine* 2013; 4: 1-9.
  30. Akhtar M and Bharatam PV: 3D-QSAR and molecular docking studies on 3-anilino-4-arylmaleimide derivatives as glycogen synthase kinase-3 $\beta$  inhibitors. *Chemical Biology and Drug Design* 2012; 79: 560-71.
  31. Shen W and Lu YH: Molecular docking of citrus flavonoids with some targets related to diabetes. *Bangladesh Journal of Pharmacology* 2013; 8: 156-170

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