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Original article

Enhancement of nutraceutical and anti-diabetic potential of fenugreek (Trigonella foenum-graecum). Sprouts with natural elicitors

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ABSTRACT

Trigonella foenum-graecum has been extensively used for centuries in traditional medicine systems for the cure of health ailments including diabetes. Improving the medicinal attributes of plants through the elicitation strategy is gaining great interest in the recent past. In the current study, an attempt is made to reveal the role and possible mechanism of action of vitamin C elicit phytochemical-rich aqueous extract of 4th day germinated IM6 genotype fenugreek sprouts in the form of lyophilized powder (IM6E) under both *in vitro* and *in vivo* conditions. The IM6E demonstrated strong α-glucosidase activity (95.24 %) and moderate α-amylase and invertase inhibition activities under *in vitro* conditions. The High Performance Thin Layer Chromatography (HPTLC) based analysis demonstrated that IM6E possess significantly higher concentration of phenolic phytochemical quercetin (0.148 %) as compared to diosgenin and trigonelline bioactive anti-diabetic nutraceuticals. In normal rats after loading with glucose and sucrose, the IM6E administration in a dose-dependent manner significantly reduced the post-prandial hyperglycemia, in a similar fashion as the anti-diabetic drug voglibose as evident from the area under curves (AUC) of oral glucose tolerance test (OGTT) and oral sucrose tolerance test (OSTT) tests. The administration of IM6E in streptozotocin (STZ) induced diabetic rats drastically improved the antioxidant activity of plasma in them as determined by Ferric Reducing Ability of Plasma (FRAP) and the effect was found to be dose-dependent. The oral administration of IM6E in diabetic rats normalized almost all the deregulated biochemical markers like liver enzymes, lipids and significantly decreased higher blood glucose levels with increasing insulin levels as compared to diabetic control. The best concentration of IM6E was found to be 300 mg/kg b.w after 21 days of experimentation. The intra-peritoneal glucose tolerance test (IPGTT) in diabetic rats responded very well to IM6E treatment and 100 mg/kg.b.w. behaved almost like the administration of 0.5U insulin/kg bw, and thus indicating the insulinotropic nature of IM6E. Our findings clearly reveal the use of IM6E for diabetes management and at the same it possesses great potential when combined with voglibose to ameliorate diabetes and its associated complications to a greater extent due to synergistic effects as compared to monotherapy. However, more clinical trials need to be performed before recommending IM6E as an anti-diabetic alternative medicine.

1. Introduction

Diabetes mellitus is a complex metabolic disorder characterised by persistent hyperglycaemia leading to impaired insulin sensitivity, insulin resistance, or perhaps both as a result of changes in carbohydrate, lipid, and protein metabolism (Bene et al., 2018). Globally, the prevalence of this condition is increasing, and is expected to reach 592 million people by 2035, with China

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representing the world's largest diabetic population (98.4 million) followed by India which has the second-largest diabetic population of around 61.5 million (Saeedi et al., 2019). This increasing trend in diabetes is due to its complex pathophysiology and lack successful preventive and therapeutic strategies against it (Kahn et al., 2014). Although, there are various treatment regimens available commercially that are used primarily to save a life and prevent long-term diabetic complications, eliminate various risk factors and increase longevity, but unfortunately due to their chemical nature, limited action and pharmacokinetic properties, associated side effects, cost, and psychological factors, their use is restricted (Zheng et al., 2018). Such therapies, although, to some extent balance the metabolic alterations associated with diabetes, but inevitably does not correct the original biochemical lesion (El-Dakak et al., 2013), and thus highlighting the dire need to develop novel hypoglycaemic agents/therapeutic strategies by methodical and scientific exploration of an enormous pool of biological and natural products, especially those derived from plants. Natural remedies derived from traditional medicinal herbs are often regarded as nontoxic, having fewer negative impacts than the synthetic ones (Malini et al., 2011). Since ancient times, several traditional medicinal plants or their extracts have been used as folklore medicine to treat diabetes. More than 1200 species of plants have been reported for their alleged hypoglycaemic activity and 200 compounds of plant origin possessing glucose-lowering property (Salehi et al., 2019). However, such medicinal plants with health claims, requires scientific backing to ascertain their effectiveness, and toxicity before recommending them as alternative medicines in therapeutic strategies against diabetes (Aly et al., 2010).

Fenugreek (*T. foenum-graecum)* with an array of bioactive chemicals in its seeds and leaves is an anti-diabetic medicinal herb that has a history of being used as folklore medicine for centuries (Laila and Murtaza, 2015). The presence of a variety of bioactive constituents (quercetin, diosgenin, trigonelline) and free amino acids (4-hydroxyisoleucine) in fenugreek seeds are primarily responsible for their pharmacological and biological activities (Mehrafarin et al., 2011). In addition, these bioactive agents are also known to be effective in decreasing the incidence of various chronic and degenerative diseases. Compared to seeds, the sprouts are reported to be more beneficial and the germinated mung bean, broccoli, sunflower, buckwheat, and chickpea sprouts have been reported to exhibit much stronger anti-diabetic activity than their seeds under *in vivo* conditions (Laila and Murtaza, 2014). Reports indicate that bioactive constituents of seeds can be improved through germination and such a process is of tremendous commercial importance (Fuller and Stephens, 2015; Geberemeskel et al., 2019). As per recent report, in contrast to genetic engineering, elicitation of bioactive compounds in sprouts through natural elicitors are a better option, as it is a cheaper and socially acceptable method for improving plant food functionality (Gawlik-Dziki et al., 2013). The present study was designed to investigate the anti-diabetic effects of vitamin C elicited phytochemical-rich lyophilized aqueous extract of 4th day germinated *T. foenum-graecum* sprouts (IM6E) using both *in vitro* and *in vivo* conditions.

2. Materials and methods

2.1. Seed treatments and germination

In the current investigation, seeds of ten different fenugreek genotypes (IM1-IM10) were collected from the Division of Vegetable Sciences, Sher-e-Kashmir University of Agricultural Sciences and Technology, Shalimar, Kashmir, India and each genotype authenticated by the Head, Division of Vegetable Sciences, SKUAST-K. All ten collected seed genotypes were treated with three different concentrations (50 μM, 100 μM, 500 μM) of Vitamin C from SRL, India prepared in aqueous solution for 4 hrs as natural phytochemical elicitor, and then germinated separately in seed germinator under a varied range of temperatures (16 °C–22 °C). The vitamin C-treated seed genotypes along with untreated controls were evaluated daily for various physicochemical quality traits as well as bioactivities till the 10th day of germination adapting standard procedures (Data unpublished). The best day of germination elicit maximum nutraceuticals with vitamin C treatment among the selected genotypes as well as the genotype showing the maximum phytochemical elicitation and bioactivity in this study was selected for further investigations.

2.2. Freeze drying and phytochemical analysis

The vitamin C treated (500 μM) 4th day IM6 sprouts that showed maximum elicitation of overall nutraceuticals were shade-dried and ground to a coarse powder. The sprout powder (1.5 kg) was extracted with 90 % double distilled water at room temperature, and then freeze-dried to yield 150gms of aqueous extract (IM6E). The diosgenin, quercetin and trigonelline in IM6E were measured by HPTLC (Gopu et al., 2008; Laila et al., 2014). The peak areas corresponding to the peak areas of standards diosgenin, quercetin and trigonelline were used for their quantification in samples using regression equations and total phenolic content according to the previously described method (Malik and Singh, 1980). The standard quercetin,trigonelline and diosgenin reference compounds were procured from Sigma chemicals (India).

2.3. In vitro anti-diabetic activity of IM6E

The α-amylase, α-glucosidase and invertase inhibition activity of IM6E was determined according to the methods described earlier (Sumner and Howell, 1935; Worthington, 1993a, 1993b).

2.4. Experimental animals and their diets

In this study, male albino Wistar-rats (150–250 g) of six to eight weeks old were procured from IIIM CSIR-lab, Jammu, India. The rats were accustomed to a standard laboratory environment (temperature 24 °C, relative humidity 55 %, 12-hour photoperiod) in stainless steel wire-bottomed cages for one week before the start of the experiment. The rats were fed a semi-purified commercial basal diet and *ad libitum*. The Institutional Animals Ethical Committee (IAEC) of the University of Kashmir ((Reg. No. 801/03/CA/CPCSEA), J&K India) approved the experimental work.

2.5. Toxicity analysis

The IM6E was evaluated for acute toxicity in 6 male Wister albino rats weighing approximately 150 to 250 g. The animals were categorised into two groups: the control group (tap water) and the experimental group (received IM6E). Rats were administrated orally up to a maximum of 3000 mg/kg b.w IM6E test dose. The rats were intensively observed for 24-hours for any fatalities and for 21 days to rule out any late acute toxicity on gross behavioural activities. Food consumption and growth of rats were also monitored on daily basis for a period of 21 days.

2.6. Oral glucose tolerance test (OGTT) and oral starch tolerance test (OSTT)

The oral glucose tolerance test was carried out on 30 normal rats fasted overnight (12 h) and divided into five experimental groups (n = 6). In the morning, blood samples were collected from

the tail of each rat by venepuncture and their blood glucose levels were determined (0 h). Next, each rat in the negative control group received equal volume of a vehicle only (water) as treatment and the positive control group received 1 mg/kg b.w. standard drug voglibose. The remaining three groups separately received IM6E 100 mg/kg b.w, or 200 mg/kg b.w or 300 mg/kg b.w, respectively. Ten minutes later, glucose load of 2 g/kg b.w was administered to each rat in five groups and blood samples were collected from each group at the intervals of 30 min, 60 min, 90 min, and 120 min. The blood glucose levels were measured by using commercial electronic one-touch glucometer (Life Scan Europe, Switzerland). The oral starch tolerance test was carried out in the same way as above, but in this test starch (3 g/kg b.w.) was used in place of glucose. The Area Under the Curve (AUC) during the OGTT and OSTT were calculated using GraphPad Prism 8.0 software (La Jolla, CA, USA).

2.7. Induction of diabetes and treatment schedule

After the acclimatisation period was over, 54 Wistar albino rats were randomly divided into nine experimental groups with six rats each per group including the Normal control group (NC), Diabetic control group (DC) and six diabetic (D) treated groups (i.e. D + Seed 300 mg/kg bw, D + 300 mg/kg bw unelicited IM6E, D + 100, 200, and 300 mg/kg bw elicited IM6E groups, respectively) and a positive control group (D + voglibose 1 mg/kg bw). Further, D + 1 mg/kg bw voglibose + 300 IM6E mg/kg bw was also included in the study to investigate the existence of synergistic effects of the extract. For induction of diabetes, the selected groups of rats were fasted for 12-hours before being given a single intraperitoneal injection of STZ (Sigma Chemicals, India) at a concentration of 60 mg/kg b.w. The STZ was prepared fresh and dissolved in citrate buffer (0.01 M, pH 4.5). The blood glucose levels of selected rats were examined before and 72- hours after STZ injection, in order to confirm the onset of diabetes. The STZ treated animals with fasting blood glucose levels > 250 mg/dl) were considered as diabetic and included in the study. The study was carried out for 21 days with a daily treatment schedule. Finally on the 21st day, blood was collected for the estimation of blood glucose and serum insulin levels. Serum insulin was estimated using a Rat Insulin ELISA kit. The liver was quickly dissected, rinsed in cold saline, dried using filter paper, and weighed. Glycogen content was determined by rapidly digesting portions of the liver (100 mg) in 2 mL of concentrated 30 % KOH solution.

2.8. Antioxidant activity assay

The antioxidant activity of IM6E was performed by FRAP assay, based on the antioxidant biomarker ferric reducing ability of plasma (Benzie and Strain, 1996). In brief, 40 µL of plasma was mixed with 1.2 mL of working FRAP solution in a test tube. At 4 min after sample-reagent mixing, the absorbance at 593 nm was measured against a reagent blank at 37 °C using a UV-1600 spectrophotometer (Shimadzu Co., Kyoto, Japan). Blood was collected by tail venipuncture and the plasma separated to determine FRAP by the standard procedure using TPTZ-reagent. The FRAP values were obtained in triplicate and expressed as µM ferric ions reduced to ferrous ions per Litre (L) of plasma. Normal Control (treated with vehicle), Diabetic Control (treated with vehicle) and Positive Control (treated with voglibose) were used for comparison.

2.9. Intra-peritoneal glucose tolerance test (IPGTT)

For this, another set of 54 rats (9 groups consisting of 6 rats each) were subjected to Intra-peritoneal glucose tolerance test (IPGTT). The known quantity of IM6E dissolved in distilled water was dialysed. After overnight fasting, IPGTT was performed by administration of an i.p. injection of glucose (2 g/kg) to the rats. Dialyzed IM6E at a respective dose of 10 mg/kg, 50 mg/ kg and 100 mg/kg was injected 10 min after i.p. injection of glucose to different groups (NC + tr 10, NC + tr 50, and NC + tr 100, D + tr 10, D + tr 50, and D + tr 100 mg/kg bw, D + Insulin 0.5U/ +10 mg/kg bw respectively). A positive control group (D + 1.5U insulin/ kgb. w), negative control group (D + tap water); and normal control NC were also included in the study. Blood glucose levels were measured as mentioned for OGTT and OSTT.

2.10. Estimation of serum insulin, serum triglycerides, serum total cholesterol levels and liver glycogen levels

Serum insulin concentrations were measured in all six experimental groups using a rat insulin Elisa kit (Merck Millipore, India). The serum total cholesterol and serum triglyceride levels were evaluated enzymatically using semi-autoanalyzer kits obtained from Agappe Diagnostic ltd (India) (RMS, India).

Glycogen content was measured in the rat liver samples according to Carroll et al method (1956).

2.11. Estimation of liver enzyme markers

On the 21st day, all the animals were slaughtered and the biochemical status of the liver enzyme indicators including serum alanine transaminase (ALT), aspartate transaminase (AST), and alkaline phosphatase (ALP) was determined using an autoanalyser and Erba diagnostic kits (Kumar et al., 2011).

2.12. Statistical analysis

For statistical analysis, SPSS IBM SPSS statics for Windows, version 23.0 (IBM Corp., Armonk, N.Y., USA) was utilised. The statistical analysis of the collected data was performed using a one-way analysis of variance (ANOVA) and correlation tests.

3. Results

3.1. Quantitative phytochemical analysis

The 500 µM vitamin C treatment on the 4th day of germination with an optimum temperature of 22 °C proved to elicit maximally selected nutraceutical contents as well as bioactivities in all the ten genotypes of fenugreek sprouts and among the ten genotypes, IM6 comparatively showed the best quality traits (Data Unpublished). Therefore, on the basis of these results, IM6 sprouts of *T. foenum-graecum* were selected for further investigations in its lyophilized form (IM6E). As shown in Table 1., the phytochemical analysis of IM6E confirmed the presence of the appreciable amount of nutraceuticals including total phenols (46.20 ± 40 mg/g), trigonelline (0.286 ± 0.008 % w/w), diosgenin (0.095 ± 0.003 % w/w) and quercetin (0.148 ± 0.004 % w/w) present in IM6E, while using the

Table 1
Phytochemical analysis of IM6E.

Constituent	Concentration
Total Phenols	46.20 ± 40 mg/g
Trigonelline	0.286 ± 0.008 %
Diosgenin	0.095 ± 0.003 %
Quercetin	0.148 ± 0.004 %

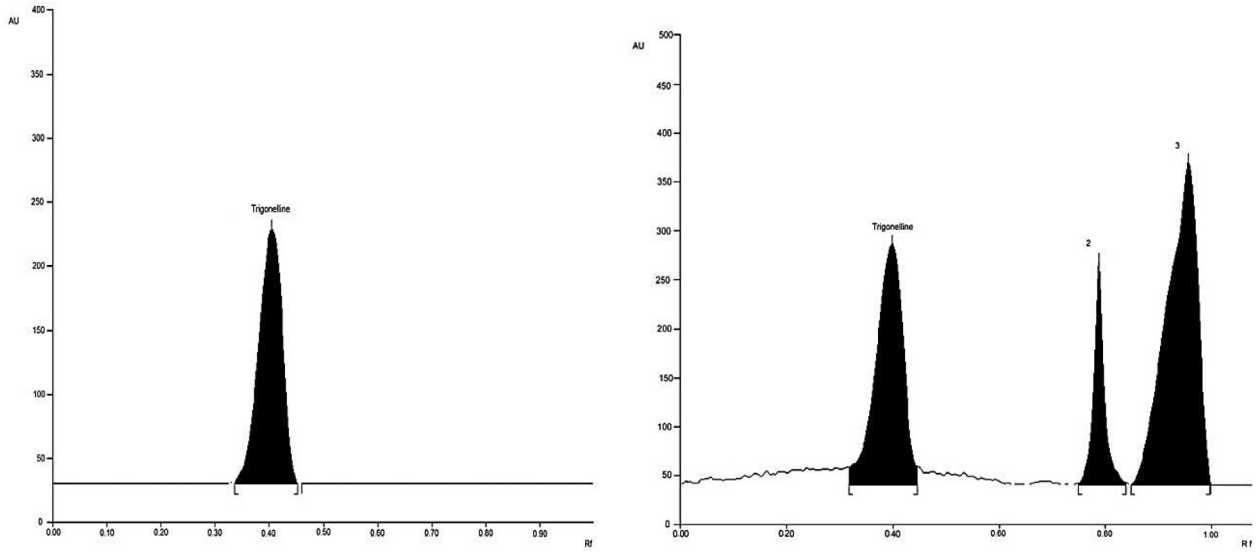

Fig. 1. HPTLC chromatogram **a**) Standard trigonelline (Conc-200 μg/ml) and **b**) 4th day geminated IM6E extract indicating the presence of trigonelline at 270 nm with its characteristic Rf value (0.40).

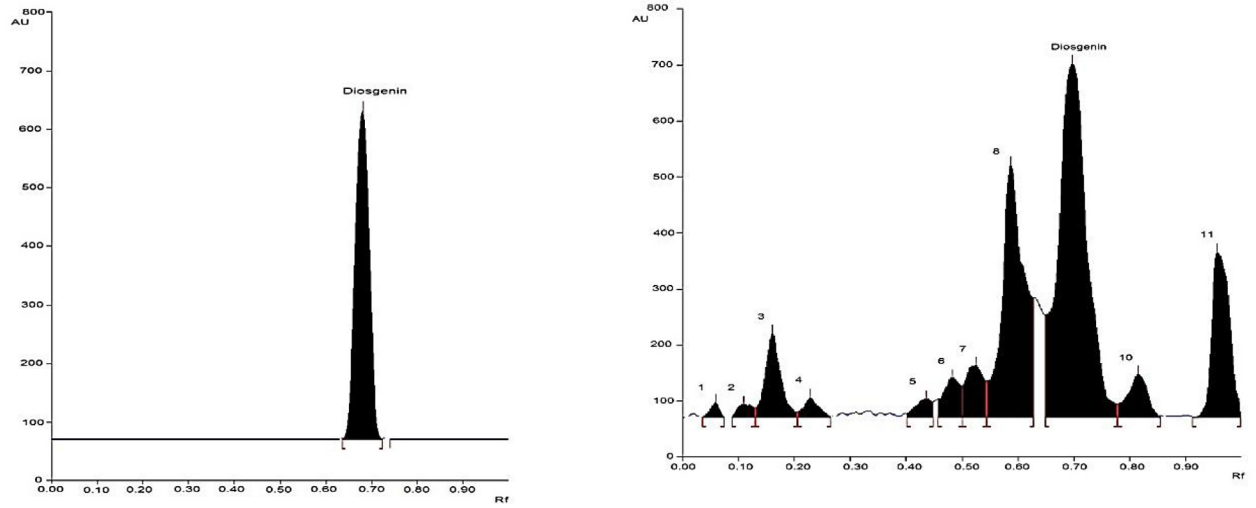

Fig. 2. HPTLC chromatogram of **a**) Standard Diosgenin and **b**) 4th day geminated IM6E indicating the presence of diosgenin at 450 nm at its characteristic Rf (0.69),

HPTLC method for the determination of the nutraceuticals (Figs. 1-3).

3.2. In vitro anti-diabetic activity

As shown in Table 2, under *in vitro* conditions the IM6E treatment showed moderate inhibition of α-amylase (41.64 %) and invertase (49.25 %) activities, but very strong α-glucosidase inhibition activity (95.24 %).

3.3. Acute toxicity analysis in albino Wistar-rats

The acute oral toxicity test showed that the IM6E did not cause any gross pharmacological/behavioural changes or manifestations of toxic symptoms in the animals (like weight loss, mortality, salivation, watery diarrhoea and nothing abnormal) even at a higher dose of 3000 mg/kg bw, and no fatality occurred up to this dosage. Therefore, the results clearly indicate that there exist no toxicological consequences, and thus suggest IM6E to be safe for conducting preclinical studies.

3.4. Effect of IM6E on OGTT & OSTT in normal rats

One week after acclimatization, OGTT was performed in normal rats to determine the effect of IM6E treatments on glucose metabolism during post-glucose administration. The results suggest that there was significant blood glucose level reduction in all the IM6E-treated groups in a dose-dependent manner (100 mg/kg bw, 200 mg/kg bw and 300 mg/kg bw) respectively. The voglibose (1 mg/kg bw) treated group also demonstrated similar results. The blood glucose reached below 100 mg/dL within 60 min as compared to the vehicle-treated group and took almost 120 mins to normalize blood glucose to this level (Fig. 4). The most effective dose of IM6E causes the maximum decline in blood glucose levels in the OGTT assessment and was found to be 300 mg/kg b.w. The results clearly indicate that 300 mg/kg b.w. of IM6E is the best dose for affecting post-prandial blood glucose levels, and even seems to be better than 1 mg/kg bw voglibose treatment. As clear from Fig. 5, the IM6E treatments significantly reduced the calculated relative area under the glucose concentration curve (AUC) for OGTT as compared to untreated control group (Fig. 5). Therefore, these

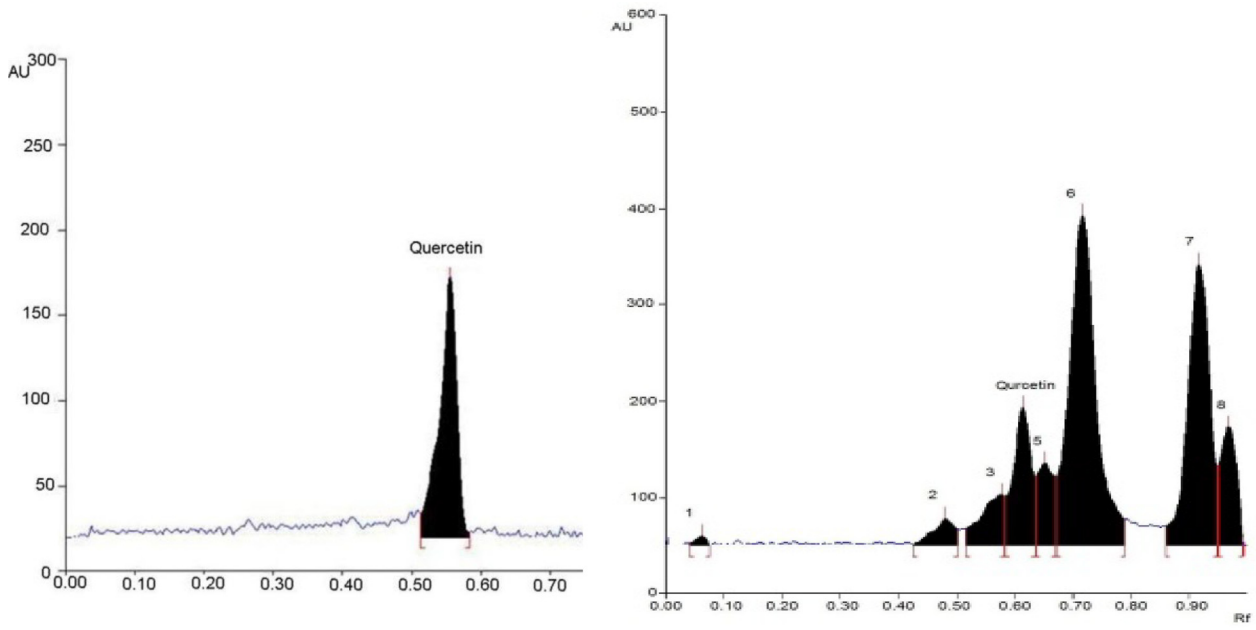

Fig. 3. HPTLC Chromatogram of **a**) Standard Quercetin and **b**) 4th day geminated IM6E indicating the presence of quercetin at 275 nm at its characteristic Rf (0.57).

Table 2
Percent enzyme inhibition of α-amylase, α-glucosidase and invertase with IM6E treatments.

Enzyme	Enzyme Inhibition
α-amylase	41.64 ± 0.93 %
α-glucosidase inhibition	95.24 ± 0.72 %
Invertase inhibition	49.25 ± 0.29 %

results confirm that IM6E possess maximum hypoglycemic effects at 300 mg/kg bw and is almost at par with voglibose treatment. Similarly, as shown in Figs. 6 & 7 in case of OSTT, a significant decrease in blood glucose levels was observed that normalized even before 120 min time interval with IM6E treatments (Fig. 6) in a dose-dependent manner (100 mg/kg bw, 200 mg/kg bw and 300 mg/kg bw) respectively, as compared to the vehicle-treated normal control group. A A Similar pattern as observed in OGTT was also found in AUC for OSTT (Fig. 7).

3.5. Effect of IM6E on plasma antioxidant activity (FRAP) in STZ-induced diabetic rats

As clear from Fig. 8, the plasma antioxidant activity of normal rats was found to be 245 μM/L FRAP and oral administrations of either IM6 seed extracts or untreated IM6 sprout treatments caused only a slight increase in plasma antioxidant activity after 21 days of experimentation. However, the IM6E doses (100, 200 and 300 mg/kg bw) though moderate, but significantly increased the plasma antioxidant activity of diabetic rats in a dose-dependent manner. The combination of 1 m/kg bw voglibose and 300 mg/kg bw IM6E almost normalized the plasma antioxidant status (241 μM/L FRAP) of diabetic rats as compared to untreated diabetic rats (150 μM/L FRAP) after 21 days of treatment, and thus indicating the combinatorial therapy to be more effective in combating oxidative stress in diabetic rats.

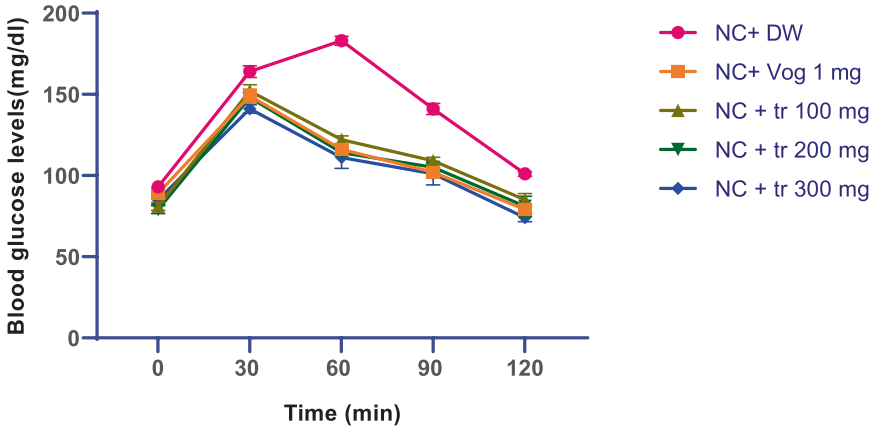

Fig. 4. Effect of IM6E on glucose tolerance in normal rats. Blood glucose curve in OGTT. Results are mean ± SD (n = 6).

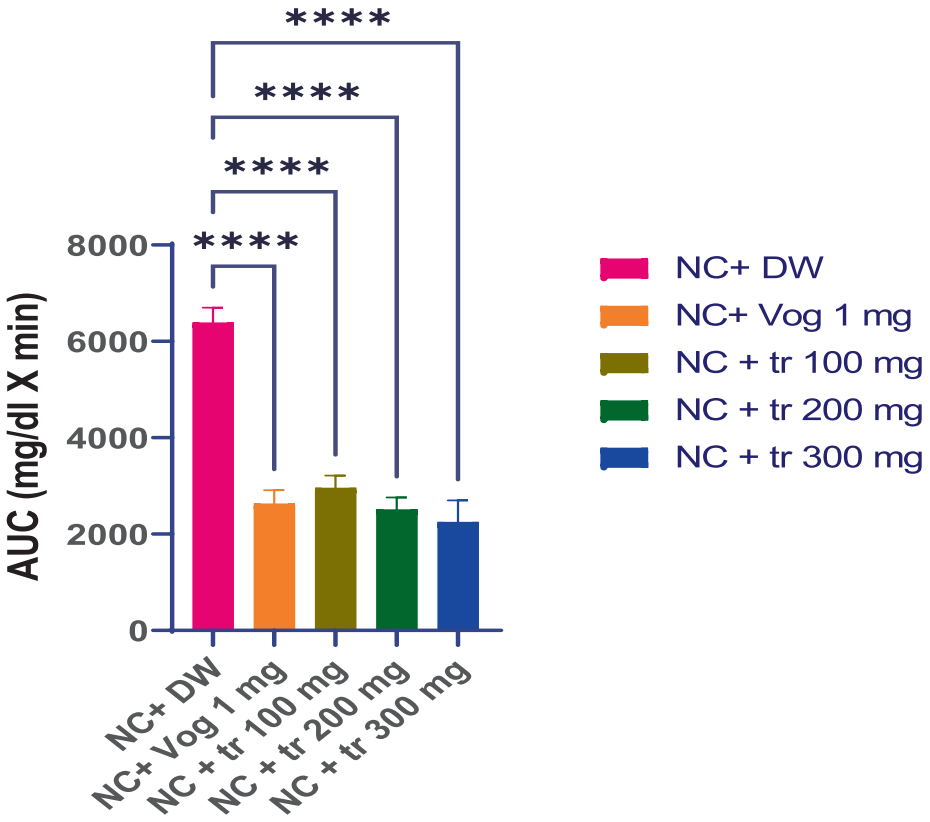

Fig. 5. Effect of IM6E on oral Glucose Tolerance Test (OGTT) in Normal rats. AUC values in OGTT. Results are mean ± SD (n = 6).

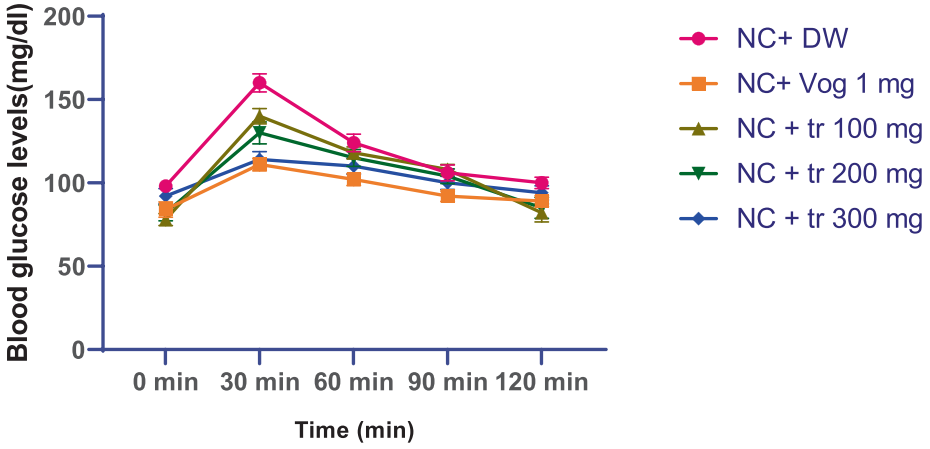

Fig. 6. Effect of IM6E on sucrose tolerance in normal rats. Blood glucose curve in OSTT. Results are mean ± SD (n = 6).

3.6. Effect of IM6E treatment on serum insulin levels in STZ-induced diabetic rats

In the present study, STZ administration caused an almost 78 % decrease in serum insulin levels in the diabetic rats as compared to normal rats (Table 3). However, oral administration of IM6E as well as I.P. administration of IM6E in diabetic rats demonstrated insulinotropic properties for IM6E as evidenced by significant dose-dependent increase in serum insulin after treatment of rats. As compared to monotherapy using voglibose (1 mg/kg b.w.), which caused only a 78 % increase in serum insulin levels of diabetic rats, the combination dose (300 mg/ kg b.w.) of IM6E along with (1 mg/ kgb.w.) voglibose caused an almost 269.5 % increase in blood insulin levels of diabetic rats. The results suggest a synergistic effect of combinational therapy, and thus indicate that such kind of strategy may thus indicate to ameliorate diabetes-related complications in a much better way.

3.7. Effect of IM6E treatment on overall blood glucose levels in STZ-induced diabetic rats

As depicted in Table 4, the hyperglycaemic blood glucose levels of STZ-induced diabetic rats reduced drastically after 21 days of IM6E treatment. As compared to the initial blood glucose levels of diabetic group, a net reduction in hyperglycaemia after 21 days of treatment was found to be 73.2 %, 78.6 %, and 86.4 % while using 100 mg/kg b.w., 200 mg/kg b.w. and 300 mg/kg b.w IM6E treatments, respectively. The 300 mg/kg bw fenugreek seeds caused a

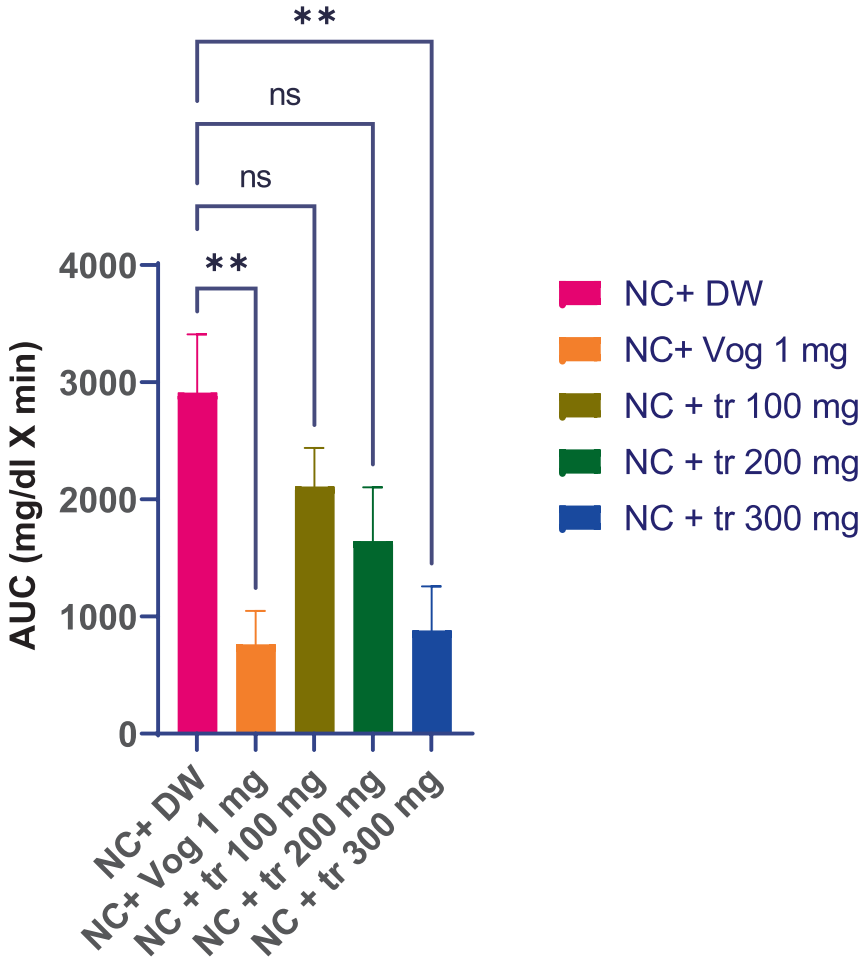

Fig. 7. Effect of IM6 sprout extract on oral starch tolerance test (OSTT) in Normal rats. AUC values in OSTT. Results are mean ± SD (n = 6).

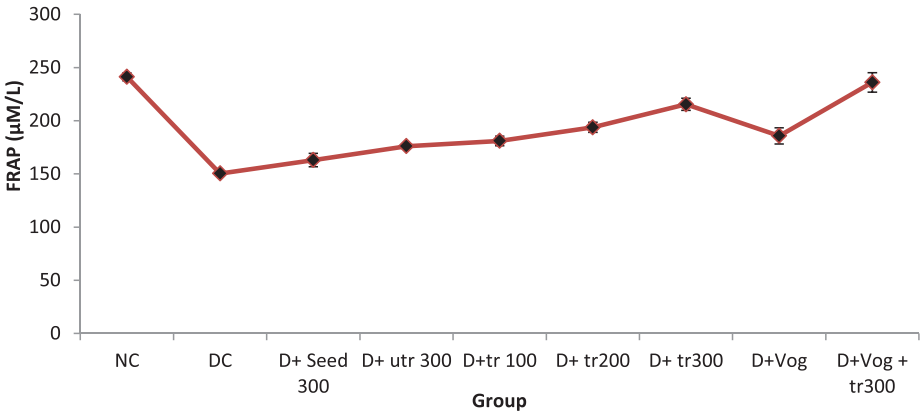

Fig. 8. Effect of IM6 seed, 4th day IM6 untreated sprouts and IM6E on antioxidant activity (FRAP) in diabetic rats. NC = Normal control; DC = Diabetic + ve control; D = Diabetic rats; utr = untreated sprouts; tr = Treated sprouts;Vog = voglibose; 100,200,300 = mg.

69.4 % reduction in overall blood glucose levels of the diabetic rats after 21 days of treatment, and which is much lower than the reduced levels (82.3 %) caused by the 300 mg/kg b.w untreated fenugreek sprouts treatment. Interestingly, 300 mg/ kg b.w. of IM6E in combination with 1 mg/kg b.w.voglibose treatment caused maximum decrease (88.09 %) in blood glucose levels of diabetic group as compared to blood glucose levels observed on 0 day. The effect seems to be due to strong synergistic effect, and thus indicating to ameliorate diabetes mellitus to a greater extent in combination as compared to monotherapy by voglibose only.

Table 3
Effect of IM6E on Insulin production in streptozotocin-induced diabetic rats at different time intervals.

Insulin (mIU/L)				
Days	**0**	**7**	**14**	**21**
NC	9.25 ± 0.03	9.29 ± 0.05	9.30 ± 0.04	10 ± 0.03
DC	2.28 ± 0.04	2.34 ± 0.04	2.29 ± 0.03	2.20 ± 0.02
D + Seed 200	2.42 ± 0.02	3.80 ± 0.05 ^{ns}	4.65 ± 0.02 ^{ns}	4.80 ± 0.03 ^{ns}
D + utr 100	2.48 ± 0.02	4.20 ± 0.03 ^{ns}	6.22 ± 0.04^{b}	8.01 ± 0.03^{b}
D + tr 100	2.50 ± 0.03	4.40 ± 0.04 ^{ns}	5.60 ± 0.02^{c}	5.93 ± 0.02^{c}
D + tr200	2.22 ± 0.02	4.40 ± 0.04^{c}	6.30 ± 0.03^{b}	7.05 ± 0.02^{b}
D + tr300	2.15 ± 0.04	4.50 ± 0.03^{b}	7.20 ± 0.03^{a}	8.05 ± 0.03^{a}
D + Vogli	1.98 ± 0.03	3.55 ± 0.06 ^{ns}	4.30 ± 0.02 ^{ns}	4.50 ± 0.03 ^{ns}
D + Vogli + tr300	1.95 ± 0.01	4.67 ± 0.02^{a}	7.41 ± 0.03^{a}	8.13 ± 0.04^{a}

Note: Values are represented as Mean ± SD; n = 6; c = P < 0.05; b = P < 0.01; a = P < 0.001; P > 0.05 is considered as non-significant (ns); NC = Normal control; DC = Normal control; DW = Distilled water; tr = Extract treated; Vog = Voglibiose.

Table 4
Effect of IM6E on blood glucose level and overall body weight in streptozotocin-induced diabetic rats after different time intervals.

Day	**Blood glucose level (mg/dL)**				**Body weight (g)**			
	0	7	14	21	0	7	14	21
NC	98 ± 2.644	87 ± 3.60	90 ± 5.29	101.09 ± 3.60	150 ± 10.00	160 ± 13.22	180 ± 8.66	210 ± 10.00
DC	445 ± 5.00	445 ± 7.00	500 ± 6.00	590 ± 5.29	140 ± 5.00	132 ± 7.21	120 ± 5.00	112 ± 7.21
D + Seed 300	580 ± 7.21	401 ± 3.60 ^{ns}	280 ± 7.21^{c}	180 ± 7.21 ^{ns}	110 ± 8.66	92 ± 11.13^{c}	100 ± 13.22^{c}	102 ± 8.00 ^{ns}
D + utr3 00	588 ± 7.06	389 ± 3.08^{c}	197 ± 4.11^{b}	104 ± 3.47^{b}	107 ± 8.14	96 ± 10.11^{b}	131 ± 12.31^{a}	160 ± 9.12^{b}
D + tr 100	598 ± 3.46	405 ± 4.58 ^{ns}	250 ± 5.00 ^{ns}	160 ± 4.35^{c}	103 ± 10.81	90 ± 10.00b	95 ± 10.00^{c}	98 ± 10.58 ^{ns}
D + tr200	582 ± 5.29	360 ± 7.21^{b}	190 ± 5.29^{b}	124 ± 3.60^{b}	110 ± 10.00	105 ± 13.22^{b}	115 ± 8.66^{b}	132 ± 7.21^{b}
D + tr3 00	590 ± 4.00	300 ± 5.29^{a}	109 ± 3.60^{a}	80 ± 5.56^{a}	105 ± 8.66	100 ± 8.66^{a}	135 ± 13.22^{a}	155 ± 13.22^{a}
D + Vogli	572 ± 4.00	390 ± 7.00^{c}	297 ± 4.58 ^{ns}	187 ± 6.24 ^{ns}	102 ± 7.21	90 ± 8.66^{b}	105 ± 8.66^{b}	114 ± 5.29^{c}
D + Vogli + tr3 00	625 ± 3.13	300 ± 5.29^{c}	113 ± 2.09^{a}	75 ± 2.16^{a}	100 ± 7.87	93 ± 7.16^{a}	111 ± 7.33^{a}	151 ± 4.14^{a}

Note: Values are represented as Mean ± SD; n = 6; c = P < 0.05; b = P < 0.01; a = P < 0.001; P > 0.05 is considered as non-significant (ns); NC = Normal control; DC = Normal control; DW = Distilled water; tr = Extract treated; utr = Extract untreated; Vogli = Voglibiose.

3.8. Effect of IM6E on overall body weight in STZ- induced diabetic rats

The overall body weight of the normal rats group significantly increased up to 40 % in 21 days as compared to the baseline body weight observed on the first day of the start of experimentation (0 day). However, on the other hand, the weight loss of the diabetic control rats group (untreated) was significantly decreased (20 %) after 21 days as compared to their initial weight (0 day). The daily oral treatment of IM6E in the diabetic rats at 200 mg/kg b.w. and 300 mg/kg b.w. considerably prevented this weight loss ($p < 0.001$) and demonstrated a net increase in total body weight to 20 % and 47.6 % higher than the weight observed in them on 0 day. Furthermore, the dose combination of 300 mg/kg bw of IM6E and 1 mg/kg bw of voglibose proportionately caused a higher increase (39.10 %) in the overall body weight of diabetic rats (Table 4).

3.9. Effect of IM6E treatment on lipid profile in STZ- induced diabetic rats

The serum triglycerides and serum total cholesterol levels were significantly elevated in STZ-diabetic control rats and the percentage of increase was 137 % and 78.26 %, respectively when compared with the normal control rats after 21 days of experimentation. The voglibose (1 mg/kg b.w.) and IM6E treatments individually decreased significantly ($p < 0.001$) the total cholesterol and serum triglyceride levels in diabetic rats, with IM6E acting in a dose-dependent manner (Table 5). However, the combination dose of IM6E (300 mg/kg b.w.) and voglibose (1 mg/kg bw) showed a remarkably more profound decrease in serum triglycerides (59.5 % decrease) and serum total cholesterol levels (41.1 % decrease) as compared to untreated diabetic control.

Table 5
Effect of IM6E on serum total cholestrol, serum triglycerides and liver glycogen content after 21 days.

Groups	**Serum total Cholesterol (mg/mL)**	**Serum triglycerides (mg/mL)**	**Liver glycogen (mg/g)**
NC	92.00 ± 2.00	124.00 ± 4.00	38.59 ± 2.77
DC	164 ± 1.73	294 ± 2.00	11.97 ± 1.31
D + Seed 300	148 ± 2.00^{c}	197.00 ± 3.00 ^{ns}	20.32 ± 1.82 ^{ns}
D + utr 300	99.01 ± 1.00 ^{a}	245 ± 2.06^{a}	23.75 ± 2.13^{b}
D + tr 100	145 ± 1.00^{c}	193 ± 1.73^{c}	24.12 ± 1.38^{b}
D + tr200	135 ± 3.00^{b}	170 ± 3.60^{b}	26.25 ± 2.10^{a}
D + tr300	107 ± 2.64^{a}	143 ± 2.64^{a}	40.32 ± 2.46^{b}
D + Vogli	155 ± 3.00 ^{ns}	225 ± 3.00 ^{ns}	28.72 ± 2.76^{c}
D + Vogli + tr300	90.07 ± 1.35 ^{a}	119.00 ± 3.12 ^{a}	43.12 ± 1.33^{b}

Note: Values are represented as Mean ± SD; n = 6; c = P < 0.05; b = P < 0.01; a = P < 0.001; P > 0.05 is considered as non-significant (ns); NC = Normal control; DC = Normal control; DW = Distilled water; tr = Extract treated; utr = Extract untreated;Vogli = Voglibiose.

4. Effects of IM6E on IPGTT in normal and hyperglycemic diabetic rats

Before IPGTT, the baseline glucose levels between the normal control group (101 mg/dL) and the positive diabetic control group (474 mg/dl) varied significantly. The intraperitoneal load of glucose (2 g/kg b.w.) raised the baseline blood glucose level of diabetic control further to almost 600 mg/dl within 135 min, as compared to the untreated normal rat group with only a slight rise in blood glucose levels, and that subsided to normal levels within 135 min (Fig. 9). The i.p. treatment of normal control rats as well as diabetic rats with IM6E after glucose load showed a remarkable decrease in blood glucose levels in a dose-dependent manner and

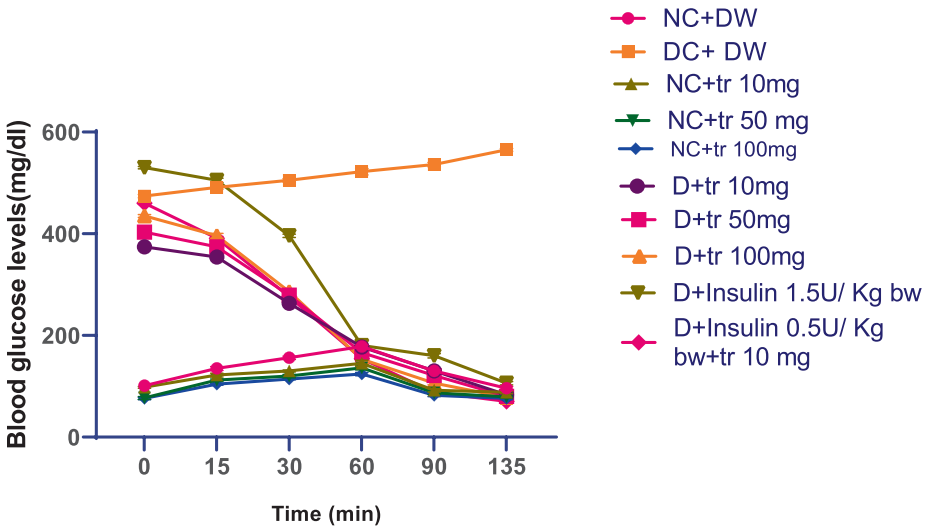

Fig. 9. Effect of IM6E on Intraperitonial glucose tolerance test (IPGTT) in normal and diabetic rats. AUC values in IPGTT. Results are mean ± SD (n = 6).

100 mg/kg bw i.p. treatment showed the most effective response. Interestingly, the combined i.p. doses of 10 mg/kg bw of IM6E with 0.5 u/kg bw insulin demonstrated higher glucose-lowering effects than the individual doses of even 1.5 u/kg bw of insulin. The results clearly indicate a potent synergistic effect of IM6E. AUC values, as depicted in Fig. 10, suggests the encouraging anti-diabetic potential of IM6E.

4.1. Effect of IM6E on liver glycogen content in STZ-induced diabetic rats

A significant ($p < 0.05$) decrease in the liver glycogen (69 %) was observed in STZ-induced control diabetic rats compared to normal rats after 21 days of experimentation (Table 4). However, the IM6E treatments significantly ($p < 0.05$) improved the liver glycogen

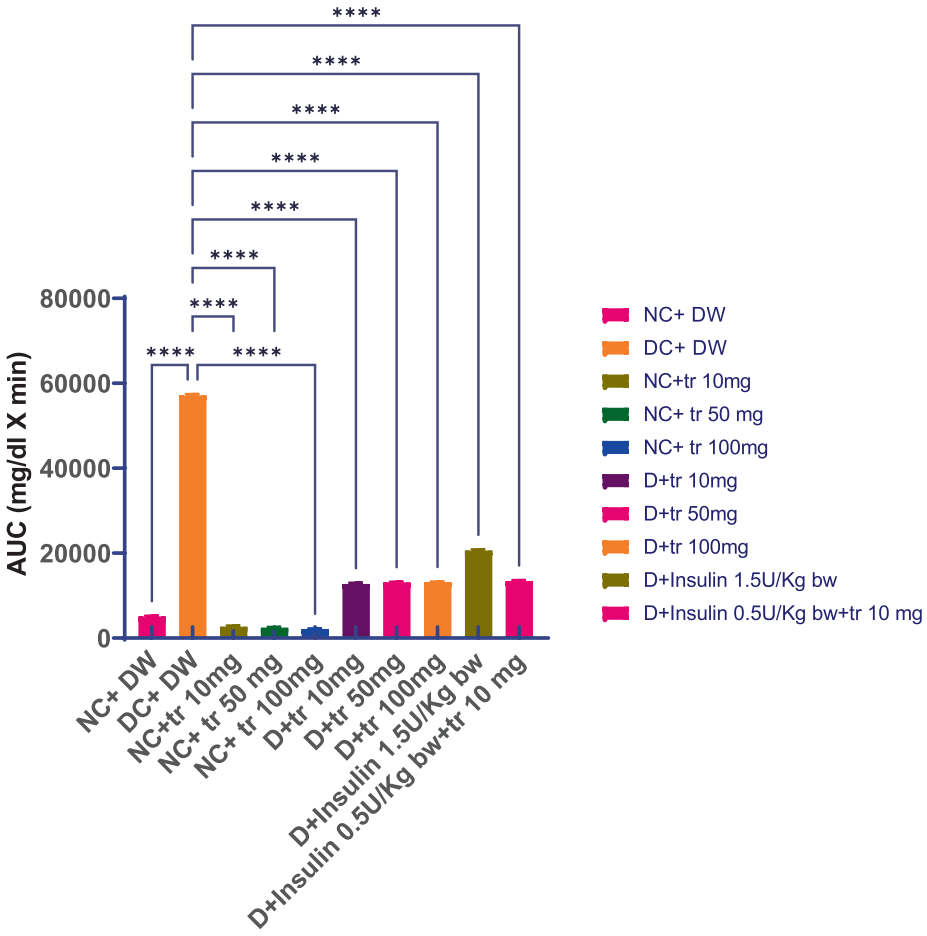

Fig. 10. Effect of IM6E on intraperitoneal glucose tolerance test (IPGTT) in male Wistar-rats.AUC values of IPGTT.

content in a dose-dependent manner. Additionally, the combination dose of IM6E (300 mg/kg bw) and voglibose (1 mg/kg bw) showed highly appreciable results with an almost 260 % increase in liver glycogen content as compared to the diabetic control rats.

4.2. Effect of IM6E treatment on liver enzyme markers in STZ- induced diabetic rats

In the present study, the diabetic rats treated with IM6E demonstrated a significant reduction in raised levels of liver marker enzymes- AST, ALT, and ALP. The percentage of decrease caused by different doses of IM6E (100,200 and 300 mg/kg bw), voglibose alone and combinatorial therapy of voglibose (1 mg/kg b.w.) + 300 mg/kg b.w. IM6E demonstrated 2.40 %, 19.27 %, 36.14 %, 39.15 % and 40.36 % decrease for AST, 3.42 %,4.03 %, 13.13 %, 15.92 %, and 17.82 % decrease for ALT and 9.09 %, 29.95 % 47.10 %, 56.30 %, and 58.06 % decrease for ALP, respectively. The results clearly indicate the synergistic effect of IM6E (Fig. 11) in lowering the crucial liver biochemical markers.

5. Discussion

Plants represent an important source of bioactive compounds, and possess great potential to be used as alternate medicines for managing several diseases like diabetes. As the conventional modern therapies adopted for managing diabetes show substantial adverse effects, hence exploring safe and low-cost phytomedicine is in need of an hour. Plants like fenugreek, with a long history of being used as traditional or folklore medicines against diabetes are being scientifically explored for the development of new drugs and expected to treat the disease in its initial phases, and thus act immensely advantageous to the diabetic patients (Laila et al., 2016; Laila and Murtaza, 2015; Maurya et al., 2021; Murtaza et al., 2013). In the recent past,natural phytochemical elicitors like vitamin C, folic acid, chitosan, lactoferrin etc are being viewed as potent agents to improve the medicinal properties of plants. Various studies have reported the anti-diabetic potency of proteins and amino acids (4 hydroxyisoleucine), alkaloids (trigonelline), saponins, coumarins, flavonoids, phenolic compounds and polysaccharides from fenugreek seeds (Fuller and Stephens, 2015; Gopu et al., 2008; Shakuntala et al., 2011).Therefore, enhancing such bioactive compounds of fenugreek for the design of novel drugs is a pressing necessity as they can play a better role in pharmaceutical or nutraceutical applications. Therefore, in the present study, we undertook a systematic approach to evaluate under *in vitro* and *in vivo* conditions, the anti-diabetic effects of a lyophilized form of aqueous extract of vitamin C pre-treated 4th day germinated phytochemical-enriched fenugreek sprouts (IM6E).

Growing evidence has shown that due to the excess generation of reactive free radicals due to hyperglycemia, the endogenous antioxidants are unable to cope and cause oxidative stress in a diabetic state, which promotes the progression of diabetes and its complications (Gavillán-Suárez et al., 2015). Several cellular, animal, and clinical studies have provided compelling evidence that plant-based antioxidant phytochemicals have therapeutic potential in the management of diabetes and its complications. Our study observed that IM6E possesses a significantly appreciable amount of antioxidant phytochemicals like quercetin, trigonelline and diosgenin than IM6 sprouts. The anti-hyperglycemic drugs, like α-glucosidase inhibitors including voglibose and acarbose have been widely used to treat diabetes, but due to some adverse side effects and high cost, there is pressing demand for searching some safer alternate means to treat diabetes (Hamnvik and McMahon, 2009; Laila and Murtaza, 2016). The *in vitro* based results of this study demonstrated that although IM6E have moderate α-amylase and invertase activities, but it possesses very strong α-glucosidase inhibition activity (95.24 %) and thus seems to be a desirable measure for the management of type II diabetes via suppressing the key enzyme (α-glucosidase), that strongly plays a crucial role in carbohydrate metabolism.

It has been reported that in addition to targeting key enzymes of carbohydrate metabolism, diosgenin, trigonelline and quercetin in fenugreek have insulin secretion modulation activity, in consortium with its polyphenolic, steroidal and alkaloidal constituents that stimulate insulin secretion (Hager et al., 2021). Quercetin is a flavonoid, present in various natural sources, which has been demonstrated *in vitro* and *in vivo* anti-diabetic properties. It improves oral glucose tolerance, as well as pancreatic β-cell function to secrete insulin. It inhibits the α-glucosidase and DPP-IV enzymes, which prolong the half-life of glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP). On the other-hand, trigonelline improves the insulin signalling pathway, attenuates the endoplasmic reticulum stress and oxidative stress in type 2 diabetic rats, and affects the regeneration of pancreatic islet β-cells, the secretion of insulin, and glucose metabolizing enzymes, and diosgenin protects the pancreatic islet β-cells and up-regulates the hepatic glucose kinase (Sun et al., 2020). (Ansari et al., 2022). In the present study, quercetin and trigonelline content of IM6E was found to be in higher concentration than diosgenin. Therefore, from the current *in vitro* studies, it seems that

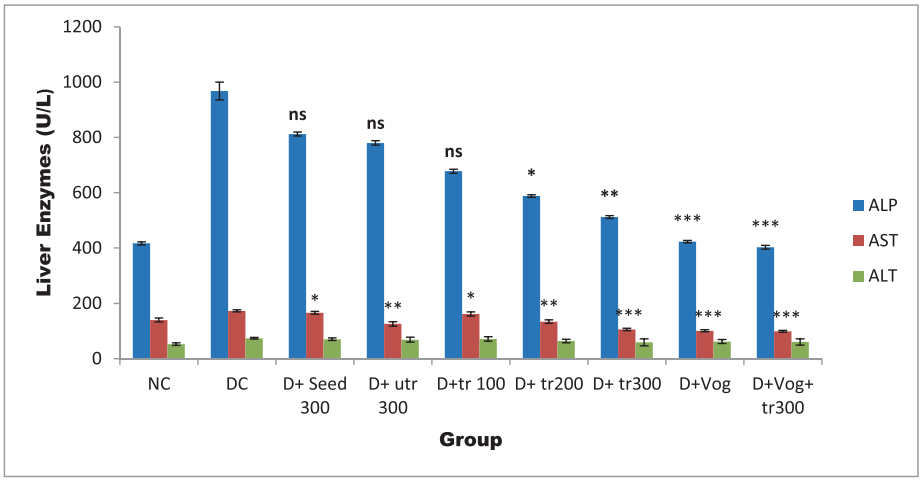

Fig. 11. Effect of IM6E treatment on Liver enzyme markers in streptozotocin- induced diabetic rats.

due to the presence of these anti-diabetic phytochemicals, IM6E can act as a potent anti-diabetic medicine.

Notably, STZ has an ability to induce diabetes in experimental rats leading to a chronic diseased state, marked by severe hyperglycemia and clinically important signs of diabetes mellitus (El-Dakak et al., 2013).As a result, it can be a valuable tool for the pharmacologic assessment of anti-diabetic foods and drugs because they directly cause irreversible damage to β-cells of pancreatic islets of Langerhans, resulting in degranulation and loss of insulin secretion (Hassan et al., 2010; Yao et al., 2020). In this study also, STZ based strategy was adopted to induce diabetes in the albino Wistar-rats to evaluate the overall effect of IM6E in them. Importantly, before suggesting any plant-based therapy for healthcare, it should be carefully examined for safety, since several plants/-plant products holding pharmacological characteristics, have been revealed to be lethal, even at low dosages (Devi et al., 2003; Silva et al., 2012). Therefore, in the current study, the toxicity test for IM6E was conducted, and it was observed that the highest dose of 3000 mg/kg bw used in the study neither caused any death nor any signs of toxicity in any animal during the entire experimentation period.

As there are three major mechanisms to manage hyperglycaemia including decreasing of post-prandial high glucose levels (Raman, 2018).Thus, in order to check the effect of IM6E on lowering high postprandial glucose levels, OGTT and OSTT tests were performed in this study using different concentrations of IM6E (100 mg/kg bw,200 mg/kg bw and 300 mg/kg bw). While administering orally different concentrations of IM6E to glucose-loaded normal rats, a dose-dependent decrease in blood hyperglycemia was observed that normalized even before 120 min of observation. The IM6E treatments significantly reduced the calculated relative area under the glucose concentration curves (AUC's) of OGTT and OSTT as compared to the untreated glucose-loaded normal control group. The study indicates that IM6E possess appreciable hypoglycemic effects at 300 mg/kg bw and is almost at par with 1 mg/kg bw of voglibose treatment. Previous studies have shown fenugreek phytochemicals including quercetin, trigonelline and diosgenin have glucose-lowering effects by targeting the carbohydrate metabolism pathway (Visuvanathan et al., 2022).The results of this study indicate that inhibiting moderately α-amylase and invertase activities, but very strongly α-glucosidase activity under *in vitro* conditions, may also mimic the same in normal rats loaded with glucose, and thus lower the hyperglycemia and AUC of OGTT and OSTT much earlier than untreated glucose-loaded normal rats.

There are reports that decreased blood glucose levels caused due to plant extract treatment may be attributed to the activation of β-cells and granulation returned to normal and insulinogenic effect (Furman et al., 2020). The present study demonstrated a decrease in blood glucose levels with increased plasma insulin levels in both oral as well as i.p., treated diabetic rats with IM6E in a dose-dependent manner compared to the diabetic control rats. This effect could be due to decreased intestinal glucose absorption probably due to strong α-glucosidase inhibition (95 %) as mentioned discussed in *in vitro* studies or due to initiation of glycogenic processes, as well as due to insulinotropic effect as well as decreased glycogenolysis and gluconeogenesis. As compared to monotherapy using the standard anti-diabetic drug voglibose (1 mg/kg b.w.), the combination of oral dose of IM6E (300 mg/ kg b.w.) or i.p. dose of IM6E(100 mg/kg bw) along with voglibose (1 mg/kg b.w.) significantly reversed deregulated biochemical markers in diabetic rats and thus suggest a synergistic effect of combinational therapy and thus suggest that such a strategy may ameliorate diabetes-related complications in a much better way.

STZ-induced diabetes is also associated with a distinctive loss of body weight, high cholesterol and triglyceride levels as well as highly raised liver enzyme levels (Asgary et al., 2012; Elberry et al., 2015). The results of this study demonstrate that diabetic rats treated with the IM6E significantly ($p < 0.001$) resulted in a considerable enhancement in overall body weight, decreased serum total cholesterol and serum triglyceride levels as compared to untreated diabetic rats. It is well-documented that administering insulin to diabetic patients increases not only lipoprotein lipase activity but also decreases plasma TG levels (Klop et al., 2013). Previous, studies have attributed the hypolipidemic activity of fenugreek due to the presence of various bioactive constituents including saponins, alkaloids and free amino acids in it, and its ability to lower serum lipid profiles is potentially attributable to the stimulation of insulin production (Bharti et al., 2018; Patel et al., 2012). The anti-diabetic properties of IM6E are also in complete agreement with the earlier studies, and the increased insulin production in IM6E-treated rats may be due to the presence of quercetin, diosgenin and trigonelline (Hager et al., 2021). The hepatic glycogen reserves are critical for whole-body glucose regulation and are greatly diminished in diabetes (Laila et al., 2016). Additionally, the increase in serum biomarkedor enzymes (AST, ALT and ALP) have been attributed to the damaged structural integrity of the liver in diabetes (Kumar et al., 2011; Zarei et al., 2015). In the present study also, a marked increase in serum AST, ALT, and ALP activities in STZ-induced diabetic control rats were observed, however, after IM6E administration a significant decrease in all the three enzymes were observed in diabetic rats. This implies the hepato-protective nature of the IM6E, as reported earlier for plant extracts by possibly inhibiting the liver damage induced by STZ or by improving the liver function (Marzouk et al., 2013).

In the insulin signalling pathway, Glycogen synthase kinase 3 beta (GSK-3β) activities are regulated by upstream components of the pathway including the level of insulin interacting at the receptor level. The insulin interaction with its receptor in turn leads to the activation of an array of protein kinases (Beurel et al., 2015; Leng et al., 2010). In the current study, IM6E administration increased significantly blood insulin levels as compared to non-treated diabetic rats probably due to the stimulation of β cells of the pancreas. The higher insulinotropic effect of IM6E could be attributed to the presence of insulinotropic compounds like quercetin, diosgenin, trigonelline and other ingredients. They target multiple molecules that are involved in the regulation of several pathways, like improving β-cell proliferation, promoting insulin secretion, reducing apoptosis, and improving hyperglycemia by regulating glucose metabolism in the liver (Al-Ishaq et al., 2019). Thus, the IM6E action mimics insulin effects as previously reported for other plant extracts to potentiate insulin production in the pancreas due to extract treatment. Therefore, from the current study, it seems that IM6E performs its hypoglycaemic effects also by re-establishing the insulin signal pathway via GSk-3 as a therapeutic target at the molecular level in diabetic rats. Since drug interaction is an important upcoming aspect studied now-a-days, and if the interactions among different herbs and medicines are explored at a mechanistic level, the optimal use of the existing herbal therapies may be accomplished, offering a beneficial therapy and remedy to the society with a low number of adverse consequences (Patel et al., 2012). Thus, in the current study, evaluation of any possible pharmacodynamics interaction of IM6E with a commercially available oral hypoglycaemic agent (voglibose) was also evaluated and interestingly a strong synergistic effect was observed in lowering the hyperglycaemia as well as and reversing the deregulated biochemical markers in a much better way in diabetic rats.

6. Conclusion

In conclusion, the results of the current investigation demonstrate that 4th day germinated fenugreek sprouts with maximally elicited phytochemicals (IM6E) by 500 µm vitamin C natural elicitor treatment inhibits three key enzymes of glucose metabolism i.e. α-amylase and invertase moderately while α-glucosidase strongly. The IM6E possess antioxidant, hypoglycaemic, hypolipidemic and insulinotropic properties under *in vivo* conditions also. The study indicates that oral administration of 300 mg/kg/bw of IM6E and i.p. administration of 100 mg/kg bw of IM6E cause maximum anti-diabetic effects as evidenced by OGTT, OSTT, IPGTT, and increased insulin and glycogen levels with decreasing levels of serum cholesterol, triglycerides, and liver enzymes ALT, AST and ALP in Wistar diabetic rats. Additionally, the results indicate that IM6E in combination with voglibose show synergistic effects and acts in a much better way to reverse the effects of diabetes as compared to monotherapy. Our findings clearly reveal the use of IM6E for diabetes management, however, clinical trials need to be performed while using higher doses of IM6E before recommending it as anti-diabetic alternative medicine.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Ethical clearance

The Institutional Animals Ethics Committee (IAEC) of the University of Kashmir (Reg. No. 801/03/CA/CPCSEA), J&K India) approved the experimental work.

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