Increase in Bioactive Compounds During Germination Improves Antioxidant and Antidiabetic Potential of Fenugreek Seeds

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Abstract- The dried seeds from Fenugreek have long been used in traditional Indian medicine against diabetes. In spite of their large medicinal use due to rich source of bioactive molecules, there has not been a single report till date that indicates the fate of such properties in them after germination. In this research we provide evidence that there is a drastic change in fenugreek seeds on germination with respect to antidiabetic potential, antioxidant activity and some antioxidants. HPTLC analysis demonstrated that total phenolic compounds involved in imparting antioxidant activity especially quercetin (flavnoid) got significantly increased during germination process in contrast to steroidal diosgenin and alkaloid trigonelline. In this in vitro mechanistic study it was shown that α amylase, α glucosidase and invertase inhibitiory properties were more prominent in sprouts extracts as compared to their seeds. A positive correlation was established for increases in total phenolic compounds with anti α glucosidase and invertase inhibitory activities respectively (r=0.664 & r=0.541). The results are of utmost importance, and provide a strong in vitro evidence for phenolic rich fenugreek sprouts to be used as preventive measure for diabetes related hyperglycemia via suppressing the two enzymes involved in carbohydrate metabolism.

Key words: Germinated fenugreek seeds, Phenols, Quercetin, Antioxidant activity, Antidiabetic activity.

I. INTRODUCTION

One of the most promising vegetable, fenugreek (*Trigonella foenum-graecum* L.) provides the treasures of secondary metabolites with numerous therapeutic properties (Thomas *et al*, 2011). It is a traditional herb and its seeds has extensively been used in India for the treatment of oxidative stress related diseases especially diabetes (Kumar *et al*, 2012). The biological and pharmacological actions of fenugreek seeds is mostly attributed to the presence of variety of bioactive constituents like quercetin, diosgenin, trigonelline, and free amino acids such as 4-hydroxyisoleucine (Mehrafarin *et al*, 2010). Trigonelline, a major alkaloidal component of fenugreek seeds has recently been suggested to exert hypoglycemic effects in healthy patients without diabetes (Monago and Nwodo, 2010).However, further study of its pharmacological activities and exact mechanism is warranted, along with the application of this knowledge to its clinical usage. Likewise, quercetin, a bioflavonoid present in fenugreek, has been reported to possess anti-inflammatory, anti-oxidant, anti-tumor, immunomodulatory, anti-ulcer, anti-cancer, antioxidant, anti-diabetic, anti-angiogenic and anti-inflammatory activities, and many other properties including the improvement of mental and physical performance (Mahmoud *et al*, 2013). Recent studies indicate that quercetin effectively ameriolates postprandial hyperglycemia and these effects are reported to be mediated through α-glucosidase inhibition (Hussain *et al*, 2012; Jo *et al*, 2009). Additionally, there is a considerable commercial interest in growing fenugreek for its high sapogenin content that is reported to demonstrate hypocholesterolemic as well as antidiabetic activity in rats (Manivannan *et al*, 2013).

As therapeutic and clinical strategies that manage type 2 diabetes is through the reduction of starch hydrolysis via blocking the pancreatic α-amylase as well as α-glucosidase activity to prevent intestinal glucose absorption (Pinto and Shetty, 2010). Recent studies have shown that seed extracts are known to be potent α-amylase inhibitors due to their rich phenolic content that bind to the reactive sites of enzymes and thus altering its catalytic activity (Ghosh *et al*, 2012). Such type of bioactive constituents as well as therapeutic value of seeds have been reported to get improved through sprouting process, which is nowadays gaining tremendous commercial importance. Further, each sprout may contain as many secondary metabolites as an entire plant. In this study we demonstrate that germinated fenugreek seeds possess higher antioxidant and anti hyperglycemic activity than their

seeds and this increase is directly related to enhanced total phenolic compounds.

II. MATERIALS AND METHODS

Chemicals and reagents

Standard diosgenin (98%), quercetin (98%), trigonelline (98%) and invertase (k=0.3) were procured from MP Biomedicals, USA. All other analytical reagent (AR) and HPLC grade chemicals used for current study were supplied by Merck (India).

Plant material

Fenugreek seeds 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 the University and grown under identical controlled environmental conditions in SKUAST-K during April to July (2012-13). The seeds collected from them were then, used for further analysis.

Seed germination

The seeds from fenugreek plants grown under identical environmental conditions 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 subjected to germination at a varied range of temperatures i.e. 16-25°C.The jars were covered with aluminium foil and the seeds germinated in dark. The germinating seeds were washed alternately with distilled water and the assay was performed daily for next 10 days. Each experiment was repeated three times and for each sample, three replicates were analyzed.

Preparation of extract

The fenugreek seeds and their respective sprouts collected on different days of germination process were surface sterilized by soaking in 0.1% sodium hypochlorite and 0.05% nonidet P-40 (NP-40) for 30 seconds and rinsed thoroughly with distilled water. Samples (fenugreek seeds and sprouts) were oven-dried and crushed to a fine powder in a grinding machine. Using 100 ml of distilled water, 10g of each sample was extracted for 30 minutes under reflux at 95°C.The extract was then centrifuged for 10 minutes and used directly for further analysis.

Quantification of phenols

The fenugreek seeds and their respective sprouts collected on different days of germination were estimated for their total phenolic content by using the modified method, as reported by Malick & Singh (1980). Aqueous seed and sprout extracts (500µl) were separately combined with 2.5ml of double distilled water followed by the addition of 0.5ml of Folin-Cioucalteau reagent and incubated for 3 minutes. Immediately, 20% sodium carbonate was added to each sample, vortexed and boiled in a water bath for approximately one minute. The absorbance of the reaction mixture was measured (650nm) against a reagent blank and catechol as standard. For each sample, three replicates were analyzed.

HPTLC based determination of bioactive constituents

The diosgenin and quercetin content of fenugreek seeds and sprouts was determined by newly developed method reported by Laila *et al* (2013). Accurately weighed seed and sprout powder (1g) from all the batches of fenugreek were taken separately in 100 ml round bottom flasks. The extraction was carried out in 50 ml of acidified absolute ethanol as an extraction solvent. The acidification of ethanol was carried out with 5ml of 1M sulphuric acid. The mixtures were refluxed for 60 minutes at 100°C in a heating block (Multi-Block, Labline Instruments,IL) to hydrolyze naturally occurring glycosylated dioscin and quercetin glycone conjugates to yield free diosgenin and quercetin in solution. After hydrolysis, the solutions were cooled, filtered and concentrated at 50°C with a rotatory evaporator (Buchi Rotavapor-R, Shivaki, India).The concentrates were made alkaline with 25% ammonia solution (pH > 12) and diluted with double volume of distilled water. Re-extraction of concentrated solutions was performed three times with dichloromethane (DCM), in order to get maximum yield of diosgenin and quercetin in the selected solvent. The pooled extracts were then washed first with 2 ml of 0.1 M sodium hydroxide to remove the free fatty acids, and then 1 ml of distilled water was added to remove the remaining hydrophilic contaminants. The washed extracts were quantitatively transferred to a 50 ml flask, and then evaporated to dryness with a rotary evaporator (Buchi Rotavapor-R, Shivaki, India) at 50°C. The dry residue obtained were dissolved in 5 ml of methanol and an aliquot of the reconstituted solution was filtered through 0.45 µm

polypropylene membrane filter (Fisher Scientific, India) before HPTLC analysis.

The trigonelline content of fenugreek seeds and sprouts was estimated by using the method reported by Gopu *et al* (2008). Powdered seed and sprout fenugreek samples (1g) were placed in separate beakers with 10 ml methanol as extracting solvent, ultrasonicated for 20 minutes, and mixed with the aid of gentle heat (50°C) in a water bath. The extract was centrifuged at 5000 rpm for 10 minutes and the supernatant was filtered through a 0.45 µm filter membrane before HPTLC analysis.

Anti-oxidant activity

The total antioxidant potential of each sample was determined by using the ferric reducing ability of plasma, FRAP assay (Benzie and Strain, 1996) as a measure of antioxidant power. In this method, a potential antioxidant reduces the ferric ion (Fe3+) to the ferrous ion (Fe2+); the latter forms a blue complex (Fe2+/TPTZ), which increases the absorption at 593 nm. Briefly, FRAP reagent consists of 10 mM 2,4,6-tripyridyl-S-triazine (TPTZ) in 40mM HCL and 20mM ferric chloride in 300 mM sodium acetate buffer (pH 3.6) in the ratio of 1:1:10 (v/v/v).To 100µl of aqueous sample extract, 3ml of FRAP reagent was added followed by thorough mixing. After standing at ambient temperature (20°C) for 4 minutes, absorbance at 593 nm was recorded against reagent blank. Calibration was done against a standard curve (50-1000 µM ferrous ion) produced by the addition of freshly prepared ammonium ferrous-sulphate. The values obtained from three replications were expressed as µM FRAP per g dry weight of sample. All determinations were performed in triplicates.

In vitro antidiabetic activity

α-amylase Inhibition Assay

In order to determine the *α*-amylase inhibition activity of fenugreek extracts under *in vitro* conditions, a method developed by Kunyanga *et al* (2011) was followed. To 100*µ*l of aqueous extract of each sample, 100*µ*l of 0.02 M sodium phosphate buffer (pH 6.9) containing *α*-amylase solution (1 unit liberates 1.9*µ*l of maltose from starch in 1 minute at pH 6.9 and temperature 25°C) and 100*µ*l of starch solution (1%) in 0.02 M sodium phosphate buffer (pH 6.9) was added followed by incubation at 25°C for 30 minutes. Immediately, the reaction was stopped using 1 ml of dinitrosalicylic acid reagent followed by incubating the test tubes in a boiling water bath for 5 minutes and finally cooling them to room temperature. The resulting reaction mixture was diluted with distilled water (10-fold) and the absorbance was measured at 540 nm. The readings obtained, were compared with the control in which sample extract is replaced with buffer solution. The percent inhibition activity of all the samples was calculated using following formula:

The % α-amylase inhibitory activity = (Ac+) - (Ac-) - (As-Ab) / (Ac+) - (Ac-) X 100
In this formula Ac+ and Ac- represents the absorbance of 100% enzyme activity (reaction mixture with enzyme but without test sample extract), and 0% enzyme activity (reaction mixture without enzyme as well as test sample), respectively. Whereas, As includes AC^+ plus sample extract and Ab includes only Ac^- devoid of sample extract, respectively.

α-glucosidase Inhibition Assay

In order to determine the *α*-glucosidase inhibition activity of extracts, a method described by Worthington (1993) was used. A total mixture of 200*µ*l aqueous extract and 200*µ*l of 0.1 M phosphate buffer (pH 6.9) containing *α*- glucosidase solution (1 unit/ml) were taken in glass tubes and incubated for 5 minutes at 25°C. After the pre-incubation, to each tube exactly 100*µ*l of 5mM *p*-nitrophenyl-*α*-D-glucopyranoside solution in 0.1 M phosphate buffer (pH 6.9) was added followed by incubation for 5 minutes (25°C). Immediately, the reaction was terminated by the addition of 0.1M sodium carbonate (Na_2CO_3) and the reaction mixtures were diluted with distilled water (10-fold). The absorbance readings obtained at 405 nm were compared with a control in which sample extract was replaced with 100*µ*l of buffer solution. The percent inhibition activity of all the samples was calculated using following formula:

The % α-glucosidase inhibitory activity = (Ac+) - (Ac-) - (As-Ab) / (Ac+) - (Ac-) X 100
In this formula Ac+ and Ac- represents the absorbance of 100% enzyme activity (reaction mixture with enzyme but without test sample extract), and 0% enzyme activity (reaction mixture without enzyme as well as test sample), respectively. Whereas, As includes AC^+ plus sample extract and Ab includes Ac^- devoid of sample extract, respectively.

Invertase inhibition assay

Invertase inhibition activity was determined using the modified method of Sumner and Howells (1935). After incubating 0.1ml of enzyme solution with 0.1/ml of water extract at 37^0C for 30 minutes, 0.9 ml of sucrose in 0.03 M

acetate buffer (pH 5.0) was added to each tube and the reaction mixture was incubated at 37 °C for 60 minutes. After the incubation period, the reaction was stopped by addition of 1 ml of dinitrosalicylic acid reagent and heated for 5 minutes in a boiling water bath. The aliquots were diluted to 10-fold with distilled water and finally the absorbance was measured at 540 nm using spectrophotometer. One unit of invertase (IU) is defined as the amount of enzyme which liberates 1µmole of glucose/minute/ml under the assay conditions.

The % invertase inhibitory activity = (Ac+) - (Ac-) - (As-Ab) / (Ac+) - (Ac-) X 100
Here Ac+ and Ac- are defined as the absorbance of 100% enzyme activity (reaction mixture with enzyme but without test sample extract), and 0% enzyme activity (reaction mixture without enzyme as well as test sample) respectively. Whereas, As represent AC^{+} including sample extract and Ab represents Ac^{-} excluding sample extract, respectively.

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.

III. RESULTS

In our study all the ten collected samples (IL1 to IL10) when subjected to varied range of temperatures (16-22^{0}C) demonstrated 22^{0}C to be the optimal temperature for raising fully grown sprouts within 4 days (Data not shown).

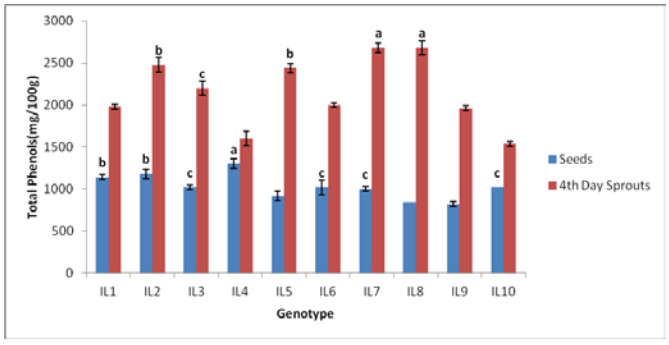


Figure 1: Effect of germination on total phenolic content of fenugreek sprouts on 4th day. Values are represented as Mean ± SD; n = 3; cP < 0.05; bP < 0.01; aP < 0.001; P > 0.05 is considered as non-significant (ns)

The Fig. 1 reveals that the water extracts from germinating fenugreek seeds demonstrated significant time dependent increase in their total phenol content. Although there existed a great level of variation with respect to phenol content among the germinating fenugreek seeds that ranged from as low as 720 mg/100g (seed) to as high as 2680 mg/100g (sprouts) however interestingly in all the cases 4th day of germination proved to be the optimal day for inducing the highest phenol content.

Our study also showed clearly (Fig. 2) that germination process drastically enhanced the total antioxidant activity of resulting fenugreek sprouts in a time dependent manner. The antioxidant activity of all the germinating seed samples reached more or less to its maximum limit within the four days, with IL8 sprout extract demonstrating 2.63 times higher antioxidant activity as compared to its un-germinated seeds. It was clear that water extracts from 4th day germinated IL8 sprouts possessed highest phenol content as well as antioxidant activity (1903.79µM/100g). The table 1, demonstrated that there existed a strong positive correlation between increase in total phenol content and enhanced total antioxidant activity (r = 0.776).

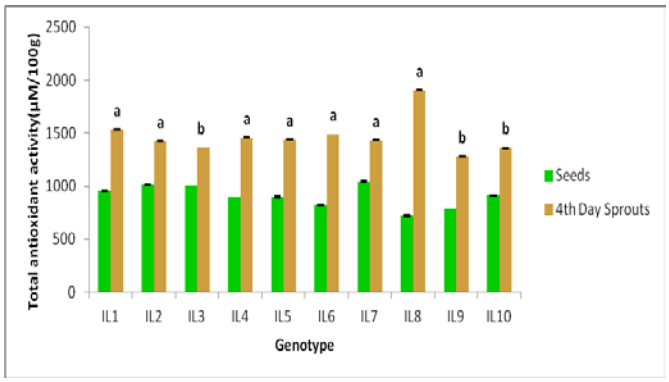
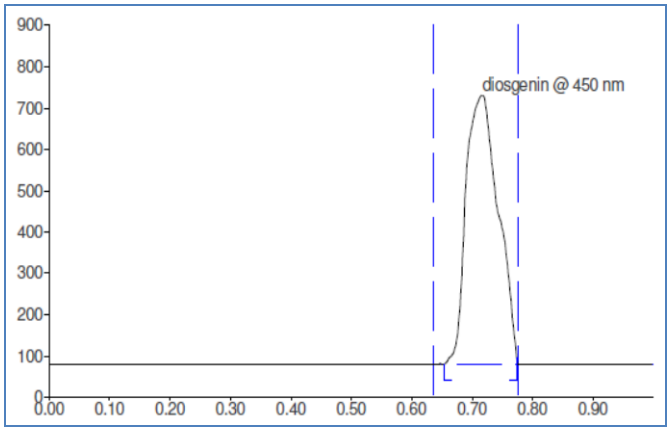


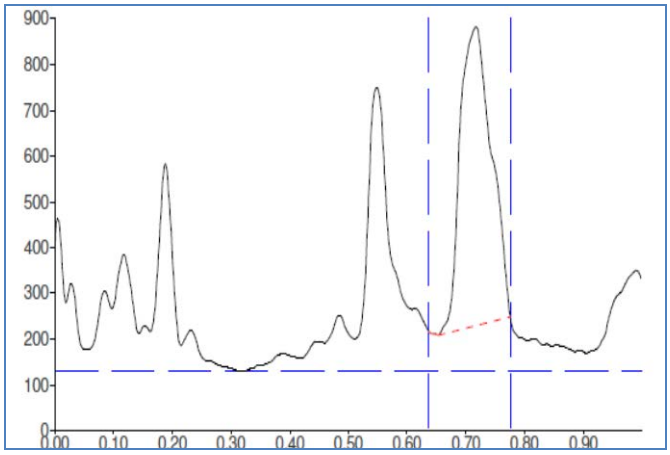
Figure 2: Effect of germination on total antioxidant activity of fenugreek sprouts on 4th day. Values are represented as Mean ± SD; n = 3; cP < 0.05; bP < 0.01; aP < 0.001; P > 0.05 is considered as non-significant (ns)

As shown in Fig. 3(a), 3(b) and 3(c) the results of peak areas for all the ten germinating seeds corresponding to peak areas of standard diosgenin, quercetin and trigonelline were used for their quantification in samples using regression equation. The results of triplicate analysis, expressed as average amount of diosgenin, quercetin and trigonelline in % w/w of each sample was found to have its characteristic Rf value of 0.69±0.02 at 450nm for diosgenin, 0.57 ±0.02 at 275 nm for quercetin and 0.40±0.02 for trigonelline, respectively. While using this method, the dried fenugreek seed samples were

found to contain diosgenin in the range of 0.0935 % to 0.135 % (w/w) and quercetin in the range of 0.00 to 0.01155 % (w/w). The fenugreek seed samples, used in the current study, were found to contain trigonelline in the range of 0.286% to 0.386 % (w/w).

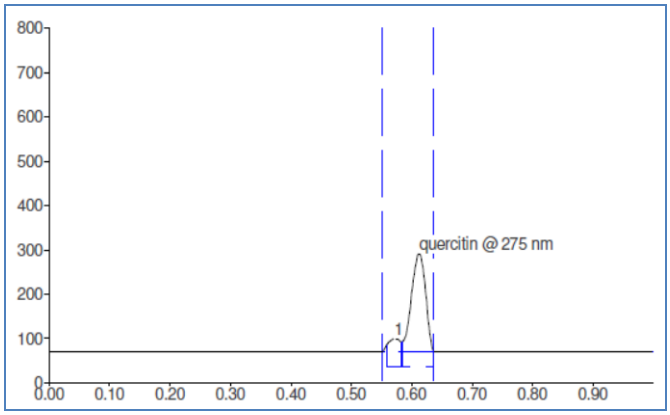


(a)

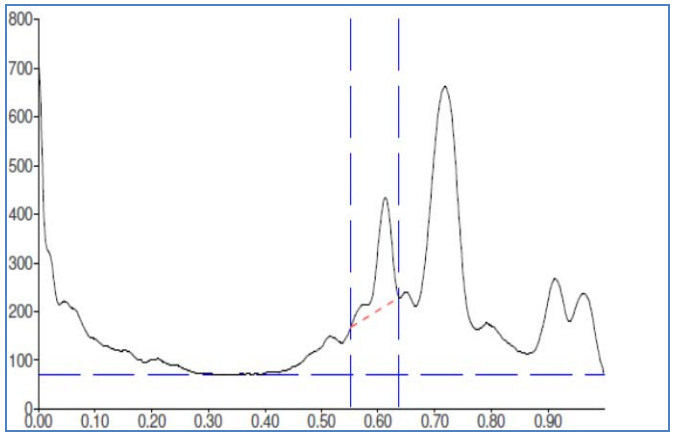


(b)

Figure 3(a) : HPTLC chromatogram of standard diosgenin (A) and IL8 seed extract (B)indicating the presence diosgenin at 450 nm after derivatisation with anisaldehyde-sulfuric acid reagent at its characteristic Rf (0.69)

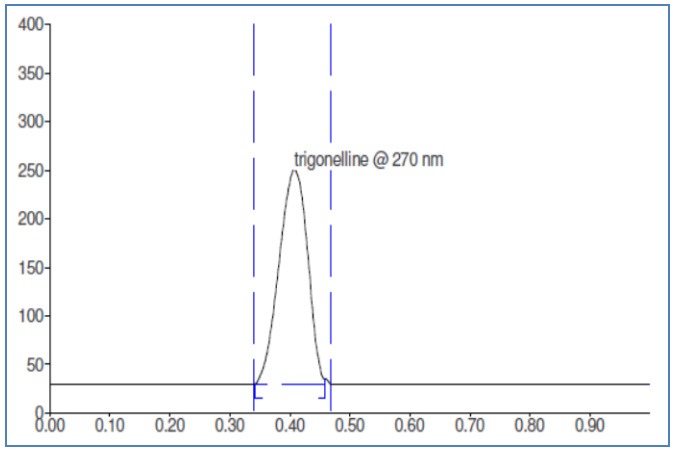


(a)

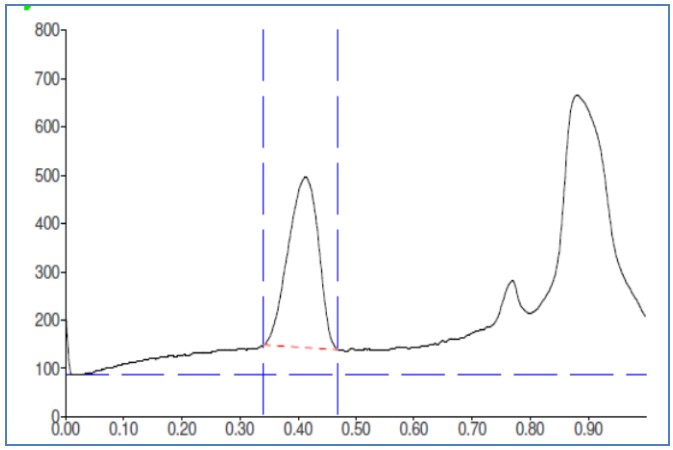


(b)

Figure 3(b): HPTLC chromatogram of standard quercetin (A) and IL8 seed extract (B) indicating the presence of quercetin at 275 nm at its characteristic Rf (0.57).



(a)



(b)

Figure 3(c): HPTLC chromatogram of A) standard trigonelline and B) IL8 seed extract indicating the presence of trigonelline at 270nm with its characteristic Rf value of 0.40±0.2.

Interestingly, our study demonstrated slightly decreasing trend in the concentrations of diosgenin and trigonelline during the germination process and thus indicating the highest concentration of these two phytochemicals to be present in seeds rather than sprouts [Fig. 4(a) and Fig. 4(b)]. The overall decrease observed during the entire germination process of fenugreek seeds, ranged in between 50.26% to 65.35% for diosgenin and 63.35% to 77.71% for trigonelline, respectively. Further, we couldn't find any correlation between decreasing trend in diosgenin and trigonelline content with total phenol content and antioxidant activity

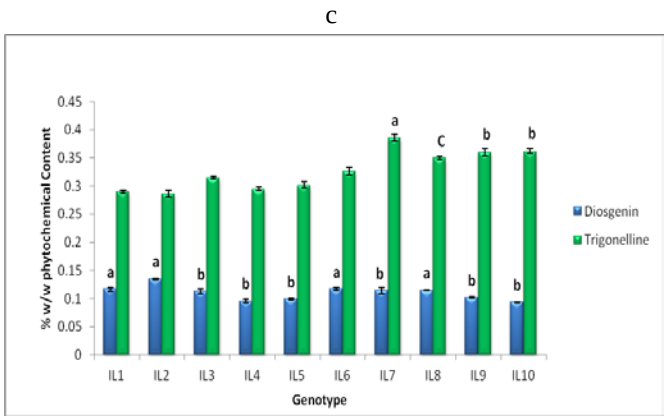


Figure 4(a): Diosgenin and trigonelline content of ten selected seed samples of fenugreek.Values are represented as Mean ± SD; n = 3; cP < 0.05; bP < 0.01; aP < 0.001; P > 0.05 is considered as non-significant (ns)

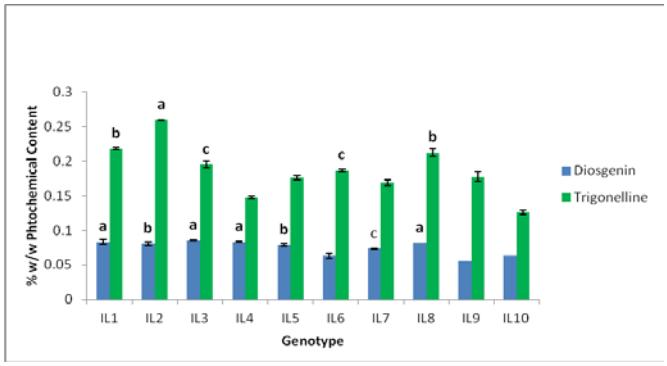


Figure 4(b): Effect of germination on diosgenin and trigonelline content of fenugreek sprouts on 4th day.Values are represented as Mean ± SD; n = 3; cP < 0.05; bP < 0.01; aP < 0.001; P > 0.05 is considered as non-significant (ns)

Interesting our research demonstrated, that quercetin a phenolic flavonoid compound increased drastically during the germination process in a time dependant manner (Fig. 5). This time dependent increase in quercetin content in fenugreek sprouts was found to correlate well with increased total phenolic content and increase in total antioxidant activity. As expected due to highest phenolic content, the maximum quercetin content (0.0417 %) was also found in the 4th day germinated IL8 sprouts. As the main aim of current research was to evaluate hypoglycemic effect of germinating fenugreek seeds, therefore in this research, the three key enzymes i.e. α-amylase, α-glucosidase and invertase involved in glucose metabolism were targeted by evaluating germinated fenugreek sprout extracts as inhibitor against them.

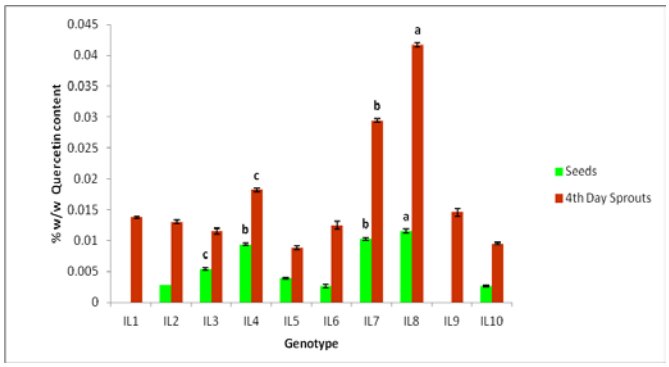


Figure 5: Effect of germination on quercetin content of fenugreek sprouts on 4th day. Values are represented as Mean ± SD; n = 3; cP < 0.05; bP < 0.01; aP < 0.001; P > 0.05 is considered as non-significant (ns)

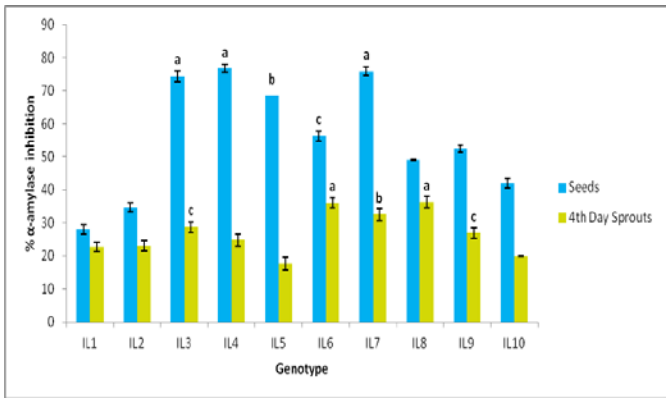


Figure 6: Effect of germination on % α-amylase inhibitory activity of fenugreek sprouts on 4th day. Values are represented as Mean ± SD; n = 3; cP < 0.05; bP < 0.01; aP < 0.001; P > 0.05 is considered as non-significant (ns)

As depicted from Fig.6, in our study, the α-amylase inhibition was exhibited higher by fenugreek seed extracts (28.015% - 76.67%) as compared to their germinated forms (5.34 % to 51.92 %). We couldn't find any significant correlation between increased α-amylase inhibition and increased total phenols (r = 0.45) or total antioxidant activity

(r = -0.022). In our study, fenugreek seed extracts demonstrated much lower (20.76% to 76.92) inhibition against α-glucosidase. However, in contrast to their respective seeds, germinated seeds showed significantly higher α-glucosidase inhibitory potential that increased in a time dependant manner during germination (Fig. 7). As compared to seeds, the 4th day germinated sprouts showed significant highest increase in α-glucosidase inhibitory property that ranged from 14.36 % to 62.37%. It was clear that in case of fenugreek seeds, the 4th day of germination proved to be the optimal day for possessing maximum α-glucosidase inhibitory power. The sprouts obtained from IL8 seeds possessed the highest α-glucosidase inhibitory activity (91.28 %). As clear from Table 1, the total phenols exhibited strong positive correlation with α-glucosidase inhibition potential (r=0.664). Similarly, a positive correlation between total antioxidant activity and α-glucosidase inhibition (r = 0.624) was established.

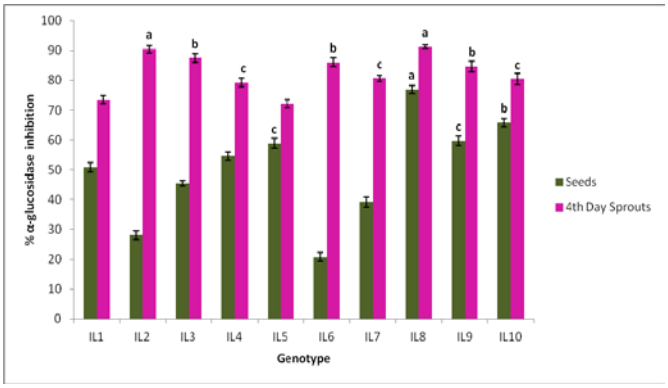


Figure 7:Effect of germination on % α-glucosidase inhibitory activity of fenugreek sprouts on 4th day .Values are represented as Mean ± SD; n = 3; cP < 0.05; bP < 0.01; aP < 0.001; P > 0.05 is considered as non-significant (ns).

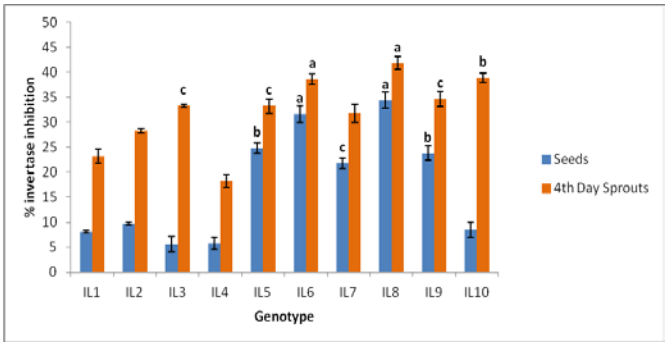


Figure 8: Effect of germination on % invertase inhibitory activity of fenugreek sprouts on 4th day.Values are represented as Mean ± SD; n = 3; cP < 0.05; bP < 0.01; aP < 0.001; P > 0.05 is considered as non-significant (ns)

Fenugreek seed extracts were found to posses lower (5.58% to 34.44%) invertase inhibitory activity than (5.72 % to 41.86%) their counterpart germinated seeds (Figure 8). One thing was clear from our results that the 4th day germinated IL8 seeds, in addition to possessing maximum phenol content, antioxidant activity and α-glucosidase inhibitory potential, also demonstrated the highest invertase inhibitory activity.

It was established that there exists a strong positive correlation (Table 1) between invertase inhibitory activity with total phenols (r=0.541) and total antioxidant activity (r =0.487). These results are quite encouraging and suggest that 4th day germinated phenol rich fenugreek sprouts especially from IL8 seeds possess strong antidiabetic property by simply inhibiting intestinal α-glucosidase and invertase enzyme activities.

IV DISCUSSION

One difficulty associated with germination process is that there exists varied optimal sprouting temperature among different cultivars of the same plant (Norouzi and Vazin, 2011). However, in our study in all the fenugreek seed genotypes, 22^0C was found to be the optimal temperature for raising fully grown sprouts within 4 days. Fenugreek seeds typically have been reported to contain mixtures of different health promoting secondary metabolites especially phenolic compounds like steroids, alkaloids and flavonoids. As far as their extraction is concerned, the maximum phenolic content has been reported to be present in water extract (Mahomoodally, 2013; Saxena *et al*, 2011). Therefore, in the present study preference was given to prepare water extracts from fenugreek seed samples. The results related to determining the optimal day for highest phenol content, orroborated well with previous reports conducted on germinating mung bean, cowpea and chickpea seeds where 4th day of germination proved to be the best (Chon, 2013; Tarzia *et al*, 2012).

It is a well-established fact that enhancement of dietary phenolic compounds as antioxidants can play a very strong role against oxidative stress related complications (Kumar *et al*, 2013). Further, there are reports that antioxidant properties of seeds increase with germination process, and such increase is positively related to total phenolic content (Liu *et al* , 2011). In this context our study also shows clearly (Fig. 2) that germination process drastically enhanced the total antioxidant activity of resulting fenugreek sprouts in a time dependent manner and reached to its maximum limit within the four days, with IL8 sprout extract

demonstrating highest antioxidant activity amoung the ten genotypes. Such type of designed food can be recommended as an excellent source of dietary natural antioxidants against various oxidative stress related diseases including diabetes.

In our earlier communication, we were able to develop a novel sensitive, fast, and reproducible HPTLC method for simultaneous analysis of diosgenin and quercetin from fenugreek (Laila *et al*, 2013). Keeping in view, the widespread use of phytochemical rich fenugreek seeds by general population for therapeutic use against diabetes, the present research was carried further ahead to evaluate HPTLC based any change in three vital antidiabetic bioactive phenolic compounds quercetin, trigonelline, and diosgenin in germinating seeds. The results of current study were found to be almost in accordance to previous reports that show fenugreek seeds contain diosgenin in the range of 0.10 to 0.90% (Snehlata and Payal, 2012) and quercetin in 0 .0 to 0.021% range (Jahan et al., 2013; Dua et al., 2013; Sharma et al., 2014). The fenugreek seed samples, used in the current study, were found to contain trigonelline in the range of 0.286% to 0.386 % (w/w) and are as such in well agreement to earlier findings that suggest fenugreek seeds contain trigonelline in the range of 0.103% to 0.288% (Hassanzadeh *et al*, 2011).

As per earlier reports diosgenin and trigonelline are involved in imparting bitterness to the fenugreek seeds and therefore, time dependent decrease in their content during the germination process makes fenugreek sprouts more acceptable for consumption. Overall, our results corroborated well with earlier documented reports showing decreasing trend in trigonelline content in germinating beans, lentils and peas (Kuo *et al*, 2004; Zheng *et al*, 2005). However, in contradiction, our results strongly opposes that germinated fenugreek seeds comparatively possesses highest steroid sapogenins (diosgenin) than their seeds (Kor *et al*, 2013). Interesting, our research demonstrated, that quercetin a phenolic flavonoid, levels increased drastically during the germination process and followed the same trend as reported by Lin *et al* (2008) in germinating buck weed sprouts. This time dependent increase in quercetin content in fenugreek sprouts was found to correlate well with increased total phenolic content and increase in total antioxidant activity. Our results are in strong agreement with the previous reports that suggest, that in lentil sprouts the quercetin reached to its peak levels on 3rd or 4th day of germination (Swieca *et al*, 2013). The study gives a clue that IL8 fenugreek sprouts possess maximum antioxidant activity due to the presence of specific phenolic compounds like quercetin.

As variety of available anti hyperglycemia therapeutic drugs can competitively inhibit α- glucosidase enzymes or bind with the reactive sites of amylase enzyme, but these drugs are often associated with adverse side effects and frequent gastrointestinal disorders such as abdominal pain, flatulence and diarrhea (Ranilla *et al*, 2008).. Among the various strategies, food-grade phenolic compounds inhibiting α-amylase activity have been reported to be potentially safer and preferred alternative for modulation of carbohydrate digestion and control of glycemic index of food products (Maiti and Majumdar, 2012). Our results are in agreement with earlier reports on Mucuna pruriens seeds, in which significant decrease has been observed in α-amylase inhibitory activity after sprouting (Randhir *et al*, 2009). The germinated fenugreek seeds are found to be much appropriate as they possess moderate α-amylase inhibitory activity as compared to their respective un-germinated seeds. The justification being that excessive inhibition of pancreatic α-amylase by antidiabetic drugs, results in the abnormal bacterial fermentation of undigested carbohydrates in the colon by colonic flora and may cause many harmful side effects in humans (Ahmed *et al*, 2014). Hence, as per our study, the lower α-amylase inhibition activity observed in fenugreek sprouts as compared to their seeds seems to be much more suitable to implement them in the dietary practice of diabetics, with minimum side effects.

In management of diabetes another specific and important class of oral hypoglycemic agents represent α-glucosidase inhibitors that can retard the rate of glucose absorption in the intestine through competitive and reversible inhibition of intestinal α-glucosidase enzyme (Vadivel and Biesalski, 2012). Several *in vitro* studies report potential α-glucosidase inhibitors from various plants like cranberry, Cuscuta reflexa, pepper, soybean extracts and guava leaf extract (Deguchi and Miyazaki, 2010, Apostolidis *et al*, 2006, Anis *et al*, 2002, Pullela *et al*, 2006, Mccue *et al*., 2005 and Georgetti *et al*., 2006). In our study, in contrast to α-amylase, the germination process significantly increased α-glucosidase inhibitory potential of sprouts that is in accordance to earlier reports on maize seeds where a significant increase in α-glucosidase inhibitory activity during germination has been reported recently (Hiran *et al*, 2013). Our study clearly indicate that 4th day germinated IL8 fenugreek seeds possess potent anti α-glucosidase inhibitory power and could prove to be effective as the antidiabetic clinical agent, when consumed in small doses on a consistent basis through the diet.

Inhibition of another key enzyme invertase (also called β-fructofuranosidase) have been previously reported from

variety of foods that in turn delayed the digestion and absorption of carbohydrates and thus, inhibited the post prandial hyperglycemia (D'Britto *et al*, 2012, Ghadyale *et al*, 2012, Deguchi and Miyazaki, 2010, Gad *et al*, 2006, Nagmoti and Juvekar, 2013). Likewise, α-glucosidase enzyme, the germination process of fenugreek seeds significantly increased inhibitory potential of resulting sprouts against invertase These results corroborate well with earlier reports that demonstrated germination of wheat and barley seeds increased their intestinal sucrase inhibitory power (Jang et al., 2012). One thing was clear that the 4th day germinated IL8 seeds, in addition to possessing maximum phenol content, antioxidant activity and α-glucosidase inhibitory potential, also demonstrated the highest invertase inhibitory activity. These results are quite encouraging and suggest that 4th day germinated phenol rich fenugreek sprouts especially from IL8 seeds possess strong antidiabetic property by simply inhibiting intestinal α-glucosidase and sucrose/invertase enzyme activities.

V. Conclusion

In summary we have established strong *in vitro* evidence that during germination process there occurs a drastic change in fenugreek seeds with respect to antidiabetic potential, antioxidant activity and some phytochemicals. The HPTLC analysis demonstrated that quercetin content gets significantly increased during germination process. Mechanistic study provides a strong rationale that α glucosidase and invertase inhibitory properties improved many fold in sprouts than their seeds. There exists a positive correlation between increases in total phenolic compounds during germination process and anti α glucosidase and invertase inhibitory activities. Such foods with high α-glucosidase/invertase inhibitor and low/medium α-amylase inhibitor activity can be considered as an ideal component of a whole food design, that can be a part of our diet to help in the proper management of hyperglycemia linked diabetes in its early stages. Although, from this study it is clear that, 4th day germinated phenolic rich IL8 sprouts are the best hypoglycemic agents among the ten selected samples. However, before recommending them as an antidiabetic medicine, they need to be re validated for their increased antidiabetic potential under *in vivo* conditions.

Table 1: Correlation matrix of variables

Variables	Total phenols	Total antioxidant activity.	α-amylase	α-glucosidase	Invertase	Trigonelline	.Diosgenin
Total antioxidant activity	0.776**						
α-amylase	0.045	0.022					
α-glucosidase	0.664**	0.624**	0.233*				
Invertase	0.541*	0.487*	0.149	0.629**			
Trigonelline	0.066	0.04	0.814**	0.385	0.195		
Quercetin	0.642**	0.615**	0.139	0.537*	0.554*	0.049	
Diosgenin	0.082	0.066	0.724	0.277	0.071	0.0850 **	0.087

N.D.:-* P < 0.05; **P < 0.001; P > 0.05 is considered as non-significant (ns).

VI Conflict of Interests

The authors declare no conflict of interests.

VII Acknowledgment

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REFERENCES

[1] Ahmed D, Younas S, Anwer-Mughal QM (2014). Study of alpha-amylase and urease inhibitory activities of *Melilotus indicus* (Linn.) All. Pak J Pharm Sci 27 (1): 57-61.

[2] Anis E, Anis I, Ahmed S, Mustafa G, Malik A, Afza N, Hai SM, Shahzad-ul-hussan S, Choudhary MI (2002). α-glucosidase inhibitory constituents from *Cuscuta reflexa*. Chem Pharm Bull 50 (1): 112–114.

[3] Apostolidis E, Kwon YI, Shetty K (2006). Potential of cranberry-based herbal synergies for diabetes and hypertension management. Asian Pac J Clin Nutr 15(3): 433–441.

[4] Benzie IF, Strain JJ (1996). The reducing ability of plasma as a measure of antioxidant power-The FRAP assay. Anal Biochem 239 (1): 70-76.

[5] Chon S (2013). Total polyphenols and bioactivity of seeds and sprouts in several legumes. Curr Pharm Des 19 (34): 6112-6124.

[6] D'Britto V, Devi PP, Prasad BLV, Dhawan A, Mantri VG, Prabhune A (2012). Medicinal plant extracts used for blood sugar and obesity therapy shows excellent inhibition of invertase activity: synthesis of nanoparticles using this extract and its cytotoxic and genotoxic effects. Int J Life Sci Pharma Res 2 (3): 61-74.

[7] Deguchi Y, Miyazaki K (2010). Anti-hyperglycemic and anti-hyperlipidemic effects of guava leaf extract. Nutr Metab 7(9): 1-10.

[8] Dua A, Vats S, Singh V, Mahajan R (2013). Protection of biomolecules against *in vitro* oxidative damage by the antioxidants from methanolic extract of *Trigonella foenum-graecum* seeds. Int J Pharm Sci Res 4(8): 3080-3086.

[9] Gad MZ, El-Sawalhi MM, Ismail MF, El-Tanbouly ND (2006). Biochemical study of the anti-diabetic action of the Egyptian plants: Fenugreek and Balanites. Mol Cell Biochem 281(1-2): 173-183.

[10] Georgetti SR, Casagrande R, Vicentini FTM, Verri Jr, WA, Fonseca MJV (2006). Evaluation of the antioxidant activity of soybean extract by different *in vitro* methods and investigation of this activity after its incorporation in topical formulations. Eur J Pharm Biopharm 64 (1): 99–106.

[11] Ghadyale V, Takalikar S, Haldavnekar V, Arvindekar A (2012). Effective control of postprandial glucose level through inhibition of intestinal alpha –glucosidase by *Cymbopogon martinii* (Roxb.). Evid Based Compl Alternat Med 1-6.

[12] Ghosh S, Ahire M, Patil S, Jabgunde A, Dusane MB, Joshi BN, Pardesi K, Jachak S, Dhavale DD, Chopade BA (2012). Antidiabetic activity of *Gnidia glauca* and *Dioscorea bulbifera*: Potent amylase and glucosidase inhibitors. Evid Based Compl Alternat Med 1-10.

[13] Gopu CL, Gilda SS, Paradkar AR, Mahadik KR (2008). Development and validation of a densitometric TLC method for analysis of trigonelline and 4-hydroxyisoleucine in fenugreek seeds. Acta Chromatographia. 4, 709–719.

[14] Hassanzadeh E, Chaichi MR, Mazaheri D, Rezazadeh S, Naghdi-Badi HA (2011). Physical and chemical variabilities among domestic Iranian fenugreek (*Trigonella foenum-graceum*) seeds. Asian J Plant Sci 10 (6): 323-330.

[15] Hiran P, Kerdchoechuen O, Laohakunjit N, Sukrong S (2013). The Inhibition of α-glucosidase for Type 2 Diabetes from Maize (Zea mays). Agric Sci J 44(2): 449-452.

[16] Hussain SA, Ahmed ZA, Mahvi TO, Aziz, TA (2012). Effect of quercetin on postprandial glucose excursion after mono- and disaccharides challenge in normal and diabetic rats. J Diabetes Mellitus 2(1): 82-87.

[17] Jahan N, Khalil-ur-Rahman, Ali S, Asi MR (2013). Phenolic Acid and flavonol contents of gemmo-modified and native extracts of some indigenous medicinal Plants. Pak J Bot 45(5): 1515-1519.

[18] Jang H-D, Zhou B, Kwon Y-I (2012). Extracts of germinated wheat and barley reduce postprandial hyperglycemia by inhibiting α-glucosidase in diabetic db/db mice. The FASEB J 26 (1025).

[19] Jo SH, Ka EH, Lee HS, Apostolidis E, Jang HD, Kwon YI (2009). Comparison of antioxidant potential and rat intestinal α-glucosidase inhibitory activities of quercetin, rutin and isoquercetin. Int J Appl Res Nat Prod 2 (4): 52-60.

[20] Kor NM, Moradi K (2013). Physiological and pharmaceutical effects of fenugreek (*Trigonella foenum-graecum* L.) as a multipurpose and valuable medicinal plant. Global J Med Plant Res 1(2): 199-206.

[21] Kumar P, Kale RK, McLean P, Baquer NZ (2012). Antidiabetic and neuroprotective effects of *Trigonella foenum-graecum* seed powder in diabetic rat brain. Prague Med Report 113 (1): 33–43.

[22] Kumar V, Ahmed D, Anwar F, Ali M, Mujeeb M (2013). Enhanced glycemic control, pancreas protective, antioxidant and hepatoprotective effects by umbelliferon-α-D-glucopyranosyl-(2I→1II)-α-Dglucopyranoside in streptozotocin induced diabetic rats. Springer plus 2 (639): 1-20.

[23] Kunyanga CN, Imungi JK, Okoth M, Momanyi C, Biesalski HK, Vadivel V (2011). Antioxidant and antidiabetic properties of condensed tannins in acetonic extract of selected raw and processed indigenous food ingredients from Kenya. J Food Sci 76(4): C560-567.

[24] Kuo Y-H, Rozan P, Lambein F, Frias J, Vidal-Valverde C (2004). Effects of different germination conditions on the contents of free protein and non-protein amino acids of commercial legumes. Food Chem 86 (4): 537-545.

[25] Laila O, Murtaza I, Abdin MZ, Ahmad S, Ganai NA, Jehangir M (2013). Development and validation of HPTLC method for simultaneous estimation of diosgenin and quercetin in fenugreekseeds (*Trigonella foenum-graceum*). ISRN Chromatogr 1-8.

[26] Lin LY, Peng CC, Yang YL, Peng RY (2008). Optimization of bioactive compounds in buckwheat sprouts and their effect on blood cholesterol in hamsters. J Agric Food Chem 56(4): 1216-1223.

[27] Liu B, Guo X, Zhu K, Liu Y (2011). Nutritional evaluation and antioxidant activity of sesame sprouts. Food Chem 129 (3): 799-803.

[28] Mahmoud MF, Hassan NA, El-Bassossy HM, Fahmy A (2013). Quercetin protects against diabetes-induced exaggerated vasoconstriction in rats: Effect on low grade inflammation. PLOS ONE 8 (5): e63784.

[29] Mahomoodally MF (2013). Traditional medicines in Africa: An appraisal of ten potent African medicinal plants. Evid Based Compl Alternat Med 1-14.

[30] Maiti D, Majumdar M (2012). Impact of bioprocessing on phenolic content and antioxidant activity of soy seed to improve hypoglycemic functionality. Asian J Plant Sci Res 2 (2): 102-109.

[31] Malick CP, Singh MB (1980). Plant enzymology and histoenzymology: A Text Manual, Kalyani publishers, New Delhi, pp 286.

[32] Manivannan J, Arunagiri P, Sivasubramanium J, Balamurugan E (2013). Diogenin prevents hepatic oxidative stress, lipid peroxidation and molecular alterations in chronic renal failure in rats. Int J Nutr Pharmacol Neurol Dis 3(3): 289-293.

[33] Mccue P, Kwon YII, Shetty K (2005). Anti-diabetic and anti-hypertensive potential of sprouted and solid-state bioprocessed soybean. Asia Pac J Clin Nutr 14(2): 145–152.

[34] Mehrafarin A, Qaderi A, Rezazadeh Sh, Naghdi-Badi H, Noormohammadi Gh, Zand E (2010). Bioengineering of important secondary metabolites and metabolic pathways in fenugreek (*Trigonella foenumgraecum* L.). J Med Plants 9(35): 1 – 18.

[35] Monago CC, Nwodo OFC (2010). Antidiabetic effect of crude trigonelline of *Abrus precatorius* Linn. seed in alloxan diabetic rabbits. J Pharm Res 3(8): 1916-1919.

[36] Nagmoti DM, Juvekar AR (2013). *In vitro* inhibitory effects of *Pithecellobium dulce* (Roxb.) Benth.seeds on intestinal α-glucosidase and pancreatic α-amylase. J Biochem Tech 4 (3): 616-621.

[37] Norouzi HA, Vazin F (2011). Modelling of the faba bean (*Vicia faba* L.) sprouting reaction to temperature in farm condition. Not Bot Horti Agrobo 39(2): 179-185.

[38] Pinto MD-S, Shetty K (2010). Health Benefits of Berries for Potential Management of Hyperglycemia and Hypertension, In: Qian MC, Rimando AM (ed), Flavor and Health Benefits of Small Fruits (Chapter 8). ACS Publications, Washington, DC, USA, pp 121–137.

[39] Pullela SV, Tiwari AK, Vanka US, Vummenthula A, Tatipaka HB, Dasari KR, Khan IA, Janaswamy MR (2006). HPLC assisted chemobiological standardization of α-glucosidase-I enzyme inhibitory constituents from *Piper longum* Linn-An Indian medicinal plant. J Ethnopharmacol 108(3): 445–449.

[40] Randhir R, Kwon YI, Shetty K (2009). Improved health-relevant functionality in dark germinated *Mucuna pruriens* sprouts by elicitation with peptide and phytochemical elicitors. Bioresour Technol 100(19): 4507 – 4514.

[41] Ranilla LG, Kwon YI, Genovese MI, Lajolo FM, Shetty K (2008). Antidiabetes and antihypertension potential of commonly consumed carbohydrate sweeteners using *in vitro* models. J Med. Foods 11(2): 337 – 348.

[42] Saxena SN, Karwa S, Saxena R, Sharma T, Sharma YK, Kakani RK, Anwer MM (2011). Analysis of antioxidant activity, phenolic and flavonoid content of fenugreek seed extracts (*Trigonella foenum-graecum* L.) seed extracts. Int J Seed Spices 1: 38-43.

[43] Sharma N, Kar A, Panda S (2014). *Trigonella foenum-graecum* seed extract enhances the antiperoxidative and antidiabetic activities of glibenclamide. Int J Pharm Sci Rev Res 24 (1): 152-156.

[44] Snehlata HS, Payal DR (2012). Fenugreek (*Trigonella foenum-graecum* L.): An Overview. Int J Curr Pharm Rev Res 2(4): 169-187.

[45] Sumner JB, Howell SF (1935). A method for determination of saccharase activity. J Biol Chem.108: 51-54.

[46] Swieca M, Gawlik-Dziki U, Jakubczyk A (2013). Impact of density of breeding on the growth and some nutraceutical properties of ready-to-eat lentil (*lens culinaris*) sprouts. Acta Sci Pol Hortorum Cultus 12(4): 19-29.

[47] Tarzia BG, Gharachorlooa M, Bahariniab M, Mortazavic SA (2012). The effect of germination on phenolic content and

antioxidant activity of chickpea. Iranian J Pharm Res 11 (4):1137-1143.

[48] Thomas JE, Bandara M, Lee EL, Driedger D, Acharya S (2011). Biochemical monitoring in fenugreek to develop functional food and medicinal plant variants. N Biotechnol 28(2): 110–117.

[49] Vadivel V, Biesalski HK (2012). Total phenolic content, *in vitro* antioxidant activity and type II diabetes relevant enzyme inhibition properties of methanolic extract of traditionally processed underutilized food legume, Acacia nilotica (L.) Wild ex. Delile. Int Food Res J 19 (2), 593-601.

[50] Worthington Biochemical Corp., 1993. Alpha amylase, In: Worthington V (ed), Worthington Enzyme Manual, Freehold, New Jersey, pp 36–41.

[51] Zheng X-q, Hayashibe E, Ashihara H (2005). Changes in trigonelline (*N*-methylnicotinic acid) content and nicotinic acid metabolism during germination of mungbean (*Phaseolus aureus)* seeds. J Exp Bot 56 (416): 1615-1623.