Abstract
PAL catalyzes the first step in the biosynthesis of phenylpropanoids, which are further modified into a wide variety of phenolic compounds. In the current study, we estimated the change in PAL enzyme activity and total phenol content in six cultivars of fenugreek (ML1-ML6) seeds and vegetative green leafy parts of their respective plants taken at early, mid and late stages of growth. The results obtained were correlated with their respective hypoglycemic activities. Our study demonstrated that, at the mid stage of growth all the cultivars showed maximum PAL activity with simultaneous increase in phenol production and hypoglycemic activities. The results of this study taken together suggest that all the fenugreek cultivars possess good hypoglycemic activity that is directly related to increase in total phenol content and PAL activity. Among six selected cultivars, ML-2 cultivar in addition to possess maximum hypoglycemic activity in terms of inhibiting \( \alpha \)-amylase (72.53 %) and \( \alpha \)-glucosidase (52 %) activities also showed maximum PAL enzyme activity (0.01008 \( \mu \)M cinnamic acid/mg protein/ min) as well as total phenol production (560 mg/100g fresh weight) at mid stage of growth. A highly significant correlation was found between PAL activity and total phenols (\( r=0.759 \)) which were significantly correlated with \( \alpha \)-amylase inhibitory activity (\( r=0.439, r=0.690 \) respectively) as well as with \( \alpha \) glucosidase inhibitory activity (\( r=0.507, r=0.552 \) respectively). It was observed that extracts from such cultivars especially that of ML2 cultivar taken at mid stage of growth when incorporated into diet could act as a potential chemopreventive functional food for better management of hyperglycemia.

Keywords: Fenugreek, Phenylalanine Ammonium Lyase, Phenols, \( \alpha \)-amylase, \( \alpha \)-glucosidase, hypoglycemic activity.
treatments with fewer side effects cause less harm to the organism (3). Therefore, apart from currently available therapeutic options, many herbal medicines have been designed for treatment of diabetes. However, the World Health Organization expert committee on diabetes has recommended that such methods of treatment should be further investigated in order to make them realistic possibilities for proper management of diabetes (4).

In plants, the phenolic metabolism is governed by an important key enzyme of phenylpropnoid metabolic pathway called Phenylalanine Ammonium Lyase (PAL). It catalysis the deamination, of L-Phenylalanine to produce the trans–cinnamic acid that is primary intermediary in the biosynthesis of phenolics (5). Recent studies have shown that PAL activity is directly correlated with the production of phenolic compounds and its high activity has been reported to be associated with the accumulation of anthocyanins and other phenolic compounds in fruit tissues of several species (6). Thus, catalytic step by PAL enzyme in plants is considered to be the first comitted step for the biosynthesis of the phenylpropanoid skelton that can be used for the synthesis of phenolics, flavonoids, phenylpropane and lignins (7) and such secondary metabolites especially phenolic constituents have been reported to be involved in retardation of $\alpha$ amylose as well as in $\alpha$ glucosidase enzyme activities (8). These two enzymes are involved in starch breakdown and intestinal glucose absorption respectively. Unfortunately, plants with such compounds have not yet gained much importance due to the lack of sustained scientific evidence, even knowing that currently available inhibitors in clinical use have their limitations, are non-specific, produce serious side effects and even elevate diabetic complications. Fenugreek (Trigonella foenum graecum) plant indigenous to the Mediterranean region, Ukraine, India and China has received a great deal of attention in medical research for its antidiabetic activity against both type I and type II diabetes (2). In addition to alkaloids and steroids, the antidiabetic activity of fenugreek has mainly been attributed to its phenolic compounds which are also involved in plant defense against various toxic insults including oxidative stress, abiotic and biotic stresses (9).

Therefore, in the current study an attempt has been made to contribute to this field of research by investigating extracts from six cultivars of fenugreek seeds as well as their respective green leafy parts at different stages of growth for their respective PAL activities as well as total phenolic compounds. Simultaneously, $\alpha$-amylase and $\alpha$-glycosidase inhibiting activities of each extract was determined and correlated with PLA and total phenols for development of functional food against diabetes.

**Materials and Methods**

**Chemicals:** All the chemicals and reagents used in this current study were of analytical reagent grade. $\alpha$-glucosidase and 4-nitrophenyl $\alpha$-D-glucopyranoside were obtained from Sigma Aldrich (India) while cinnamic acid, catechol, phenylalanine and dinitrosalicylic acid (DNSA) from SRL Pvt. Ltd. (Mumbai, India). Starch, sodium carbonate ethanol, sodium phosphate monobasic (Na$_2$HPO$_4$), sodium phosphate dibasic, sodium potassium tartrate, and sodium hydroxide (NaOH), were purchased from Qualigens, (Mumbai, India). Porcine pancreatic $\alpha$-amylase and sodium chloride (NaCl) were obtained from Hi Media Laboratories (Mumbai, India).

**Plant Material:** For the current investigation, 100% pure and promising seeds belonging to six different cultivars of fenugreek were collected from the local market as well as from the Division of Vegetable Sciences, Sher-e-Kashmir University of Agricultural Sciences and Technology, Shalimar, Kashmir, India. The seeds collected from SKUAST-K were ML1 (Methi Shalimar), ML2 (6AGR), ML3 (Shalimar improved), ML4 (SAW) and ML5 (Methi local). Whereas ML6 (Kasuri methi) was collected from local market. All the seeds were identified by Imtiyaz Murtaza et al.
subject expert from the Division of Vegetable Sciences, SKUAST-K, Shalimar. These selected seeds were grown under controlled atmospheric conditions for further studies and the vegetative green leafy samples from each cultivar collected at an early (40 days after sowing), mid (62 days after sowing) and late stage (105 days after sowing) of growth. The collected samples were subjected for further analysis and the measurements were conducted in triplicates for each sample.

**PAL extraction and assay:** Approximately, 500 mg of seed as well as leafy samples collected randomly from five plants each was homogenized in 15 ml of 5 Mm Tris-HCl buffer (pH 8.5) containing 1.4 mM β-mercaptoethanol and the resulting slurry was filtered through two layers of cheese cloth. The filtrate was centrifuged at 12000 g for 15 minutes at 4°C and stored on ice. The resulting supernatant was directly used in the estimation of total protein as well as for PAL enzyme assay as crude extract. The protein content was measured by Bradford method (10). The PAL activity in these extracts was measured by the method developed by Khan et al (11). 0.1 ml of enzyme extract was combined with 1 ml of 50 mM Tris HCl, 0.5 ml of 20 mM L-phenylalanine and 0.4 ml of double distilled water and the resulting reaction mixture was incubated for 60 minutes at 30°C. The reaction was stopped by the addition of 250 μl of 2 N HCl and the cinnamic acid formed in reaction mixture was extracted in 2 ml of toluene. In one ml of separated toluene layer, a pinch of anhydrous sodium sulphate was added and absorbance was measured at 290 nm. The standard curve was established using cinnamic acid as standard. The specific activity of the enzyme was expressed as μ moles of cinnamic acid produced /min/mg protein.

**Total Phenol assay:** Total phenolic content in fenugreek samples was determined according to method reported by Malick et al (12). Approximately, 500mg of each sample was homogenized in ten time volume of 80% ethanol and centrifuged at 11900 g. Supernatant was collected and the residue was again re-extracted with five times the volume of 80% ethanol. The pooled supernatants were evaporated to dryness and finally reconstituted with a known volume of double distilled water. To determine phenol content, 500μl of the reconstituted extract was combined with 2.5ml of double distilled water and 0.5ml of Folin cioucalteau reagent. After 3 minutes of incubation period, 20% sodium carbonate was added to each sample, vortexed and boiled in a water bath for exactly one min. The absorbance was measured at 650nm against reagent blank. A standard curve was established using catechol as standard. Absorbance values were converted to milligram of phenolics per 100g of fresh tissue. For each cultivar three replicates were analyzed

**α-Amylase Inhibition Assay:** The α-amylase inhibition activity of fenugreek extracts was measured by following the method reported by Catherine Nkirote et al (13). 100 μL of 1% starch solution in 0.02 M sodium phosphate buffer (pH 6.9) was added to mixture of 100 μL of ethanolic extract and 100 μL of 0.02 M sodium phosphate buffer (pH 6.9) containing α-amylase solution (1 unit liberates 1.9 μL of maltose from starch in 1 min at pH 6.9 and temperature 25°C), and was incubated at 25°C for 30 minutes. After the incubation, the reaction was stopped with 1 mL of DNSA reagent. The test tubes were then incubated in a boiling water bath for 5 min and cooled to room temperature. The reaction mixture was then diluted to 10-fold with distilled water and the absorbance was measured at 540 nm. The readings were compared with the control, which contained buffer instead of sample extract. Based on the absorbance value, the percent inhibition activity was calculated for all the samples (8).

Absorbance was calculated by using following formula:

\[
\text{Absorbance} = \frac{(Ac+)-(Ac)}{(Ac+)-(Ac-)} \times 100
\]

“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. Where “As” represent “AC+” including sample extract and “Ab” represent “Ac-” excluding sample extract) respectively.

**α-Glucosidase Inhibition Assay:** The α-glucosidase inhibition activity was determined according to the method described by Worthington (14). A total of 100 μL of ethanolic extract and 200 μL of 0.1 M phosphate buffer (pH 6.9) containing α-glucosidase solution (1 unit/mL) were taken in tubes and incubated at 25°C for 5 min. After the pre-incubation, 100 μL of 5 mM p-nitrophenyl-α-D-glucopyranoside solution in 0.1 M phosphate buffer (pH 6.9) was added to each tube and the reaction mixture was incubated at 25°C for 5 minutes. After the incubation period, the reaction was stopped by addition of 0.1 M Na2CO3 and the aliquots were diluted to 10-fold with distilled water, and the absorbance readings recorded at 405 nm and compared to a control that had 100 μL of buffer solution in place of the extract. The results were calculated and expressed as percentage of α-glucosidase inhibition.

Absorbance was calculated by using following formula (8):

\[
\text{The } \% \text{ α-glucosidase inhibitory activity} = \frac{(Ac+) - (Ac-) - (As-Ab)}{(Ac+) - (Ac-)} \times 100
\]

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 represent “Ac-” excluding sample extract respectively.

**Statistical Analysis:** Statistical analysis of data was performed by using one way analysis of variance (ANOVA) and correlation tests. The data was analyzed by using comprehensive statistical package SPSS (Version 20) for windows.

**Results and Discussion**

Plants synthesize a vast range of secondary metabolites including phenolic and flavonoid antioxidants that are currently being aggressively exploited to develop preventive and treatment measures for common oxidative stress linked degenerative diseases such as diabetes, cardiovascular disease, certain cancers and even aging (15). Among plants, fenugreek has been viewed as miraculous herb due to its unprecedented health promoting activities. In the current study, six different cultivars of fenugreek possessing varied phenotypic and genotypic characteristics were analyzed for their phenol production and their correlation to PAL enzyme activity. As during growth, there exists change in different type of secondary metabolites including total phenols, therefore in order to determine which stage of growth had the highest level of phenolics and PAL activity, six cultivars of fenugreek (ML1-ML6) were analyzed and compared with each other. A high variation in terms of phenolic content (95-560 mg / 100g fw) was observed in the selected fenugreek seed cultivars as well as their respective green leafy parts collected at different stages of growth (Fig. 1B-4B). Such significant differences among the different cultivars have been reported to be likely due to genotypic and environmental differences (namely, climate, location, temperature, fertility, diseases and pest exposure) within species, choice of parts tested, time of taking samples and determination methods (16). Further, a steady increase in phenols was observed while moving from seed stage to mid stage of growth. However, towards late stage of growth there was a gradual decrease in total phenol content attributed to decreased metabolic activities during this phase. In production of phenolic compounds, Phenylalanine Ammonium Lyase (PAL) enzyme plays a very crucial role. It catalyses the first step in the biosynthesis of phenylpropanoids, that are further modified into a wide variety of phenolic secondary metabolites. Therefore, for various scientific interventions including metabolic engineering and hyperexpression of phenols and their derivatives,
this enzyme has recently gained much interest. In the current study, we also evaluated the changes in PAL enzyme activity in all the six cultivars of fenugreek (ML1-ML6 series) at different stages of growth. PAL activity was found to follow the same trend as found in case of phenols and was found to lie in between $0.00017-0.01008 \mu M$ cinnamic acid/min/mg protein (Fig. 1A-4A). There was gradual increase in PAL activity from seed stage till mid stage that was followed by slight decrease while moving towards late stage of growth. Among all the six selected cultivars, ML2 possessed the highest PAL activity ($0.01008 \mu M$ cinnamic acid/min/mg protein) whereas ML4 the lowest i.e $0.00290 \mu M$ cinnamic acid/min/mg protein at mid stage of growth.

Overall, in this study it was found that at mid stage of growth phase all the cultivars possessed maximum PAL activity as well as maximum phenol production (Fig. 1-4). These results corroborated well with the previous reports indicating that PAL enzyme plays a key role in synthesis of phenolics in plant system (5). Thus, among these selected cultivars, ML-2 showed the maximum PAL enzyme activity as well as total phenol content ($560mg/100g fw$) at mid stage of growth (Fig. 3A). Phenolic compounds are currently been viewed as strong antioxidants therefore, current study clearly suggests that at mid stage of growth, ML2 cultivar of fenugreek can act as a potent antioxidant rich food.

**Fig. 1.** (A) PAL activity of different cultivars of fenugreek seeds. (B) Total phenol content, $\alpha$ amylase inhibition and $\alpha$ glucosidase inhibition shown by different cultivars of fenugreek seed extracts.

**Fig. 2.** (A) PAL activity of different cultivars of fenugreek at early stage of growth. (B) Total phenol content, $\alpha$ amylase inhibition and $\alpha$ glucosidase inhibition shown by different cultivars of fenugreek extracts at early stage of growth.
In previous studies, it has been reported that production of PAL can get increased due to number of reasons including sprouting, physical wounding as well as by modern biotechnological interventions resulting in many fold increase in total phenol content or their precursors (17). Since, in our investigation, all the cultivars were grown under same environmental conditions as well as by all means followed same procedural evaluations. Therefore, the variation observed in PAL activity and total phenol content among different cultivars can be mostly attributed to genetic differences and not to environmental influences. It has been reported that high concentration of phenolics can cause astringent undesirable effects and thus make that food unacceptable for consumption (18). Therefore, our current study moves in different direction and in addition to evaluate PAL and Phenol content the potential functionality of these samples in terms of hypoglycemic property of phenol was evaluated.

The study was carried out by targeting two Key enzymes viz pancreatic alpha-amylase and intestinal alpha-glucosidase, involved in the enzymatic breakdown of starch and absorption of complex carbohydrates respectively. The inhibition of their activities has been viewed as potential avenues for modulation of type 2 diabetes-associated post-prandial hyperglycemia (19).

As per previous reports, it has been shown that individual phenolic compounds present in plants possess hypoglycemic activity and can thus act as therapeutic agents. Recently, it has been cited that the presence of certain bioactive compounds of phenolic nature like quercetin, pose to be α-amylase and α-glucosidase inhibitors, and thus play an important role in treatment of managing hyperglycemia and related complications with minimum side effects as compared to currently available therapeutic regimes (8, 20-23). However, instead of using such compounds individually, it is suggested that they perform much better in combination due to synergistic effects and varied bioavailabilities. In our study, ethanolic extracts of fenugreek demonstrated very effective α-amylase inhibitory activity that varied in between 26.72% to 72.53% at different stages of growth (Fig. 1- 4). As the main issue to attempt to manage diabetes at first step in hyperglycemic patients is to target this potent enzyme found in saliva as well as pancreatic juice (20). Therefore, our study demonstrates very encouraging results by inhibiting α-amylase activity especially by fenugreek extracts prepared from leaves collected at mid stage of growth and thus indicate to play a great role in controlling fluctuating blood glucose levels. As discussed above, the inhibitory activities of this enzyme are often linked to certain phenolic compounds present in plant foods. Therefore, the inhibition of this enzyme could vary by being high or low depending on the presence of phenolic phytochemicals present in specific food that modulate its activity (24,25). Extracts from ML2 cultivar possessing maximum phenol content and high PAL activity at mid stage of growth demonstrates highest inhibition activity (72.5 %) against this potent chemotherapeutic target enzyme (Fig. 3B) followed by other cultivars. These food based phenolic compounds have been reported to bind to the reactive sites of enzymes and alter their catalytic activity (2). Therefore, the maximum inhibition of α-amylase by extracts possessing high phenolic content may also occur through the direct blockage of the active centre at several subsites of the enzyme (26).

In diabetes, in addition to target α amylase it is a well-established fact that therapeutic approach to decrease postprandial hyperglycemia in this disease is by slowing the absorption of glucose, by inhibition of α-glucosidase enzyme in digestive system (27). Thus, it is important that dietary α-glucosidase inhibitors are present in sufficient quantity, to prevent the absorption of glucose in small intestine (28). In the current study, ethanolic extracts of fenugreek under in vitro conditions also demonstrated α-glucosidase inhibitory activity that varied between 20.08% to 52 % (Fig. 3B). Interestingly, among

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A    Fenugreek Cultivars

Fig. 3. (A) PAL activity of different cultivars of fenugreek at mid stage of growth. (B) Total phenol content, α− amylose inhibition and α−glucosidase inhibition shown by different cultivars of fenugreek extracts at mid stage of growth.

B    Fenugreek Cultivars

Fig. 4. (A) PAL activity of different cultivars of fenugreek at late stage of growth. (B) Total phenol content, α− amylose inhibition and α−glucosidase inhibition shown by different cultivars of fenugreek extracts at late stage of growth.

Table 1. Correlation matrix of variables

<table>
<thead>
<tr>
<th>Variables</th>
<th>PAL Activity</th>
<th>Total Phenols</th>
<th>α-amylase inhibition</th>
<th>α-glucosidase inhibition</th>
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<td>PAL activity</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Total Phenols</td>
<td>0.759**</td>
<td>0.690*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-amylase inhibition</td>
<td>0.439*</td>
<td>0.552*</td>
<td>1</td>
<td>0.656*</td>
</tr>
<tr>
<td>α-glucosidase inhibition</td>
<td>0.507*</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

** Highly significant
* Significant

PAL enzyme activity
the six different cultivars, ML2 possessing highest $\alpha$-amylase activity also showed the maximum inhibitory $\alpha$-glucosidase inhibitory activity (52 %) among the selected cultivars at mid stage of growth. Thus, extract from ML2 can act as a potent health food to regulate carbohydrate metabolism and manage hyperglycemia. Interestingly, a direct correlation between increase in phenolic content and inhibition of $\alpha$-amylase and $\alpha$-glucosidase was observed. It has been reported that such type of food grade materials offer cost effective and locally based strategies to control postprandial hyperglycemia with minimum side effects (23).

The statistical analysis data (Table 1) indicates that there exists highly significant correlation between increase in PAL activity and total phenols ($r=0.759$) that were in turn significantly related to $\alpha$-amylase inhibitory activity ($r=0.439$, $r'=0.690$ respectively) as well as with $\alpha$ -glucosidase inhibitory activity ($r=0.507$, $r=0.552$ respectively). Overall mean along with standard deviation for PAL activity, total phenols, $\alpha$ amylase inhibitory activity and $\alpha$ -glucosidase inhibitory activity was recorded as $0.00312 \pm 0.00272$, $328.2 \pm 147.07$, $47.03 \pm 12.301$ and $33.73 \pm 7.75$ respectively. Using ANOVA it was found that ML2 cultivar with respect to phenols, $\alpha$-amylase inhibitory activity and $\alpha$-glucosidase inhibitory activity shows highest and significant results of 382.25, 62.50 and 40.006 respectively and among stages, mid stage shows significant results for the above variables with 476.67, 56.313 and 39.625 respectively. Through this study, we were able to provide the strong rationale for determining the best cultivar and best stage of growth in fenugreek to be used as potential chemopreventive food against diabetes. Further such type of study paves way to identify promising genotypes for breeding and industrial use.

**Conclusion**

In this study, we observed that hypoglycemic activity varies in fenugreek at each stage of growth that is directly related to corresponding change in phenol production as well as in PAL activity. Therefore, through this novel approach, we identified in this important crop that stage of growth which possesses maximum PAL activity as well as total phenol content and that in turn lowers the glycemic index and controls the post-prandial hyperglycemia by targeting $\alpha$-amylase and $\alpha$-glucosidase enzymes involved in carbohydrate metabolism. Thus, it is suggested that extracts of such fenugreek cultivars, especially from ML2 cultivar taken at mid stage of growth when incorporated into diet could act as a potential chemopreventive food for better management of hyperglycemia related to diabetes. However, despite such encouraging results, it is suggested that more research in this direction is required for developing such food based effective and valuable functional food and herbal formulations for anti-diabetic therapy.

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**References**


