Phenolic acid profiling and antiglycation studies of leaf and fruit extracts of tyrosine primed *Momordica charantia* seeds for possible treatment of *diabetes mellitus*

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Abstract: The increasing risk of variety of fatal diseases including *diabetes mellitus* is imposing serious challenge to chemist, biologists and clinicians. Due to the side effects of the chemotherapy, worldwide it is thinking that phyto-medicine are more effective to cope continuously increasing risk of fatal diseases without any side effect. Seed priming is a strategic pre-sowing semi-bioengineering technique which has ability to improve the growth rate and biologically active compounds in short time. Among seed priming techniques, tyrosine seed priming most frequently used because amino acids provide best growth media for nutritional food crops. Seeds of *Momordica charantia* were subjected to the pre-sowing tyrosine solution. Different growth parameters including growth emergence rate, seedling vigor, growth and weight of root, shoot and leaf were studied. The results showed positive effect on *Momordica charantia* seed growth and phenolic acids production i.e. ferulic acid – 43.95 ppm and sinapic acid – 18.39 ppm. The antiglycation assay showed 23.45±1.23% antiglycation activity of primed-seed fruit extract as compare to control seed fruit extract (0.87±0.03%). On the basis of the results, it is concluded that tyrosine primed seed fruit extract could effectively be further tested for pre-clinical and clinical studies to manage *diabetes mellitus* disease.

Keywords: Tyrosine priming, antiglycation activity, *diabetes mellitus*, antioxidants, phenolic acids.

INTRODUCTION

Seed priming is a strategic pre-sowing semi-bioengineering technique used for the improvement of germination speed, uniform growth and chemical composition (Khalil *et al.,* 2010). In recent years lot of scientific exercises are being done to explore the medicinal values of flora all over the world which explored numerous chemicals for therapy of infectious and malignant diseases, promisingly. However, the isolation rate of these medicinal compounds from the plants depends on their production in plants. To manage the ever-increasing cases of infectious and malignant diseases it is priority order challenge for chemists, biochemists, technologists and biologists to develop semi-bioengineering techniques to improve the uniform growth rate with increased production of biologically active compounds.

Bitter gourd (*Momordica charantia)* is a medicinal plant belonging to family *Cucurbitaceae* and is a good source of minerals, vitamins, protein and biologically active ingredients. Different processing techniques are in practice to make the bitter gourd in use for long time in order to take the advantage of its mineral profile and biological active ingredients. Minimal processing, drying and canning processing is used to maintain the quality of bitter gourd for a long time (Kumar *et al*., 2016). It is effectively used for the treatment of metabolic disorder, infections and *diabetes mellitus* diseases that render the attention of scientists to explore other medicinal utilizations (Kumar *et al*., 2016). Chemical priming at different stages of plant life was previously investigated and was found positive impact on biochemical and biological profile of plants which in some cases appear to improve antiglycation activity (Kosseva, 2017), however, the technique uses variety of sets of chemical compositions which produce side effect along with the aforementioned roles (Zhang. *et al.,* 2015).

The aim of this study is to investigate antiglycation activity of *Momordica charantia* by seed priming with tyrosine solution Tyrosine (4-hydroxyphenylalanine) is one of the 20 standard amino acids, has its role in protein synthesis, photosynthesis, signal transduction processes. It is hypothesized that the priming with naturally occurring tyrosine amino acid will enhance the antiglycation activity of the bitter gourd remarkably.

MATERIALS AND METHODS

Collection of sample

Taxonomically identified and confirmed seeds of *Momordica charantia* were obtained from Ayub

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Agriculture Research Institute (AARI), Faisalabad, Pakistan. Germination experiment was conducted in the fields of Government College University, Faisalabad.

Seed priming with tyrosine and germination parameters studies

The *Momordica charantia* seeds were primed with water which taken as control (non-primed seeds) and tyrosine solution of three different concentrations i.e. 0.1%, 0.2% and 0.3% for 12 hours (primed-seeds). After priming, seeds were washed with distilled water, covered with filter paper and left to dry in air. Primed and non-primed seeds were sowed in field of botanical garden at GC University, Faisalabad, at different distances. Experiments were designed with three replicates. Seed germination data were recorded after 7^{th} day of sowing the seeds using following parameters;

- Germination percentage was calculated at the end of 7^{th} day by using the formula given below reported earlier (Ijaz *et al*., 2012).
  $Gp = (N_g/N_p) \times 100$
  "N_g" is the last number of emerged seeds and "N_p" is the total number of seeds sown.

- Mean growth time (MGT) was calculated according to as follow.
  $MGT = \sum(Dn) / \sum n$
  "n" is the number of seeds germinated on day D, and D is number of days counted from the beginning of the germination test. Seedling vigor was calculated by following the formula described earlier (Vashisth and Nagarajan, 2010).
  Vigor index I= germination (%) × seedling length (root + shoot)
  Vigor index II= germination (%) × seedling dry weight (root + shoot)

- Similarly, primary root length, shoot length, leaf area, total fresh weight of root, shoot and leaf was also measured.

Phenolic profiling through high-performance liquid chromatography

HPLC was done for the investigation qualitative and quantitative phenolic acids profile following the procedure reported by Hussain *et al*., (2012) with slight modification. Varian HPLC using ODS (C18) reversed phase column was used for the identification of phenolic acids present in extracts. In order to separate different phenolic acids two solvents i.e. solvent A (70% acetonitrile in methanol) and solvent B (0.5% glacial acetic acid) were used as mobile phase with a constant flow rate of 1 mL/min in gradient mode. The gradient scheme is as follow; solvent A (100%) for first 5 minutes, solvent A (95%) and solvent B (5%) for 5-10 minutes, solvent A (5%) and solvent B (95%) for 10-30 minutes, solvent A (100%) from 30-35 minutes. Twenty microliter sample was loaded through HPLC sample port using microsyringe. Detection was carried out with built in UV-visible spectroscopic detector at 275 nm. Identification of phenolic acids was performed by correlating their relative retention times with those of standard mixture chromatogram. The amounts of individual compounds were measured on the basis of the area under peak, relative to the corresponding standard phenolic acid concentration peak area relation.

Antiglycation activity assay

Took 1g sample and add 1.5mL methanol (50%), centrifuged at 1500 rpm for 10 min. Now supernatant used for the analysis of antiglycation activity following the procedure reported previously (Matsuda *et al.,* 2003). Briefly, the reaction mixture comprises of 150µL D-glucose, 150µL bovine serum albumin (BSA in 1mL sodium phosphate buffer pH 7.2) and 150µL samples were incubated at room temperature for 7 days. The absorbance was measured using a spectrophotometer at a wavelength of 440 nm. The reaction mixture without D-glucose was used as a blank solution. Measurements were performed in triplicate. The percent inhibition of glycation was calculated with following expression:

$$\text{Inhibition of glycation (\%)} = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 10$$

STATISTICAL ANALYSIS

All determination was made in complete randomize experiments. The presence or absence of significant difference among different factors was as curtained with the analysis of variances (ANOVA). The means were compared to final significant difference using LSD testing the cases of all. Overall interaction of all the factors was checked for significance using computer software COSTAS (Cohort software, 2003).

RESULTS

Tyrosine priming effect on seed growth

Growth emergence rate and seedling vigor

The rate of final growth emergence of primed *Momordica charantia* seeds was studied at different tyrosine concentrations such as 0.1, 0.2 and 0.3% solution. The growth emergence rate was calculated as mean of triplicate experiments and standard deviation (mean ± S.D) as shown in fig. 1a. The significant difference in treatment using different tyrosine solutions was calculated at p value <0.05. The mean growth emergence rate using different parameters i.e. root, shoots and leaves, calculated from the data obtained from growth emergence rate as shown in the fig. 1b. Similarly, seedling vigor also studied by treating the *Momordica charantia* seeds with 0.1%, 0.2% and 0.3% tyrosine solution. The seedling vigor of primed-seeds was compared with control groups.

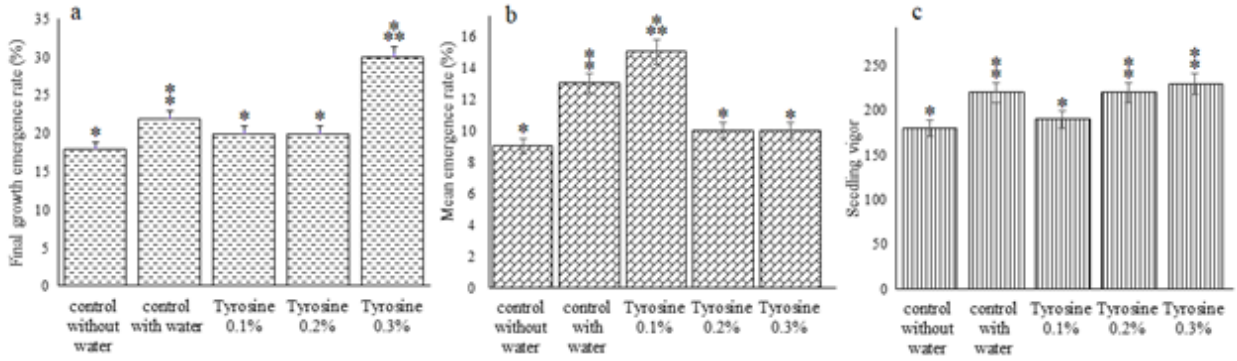

Fig. 1: Treatment of *Momordica charantia* seeds with Tyrosine solution of different concentration and control solution. Where a: final growth emergence rate, b: mean emergence rate and c: seedling vigour

Table 1: Phenolic acid profile of control and tyrosine primed *Momordica charantia* seed leaf and fruit extracts using high performance liquid chromatography analysis

S No.	Phenolic acids	Retention time (min)	Concentration (ppm)			
			Control leaf extract	Tyrosine treated leaf extract	Control fruit extract	Tyrosine treated fruit extract
1	Querecetin	2.82	3.77	2.73	2.96	2.22
2	Benzoic acid	14.65	1.64	×	×	×
3	Chlorogenic acid	15.87	5.19	0.95	×	×
4	Syringic acid	16.55	3.31	×	×	2.61
5	m-Coumeric acid	19.73	5.81	×	×	×
6	p-Coumeric acid	17.64	X	0.77	×	×
7	Cinamic acid	24.71	16.25	15.29	11.58	×
8	Ferulic aid	21.83	×	12.38	14.78	43.95
9	Sinapic acid	26.19	×	×	×	18.39
10	Vanillic acid	13.87	×	×	×	24.36

The results obtained from these investigations are shown in fig. 1c.

Growth and weight study of root, shoot and leaf

The growth attribute parameters such as growth of roots, shoots and leaves, and weight of roots, shoots and leaves of control and primed-seeds were investigated. The results of these two parameters studying using roots, shoots and leaves are shown in fig. 2(a-f). The effect of tyrosine priming was expressed in term of significant difference (p <0.05); the study points with similar signs, † or ‡, indicates non-significant difference while with different signs indicates significant difference and consequently significant effect of treatment.

HPLC analysis for phenolic profiling

The qualitative and quantitative HPLC analysis of control and primed-seeds leaf and fruit extracts was carried out using gradient elution system. Prior to the sample elution, the solution of standard phenolic acids i.e. querecetin, benzoic acid, chlorogenic acid, syringic acid, m-coumaric acid, p-coumaric acid, cinamic acid, ferulic acid, sinapic acid and vanillic acid were eluted and leaf and fruit extracts of control and primed-seed were also eluted under same conditions of temperature, pressure, flow rate and solvent system. The qualitative information about the phenolic acid profiling of control leaf and fruit extract are shown in the HPLC chromatograms fig. 3 chromatogram "a" and "c", respectively. While in the fig. 3 the chromatograms "b" and "d" shows the HPLC results of primed-seeds leaf and fruit extracts, respectively. The quantification was carried out by calculating the area under each peak and comparing the standard phenolic acid peaks as shown in the table 1.

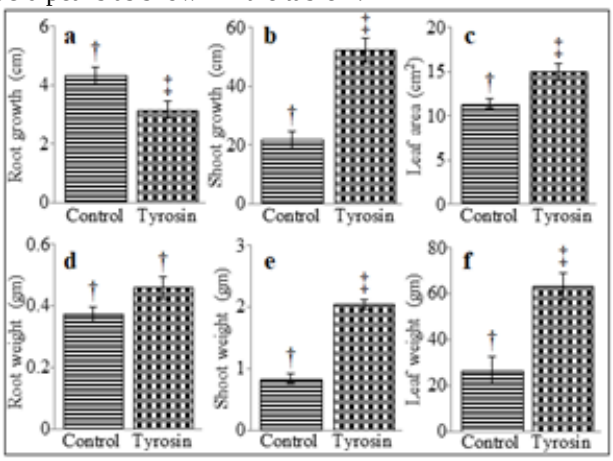

Fig. 2: Effect of tyrosine seeds priming upon growth attribute of *Momordica charantia* L. yield; a) root growth, b) shoot growth, c) leaf area, d) root weight, e)

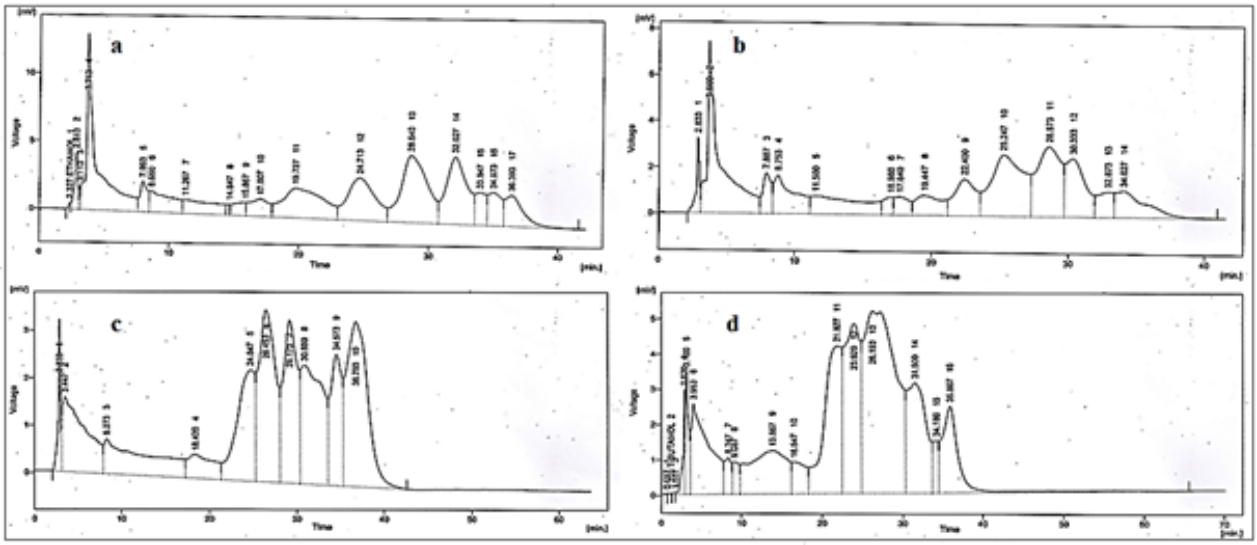

Fig. 3: HPLC analysis of controlled and primed-seed sample; a) controlled leaf extract sample, b) primed-seed leaf extract, c) controlled fruit extract, and d) primed-seed fruit extract analysis of *Momordica charantia* L. for the phenolic profile.

shoot weight and f) leaf weight different signs "† and ‡" show significant difference between the two results.

Antiglycation study

Antioxidants have ability to show antiglycation potential and consequently the *diabetes mellitus*. As the primed-seeds fruits showed promising increase in phenolic acid production – so the primed-seed fruit extracts was used to study the antiglycation activity comparing to control fruit extract activity. The results of the study are shown in fig. 4. The increased antiglycation level (23.45±1.23%) was noted with tyrosine primed-seed fruit extracts; which was significantly ($p<0.05$) higher than control seed fruit extract (0.87±0.03%).

DISCUSSION

This study aimed to test the growth and phenolic composition of *Momordica charantia* (a well-known plant for its medicinal values in broad range) by priming its seeds using tyrosine amino acid solution prior to sowing. The selection of tyrosine amino acid priming play criticle role on plant physiology and biochemistry in a number of ways; they act as buffers, synthesize other organic compounds like proteins, vitamins, enzymes, terpenoids (El-Aziz, *et al.,* 2007). It involves the stimulation of certain biochemical processes of seeds that play a key role in dormancy breakdown and mobilization of reserved food of seeds. It also includes better enzymatic activity leading towards the early emergence of the embryonic part during germination with better synchrony. Tyrosine as priming amino acid has been reported for improved growth of maize in adverse environmental conditions and medicinal plant *Trachyspermum ammi* L. under normal field conditions (Mahmood *et al.,* 2017).

The effect of tyrosine seed priming on growth was studied using different concentrations of tyrosine amino acid i.e. 0.1, 0.2, and 0.3% solution. The results indicate that 0.3% solution of primer enhanced the growth rate of the seeds significantly ($p<0.05$). The lower concentrations didn't show the significant effect as compared to the control growth, however, the mean growth rate showed 0.1% solution is more compatible ($p<0.05$) to gain uniform speed growth rate. The seedling vigor i.e. the strength of the growing plant, was more affected by 0.2 and 0.3% tyrosine solution ($p<0.05$) as represented in fig. 1. The priming effect was also further investigated using growth and weight parameters of root, shoot and leaf. In all cases except root growth the priming effect remained significant ($p<0.05$) as shown in fig. 2. The reverse effect was recorded in case of growth of root, where the growth of root reduced significantly (fig. 2a). The similar effect was noted using the tyrosine for priming maize seeds (Mahmood *et al.,* 2017).

The basic aim of priming effect was to sort out the phenolic profiling and antiglycation potential of the controlled and primed-seed growing plants. The true fig. of phenolic composition with-out and with primed-seed growth plant was analyzed using state of the art HPLC technique. The phenolic profile was assessed qualitatively and quantitatively by running standard phenolic acids (querecetin, benzoic acid, chlorogenic acid, syringic acid, m-coumeric acid, p-coumeric acid, cinamic acid, ferulic acid, sinapic acid and vanillic acid) prior to controlled and primed sample (leaf and fruit extracts) through HPLC under similar conditions of temperature, pressure, solvent system and flow rate. The results showed dual effect on phenolic profile i.e. suppression effect in case of primed-seed leaf and up-lifting effect in case of primed seed fruit. The leaf is commonly less known for phenolic

compounds; however the fruits are considered the major source of phenolic components of medicinal plants.

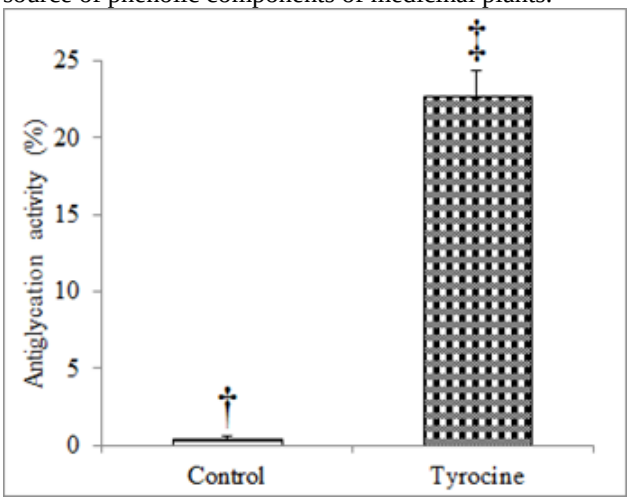

Fig. 4: Effect of tyrosine seeds priming upon antiglycation activity of *Momordica charantia* L.- different signs "† and ‡" show significant difference between the two results

In present study phenolic acids; querecetin, benzoic acid, chlorogenic acid, syringic acid, m-coumeric acid, and cinamic acid was found in controlled leaf extracts in the range of (1.64 - 16.25 ppm) while querecetin, chlorogenic acid, p-coumeric acid and cinamic acid was found relatively in less concentration as compared to the controlled leaf extract sample. The p-coumeric acid and ferulic acid was not detected in controlled but primed-seed leaf extract showed 0.77 and 12.38 ppm, respectively. But in case of fruit extract analysis; the controlled extract sample showed querecetin, cinamic acid and ferulic acid 2.96 ppm, 11.58 ppm and 14.78 ppm, respectively. The primed-seed fruit extract sample, in contrast, showed querecetin-2.22 ppm, syringic acid - 2.61 ppm, ferulic acid-43.95 ppm, sinapic acid-18.39 ppm and vanillic acid-24.36 ppm. The major breakthrough was seen in case of ferulic acid, as it was detected 14.78 ppm in controlled sample while experimental sample showed almost three-times increase in concentration (43.95 ppm). The other prominent effect of priming was appeared in case of sinapic acid and vanillic acid which was found absent in controlled sample but in primed-seed fruit extract these two phenolics were found 18.39 ppm and 24.36 ppm concentration, respectively.

It is well-explored fact that phenolic acid have a linear correlation with antioxidant capacity of plants and its concentration is known as the index of medicinal potential of the plants (Sahar *et al.*, 2013, Khan *et al*., 2014, Asghar *et al.*, 2016, Asif *et al.*, 2017). Tyrosine priming, as found dominantly in case of fruits extract in present study, appeared to enhance the total soluble phenolics which also confirm the previous findings (Mahmood *et al.*, 2017). Tyrosine is actually one of the three amino acids that act as a precursor of phenylpropanoid pathway through which majority of medicinal plant secondary metabolites including phenolics produces (Kallscheuer, *et al.*, 2017).

Natural products with antioxidant activity are often strong antiglycating agents (Ghous, *et al*., 2015). Glycation, as reported, is the major pathway to *diabetes mellitus* (Neves, 2013). The *diabetes mellitus* can be avoided through intake of antioxidant foods. The enhanced phenolic acid concentration in primed-seed fruit extract was subjected to antiglycation assay and it was found that the fruit extract showed credible strength to suppress glycation process. The controlled sample showed 0.87± 0.03% antiglycation activity while tyrosine primed-seed fruit extract showed 23.45±1.23% antiglycation activity; which was significantly ($p<0.05$) higher than control seed fruit extract.

CONCLUSION

Tyrosine is a precursor of phenylpropanoid pathway which is responsible for the production of plant's secondary metabolites, especially phenolic acid. Our study revealed the positive effect of tyrosine priming on growth rate of medicinally positive plants and consequently on the enhanced production of phenolic acids. From this study we concluded that seed priming with naturally occurring amino acid have potential to grow plants in uniform speed with increased concentration of biologically active components that can be isolated in good quantity to cope variety of malignancies. At a spot, the difference in antiglycation activity of controlled and primed extract reveals the strategy is fruitful for enhancing the antiglycation activity of medicinal plants and consequently to manage the rate of appearance of *diabetes mellitus*. The data and approach of this study can also be used to further studies and pre-clinical trials using animal model before jumping to clinical study.

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