ASIAN JOURNAL OF PHARMACEUTICAL AND CLINICAL RESEARCH

INNOVARE ACADEMIC SCIENCES
Knowledge to Innovation

Vol 9, Issue 2, 2016

Online - 2455-3891
Print - 0974-2441

Research Article

ASSESSMENT OF THE EFFECT OF NITRIC OXIDE AND CALCIUM ION ON THE THERAPEUTIC POTENTIAL AND OXIDATIVE STRESS STATUS OF FENUGREEK SPROUTS

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Received: 07 January 2016, Revised and Accepted: 15 January 2016

ABSTRACT

Objective: The aim of the present study was to investigate the effect of priming with exogenous sources of calcium ion and nitric oxide on the antioxidant activity, antidiabetic activity, and related phenolic contents along with the histochemical status of fenugreek sprouts.

Methods: The fenugreek seeds were primed with calcium chloride (CC), calcium chelator ethylene glycol-bis (2-aminoethylether) -N, N, N′, N tetra acetic acid (EG), sodium nitroprusside (SNP) and 2-(4-carboxyphenyl) -4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (CP) and germinated for 72 hrs. The sprout extracts were investigated for their antioxidant potential by 2,2-diphenyl-1-picrylhydrazyl, 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid, reducing power as well as beta-carotene bleaching assays along with *in vitro* antidiabetic activity by α-amylase and α-glucosidase inhibition. Along with this, phytochemicals such as phenolics, flavonoids, and carotene content were also estimated, and the histochemical detection of reactive oxygen species in roots was performed.

Results: The results demonstrated that the seeds pre-treated with CC and SNP showed enhanced antioxidant as well as antidiabetic potential over control; on the other hand, their action was reversed by their antagonists, EG, and CP. A similar trend was observed in the phytochemical contents of the sprouts. Furthermore, it was evident from the histochemical detection of H_2O_2 and superoxide localization as well as lipid peroxidation and plasma membrane integrity that the exogenous supply of calcium ion and nitric oxide exhibited protective role in the germinating seedlings.

Conclusions: The study suggests the active involvement of the signal molecules, Ca^{2+}, and nitric oxide in signaling pathways associated with related phenolic compounds and oxidative stress management.

Keywords: Antidiabetic, Antioxidant, Calcium ion, Germination, Histochemical localization, Phenolics, Nitric oxide, *Trigonella foenum-graecum*.

INTRODUCTION

The fact that the consumption of antioxidant-rich food is extensively associated with reduced risk of various disorders and diseases has been suggested by several researchers [1]. Natural resources such as fruits, vegetables, and also sprouts have been found to be a potential source of bioactive compounds such as polyphenols, ascorbic acid, β-carotene, etc., which possess high antioxidant activity. Therefore, the habitual intake of sprouts, fruits, and vegetables is highly recommended by the nutritionists, as they provide long-term health benefits [2].

Free radicals, such as reactive nitrogen species (RNS) and reactive oxygen species (ROS), are considered to be a fundamental part of normal physiology. Oxidative stress occurs as a consequence of the imbalance in the antioxidant defense system and free radical production [3,4]. These free radicals are mainly responsible for several oxidative stresses mediated chronic diseases and disorders in the human body system, including diabetes [5]. Excessive production of ROS leads to degradation of the pancreatic β-cells, thus causing Type 1 diabetes and Type 2 diabetes with insulin resistance. The inhibiting agents of α-amylase and α-glucosidase (AG), which are responsible for post-prandial hyperglycemia, have gained more attention for diabetic treatment [6]. Antioxidant compounds play a vital role in preventing the cellular damages, against the highly unstable chemical components such as free radicals and ROS, which are constantly produced by the cell metabolism and their concentration increases under stress conditions [7]. It has also been reported that the natural sources of antioxidants can efficiently control the post-prandial hyperglycemia via inhibition of α-amylase and AG without any negative effects [8].

Trigonella foenum-graecum commonly known as fenugreek has been reported to possess several pharmacological and folkloric applications. Its leaves have been reported to show potential antioxidant property, antimicrobial as well as antidiabetic activity. The *in vivo* hypoglycemic activity of fenugreek seeds has been established in various animal model systems. In addition, fenugreek seeds possess potential hypocholesterolemic effect, antioxidant property and are also very effective in the treatment of diabetic disorders [9].

Nitric oxide is a bioactive molecule, which functions both as a pro-oxidant as well as antioxidants in plant system [10]. The chemical properties of nitric oxide make it a versatile signaling molecule that functions via interactions with several cellular components [11]. It is also considered as an RNS and its concentration-dependent impacts on different systems were reported to be either protective or toxic [12].

Calcium (Ca^{2+}) is another important secondary messenger and signaling molecule which is actively involved in various physiological and developmental processes. In the cited literature, Ca^{2+} has also shown a protective effect against stress by mitigation of oxidative damages and membrane stabilization [13].

Seed priming is a pre-sowing technique in which seeds are subjected to the low external water potential that limits hydration which does not allow the protrusion of radicle through the seed coat. This technique is known to enhance the primary development of seeds under unfavorable environment [14,15]. The priming of seeds with various substances such as water, inorganic salts, osmolytes, and hormones has been successful and reported as a cost-effective strategy to enhance tolerance under saline conditions [16].

Sprouting has been considered as the effective means by which the nutritional quality of the seeds is enhanced. During germination process, mobilization of complex macromolecules such as stored

carbohydrates and protein reserve takes place [17]. In several legumes, sprouting is found to improve the soluble protein and fiber content and reduce the phytic, tannic acid, and trypsin inhibitors, consequently enhancing their nutraceutical quality [18].

However, the literature suggests that reports on comparative account on the role of calcium ion as well as nitric oxide molecule and the effect of their exogenous application on the therapeutic potential of fenugreek sprouts during early developmental phases is not studied until date. Therefore, a study on the influence of calcium chloride (CC)and nitric oxide priming on therapeutic potential and oxidative stress status may provide insight to their role in regulating these processes. Considering this fact, the present study was designed to investigate the effect of priming with exogenous sources of calcium ion and nitric oxide on the free radical scavenging activity, antidiabetic activity, and related phenolic contents along with the histochemical status of fenugreek sprouts.

METHODS

Materials and treatment

The fenugreek seeds were subjected to surface sterilization with 0.1% sodium hypochlorite solution. The sterilized seeds were washed thrice with distilled water and pre-treated with the solutions of CC; a calcium chelator: Ethylene glycol-bis(2-aminoethylether)-N,N,N´,N, tetra acetic acid (EG); sodium nitroprusside (SNP) as an exogenous source of nitric oxide and 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (CP), a nitric oxide scavenger. For control set, seeds were primed with normal water and kept in a rotary shaker along with the treated seeds. After priming for 24 hrs, the seeds were washed thrice with sterile water and kept in the seed germinator for germination for 72 hrs.

Preparation of extracts

The sprouts of fenugreek of three different stages: 24, 48, and 72 hrs were extracted with methanol through soxhlet extraction apparatus. The refluxed samples were separated from the residues by filtering through Whatman No. 1 filter paper, and the extract was concentrated to a definite concentration of 1 g/ml using a vacuum rotary evaporator. The obtained methanolic extracts were stored in brown bottles and kept in the refrigerator for further experimental analysis.

2,2-diphenyl-1-picrylhydrazyl (DPPH) based free radical scavenging activity

The radical scavenging activity of the sprout extracts was measured by DPPH method [19]. The reaction mixture contained 1.8 ml of 0.1 mM DPPH and 0.2 ml of methanolic extracts. The reaction mixture was vortexed and left in the dark at room temperature for 30 min. The absorbance was measured at 517 nm. A reaction mixture without test sample was considered as control.

Radical scavenging activity was expressed as percent inhibition from the given formula:

Percentage inhibition of DPPH radical = $[(A_0-A_1)/A_0]\times 100$

Where, A_0: Absorbance of the control and A_1: Absorbance of the extract or standard. Then, percentage inhibitions were plotted against the concentration and from the graph, inhibitory concentration 50% (IC_{50}) was calculated.

Ferric reducing antioxidant power assay (RP)

The ferric reducing antioxidant power of methanolic extracts was determined by the standard method [20]. Different concentrations of 1 ml of methanolic extracts were mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide ($K_3Fe(CN)_6$) (2.5 ml, 1%). The mixture was incubated at 50°C for 20 minutes. Then, 2.5 ml of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged for 10 minutes at 3000 rpm. The upper layer of solution (2.5 ml) was mixed with distilled water (2.5 ml) and $FeCl_3$ (0.5 ml, 0.1%). The absorbance was measured at 700 nm against a blank using ultraviolet-visible spectrophotometer.

2,2′-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid ($ABTS^+$) radical cation(s) decolorization assay

The spectrophotometric analysis of $ABTS^+$ radical cation(s) scavenging activity was determined according to Re *et al.* [21] method with some modifications. This method is based on the ability of antioxidants to quench the $ABTS^+$ radical cation, a blue/green chromophore with characteristic absorption at 734 nm. The $ABTS^+$ was obtained by reacting 7 mM $ABTS^+$ radical cation(s) in H_2O with 2.45 mM potassium persulfate ($K_2S_2O_8$), stored in the dark at room temperature for 6 hrs. Before usage, the $ABTS^+$ solution was diluted to get an absorbance of 0.750±0.025 at 734 nm with sodium phosphate buffer (0.1 M, pH 7.4). Then, 2 ml of $ABTS^+$ solution was added to 1 ml of the methanolic extract. After 30 minutes, percentage inhibition at 734 nm was calculated for each concentration. Solvent blanks were run in each assay.

The $ABTS^+$ scavenging was calculated using the following formula:

$ABTS^+$ scavenging effect (%)= $[1-(A_S/A_C)]\times 100$

Where, A_C is the initial concentration of the $ABTS^+$ radical cation(s), and A_S is absorbance of the remaining concentration of $ABTS^+$ radical cation(s) in the presence of the extract.

β-carotene-linoleate bleaching (BCB) assay

The AA was evaluated based on the BCB protective method [22]. β-carotene (0.2 mg in 1 ml chloroform), linoleic acid (0.02 ml), and tween 20 (0.2 ml) were transferred into a round-bottomed flask. Chloroform was totally evaporated at room temperature under vacuum at reduced pressure using a rotary evaporator. After evaporation, 50 ml of distilled water was added to the mixture, and then shaken vigorously to form an emulsion. 2 ml of emulsion was then added to 0.2 ml of methanolic extract or methanol (as control) into test tubes and immediately placed in a water bath at 50°C. The absorbance was read at 30 minutes intervals for 2 hrs at 470 nm.

Degradation rate (DR) was calculated according to first order kinetics, based on equation;

$$\ln\frac{a}{b}\times\frac{1}{t}=DR_{sample}\text{ or }DR_{standard}$$

Where, ln is natural log, a is the initial absorbance (470 nm) at time 0, b is the absorbance (470 nm) at 30, 60, 90, or 120 minutes, and t is the initial absorbance (470 nm) at time 0.

AA was expressed as inhibition percentage relative to the control, using the formula:

$AA = [(DR_{control}-DR_{sample})/DR_{control}]\times 100$

Total phenolic content (TPC)

TPCs of sprout extracts were evaluated according to the standard protocol [23]. 1 ml of the methanolic extract was added to a mixture containing 1 ml of 95% ethanol, 5 ml of distilled water, and 0.5 ml of 50% Folin-Ciocalteu reagent. After 5 minutes incubation, 1 ml of 5% Na_2CO_3 was added. It was mixed thoroughly and further kept for 1 hr of incubation. Finally, the absorbance of the colored reaction mixture was measured at 765 nm against the reagent blank. The TPC was determined as mg of gallic acid equivalent per g fresh weight.

Total flavonoid content (TFC)

The flavonoid content was evaluated by performing a standard spectrophotometric method [24]. 1 ml of extract was diluted with distilled 4 ml water in a volumetric flask. Initially, 0.3 ml $NaNO_2$ solution (5%) was added to each volumetric flask; after 5 minutes, 0.3 ml $AlCl_3$ (10%) was added; and then again after 6 minutes, 2 ml NaOH (1 M) was added to the mixture. The absorbance of the reaction mixture was taken after adding 2.4 ml of distilled water at 510 nm. The TFC in different extracts was expressed as quercetin equivalent per g fresh weight.

Total carotene content (TCC)
TCC was evaluated according to the standard protocol [25]. Initially, the methanolic extract was dried and converted to aqueous which was further partitioned with an equal volume of ether using a separating funnel. The ether layer was collected and evaporated and then again reconstituted with ethanol. 0.1 ml of 60% aqueous KOH solution was added to the ethanolic extract and heated for 5 minutes in a water bath. The mixture was incubated in dark condition for overnight. Next day, the mixture was again partitioned with ether, the ether layer was further evaporated and reconstituted with ethanol, and the absorbance was recorded at 450 nm. The carotene content was determined using a calibration curve constructed against pure β-carotene.

***In vitro* α-amylase inhibitory activity (AA)**
The α-amylase inhibition potential of the extract was estimated by standard spectrophotometric method [26]. About 0.5 ml of aqueous extract was reacted with 0.5 ml of α-amylase solution and incubated at 37°C for 5 minutes. After incubation, 0.5 ml starch solution (1%) was added and was further incubated for 10 minutes. To the above reaction mixture, 1 ml of dinitrosalicylic acid reagent was added to terminate the reaction and was heated for 10 minutes in a hot water bath till the color of reaction mixture color changed to orange-red. After a change in color, the reaction mixture was cooled and diluted up to 5 ml with distilled water. The absorbance value was measured at 540 nm. The α-amylase inhibitory activity was estimated by measuring the concentration of inhibitor required to inhibit 50% of the enzyme activity.

***In vitro* AG inhibitory activity**
The AG inhibitory property of the sample extract was assayed as suggested by Jung *et al*., [27] with slight modification. The different concentrations of extract were prepared by adding 0.2 mM phosphate buffer (pH 6.8). After that 0.1 ml of enzyme solution was added and kept for incubation at 37°C. Then, 0.25 ml pNPG (3 mM) was added, and the reaction was terminated by the adding 4 ml of Na_2CO_3 (0.1 M). The AG inhibition activity was estimated by determining the kinetics of release of pNPG at 405 nm. The control contained all the reagents without the sample extract.

***In situ* ROS detection**
For detection of hydrogen peroxide localization in the roots, the fenugreek seedlings were stained for about 40-45 minutes in potassium iodide/starch reagent (4% w/v starch and 0.1 M potassium iodide solution) [28].

Plasma membrane integrity (PMI) of the seedlings was detected by the method suggested by Yamamoto *et al*. [29]. First, roots were stained with Evans blue solution (0.025% w/v, in 100 mM $CaCl_2$) for 30 minutes, then after the roots were rinsed thrice in distilled water to remove the extra stain.

The localization of superoxide in the tissue was performed by illumination of the roots dipped in the nitro blue tetrazolium salt solution in fluorescent lamp [30].

The histochemical detection of membrane lipid peroxidation (MLP) was performed according to Pompella *et al*. [31]. Schiff's reagent was used to stain the freshly harvested roots; the roots were stained until the red color developed on the roots. The extra stain imparted by Schiff's reagent was removed by rinsing the stained roots with a potassium sulfite solution (0.5% w/v, in 0.05 M HCl). Stained roots were photographed under a Nikon SLR camera (Model: D3200).

Statistical analysis
Each experiment was performed in triplicate. The software package, MS Excel 2007 (Microsoft, Redmond, WA, USA) was used for comparing the antioxidant attributes of different developmental stages of the fenugreek sprouts. The different group means were compared by Duncan's multiple range test through DSAASTAT software (version 1.002; DSAASTAT, Peruglia, Italy); $p<0.05$ was considered significant in all cases. Smith's Statistical Package version 2.5 (prepared by Gary Smith, CA, USA) was used for determining the IC_{50} values of antioxidants and their standard error of estimates.

RESULTS AND DISCUSSION

Antioxidant activity
The phenolic compounds which possess potent antioxidant property are known to prevent several oxidative stress mediated disorders in living systems. The assessment of the antioxidant potential of plant extracts is very essential for determining its nutritional value [32]. The present research aimed at enhancement of antioxidant potential and related phenolic content of fenugreek through elicited sprouting. The antioxidant property of the sprouts was determined in terms of their free radicals scavenging potential such as DPPH and $ABTS^+$; ferric RP, and β-carotene protective property. To evaluate the effect of Ca^{2+} and nitric oxide molecule on the therapeutic potential, the fenugreek seeds were primed with CC, SNP and their antagonists EG and CP, respectively.

From the result, it was observed that the sprouts, which were primed with CC and SNP exhibited enhancement in free radical scavenging property (Fig. 1) and RP (Table 1) in comparison to the control sprouts. On the other hand, those sprouts pre-treated with EG and CP showed a loss in their AA indicating the involvement of nitric oxide and Ca^{2+} in improving antioxidant properties of fenugreek sprouts.

Phytochemical analysis
The phenolic compounds, by virtue of their capability to react as competent reducing agents and singlet oxygen molecule quenchers, play a vital role as an antioxidant factor and hence possess potential human health benefits [33]. So, the bioactive phytochemical, such as total phenols and flavonol content, was evaluated for the fenugreek sprouts. The phenol and flavonol content of fenugreek sprouts was estimated for 3 days of germination. Among all the elicitors applied, CC exhibited the maximum increase in phenolic content followed by SNP, which was found to be 42% and 27%, respectively (Fig. 2). Our result is in agreement with the earlier report which suggested an increase in the phenol content in CC primed seedlings of *Cucumis sativus* [16].

In the case of flavonol estimation, it was observed that SNP exhibited the maximum increase followed by CC, which was found to be 50% and 22% than the control sprouts, respectively (Fig. 2). A similar trend was observed in the case of carotene content (Fig. 2).

Table 1: RP and β-carotene protective activity of fenugreek sprouts under the influence of different elicitors

Priming agents	24 hrs stage		48 hrs stage		72 hrs stage	
	RP (AAE mg/g fwt)	BCB (%)	RP (AAE mg/g fwt)	BCB (%)	RP (AAE mg/g fwt)	BCB (%)
Control	406.06±39.85^{b}	30.16±5.12^{b}	495.6±13.83^{c}	31.44±2.36^{b}	346.83±116.59^{ab}	25.59±2.56^{b}
CC	662.88±39.85^{a}	50.35±12.9^{a}	1149.7±61.97^{a}	47.7±3.58^{a}	500.17±69.08^{a}	36.6±0.65^{a}
EGTA	342.32±36.57^{bc}	12.87±3.22^{c}	375.73±0.39^{d}	23.57±1.77^{c}	305.62±45.01^{b}	11.19±1.82^{c}
SNP	622±59.08^{a}	31.26±4.83^{b}	666.6±65.19^{b}	42.44±2.82^{a}	490.38±61.19^{a}	28.95±3.8^{b}
CP	295.72±12.05^{c}	8.85±1.66^{c}	355.48±8.15^{d}	19.22±1.82^{c}	264.54±16.56^{b}	7.55±1.12^{c}

Results are represented as mean±SEM, n=3. Values with different letters (a, b, c, d) are significantly ($p<0.05$) different from each other by DMRT. RP: Reducing power, AAE: Ascorbic acid equivalent, BCB: β-carotene-linoleate bleaching, SEM: Standard error of mean, DMRT: Duncan's multiple range test, CC: Calcium chloride, SNP: Sodium nitroprusside, EGTA: Ethylene glycol tetraacetic acid

***In vitro* antidiabetic activity**

The key enzymes in the digestive system, α-amylase, and AG are actively involved in the release of glucose in the blood via breakdown of starch. Hence, the inhibition of these enzymes would minimize the breakdown of starch, which may lead to a reduction in the post-prandial hyperglycemia level [34]. For *in vitro* antidiabetic activity, the potential of the extract to inhibit the activity of these enzymes, AG, and α-amylase was determined. The inhibition capacity of the sprouts was expressed in terms of IC_{50} values. The results revealed that the fenugreek sprouts have potential antidiabetic property, and interestingly, it was also observed that the exogenous supply of Ca^{2+} and nitric oxide resulted in the enhancement of this activity in fenugreek sprouts. Both the AG and α-amylase inhibition capacity was enhanced by CC and SNP (Fig. 3).

The phenolics and flavonoid compounds have been known to be efficient inhibitors of AG, and also regulators of hyperglycemia and other diabetic related complications arising from oxidative stress [35]. In agreement to the earlier cited statement, it was observed that the set of sprouts having higher phenolic content exhibited stronger α-amylase and AG activity thus suggesting the probable role of phenolics in the management of diabetic disorders.

Effect of nitric oxide scavenger (CP) and calcium chelator (EG) was also assessed on the antioxidant, antidiabetic as well as related phytochemical content of the fenugreek sprouts. The effect of these elicitors was found to be negative on both the aspects of the sprouts, i.e. therapeutic potential and phytochemical content.

Histochemical detection

After a detailed analysis of antioxidant and antidiabetic property along with the phytochemical content, histochemical detection was performed for studying the specific localization of different free radicals and their pattern of accumulation in the tissue.

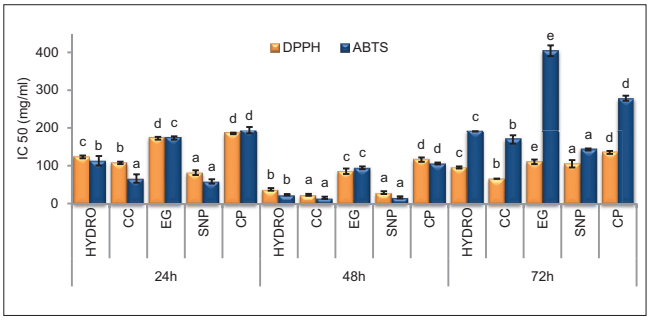

Fig. 1: 2,2-diphenyl-1-picrylhydrazyl and 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid-free radical scavenging activity of fenugreek sprouts under the influence of different elicitors. Results are represented as mean ± standard error of mean, n=3. Values with different letters (a, b, c, etc.) are significantly (p<0.05) different from each other by Duncan's multiple range test

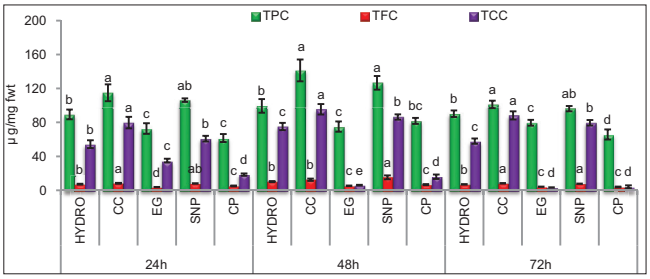

Fig. 2: Phytochemical contents of fenugreek sprouts under the influence of different elicitors. Results are represented as mean ± standard error of mean, n=3. Values with different letters (a, b, c, etc.) are significantly (p<0.05) different from each other by Duncan's multiple range test

For detecting the localization of H_2O_2 molecules, potassium iodide-starch solution was used. As a result, it was observed that no accumulation of H_2O_2 took place in the control roots (Fig. 4a); the seedlings treated with CC and SNP were found to be resistant, but significant H_2O_2 localization was observed at the growing region and those treated with CP and EG were found to be very much sensitive; also a significant accumulation of the H_2O_2 molecule at the growing region of the roots was detected. It has been suggested that nitric oxide serves as a part of signaling cascade and exhibits AA by quenching the ROS molecules and protecting the cell against oxidative damages [36]. Similarly, the reduction in the accumulation of H_2O_2 in the roots of seedlings primed with the SNP and CC indicates the protective role of Ca^{2+} and nitric oxide. Similar patterns of superoxide molecule localization were observed during the histochemical detection (Fig. 4b).

The Evans blue reagent was applied for determining the status of PMI of fenugreek seedlings. The membrane integrity remained consistent for the control set; the roots pre-treated with CP and EG exhibited darker stain against Evans blue signified occurrence of injuries to the plasma membrane; the one pre-treated with NO donor, i.e. SNP the membrane integrity remained more or less stable showing less negative effects on the roots and the plasma membrane of those primed with CC was found to be undisturbed as evident from lighter stain exhibited against the dye (Fig. 4c). In agreement to our result, the protective role of Ca^{2+} was reported in case of *Amaranthus lividus* [37]. In addition, Rubbo *et al.* [38] has also claimed a potent inhibitory role of NO in the accumulation of malondialdehyde, thus leading to prevention of MLP.

For determining the degree of MLP, the response of the seedlings to Schiff's reagent was studied. The accumulation of malondialdehyde was observed in the growing region of the control roots as they

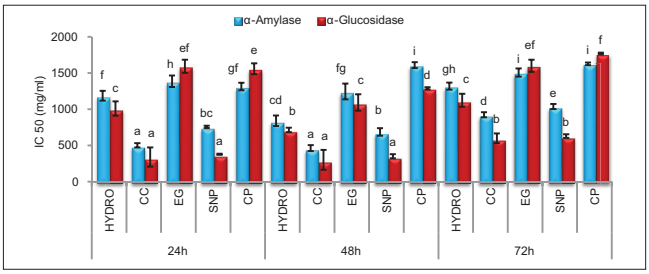

Fig. 3: α-amylase and α-glucosidase inhibition activity of fenugreek sprouts under the influence of different elicitors. Results are represented as mean ± standard error of mean, n=3. Values with different letters (a, b, c, etc.) are significantly (p<0.05) different from each other by Duncan's multiple range test

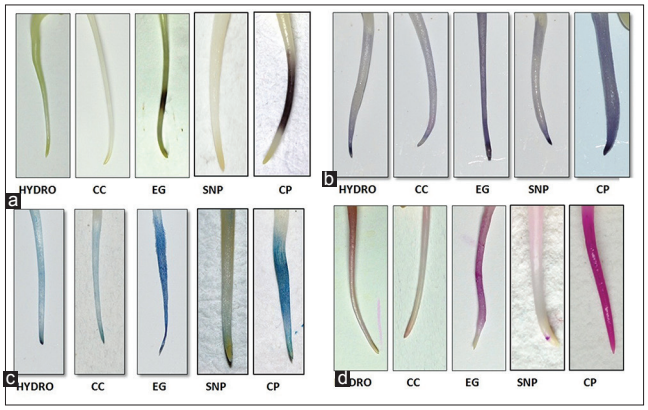

Fig. 4: Histochemical studies showing the effect of different elicitors on (a) H_2O_2 localization; (b) superoxide localization; (c) plasma membrane integrity; and (d) membrane lipid peroxidation of fenugreek seedlings

exhibited some stain; the occurrence of lipid peroxidation of the plasma membrane was observed at a much higher degree in those seedlings pre-treated with CP and EG as evident from a much darker stain. The roots primed with CC exhibited much lighter stain followed by SNP, thus proving their protective effect against peroxidation of membrane lipids (Fig. 4d).

Malondialdehyde accumulation is reported to damage the biological membrane by forming free radicals which are responsible for peroxidation of poly-unsaturated fatty acids [39]. In the present study, the inhibitory action of both nitric oxide and calcium ion against malondialdehyde accumulation was observed after priming in fenugreek seedlings. In agreement to our study, nitric oxide and calcium ion are reported to mitigate the various oxidative stress mediated damages such as lipid peroxidation and electrolyte leakage in several plant samples [38,40,41].

Accumulation of malondialdehyde is usually associated with injury to biological membranes due to peroxidation of poly-unsaturated fatty acids [39]. Consequently, membrane integrity is altered due to peroxidation of lipid and the ultimate fate is electrolyte leakage from plant tissue. Since both the signaling molecules, i.e. calcium ion and nitric oxide are known to inhibit lipid peroxidation [13,40], these molecules can also uphold the integrity of membranes and hinder leakage of ions. Similar phenomena were observed in the present study as those roots which were sensitive to the accumulation of malondialdehyde were equally found to exhibit loss of membrane integrity and those with less degree of peroxidation responded least to the Evans blue stain.

The enhancement in the therapeutic property and related bioactive phytochemicals in exogenous Ca^{2+} and nitric oxide primed seedlings and subsequent improvement in membrane lipid and membrane integrity status of the plasma membrane further reinforces the hypothesis suggesting the crucial involvement of both the molecules in the signaling pathways associated with synthesis of these bioactive phytochemicals during germination phase in fenugreek leading to betterment in the nutraceutical properties as well as oxidative stress management.

Principal component analysis (PCA)

PC factor loadings biplot was used to determine the importance of different therapeutic and phytochemical attributes along with the elicitors applied and their contribution in the enhancement of the functional value of fenugreek sprouts. The loadings of first (PC1) and second (PC2) PC accounted for 87.12% and 5.52% of the total data variance, respectively (Fig. 5). Interestingly, almost all antioxidants (IC_{50} values for scavenging different free-radical species) and antidiabetic (IC_{50} values for inhibition of digestive enzymes) attributes were clustered together along with the histochemical factors with negative loadings on PC1, whereas the reducing potential, β-carotene protective activity, and phenolic components exhibited strong positive loadings on the same component. This indicates that the secondary metabolites synthesized through phenylpropanoid pathway principally contributed for the antioxidant as well as antidiabetic property of fenugreek. Previously, similar observations were reported for *Punica granatum* cultivars, where antioxidant and phytochemical attributes were loaded on PC1 at opposite coordinates [42]. Similar relevant findings were

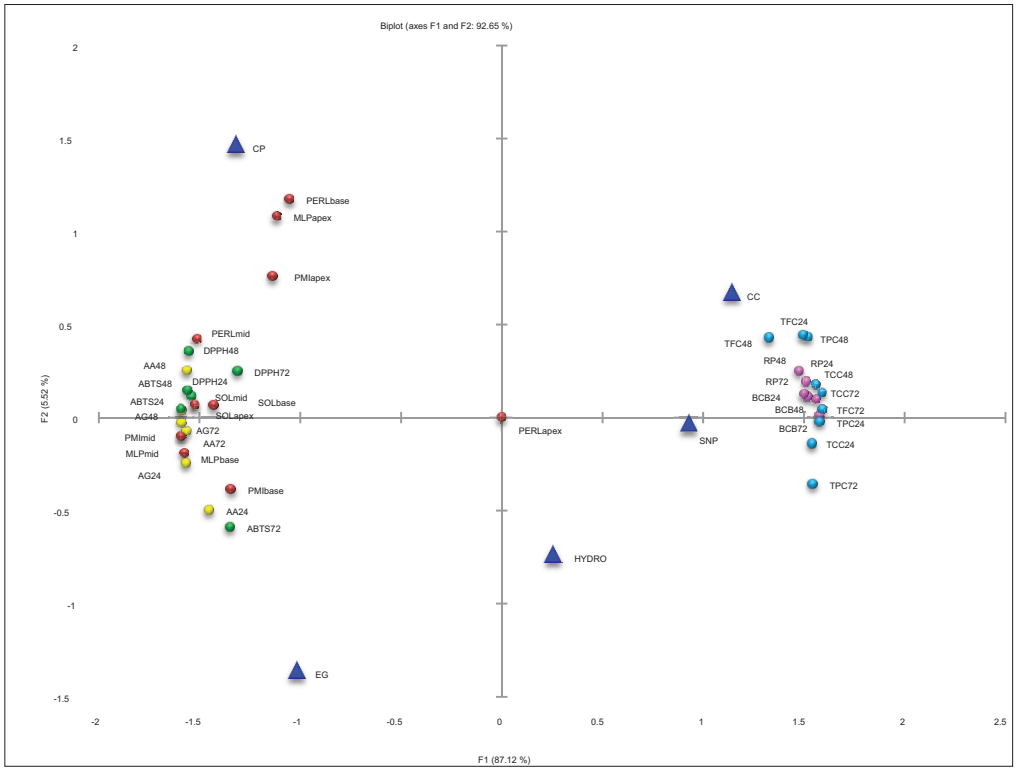

Fig. 5: Principal component analysis of different attributes. The green dot signifies free radical scavenging activity; pink dot signifies reducing power (RP) and β-carotene-linoleate bleaching activity (BCB); yellow dot signifies antidiabetic activity; sky blue dot signifies phytochemical content and red dot signifies histochemical status. (2,2-diphenyl-1-picrylhydrazyl [DPPH] 24, DPPH48, DPPH72 - DPPH scavenging activity at 24 hrs, 48 hrs, and 72 hrs; 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid [ABTS] 24, ABTS48, ABTS72 - ABTS scavenging activity at 24, 48, and 72 hrs; RP24, RP48, RP72 - RP at 24, 48, and 72 hrs; BCB24, BCB48, BCB72 - BCB at 24, 48, and 72 hrs; α-amylase inhibitory activity [AA] 24, AA48, AA72 - AA at 24, 48, and 72 hrs; α-glucosidase inhibitory activity [AG] 24, AG48, AG72 – AG at 24, 48, and 72 hrs; total phenolics content [TPC] 24, TPC48, TPC72 – TPC at 24, 48, and 72 hrs; total flavonol content [TFC] 24, TFC48, TFC72 - TFC at 24, 48, and 72 hrs; total carotene content [TCC] 24, TCC48, TCC72 - TCC at 24, 48, and 72 hrs; superoxide localization (SOL) apex, SOLmid, SOLbase - SOL at apex, middle and basal region; peroxide localization (PERL) apex, PERLmid, PERLbase - PERL at apex, middle and basal region; membrane lipid peroxidation (MLP) apex, MLPmid, MLPbase - MLP at apex, middle and basal region; plasma membrane integrity (PMI) apex, PMImid, PMIbase –PMI at apex, middle and basal region)

also recorded during analysis of AA of *Couroupita guianensis* [43]. The similarities and differences among the different elicitors were also analyzed. The distance between the locations of any two elicitors on the score plot is directly related to the degree of similarity between them. PCA showed that the CC, SNP, EGTA, and CP were located in four different coordinates, whereas hydro was situated near the origin and were heavily loaded on PC2 (Fig. 5). Among four different elicitors, EGTA and CP were situated on the left side of PC1, whereas the CC and SNP were placed on opposite co-ordinate (right side) of PC1. This indicates that the effect of Ca^{2+} and nitric oxide was constructive on the different therapeutic properties, whereas the effect of their antagonists EGTA and CP was deteriorative in comparison to control, i.e. hydroprimed (hydro).

Overall, it was observed that among all treatments, CC and SNP priming exhibited enhancement in their antioxidant and antidiabetic potential along with related phenolic content, among which CC was found to exhibit the best result. It is considered that the efficiency of the plant phenolic compounds in overcoming the oxidative stress mediated damage is attributed to their reactivity toward the free radicals. The reduction of phenoxyl radicals by the cellular reducing factors recycles the antioxidative phenolics, thus enhancing the antioxidant potential in the system [44]. The best antioxidant, as well as the antidiabetic activity of sprouts during 48 hrs stage, indicates that the factors responsible for the therapeutic potential were elicited appropriately at this stage. Another reason might be that at early germination stage there must be high oxygen demand, and consequently, the phenolics might be involved in protection from probable oxidative stress-induced damages [45].

CONCLUSION

It can be suggested that the increase in the phenolic compounds was found to be responsible for the enhancement in antioxidant and antidiabetic properties of sprouts. The negative effects of the antagonists of Ca^{2+} and nitric oxide signaling indicate that the flux of these molecules within the cellular system is essential which plays a vital role during germination phases to regulate the mechanisms responsible for the synthesis of antioxidative and antidiabetic phytochemical compounds. Further, the efficiency of these signaling molecules for oxidative stress management was reinforced by the histochemical study, where it was observed that a decrease in the availability of these molecules resulted in the oxidative damage to the seedlings. Thus, this knowledge can be used to design the priming based sprouting techniques which might have potential application in improving the nutraceutical quality of legume sprouts along with oxidative stress management.

ACKNOWLEDGMENT

The authors gratefully acknowledge the Department of Science and Technology for providing financial support for this research work under INSPIRE Scheme.

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