Involvement of Calcium ion in Enhancement of antioxidant and antidiabetic Potential of Fenugreek Sprouts

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ABSTRACT

Introduction: Calcium ion (Ca^{2+}) is considered as a key signal transducer in plants which is involved in various physiological processes. The aim of the present study was to evaluate the influence of Ca^{2+} in enhancement of antioxidant as well as anti-diabetic activity of the fenugreek seedlings during developmental phase. **Methods:** The fenugreek seeds primed with Calcium chloride ($CaCl_2$), Calcium chelator EGTA [Ethylene glycol-bis(2-aminoethylether)-N,N,N´, N, tetra acetic acid] and calcium channel blocker $LaCl_3$ (lanthanum chloride) and germinated for 3 days. The sprout extracts were investigated for their antioxidant potential by DPPH, $ABTS^+$, metal chelating, reducing power, nitric oxide scavenging capacity and anti-lipid peroxidation as well beta-carotene bleaching assays along with *in vitro* antidiabetic activity by α-amylase and α-glucosidase inhibition. Along with this, phytochemicals such as phenol, flavonol and carotene were also estimated. **Results:** The results demonstrated that the seeds treated with calcium chloride showed enhanced antioxidant as well as antidiabetic potential over control; on the other hand the action was reversed by EGTA and $LaCl_3$. Similar trend was observed in the phytochemical contents of the sprouts. **Conclusion:** Our data suggested that the improvement in nutraceutical value of fenugreek sprouts by calcium chloride could be due to the involvement of Ca^{2+} in signaling pathways associated with related phenolic compounds.

Key words: Antioxidant, Antidiabetic, Calcium ion, Germination, Phenolics.

INTRODUCTION

Several pharmacological as well as experimental studies have suggested that consumption of foods rich in antioxidant is significantly associated with reduced risk of various disorders and human diseases, including diabetes.[1] Some of the food types such as fruits, vegetables and sprouts and herbal drugs have been found to be very rich in bioactive compounds such as polyphenols, vitamins C and E, β-carotene etc, which posses potential antioxidant activity. Therefore, in recent times the regular consumption of sprout or germinated seeds, fruits and vegetables, is highly recommended as they are considered to provide long term health benefits.[2]

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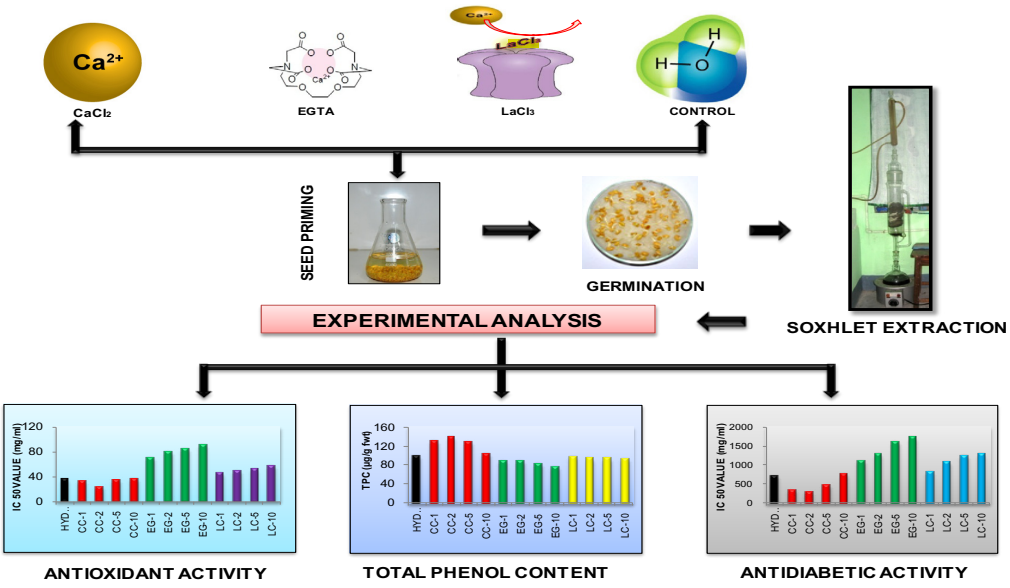
Reactive oxygen species (ROS) or free radicals leaked during the process of metabolism have been the major source for the oxidative stress in the living system. These free radicals are found to be responsible for several chronic diseases and disorders in human body system.[3] The increased level of ROS has been associated with the degradation of pancreatic beta-cells leading to type 1 diabetes and the onset of type 2 diabetes by insulin resistance.[4] The inhibitors of α-amylase and α-glucosidase, which are responsible for post-prandial hyperglycemic conditions have gain more attention by the medical practitioner for the diabetic treatment. The antioxidant compounds present in these food sources and herbal drugs could be attributed to their properties such as hydrogen donors, reducing agents and metal ion-chelators.[5] It has also been reported that the natural sources of antioxidants can play vital role in controlling the post-prandial hyperglycemic conditions via inhibition of the key enzymes α-amylase, α-glucosidase which is a potential approach towards diabetic treatment.[6] *Trigonella foenum-graecum* commonly known as fenugreek, the different plant



Graphical Abstract

parts has been reported to possess pharmacological and folkloric significance. Its leaves have been found to exhibit potential antioxidant property, antibacterial activity and anti-diabetic. The in vivo hypoglycemic activity of fenugreek seeds has been reported in various animal model systems. In addition, it is suggested that the fenugreek seeds possess potential hypocholesterolemic effect, antioxidant property and also can be very much effective in the treatment and/or prevention of diabetes.[7]

Calcium (Ca^{2+}) has emerged as an important key secondary messenger and signal transducer which have been reported to be involved in various physiological processes. It has been considered to be significantly involved in linking stress perception and regulation of adaptive cellular responses.[8] In the cited literature Ca^{2+} has also exhibited protective response against stress by regulating mitigation of oxidative damages and stabilization of membranes.[9] Sprouting has been considered as the effective means by which the nutritional quality of the seeds is enhanced. During germination process mobilisation of complex macromolecules such as stored carbohydrates and protein reserve takes place.[10] In several legumes sprouting is found to improve the soluble protein and fibre content and reduce the phytic, tannic acid and trypsin inhibitors, consequently enhancing their nutraceutical quality.[11]

The present work has been designed considering the fact that till date no work has been performed on the effect of the elicitors of calcium signalling on the antioxidant as well as antidiabetic property of fenugreek sprouts. Therefore the aim of the present study was to evaluate the antioxidant as well as antidiabetic activity of the fenugreek under the influence of Calcium chloride, EGTA a calcium chelator and lanthanum chloride a calcium channel blocker, during post germination phase.

MATERIALS AND METHODS

Chemicals

Sodium hypochlorite, calcium chloride, EGTA [Ethylene glycol-bis (2-aminoethylether)-N,N,N´,N, tetra acetic acid], LaCl3 (lanthanum chloride), Methanol, 2,2-diphenyl-1-picryl hydrazyl (DPPH), potassium persulphate, 2,2-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), sulfanilamide, glacial acetic acid, napthyl ethylenediamine dihydrochloride (NED), potassium ferricyanide, thiobarbituric acid (TBA), trichloroacetic acid (TCA), ferrous sulphate, potassium hydroxide (KOH), potassium dihydrogen orthophosphate (KH_2PO_4), ferric chloride ($FeCl_3$), ferrous chloride ($FeCl_2$), ferrozine, sodium nitroprusside, β- carotene, chloroform, linoleic acid, gallic acid, quercetin, folin-ciocalteau reagent, sodium carbonate (Na_2CO_3), sodium nitrite ($NaNO_2$), aluminium chloride ($AlCl_3$), sodium hydroxide (NaOH), ether, ethanol, alpha-amylase, starch, DNSA, Rochelle salt, alpha-glucosidase, p-nitrophenyl-α-D-glucopyranoside. All these chemicals were either purchased from Himedia, India or of Merck, Germany made.

MATERIALS AND TREATMENT

The fenugreek seeds were sterilized with 0.1% sodium hypochlorite solution. The sterilized seeds were treated with the solutions of calcium chloride, EGTA a calcium chelator

and lanthanum chloride a calcium channel blocker, and kept in rotary shaker for 24 h. For control set, seeds were primed with normal water and kept in rotary shaker along with the treated seeds. After treatment, the seeds were washed thrice with sterile water and kept in the seed germinator for 72 h.

Preparation of extract

The germinated seedlings of fenugreek of 3 different stages: 24 h, 48 h & 72 h were crushed in mortar-pestle and processed through soxhlet extraction apparatus with methanol. The refluxed samples were separated from the residues by filtering through Whattman 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 refrigerator for further experimental analysis.

Animal material

Goat liver, used for anti-lipid peroxidation assay, was collected from slaughter house immediately after slay and the experiment was conducted within an hour after collection.

DPPH based free radical scavenging activity (DPPH)

The DPPH free radical scavenging activity of the sprout extracts were determined following the method.[12] The reaction mixture contained 1.8 ml of 0.1 mM DPPH and 0.2 ml of methanolic extracts. The absorbance of the reaction mixture was measured at 517 nm after an incubation of 30 min. A reaction mixture without test sample was considered as control.

$ABTS^+$ radical cation(s) decolorization assay

The spectrophotometric analysis of $ABTS^+$ radical cation(s) scavenging activity was measured according to Re *et al.*[13] method. 1 ml of $ABTS^+$ solution was reacted with 0.5 ml of methanolic extract. After 30 min of incubation at room temperature the absorbance was recorded at 734 nm. Solvent blanks were run in each assay.

Reducing antioxidant power (FRAP) Assay

The reducing antioxidant power of the sprout extracts was estimated by the standard method.[14] The extracts were mixed with 0.2 M of phosphate buffer (2.5 ml, pH 6.6) and 2.5 ml of 1% potassium ferricyanide. The reaction mixture was kept safely for incubation at 50°C for 20 min. After incubation, 2.5 ml of 10% trichloroacetic acid was added to reaction mixture and centrifuged at 3000 rpm. The supernatant (1 ml) was taken and diluted with distilled water (1 ml) and 0.2 ml of $FeCl_3$ (0.1%) added to it. The increase in OD value was recorded at 700 nm against a blank.

Metal chelating activity (MC)

The chelating capacity of the extracts for ferrous ions was evaluated according to the method of Dinis *et al.*,[15] with some modifications. The 400 µl of sprout extract was diluted with 1600 µl of methanol and was reacted with 40 µl of ferrous chloride (2 mM). After duration of 30s, 0.8 ml of ferrozine solution (5 mM) was added. After 15 min of incubation, the change absorbance was recorded at 562 nm.

Nitric oxide scavenging activity (NOS)

The spectrophotometric analysis of nitric oxide scavenging activity was measured by the Greiss reaction.[16] 320 µL of methanolic extract was reacted with 360 µL sodium nitroprusside-PBS solution (5 mM), 216 µL of Greiss reagent (1% sulphanilamide, 2% H_3PO_4 and 0.1% napthyl ethylenediamine dihydrochloride) and incubated at for one hour at 25°C. Finally 2 ml distilled water was added and absorbance was taken at 546 nm.

Anti-lipid peroxidation (ALP) assay

The anti-lipid peroxidation activity of the sprout extracts of different developmental stages was estimated by the standard method[17] followed by some modification. 0.1 ml sample extract was added to a mixture of 2.8 ml of 10% goat liver homogenate and 0.1 ml of 50 mM $FeSO_4$. The reaction mixture was kept for incubation for 30 min at 37°C. After that 2 ml of 10% TCA-0.67% TBA in 50% acetic acid was added to 1 ml of reaction mixture for terminating the reaction. Mixture was kept in water bath for 1 hour and centrifuged at 10,000 rpm for 5 minutes. The absorbance of supernatant was read at 535 nm.

ALP % was calculated using the following formula:

$$\text{ALP percent} = \frac{\text{Abs. of } Fe^{2+} \text{ induced peroxidation} - \text{Abs. of sample}}{\text{Abs. of } Fe^{2+} \text{ induced peroxidation} - \text{Abs. of control}} \times 100$$

β-Carotene–linoleate bleaching (BCB) assay

The antioxidant activity was estimated based on the β- carotene bleaching protective capacity developed by Velioglu *et al.*[18] β-Carotene (0.2 mg in 1 ml chloroform), linoleic acid (0.02 ml) and Tween 20 (0.2 ml) were mixed in a round bottomed flask. Then chloroform was totally evaporated at reduced pressure using a rotary evaporator. After evaporation, 50 ml of distilled water was added to

the dried residue, and then shaken vigorously to form an emulsion for further use. 0.2 ml of methanolic extract was reacted with 2 ml of emulsion into test tubes and placed in a water bath at 50°C. The absorbance was recorded at 30 min intervals for 2 h at 470 nm.

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

$$Ln\ a/b \times 1/t = DR_{sample}\ or\ DR_{standard}$$

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 min and t is the initial absorbance (470 nm) at time 0.

Antioxidant activity (AA) was expressed as percent of inhibition relative to the control, using the following formula:

$$AA = \frac{DR_{control} - DR_{sample\ or\ standard}}{DR_{control}} \times 100$$

Total phenolic content (TPC)

Total phenolic contents of methanolic extracts were measured according to the standard protocol.[19] 1 ml of the methanolic extract was reacted with a mixture containing 1 ml of 95% ethanol solution, 5 ml of distilled water and 500 µl of folin-ciocalteau reagent (50%). After incubation period of 5 min, 1 ml of 5% Na_2CO_3 was added. It was mixed thoroughly on a vortex shaker and further kept for 1h of incubation at room temperature. Finally the absorbance of coloured reaction mixture was recorded at 765 nm against the reagent blank. The total phenolic content was measured as mg of Gallic acid equivalent per gram fresh weight.

Total flavonoid content (TFC)

The total flavonoid content was measured by performing a standard spectrophotometric method.[20] 1 ml of sprout extract was diluted with 4 ml distilled water in a volumetric flask. Initially, 300 µl $NaNO_2$ solution (5%) was added to each volumetric flask and after 5 min, 0.3 ml $AlCl_3$ (10%) was also added. After 6 min of incubation, 2 ml NaOH (1 M) was added to the mixture. Absorbance of the reaction mixture was measured at 510 nm after adding 2.4 ml of distilled water. The flavonoid content in different extracts was estimated as quercetin equivalent (QE) per gm fresh weight.

Total carotene content (TCC)

Total carotene contents (TCC) were determined according to the standard protocol.[21] Firstly, the methanolic sample was dried and converted to aqueous extract which was further partitioned thrice with equal volume of peroxide free ether using a separating funnel. The ether layer was collected and evaporated and then after reconstituted with ethanol. To the ethanolic extract 0.1 ml of 60% aqueous KOH was added and heated for 5 min with test tubes covered with marbles. The mixture was kept in dark for overnight. Next day again the mixture was partitioned with ether, the ether layer was evaporated and reconstituted with ethanol and the absorbance of the ethanolic mixture was recorded at 450 nm. The carotene content was calculated using a calibration curve prepared against pure β-carotene.

Table 1: Free radical scavenging activity of fenugreeks sprouts treated with calcium chloride, lanthanum chloride and EGTA at 24 h stage

	24 HOURS GERMINATION						
TREATMENT (mM)	DPPH (mg/ml)	ABTS (mg/ml)	MC (mg/ml)	RP (AAEµg/g fwt)	NO (mg/ml)	ALP (mg/ml)	BCB (%)
HYDRO	123.26 ± 3.63^{de}	113.17± 12.54^{cd}	163.93 ± 2.68^{b}	406.06± 39.85^{def}	1208.86 ± 3.80^{d}	784.84 ± 2.74^{b}	30.16 ± 5.12^{b}
CC-1	85.69 ± 1.30^{a}	53.91 ± 9.87^{a}	154.20 ± 3.02^{a}	567.79 ± 11.88^{b}	931.35 ± 7.13^{c}	564.15 ± 6.21^{a}	56.79 ± 9.93^{a}
CC-2	107.45 ± 3.22^{b}	66.02 ± 11.20^{ab}	161.62 ±1.30^{ab}	662.88 ± 39.85^{a}	896.19 ± 6.45^{b}	961.69 ± 0.20^{d}	50.35 ± 12.9^{a}
CC-5	113.17 ± 1.12^{bc}	72.83 ± 16.63^{b}	163.32 ± 7.34^{b}	490.98 ± 55.80^{c}	719.55 ± 11.45^{a}	1008.62 ± 6.01^{e}	45.53 ± 8.24^{a}
CC-10	120.02 ± 1.97^{cd}	102.7 ±3.12^{c}	165.76 ±6.97^{b}	451.35 ± 60.02^{cd}	1200.19 ± 0.93^{d}	1453.13 ± 2.54^{h}	15.11 ± 2.9^{cd}
EG-1	157.31 ± 7.52^{g}	159.93 ± 2.77^{fg}	286.16 ±1.47^{e}	339.67 ±31.88^{ef}	1411.70 ±1.91^{f}	1109.38 ± 6.82^{g}	14.89 ± 1.8^{cd}
EG-2	169.97 ± 3.01^{h}	164.19 ± 4.53^{g}	309.29 ±1.33^{f}	338.61 ± 44.23^{ef}	1561.06 ± 5.28^{i}	944.25 ± 3.67^{c}	13.01 ± 1.8^{cd}
EG-5	172.82 ± 3.76^{h}	173.34 ± 4.29^{g}	324.19 ± 2.15^{g}	342.32 ±36.57^{ef}	1657.41 ± 7.44^{j}	3360 ± 9.35^{j}	12.87 ± 3.22^{d}
EG-10	192.35 ± 5.24^{i}	233.88 ± 2.67^{h}	428± 1.50^{h}	324.79 ± 2.81^{f}	1764.39 ± 6.23^{k}	NA	12.18 ± 2.9^{d}
LC-1	131.43 ± 5.19^{ef}	121.40 ±4.22^{d}	183.98 ± 0.98^{c}	411.21±22.51^{de}	1251.15 ± 9.35^{e}	1083.33 ± 6.68^{f}	27.09 ± 6.19^{bc}
LC-2	138.32 ± 5.91^{f}	145.86 ± 6.84^{ef}	222.62 ± 0.47^{d}	389.28 ± 10.63^{def}	1502.61 ± 2.55^{g}	1082.32 ± 4.88^{f}	29.86 ± 4.64^{b}
LC-5	145.44 ± 1.43^{f}	139.11 ± 7.83^{e}	289.15 ± 5.40^{e}	342.96 ± 15.62^{ef}	1535.64 ± 7.29^{h}	1716.35 ± 8.15^{i}	19.79 ± 5.60^{bcd}
LC-10	151.88 ± 8.28^{g}	143.98 ± 4.06^{ef}	307.48 ± 3.02^{f}	335.18 ± 4.38^{ef}	1788.26 ± 4.61^{l}	NA	5.71 ± 1.05^{d}

Results are expressed as Mean ± SEM of triplicate determinations. Values with different letters (a, b, c, etc.) differ significantly (p≤0.05) by Duncan's Multiple Range Test (DMRT).

Table 2: Free radical scavenging activity of fenugreeks sprouts treated with calcium chloride, lanthanum chloride and EGTA at 48 h stage

	48 HOURS GERMINATION						
TREATMENT (mM)	DPPH (mg/ml)	ABTS (mg/ml)	MC (mg/ml)	RP (AAEμg/g fwt)	NO (mg/ml)	ALP (mg/ml)	BCB (%)
HYDRO	37.31 ± 10.53^{abc}	23.24 ± 1.71^{a}	89.55 ± 3.03^{b}	495.60 ± 13.83^{d}	983.57 ± 12.01^{bc}	919.07± 32.53^{abc}	31.44 ± 2.36^{def}
CC-1	32.23 ± 6.36^{ab}	20.99 ± 2.3^{a}	80.5 ± 4.09^{a}	735.10 ± 33.68^{b}	801.68 ± 31.93^{ab}	666.05 ±129.27^{a}	64.21 ± 4.82^{a}
CC-2	22.83 ± 10.56^{a}	14.91 ±3.10^{a}	78.06 ± 4.72^{a}	1149.70 ± 61.97^{a}	662.74 ± 27.83^{a}	655.45 ±5.99^{a}	47.70 ± 3.58b
CC-5	34.61 ± 7.37^{abc}	21.71 ± 0.95^{a}	88.58 ± 4.00^{b}	760 ± 12.66^{b}	653.56 ± 49.55^{a}	737.80 ± 278.09^{ab}	41.90 ± 3.14^{c}
CC-10	37.40 ± 9.68^{abc}	23.51 ± 8.23^{a}	89.28 ± 3.75^{b}	643.3 ± 37.43^{c}	979.01 ± 29.03^{bc}	752.9 ± 301.85^{ab}	27.25 ± 2.04^{def}
EG-1	70.93 ± 5.59^{ef}	81.07 ± 1.53^{c}	114.99 ± 1.50^{d}	420.71 ± 15.55^{ef}	1170.77 ± 279.72^{c}	1068.17 ± 222.98^{bc}	27.23 ± 2.04^{efg}
EG-2	81.06 ± 7.77^{fg}	92.55 ± 2.5^{d}	155.97 ± 4.17^{f}	424.18 ± 24.23^{ef}	1344.86 ± 14.66^{d}	1115.75 ± 27.11^{c}	25.94 ± 1.95^{fg}
EG-5	85.39 ± 7.20^{fg}	94.47 ± 9.61^{d}	208.16 ± 2.58^{i}	375.73 ± 0.39^{f}	1508.76 ±13.44^{de}	1180 ± 187.71^{c}	23.57 ± 1.77^{g}
EG-10	90.63 ± 2.08^{g}	133.63 ± 1.42^{e}	258.77 ± 2.57^{j}	364.53 ± 4.85^{f}	1594.15 ± 47.64^{e}	NA	21.56 ± 1.62^{g}
LC-1	45.86 ± 4.32^{bcd}	42.52 ± 8.49^{b}	98.39 ± 1.87^{c}	469.70 ± 21.65^{de}	1014.25 ± 11.01^{c}	1038.27± 43.04^{bc}	36.26 ± 2.72^{d}
LC-2	49.36 ± 6.16^{bcd}	44.21 ± 5.74^{b}	141.44 ± 4.72^{e}	442.01 ± 23.99^{de}	1030.35 ± 29.93^{c}	1145.45 ± 44.39^{c}	32.25 ± 2.42^{de}
LC-5	52.47 ± 9.86^{cd}	45.73 ± 1.81^{b}	169.45 ± 3.07^{g}	416.5 ± 22.51^{ef}	1057.14 ± 39.04^{c}	1150.82 ± 58.12^{c}	31.03 ± 2.33^{efg}
LC-10	57.47 ± 9.55^{de}	45.55 ± 7.80^{b}	183.40 ± 3.71^{h}	414.04 ± 18.83^{ef}	1091.22 ± 59.06^{c}	1197.85 ± 95.54^{c}	22.88 ± 1.72^{g}

Results are expressed as Mean ± SEM of triplicate determinations. Values with different letters (a, b, c, etc.) differ significantly ($p \leq 0.05$) by Duncan's Multiple Range Test (DMRT).

Table 3: Free radical scavenging activity of fenugreeks sprouts treated with calcium chloride, lanthanum chloride and EGTA at 72 h stage

	72 HOURS GERMINATION						
TREATMENT (mM)	DPPH (mg/ml)	ABTS (mg/ml)	MC (mg/ml)	RP (AAEμg/g fwt)	NO (mg/ml)	ALP (mg/ml)	BCB (%)
HYDRO	95.27 ± 2.93^{cd}	190.97 ± 0.38^{abc}	240.71 ± 1.2^{d}	346.83 ± 116.59^{bcd}	1487.51 ± 1.4^{e}	1295 ± 26.42^{bc}	25.59 ± 2.56bc
CC-1	89.41 ± 1.24^{b}	176.49 ± 4.91^{ab}	167.41 ± 3.21^{a}	501.66 ± 7.5^{a}	1040.65 ± 6.87^{b}	1149.07 ± 26.19^{a}	31.03 ± 2.35ab
CC-2	65.58 ± 0.45^{a}	169.52 ± 11.23^{a}	227.88 ± 5.05^{b}	500.17 ± 69.08^{ab}	1062.32 ± 3.42^{c}	1289.46 ± 23.33^{bc}	36.6 ± 0.65^{a}
CC-5	93.91 ± 0.18^{bc}	188.64 ± 0.69^{abc}	234 ± 3.65^{bc}	457.38 ± 100.97^{abc}	925.39 ± 7.51^{a}	1233.56 ± 36.33^{b}	27.07 ± 2.74^{bc}
CC-10	94.64 ± 2.47^{cd}	189.46 ± 0.08^{abc}	238.37 ± 0.7^{cd}	358.38 ± 55.64^{bcd}	1318.11 ± 1.61^{d}	1349.91 ± 36.55^{cd}	17.32 ±0.54^{def}
EG-1	102.04 ± 2.96^{e}	315.51 ± 20.37^{d}	269.02 ± 2.34^{e}	318.07 ± 8.44^{cd}	1938.44 ± 5.52^{g}	1437.5 ± 35.96^{e}	14.76 ± 0.53^{ef}
EG-2	104.41 ± 2.84^{e}	338.16 ± 19.50^{e}	336.92 ± 1.74^{g}	310.77 ± 50.64^{cd}	1978.93 ± 4.02^{h}	1444.47 ± 36.23^{e}	13.69 ± 2.03^{f}
EG-5	110.4 ± 6.06^{f}	404.31 ± 14.12^{f}	345.22 ± 3.98^{h}	305.62 ± 45.01^{cd}	2075.16 ± 3.64^{i}	1524.44 ± 46.53^{f}	11.19 ±1.82^{f}
EG-10	282.44 ± 0.27^{h}	508.94 ± 13.45^{g}	376.06 ± 2.51^{j}	283.11 ± 61.89^{d}	2627.24 ± 2.59^{l}	NA	10.77 ± 1.67^{f}
LC-1	99.52 ± 0.72^{de}	193.93 ± 7.4^{bc}	270.16 ±1.71^{e}	335.77 ± 39.39^{cd}	1591.21 ± 0.87^{f}	1382.92 ± 33.45^{de}	24.59 ±2.1^{bc}
LC-2	99.82 ± 0.06^{de}	194.76 ± 1.76^{bc}	328.75 ± 1.91^{f}	334.18 ± 99.4^{cd}	1597.6 ± 0.2^{f}	1441.63 ± 35.71^{e}	22.52 ± 6.82^{cd}
LC-5	101.3 ± 0.99^{e}	201.04 ± 4.64^{c}	331.01 ± 3.98^{fg}	325.7 ± 64.71^{cd}	2226.64 ± 0.41^{j}	1606.28 ± 64.83^{f}	21.08 ±2.19^{cde}
LC-10	121.29 ± 0.51^{g}	212.03 ± 1.19^{c}	355.77 ± 3.31^{i}	325.26 ± 72.83^{cd}	2512.1 ± 1.73^{k}	1602.97 ± 29.45^{f}	20.74 ± 5.41^{cde}

Results are expressed as Mean ± SEM of triplicate determinations. Values with different letters (a, b, c, etc.) differ significantly ($p \leq 0.05$) by Duncan's Multiple Range Test (DMRT).

In vitro α-amylase inhibitory activity

The alpha-amylase inhibition potential of the extract was estimated by standard spectrophotometric method.[22] 0.5 ml of aqueous extract was reacted with 0.5 ml of α-amylase solution and incubated at 37°C for 5 min. After incubation, 0.5 ml starch solution (1%) was added and was further incubated for 10 min. To the above reaction mixture, 1 ml of DNSA reagent was added to terminate the reaction and was heated for 10 min in a hot water bath till the colour of reaction mixture colour changed to orange-red. After change in colour, the reaction mixture was cooled and diluted up to 5 ml with distilled water. The OD 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 α-glucosidase inhibitory activity

The alpha-glucosidase inhibitory property of the sample extract was assayed as suggested by Jung *et al*.,[23] (2006) 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 α-Glucosidase 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. The α-glucosidase inhibitory activity was estimated by following equation:

$$Inhibitory\ ratio\ \% = [1- (As- Ab)/ Ac] \times 100$$

Table 4: Phytochemical contents of fenugreeks sprouts treated with calcium chloride, lanthanum chloride and EGTA at 24 h stage

24 HOURS GERMINATION			
TREATMENT	TPC (µg/g fwt)	TFC (µg/g fwt)	TCC (µg/g fwt)
HYDRO	89.37 ± 5.74^{bc}	7.16 ± 0.48^{c}	54.36 ± 4.65^{b}
CC-1	95.91 ± 8.31^{b}	8.25 ± 0.55^{b}	76.12 ± 6.52^{a}
CC-2	115.01 ± 9.96^{a}	7.65 ± 0.51^{bc}	79.75 ± 6.83^{a}
CC-5	90.13 ± 4.81^{bc}	9.62 ± 0.64^{a}	72.49 ± 6.21^{a}
CC-10	90.10 ± 4.69^{bc}	7.60 ± 0.51^{bc}	59.19 ± 5.07^{b}
EG-1	87.24 ± 5.12^{bc}	4.34 ± 0.29^{fg}	87.24 ± 5.12^{ef}
EG-2	83.75 ± 4.15^{cd}	4.78 ± 0.32^{ef}	83.75 ± 4.15^{f}
EG-5	72.78 ± 6.24^{de}	3.74 ± 0.25^{gh}	72.78 ± 6.24^{fg}
EG-10	65.86 ± 5.78^{e}	2.76 ± 0.18^{h}	65.86 ± 5.78^{g}
LC-1	87.46 ± 4.12^{bc}	5.85 ± 0.39^{d}	35.01 ± 3.00^{c}
LC-2	86.97 ± 3.53^{bc}	5.31 ± 0.35^{de}	28.97 ± 2.48^{cd}
LC-5	86.86 ± 3.97^{bc}	5.03 ± 0.33^{def}	21.71 ± 1.86^{de}
LC-10	84.84 ± 3.88^{bc}	4.76 ± 0.32^{ef}	19.29 ± 1.98^{de}

Results are expressed as Mean ± SEM of triplicate determinations. Values with different letters (a, b, c, etc.) differ significantly ($p \leq 0.05$) by Duncan's Multiple Range Test (DMRT).

Table 5: Phytochemical contents of fenugreeks sprouts treated with calcium chloride, lanthanum chloride and EGTA at 48 h stage

48 HOURS GERMINATION			
TREATMENT	TPC (µg/g fwt)	TFC (µg/g fwt)	TCC (µg/g fwt)
HYDRO	99.46 ± 8.09^{bc}	10.2 ± 0.58^{c}	75.24 ± 4.24^{c}
CC-1	130.88 ± 11.95^{a}	10.11 ± 0.72^{c}	91.09 ± 6.44^{a}
CC-2	141.24 ± 12.9^{a}	12.45 ± 1.32^{a}	95.46 ± 6.21^{a}
CC-5	129.24 ± 11.8^{a}	11.43 ± 1.17^{b}	84.58 ± 5.5^{b}
CC-10	104.04 ± 9.5^{b}	10 ± 0.71^{c}	72.49 ± 4.71^{c}
EG-1	88.66 ± 7.21^{bcd}	6.4 ± 0.46^{ef}	48.31 ± 3.14^{f}
EG-2	88.49 ± 7.19^{bcd}	5.69 ± 0.41^{f}	31.39 ± 2.04^{g}
EG-5	82.22 ± 7.51^{cd}	5.36 ± 0.33^{f}	24.13 ± 1.57^{g}
EG-10	75.02 ± 6.1^{d}	5.25 ± 0.32^{f}	5.99 ± 0.39^{h}
LC-1	97.22 ± 7.9^{bc}	7.54 ± 0.54^{de}	62.82 ± 4.08^{d}
LC-2	94.99 ± 7.72^{bcd}	7.49 ± 0.53^{de}	60.4 ± 3.93^{d}
LC-5	94.39 ± 7.67^{bcd}	6.72 ± 0.48^{def}	57.98 ± 3.77^{de}
LC-10	91.55 ± 7.44^{bcd}	5.47 ± 0.39^{f}	49.52 ± 3.22^{ef}

Results are expressed as Mean ± SEM of triplicate determinations. Values with different letters (a, b, c, etc.) differ significantly ($p \leq 0.05$) by Duncan's Multiple Range Test (DMRT).

where As, Ab and Ac represent the OD value of the sample, blank and control reaction mixture respectively.

RESULTS AND DISCUSSION

Antioxidant activity

The assessment of antioxidant activity of plant extract is very much important for determining its nutritional value.[24] The mode of action of antioxidant compounds responsible for defence mechanisms in different stages of plant, depends on plant's growth and development.[25] The antioxidant property of the sprout extracts was evaluated in terms of their free radicals such as DPPH, ABTS, and nitric oxide quenching potential, ferric reducing power, metal chelation, lipid peroxidation and β-carotene protective activity. To evaluate the effect of Ca^{2+} on the antioxidant property of the fenugreek seeds, the seeds were primed with calcium chloride solution as an exogenous source of Ca^{2+}. It was evident from Table 1-3 there was significant enhancement in the free radical scavenging activity of sprouts pre-treated with calcium chloride indicating the involvement of Ca^{2+} in improving antioxidant potential. The elicitation of fenugreek seeds with calcium chloride showed a gradual increase in free radical scavenging activity from 24 h stage to 48 h stage and then decreased towards 72 h stage.

Table 6: Phytochemical contents of fenugreeks sprouts treated with calcium chloride, lanthanum chloride and EGTA at 72 h stage

72 HOURS GERMINATION			
TREATMENT	TPC (µg/g fwt)	TFC (µg/g fwt)	TCC (µg/g fwt)
HYDRO	90.24 ± 3.96^{bc}	6.89 ± 0.31^{c}	57.98 ± 3.19^{d}
CC-1	99.08 ± 4.35^{a}	8.8 ± 0.4^{a}	66.45 ± 3.65^{c}
CC-2	101.2 ± 4.44^{a}	8.2 ± 0.37^{b}	88.21 ± 4.85^{a}
CC-5	93.73 ± 4.11^{bc}	7.87 ± 0.35^{b}	76.12 ± 4.19^{b}
CC-10	94.77 ± 4.16^{bc}	7.27 ± 0.33^{c}	62.82 ± 3.46^{cd}
EG-1	88.39 ± 3.88^{bcd}	4.87 ± 0.22^{ef}	14.46 ± 0.8^{fg}
EG-2	83.69 ±3.67^{cd}	4.82 ± 0.22^{ef}	12.04 ± 0.66^{g}
EG-5	80.26 ± 3.52^{cd}	4.32 ± 0.19^{fg}	4.79 ± 0.26^{h}
EG-10	79.6 ± 3.49^{d}	4.11 ± 0.18^{g}	3.28 ± 0.2^{h}
LC-1	88.88 ± 3.9^{bcd}	6.02 ± 0.27^{d}	20.5 ± 1.13^{e}
LC-2	84.79 ± 3.72^{cd}	6.02 ± 0.27^{d}	19.3 ± 1.06^{ef}
LC-5	82.17 ± 3.61^{cd}	5.31 ± 0.24^{e}	12.04 ± 0.66^{g}
LC-10	80.09 ± 3.52^{d}	5.14 ± 0.23^{e}	4.79 ± 0.26^{h}

Results are expressed as Mean ± SEM of triplicate determinations. Values with different letters (a, b, c, etc.) differ significantly ($p \leq 0.05$) by Duncan's Multiple Range Test (DMRT).

Phytochemical analysis

The phenolic compounds, being well-equipped with the ability to act as efficient hydrogen donors, reducing agents and quenchers of singlet oxygen, play a vital role as an antioxidant component in the living system and hence possess potential human health benefits.[26] For phytochemical analysis, total phenol, flavonol, and carotene content were measured for three days of germination phase. The significant enhancement of these all bioactive phytochemicals was observed in the sprouts pre-treated with calcium chloride. The phenolic content was found to increase by 42%, flavonol by 22%, and also significant enhancement was observed in carotene content which was found to be 26.87%. In agreement to present work Joshi *et al*,[27] has also reported increase in phenolics in the seeds of cucumber after priming with calcium chloride.

Effect of calcium channel blocker ($LaCl_3$) and calcium chelator (EGTA) was also assessed on the antioxidant as well

Table 7: *In vitro* α-amylase and α-glucosidase inhibitory activity of fenugreek sprouts of different developmental phases (24, 48 and 72 hours)

Treatments (mM)	α-Amylase inhibitory activity (mg/ml)			α-Glucosidase inhibitory activity (mg/ml)		
	24h	48h	72h	24h	48h	72h
HYDRO	1186.7 ± 68^{c}	840.4 ± 73.4^{cd}	1319.3 ± 49.2^{c}	1010 ± 97.8^{c}	715.2 ± 31.2^{b}	1122.8 ± 89.9^{b}
CC-1	619.2 ± 35.5^{a}	607.2 ± 53^{ab}	1320.2 ± 49.2^{c}	336.5 ± 41.4^{a}	330 ± 72.4^{a}	717.5 ± 121.9^{a}
CC-2	522.7 ± 29.9^{a}	463.7 ± 40.5^{a}	923.7 ± 34.4^{a}	339.4 ± 132.8^{a}	301.1 ± 137.7^{a}	599.8 ± 67.1^{a}
CC-5	502.5 ± 28.8^{a}	669.2 ± 58.4^{bc}	1145.3 ± 42.7^{b}	348.9 ± 26.69^{a}	464.7 ± 93.1^{a}	795.4 ± 78.6^{a}
CC-10	985.5 ± 56.5^{b}	1345.4 ± 117.5^{g}	1576.1 ± 58.8^{d}	566.4 ± 26.7^{b}	773.2 ± 85^{b}	1176.2 ± 67^{b}
EG-1	1202.9 ± 68.9^{c}	1245.7 ± 108.7^{fg}	1824.3 ± 68^{fg}	1055.2 ± 68.9^{c}	1092.7 ± 113.9^{c}	1600 ± 84.5^{c}
EG-2	1386.2 ± 79.4^{d}	1121.43 ± 98^{ef}	1626.8 ± 60.6^{de}	1593.3 ± 91.1^{d}	1289 ± 146.5^{c}	1869.8 ± 139.9^{d}
EG-5	1291.2 ± 73.9^{cd}	1272.5 ± 111.1^{fg}	1506 ± 56.2^{d}	1634.5 ± 75.4^{d}	1610.7 ± 18.3^{d}	1906.3 ± 153.1^{d}
EG-10	1707.5 ± 97.8^{e}	1299.8 ± 113.4^{fg}	1713.4 ± 64^{ef}	2276.6 ± 97.7^{e}	1732.1 ± 225.4^{d}	2284.5 ± 203.7^{e}
LC-1	1199.3 ± 68.7^{c}	953.6 ± 83.3^{de}	1519.3 ± 56.7^{d}	1047.4 ± 42.2^{q}	832.8 ± 87.8^{b}	1326.9 ± 156.8^{b}
LC-2	1284.2 ± 73.6^{cd}	1209.6 ± 105.6^{fg}	1798.7 ± 67.1^{fg}	1141.5 ± 164.9^{c}	1075.2 ± 102.4^{c}	1598.9 ± 54.7^{c}
LC-5	1287.1 ± 73.7^{cd}	1410.4 ± 123.1^{g}	1885.7 ± 70.3^{g}	1148.1 ± 130.9^{c}	1258.2 ± 111.9^{c}	1738.04 ± 92.1^{cd}
LC-10	1679.7 ± 96.2^{e}	1113.3 ± 97.2^{ef}	1632.7 ± 61de	2545 ± 133.3^{f}	1294.6 ± 124.5^{c}	1700.8 ± 91.4^{cd}

Results are expressed as Mean ± SEM of triplicate determinations. Values with different letters (a, b, c, etc.) differ significantly ($p \leq 0.05$) by Duncan's Multiple Range Test (DMRT).

as related phytochemicals content of the fenugreek sprouts. The effect of these elicitors was found to be negative on both the aspects of the sprouts (Table 4-6). EGTA had the greatest effect on reduction in the antioxidant property of the sprouts with corresponding loss in the synthesis of relative phytochemicals followed by $LaCl_3$.

The enhancement in the phenolic in Ca^{2+} primed seedlings and subsequent improvement in antioxidant as well as anti-diabetic potential of the sprouts further reinforces the hypothesis suggesting the vital involvement of Ca^{2+} in some signaling pathway during germination phase in fenugreek leading to betterment in the nutraceutical properties.

In vitro Anti-diabetic activity

The key enzymes of the digestive system, α-amylase and α-glucosidase 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 reduction in the postprandial hyperglycemia level.[28] On assessment of in-vitro antidiabetic activity similar trend was observed in the α-amylase and α-glucosidase inhibitory activity of the fenugreek sprouts primed with different elicitors of Ca^{2+} signalling. The α-amylase as well as α-glucosidase inhibitory activity was found to be highest in the sprouts per-treated with calcium chloride and those treated with $LaCl_3$ and EGTA exhibited decline in the anti-diabetic activity when compared with the control set (Table 7).

RPreviously several researchers have demonstrated the relationship between free-radical scavenging potential and bioactive phytochemicals. In the present study, interestingly significant positive correlation was derived between antioxidant and phenolics (r^2=0.639) as well as anti-diabetic and phenolics (r^2=0.691) from Pearson's correlation analysis indicating active participation of these phenolic compounds in the nutraceutical property of the fenugreek sprouts. The phenolics and flavonoids have been reported to be efficient inhibitors of α-glucosidase, and also regulators of hyperglycemic disorders and other diabetic associated complications arising from oxidative stress.[29] A highly significant correlation between free radical scavenging activity and *in vitro* anti-diabetic activity indicates an existence of strong cohesiveness among antioxidant and anti-hyperglycemic properties of the plant extract. Supporting such correlation several hypothesis and mechanisms have suggested by different authors.[6] In a nut shell, it was observed that among all treatments, the significant enhancement of anti-diabetic activity, antioxidant activity and related phenolics was found in the sprouts primed with calcium chloride and on the other hand $LaCl_3$ and EGTA exhibited reverse effect on the bioactivity of the fenugreek sprouts. Thus it can be suggested that a flux of calcium ion is required within the cellular system which comes into play during germination to regulate some mechanism responsible for synthesis of antioxidative as well as anti-diabetic phytochemical compounds.

CONCLUSION

In conclusion, the present study suggested that priming with $CaCl_2$ as exogenous source of Ca^{2+} was found to be effective in enhancement of antioxidant as well as anti-diabetic activity and related phenolics fenugreek sprouts.

When analyzing the effect of Ca^{2+} flux during time course of germination the stimulatory effect of $CaCl_2$ was most pronounced at the initial phase i.e. from 24 h to 48 h and then the action was declined during further extension of post-germination phases. This knowledge can be used to design the sprouting techniques which might have potential application in improving the nutraceutical quality of legume sprouts along with health relevant functional values.

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CONFLICTS OF INTEREST

Authors claim no conflicts of interest.

Highlights of Paper

- *Trigonella foenum-graecum* is an important medicinal legume with wide spectrum of pharmacological and folkloric significance.
- Priming with calcium chloride resulted in enhancement of therapeutic potential and related phytochemical contents of fenugreek sprouts.
- Priming with EGTA and Lanthanum chloride reversed the action of Calcium ion.
- Thus, the involvement of Ca^{2+} in signaling pathways associated with related phenolic compounds is suggested.

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