

Original article

Evaluation of *in vitro* antioxidant property and phytochemical contents in different genotypes of fenugreek (*Trigonella foenum graecum* L.)

Naveen Chandra Pant, Karishma Joshi*, Manoj Kumar**, J.P.Singh*** and Sanjeev Agrawal**

Department of Agriculture, Dolphin (P.G.) Institute of Biomedical and Natural Sciences, Dehradun-248007, Uttarakhand, India

*Department of Biochemistry, Dolphin (P.G.) Institute of Biomedical and Natural Sciences, Dehradun-248007, Uttarakhand, India

**Department of Biochemistry, College of Basic Sciences and Humanities, G. B. P. U. A. and T., Pantnagar-264145, Uttarakhand, India

***Department of Vegetable Sciences, College of Agriculture, G. B. P. U. A. and T., Pantnagar-264145, Uttarakhand, India

Received October 3, 2017; Revised November 24, 2017; Accepted November 30, 2017; Published online December 30, 2017

Abstract

Polyphenol content and antioxidant activity in methanolic extracts of 10 fenugreek (*Trigonella foenum graecum* L.) genotypes from north India were evaluated, using different *in vitro* assays, together with total carotenoid, α -tocopherol and total ascorbate content. The antioxidant activity was evaluated using total antioxidant activity (Phosphomolybdenum method), ferric reducing antioxidant power (FRAP) assay, 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay, 2,2'-azinobis (3-ethylbenzothiazoline)-6-sulphonate (ABTS) assay, hydroxy radical scavenging assay, superoxide radical scavenging assay, nitric oxide scavenging and β -carotene-linoleate bleaching assay. The total phenol content (TPC) varied from $(48.766 \pm 0.042 \text{ mg GAE g}^{-1} \text{ extract})$ in RMT-303 to $(36.204 \pm 0.371 \text{ mg GAE g}^{-1} \text{ extract})$ in UM-265. RMT-303 also showed higher flavonoid $(4.682 \pm 0.105 \text{ mg QE g}^{-1} \text{ extract})$, α -tocopherol $(171.918 \pm 11.722 \mu\text{g g}^{-1} \text{ dry wt.})$ and total ascorbate content $(359.884 \pm 30.886 \mu\text{g g}^{-1} \text{ dry wt.})$. Higher free radical scavenging activity, viz., ABTS radical $(\text{IC}_{50} = 303.380 \pm 4.170 \mu\text{g ml}^{-1} \text{ extract})$, superoxide radical $(\text{IC}_{50} = 152.040 \pm 3.290 \mu\text{g ml}^{-1} \text{ extract})$ and nitric oxide scavenging $(\text{IC}_{50} = 142.160 \pm 0.320 \mu\text{g ml}^{-1} \text{ extract})$ was observed in RMT-1. The genotypes showed significant difference ($p \geq 0.05$) with respect to phytochemical content and corresponding antioxidant activity. The phytochemicals, viz., α -tocopherol and total ascorbate content within the genotypes showed a significant positive correlation (*i.e.*, 0.517 and 0.546) with antioxidant activity (TAA) exhibited by the genotypes. Significant positive correlation (0.659) was observed between phenol and flavonoid content within the genotypes. The findings of the present investigation clearly show variation in phytochemical content and antioxidant activity among fenugreek genotypes. Thus, variation in antioxidant property among the genotypes may be attributed to the variation in phytochemical content which may be due to genetic differences among the fenugreek genotypes.

Keywords: *Trigonella foenum graecum* L., reactive oxygen species, polyphenols, antioxidants.

1. Introduction

Under normal physiological condition, cellular systems are incessantly challenged by stress arising from both internal and external sources. The aerobic metabolism results in generation of reactive oxygen species (ROS) which include free radicals such as superoxide anion, hydroxyl radical as well as non-radical molecules such as hydrogen peroxide and singlet oxygen (Tanou *et al.*, 2009). Under oxidative stress, the oxidative and non-oxidative systems of the cells and tissues lost balance between each other which results into over production of oxidative free radical and ROS (Rani *et al.*, 2016). Antioxidants are compounds which have the ability to quench ROS and, thus, prevent the oxidation of other molecules and may, therefore, play an important role in the prevention of degenerative diseases (Biglari *et al.*, 2008). Current emphasis is now directed

towards finding naturally occurring antioxidants of plant origin. The polyphenolics have been reported as the major components having health supremacy and outstanding connection between advantages and measured antioxidant values (Farzaneh and Carvalho, 2015). They are widely distributed in plants and it is widely known that of plants synthesize polyphenolics which are used as a natural source of antioxidants, having health promoting effects (Traka and Mithen, 2011; Mandegary *et al.*, 2012; Niciforovic *et al.*, 2010).

Fenugreek (*Trigonella foenum graecum* L.) is one of oldest medicinal plants, recorded in history (Lust, 1986). It is a legume which has been used as a spice to boost the sensory quality of food throughout the world (Wani and Kumar, 2016). Seeds of Fenugreek has been attributed with antidiabetic, hypercholesterolemic, antileukemic, antipyretic, antioxidant, hepatoprotective and antimicrobial properties (Vyas *et al.*, 2008; Kawabata *et al.*, 2011; Bahmani *et al.*, 2015; Subhashini *et al.*, 2011 and Hannan *et al.*, 2003). It is also used as a food stabilizer due to its high fiber, protein content (Srinivasan, 2006). The plants of Fenugreek contain various compounds like polyphenols, alkaloids, steroids, glycosides and amino acids, etc. Seeds of fenugreek are reported to contain saponin, diosgenin, trigogenin, gitongenin, rhaponticin, isovitexin and

Author for correspondence: Dr. Naveen Chandra Pant
Assistant Professor, Department of Agriculture, Dolphin (P.G.) Institute of Biomedical and Natural Sciences, Dehradun-248007, Uttarakhand, India

E-mail: naveen_cpant@rediffmail.com

Tel.: +91-9639653975

Copyright © 2017 Ukaaz Publications. All rights reserved.

Email: ukaaz@yahoo.com; **Website:** www.ukaazpublications.com

proteins, *etc.* (Wani and Kumar, 2016, He *et al.*, 2015). India is one of the largest producers of Fenugreek in the world with different cultivars currently being grown in different regions. In recent years, due to growing consciousness for foods with additional health properties (functional foods), there is an eminent need for better genotypes with high antioxidant properties, therefore, fenugreek genotypes were screened on the basis of their antioxidant activities employing different *in vitro* assay methods.

The main objective of this study was to evaluate Fenugreek genotypes from Northern India, in order to screen potential fenugreek genotypes with better antioxidant property and phytochemical content. The present study could prove to be of immense value for the breeders to develop cultivars with outstanding quality attribute as well as provide a basis on which any future selections (genotypes) may be evaluated and compared.

2. Materials and Methods

2.1 Samples

The seeds of 10 fenugreek genotypes (under varietal trials of All India Coordinated Research Project (AICRP) on Spices) AM-316, UM-222, UM-265, IC-143847, IC-066843, RMt-303, Pusa early bunching, RMt-1, GM-2 and Pant ragini were collected from Vegetable Research Centre (VRC), GBPUA and T, Pantnagar, district Udham Singh Nagar (Uttarakhand, India). The samples were selected homogeneously in terms of maturity, three replicates 10 g each were used for sample preparation for each genotype.

The seed samples were oven dried at $50 \pm 5^\circ\text{C}$. The dried seeds were crushed with pestle and mortar and seed powder of respective genotypes was used for estimation of phytochemicals and antioxidant activity. Methanolic extract of seeds was prepared for quantification of various polyphenols and antioxidant activity except carotenoid, total α -tocopherol and ascorbic acid estimation. For methanolic extract preparation, seed powder of respective genotype was mixed with 50 ml methanol and kept overnight in a shaker. The next day, mixture was filtered with Whatmann filter paper No. 2 and extracted twice with 20 ml methanol. Methanol was evaporated by rotatory evaporator at $60 \pm 3^\circ\text{C}$ and the extract was weighted and redissolved in methanol in order to make suitable stock solution. The methanolic extract was stored at -20°C and used for further investigation.

2.2 Chemicals and reagents

The compounds 2,2'-azinobis (3-ethylbenzothiazoline)-6-sulphonate disodium salt (ABTS), 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,4,6 tripyridyl-S-triazine (TPTZ) and quercetin were purchased from Sigma-Aldrich (St. Louis, MO, USA). All chemicals and reagents used were of analytical grade.

2.3 Phytochemical estimation

2.3.1 Total phenolic content

The total phenolic content was estimated by Folin-Ciocalteu method of Swain and Hills (1959). The aliquot (200 μl) was mixed with 1.5 ml of Folin-Ciocalteu reagent, and allowed to stand at room temperature for 5 min; then 1.5 ml of saturated sodium bicarbonate solution was added to the mixture. After 90 min, absorbance was measured at 725 nm against blank. A calibration curve with gallic acid was prepared and the results were expressed as gallic acid equivalents (mg GAE g^{-1} extract).

2.3.2 Total flavonoid content

Total flavonoid content was determined according to Kim *et al.* (2003). 0.3 ml sodium nitrite solution (5%) was added to 1 ml of the seed extract, followed by 0.3 ml of 10 % aluminum chloride solution. The test tubes were incubated at ambient temperature for 5 min, and then 2 ml of 1M sodium hydroxide were added to the mixture. The mixture was thoroughly vortexed and the absorbance was read at 510 nm against blank. Flavonoid content was expressed as quercetin equivalent (mg QE g^{-1} extract).

2.3.3 Tannin content

Tannins were estimated by using Folin-Denis reagent described by Swain and Hills (1959). Tannins reduced phosphotungstomolybdic acid in alkaline solution and generated a blue colored solution. The 0.2 ml (1mg ml^{-1}) extract of each plant part was mixed with 0.5 ml Folin-Denis reagent and 1 ml saturated Na_2CO_3 was added sequentially. Mixture was incubated for 30 min at room temperature and absorbance was read at 760 nm. Experiment was carried out in three replicates. Amount of tannins was estimated as mg tannic acid g^{-1} extract from calibration curve of tannic acid standard solution.

2.3.4 Total carotenoids

The total carotenoids were extracted in organic solvent (acetone) on the basis of their solubility (Jensen, 1978). Distilled acetone (20 ml) was added to 1 gram grounded sample and extracts were filtered through filter paper. The combined filtrates were partitioned thrice with equal volume of peroxide-free ether. The pooled ether fraction (containing carotenoids) was evaporated under reduced pressure at 35°C in a rotary evaporator. The residue was dissolved in ethanol and treated with 60 % aqueous KOH. The resulting mixture was boiled for 5-10 min and partitioned thrice with ether. The combined ether fraction was evaporated and the residue was dissolved in ethanol. The absorbance of ethanolic solution was measured at 450 nm. The carotenoid content ($\mu\text{g g}^{-1}$ fresh wt.) was calculated using a standard curve (10-200 μg) prepared by using β -carotene as standard.

2.3.5 Total α -tocopherol (vitamin E)

α -Tocopherol was estimated as described by Backer *et al.* (1980). 500 mg of seed sample was homogenized with 10 ml of a mixture of petroleum ether and ethanol (2:1.6 v/v) and the extract was centrifuged at 10,000 rpm for 20 min and the supernatant was used for estimation of α -tocopherol. To 1 ml of extract, 0.2 ml of 2 % 2, 2-dipyridyl in ethanol was added and mixed thoroughly and kept in the dark for 5 min. The resulting red colour was diluted with 4 ml of distilled water and mixed well. The resulting colour in the aqueous layer was measured at 520 nm. The α -tocopherol content ($\mu\text{g g}^{-1}$ fresh wt.) was calculated using a calibration curve of α -tocopherol.

2.3.6 Ascorbic acid

Ascorbic acid was estimated by the method of Laws *et al.* (1983). To 500 mg powdered seed samples, 10 % (w/v) trichloroacetic acid was added. After vortex-mixing, it was allowed to stand in ice for 5 min. 10 μl NaOH was added and the mixture was centrifuged for 2 min in a microfuge. To 200 μl of supernatant sample, 900 μl 200 mM NaH_2PO_4 buffer (pH 7.4), and 200 μl of 1.5 mM-dithiothreitol was added, after thorough mixing the reaction mixture was left at room temperature for 30 min, 200 μl of 0.5 % (w/v) N-ethylmaleimide (to remove excess DTT) was added. The samples were vortex-mixed and incubated at room temperature. Colour was

developed by adding 1000 µl of 10 % (w/v) trichloroacetic acid, 800 µl of 42 % (v/v) O-phosphoric acid, 800 µl of 65 mM 2, 2'-bipyridyl in 70 % (v/v) ethanol and 400 µl of 3 % (w/v) FeCl₃. After vortex-mixing, samples were incubated at 42°C for 60 min and the absorbance measured at 525 nm. The ascorbic acid content (µg g⁻¹ fresh wt.) was calculated using a standard curve of ascorbic acid.

2.4 Antioxidant activity

The methanolic extract of 10 Fenugreek genotypes at different concentrations ranging from 200-800 µg were tested for their ability to scavenge free radicals generated by different *in vitro* systems. The IC₅₀ values were calculated from the inhibition percents of the respective assay systems.

2.4.1 Determination of total antioxidant capacity

The antioxidant activity of the extracts of was evaluated by phosphomolybdenum method (Prieto *et al.*, 1999). The assay is based on the reduction of Mo (VI) to Mo (V) by the extract and subsequent formation of a green phosphate/Mo (V) complex at acidic pH. 0.3 ml extract was combined with 3 ml of reagent solution (0.6 M sulfuric acid, 28 M sodium phosphate and 4 mM ammonium molybdate). The tubes containing the reaction solution were incubated at 95°C for 90 min. The absorbance of the solution was measured at 695 nm against blank after cooling to room temperature. Methanol (0.3 ml) in the place of extract was used as the blank. The antioxidant activity was expressed as the trolox equivalents (µmol TE g⁻¹ extract).

2.4.2 Ferric reducing antioxidant potential (FRAP) assay

The ability to reduce ferric ions was measured using modifying methods of Wong *et al.* (2006) and Benzie and Strain (1996). The assay is based on reduction of the 2,3,5-triphenyl-1,3,4-triaza-2-azoniacyclopenta-1,4-diene chloride (TPTZ)-ferric iron complex to the ferrous form at low pH. The FRAP reagent was made by combining 25 ml of acetate buffer (300 mM, pH 3.6), 2.5 ml TPTZ solution (10 mM TPTZ in 40 mM HCl), 2.5 ml FeCl₃ (20 mM) in a ratio of 10:1:1 (v/v). An aliquot (200 µl) of the extract with appropriate dilution was added to 3 ml of FRAP reagent and the reaction mixture was incubated in a water bath at 37°C. The increase in absorbance at 593 nm was measured after 30 min against blank. Results were expressed as ascorbic acid equivalents (µmol AAE g⁻¹ extract) by calibration curve of ascorbic acid.

2.4.3 DPPH free radical scavenging activity

The free radical scavenging ability of methanolic extract of Fenugreek against 1, 1-diphenyl-2-picrylhydrazyl (DPPH) free radical was evaluated by method of Braca *et al.* (2001). Briefly, 1 ml of the extract was mixed with 1 ml of 0.4 mM methanolic solution containing DPPH radicals. Similarly, a reference solution of methanol and DPPH was prepared in just the same way. Both solutions were kept in a dark chamber for 30 min before measuring the absorbance at 517 nm. Free radical scavenging ability was calculated as follows:

$$\% \text{ DPPH radical scavenging} = \left[1 - \left(\frac{\text{Sample}_{\text{Abs}}}{\text{Control}_{\text{Abs}}} \right) \right] \times 100$$

2.4.4 ABTS radical cation-scavenging assay

The assay was performed by a slightly modified protocol of Re *et al.* (1999). ABTS solution (7 mM) was reacted with ammonium

persulphate (2.45 mM) solution and kept for 12–16 h in the dark, to produce a dark coloured solution containing ABTS radical cations. The initial absorbance was measured at 745 nm. This stock solution was diluted with methanol to give a final absorbance value of about 0.7 (± 0.02) (Mensor *et al.*, 2001) and equilibrated at 30°C. Different concentrations of the sample (100 – 800 µg/ml) were prepared by dissolving the extracts in water. About 0.3 ml of the sample was mixed with 3 ml of ABTS working standard in a microcuvette. The decrease in absorbance was measured exactly one minute after mixing the solution, then up to six minutes. The final absorbance was noted. The percentage inhibition was calculated according to the formula:

$$\% \text{ ABTS radical scavenging} = \left[1 - \left(\frac{\text{Sample}_{\text{Abs}}}{\text{Control}_{\text{Abs}}} \right) \right] \times 100$$

2.4.5 Hydroxyl radical scavenging activity

Free radical dependent 2-deoxyribose degradation was studied using the Fenton oxidant reaction mixture of Fe²⁺/ascorbic acid and H₂O₂ by Halliwell *et al.* (1995). Hydroxyl radical scavenging ability of *T. foenum-graecum* extract was measured by studying the competition between deoxyribose and the test compounds for hydroxyl radicals generated from the Fe³⁺/ascorbate /EDTA/H₂O₂ system. The reaction mixture containing deoxyribose (2.8 mM), FeCl₃ (0.1 mM), EDTA (0.1 mM), H₂O₂ (1 mM), ascorbic acid (0.1 mM) KH₂PO₄ – KOH buffer (20 mM, pH 7.4) and the extract (0-3000 µg/ml) in a final volume of 1.0 ml was incubated for 1 h at 37°C. Deoxyribose degradation was measured as thiobarbituric acid reactive substances (TBARS) by the method of Ohkawa *et al.* (1979), as modified by Liu *et al.* (1990). Briefly, 1.5 ml of 20 % acetic acid (pH 3.5), 1.5 ml of 0.8 % thiobarbituric acid (TBA) and 0.2 ml of 8.1 % sodium dodecyl sulphate (SDS) prepared in distilled water and the mixture incubated at 100°C for 1 h, cooled and 2 ml of trichloroacetic acid added. The mixture was vortexed vigorously and centrifuged at 3000 g for 10 min. and the absorbance of the supernatant read at 532 nm wavelength. Concentration of TBARS was determined using its molar extinction. Hydroxyl radical scavenging of extract was calculated relative to control.

$$\% \text{ Hydroxy radical scavenging} = \left[\left(\frac{\text{Sample}_{\text{Abs}}}{\text{Control}_{\text{Abs}}} \right) \right] \times 100$$

2.4.6 Superoxide anion radical scavenging assay

Superoxide anion radical scavenging was estimated according to the method of Nishikimi *et al.* (1972). About 1 ml of nitroblue tetrazolium (NBT) solution (156 µM prepared in 100 mM phosphate buffer, pH 7.4), 1 ml of NADH solution (468 µM prepared in 100 mM phosphate buffer pH 7.4) and test samples concentrations (100-500 µg/ml) were mixed and the reaction started by adding 100 µl of phenazine methosulphate (PMS) solution (60 µM) prepared in phosphate buffer (100 mM, pH 7.4). The reaction mixture was incubated at 25°C for 5 min and the absorbance at 560 nm was measured against the control samples. Quercetin was used as reference compound.

$$\% \text{ Superoxide radical scavenging} = \left[100 - \left(\frac{\text{Sample}_{\text{Abs}}}{\text{Control}_{\text{Abs}}} \right) \right] \times 100$$

2.4.7 Nitric oxide scavenging assay

The method of Garrat (1964) was adopted to determine the nitric oxide (NO) radical scavenging activity of aqueous extract of *T. foenum-graececum* L. Sodium nitroprusside in aqueous solution at physiological pH spontaneously generate nitric oxide which interacts with oxygen to produce nitrite ions determined by the use of Griess reagents. To 2 ml of 10 mM sodium nitroprusside dissolved in 0.5 ml phosphate buffer saline (pH 7.4) was mixed with 0.5 ml of plant extract at various concentrations (0.2 - 0.8 mg/ml). The mixture was incubated at 25°C. After 150 min, 0.5 ml of incubation solution was withdrawn and mixed with 0.5 ml of Griess reagent (1.0 ml sulfanilic acid reagents (0.33 % in 20 % glacial acetic acid) at room temperature for 5 min with 1 ml of naphthylethylenediamine dichloride (0.1 % w/v). The mixture was incubated at room temperature for 30 min. The absorbance was measured at 540 nm. The amount of nitric oxide radical was calculated following this equation:

$$\% \text{ Nitric oxide radical scavenging} = \left[1 - \left(\frac{\text{Sample}_{\text{Abs}}}{\text{Control}_{\text{Abs}}} \right) \right] \times 100$$

2.4.8 β -Carotene linoleate bleaching assay

The antioxidant activity of methanolic extracts of Fenugreek genotypes, was evaluated by the β -carotene method (Adegako *et al.*, 1998). β -Carotene (0.2 mg) in 0.2 ml of chloroform, linoleic acid (20 mg), and tween-40 (polyoxyethylenesorbitan monopalmitate) (200 mg) were mixed. Chloroform was removed at 40°C under reduced pressure (40 mm Hg), and the resulting mixture was diluted with 10 ml of water. After that, 40 ml of oxygenated water was added to the emulsion. Aliquots (4 ml) of the emulsion were transferred into different test tubes containing 0.2 ml solutions of Fenugreek extracts and BHA of different concentrations (50, 100, and 200 μ g). A control containing 0.2 ml of ethanol and 4 ml of the above emulsion was prepared. The tubes were placed in a water bath maintained at 50°C, and the absorbance was measured at 470 nm at zero time ($t = 0$). Measurement of absorbance was continued until the color of the β -carotene disappeared in the control tubes ($t = 180$ min) at intervals of 15 min. A mixture prepared as above

without β -carotene served as the blank. All determinations were carried out in triplicate. The antioxidant activity (AA) of the extracts was evaluated in terms of bleaching of the β -carotene using the following formula:

$$\text{Antioxidant activity} = (1 - (A_0 - A_t)(A_0^0 - A_t^0))$$

where, A_0 and A_0^0 are the absorbance values measured at zero time of the incubation for test sample and control, respectively, and A_t and A_t^0 are the absorbance values measured in the test sample and control, respectively, after incubation for 180 min.

3. Statistical analysis

Analysis of variance (ANOVA) and Duncan post hoc test were carried out on the values obtained in the experiment. Correlation analysis (bivariate) was also carried out to determine the relationship between free radical scavenging activity in different *in vitro* assay models and the non-enzymatic antioxidants (total phenol, flavonoid, total carotenoids, α -tocopherol and total ascorbate) present in different Fenugreek genotypes. The software IBM SPSS Statistics 20 (IBM Corporation) and SigmaPlot for Windows 11.0 (Systat Software, Inc.) were used to perform statistical analysis and graphing respectively. Results are expressed as mean \pm SE \bar{x} ($n = 3$). A statistical difference at $p \leq 0.05$ was considered to be significant.

4. Results

4.1 Phytochemical analysis

4.1.1 Total phenol content

Total phenol content was expressed as mg gallic acid equivalents g^{-1} extract. Highest phenol content was observed in RMT-303 followed by IC-143847, *i.e.*, 48.766 ± 0.042 and 47.847 ± 0.730 mg GAE g^{-1} extract (Table 1). Lowest phenol content was observed in Pusa early bunching, *i.e.*, 40.237 ± 0.480 mg GAE g^{-1} extract. No significant difference in phenol content was observed in Pant ragini, RME-1 and AM-316, *i.e.*, 42.346 ± 0.544 , 42.168 ± 0.258 and 41.930 ± 0.720 mg GAE g^{-1} extract, respectively. These genotypes differed significantly w.r.t. phenol content from other genotypes under investigation showing variable phenol content.

Table1: Phytochemical content in different Fenugreek (*Trigonella foenum graecum* .L) genotypes

Genotypes	Phenol content (mg gallic acid equivalents g^{-1} extract)	Flavonoid content (mg quercetin equivalents g^{-1} extract)	Tannin content (mg tannic acid equivalents g^{-1} extract)	Total carotenoid content (μ g β -carotene equivalents g^{-1} seed)	α -Tocopherol content (μ g α -tocopherol equivalents g^{-1} seed)	Total ascorbate content (μ g ascorbic acid equivalents g^{-1} seed)
AM-316	41.930 \pm 0.720 ^d	2.736 \pm 0.035 ^g	0.749 \pm 0.004 ^a	21.098 \pm 0.482 ^f	19.634 \pm 5.861 ^h	225.391 \pm 24.565 ^d
UM-222	47.286 \pm 0.352 ^b	3.952 \pm 0.070 ^b	0.391 \pm 0.001 ^{cd}	85.384 \pm 0.317 ^a	75.472 \pm 8.792 ^{ef}	290.318 \pm 4.016 ^c
UM-265	36.204 \pm 0.371 ^f	3.496 \pm 0.087 ^{cd}	0.362 \pm 0.003 ^e	32.417 \pm 4.377 ^{bc}	90.700 \pm 5.861 ^{de}	293.797 \pm 14.057 ^c
IC-143847	47.847 \pm 0.730 ^{ab}	3.800 \pm 0.157 ^{bc}	0.419 \pm 0.003 ^b	27.272 \pm 0.915 ^{de}	121.401 \pm 0.517 ^{bc}	68.472 \pm 0.670 ^f
IC-066843	44.128 \pm 0.808 ^c	3.679 \pm 0.087 ^{bc}	0.398 \pm 0.003 ^c	23.516 \pm 0.126 ^{ef}	105.928 \pm 8.792 ^{cd}	49.159 \pm 1.338 ^f
RMT-303	48.766 \pm 0.042 ^a	4.682 \pm 0.105 ^a	0.384 \pm 0.002 ^d	25.164 \pm 0.063 ^{ef}	171.918 \pm 11.722 ^a	359.884 \pm 30.886 ^b
Pusa Early Bunching	40.237 \pm 0.480 ^e	3.344 \pm 0.140 ^{de}	0.384 \pm 0.002 ^d	29.340 \pm 0.190 ^{cd}	146.538 \pm 20.514 ^{ab}	170.898 \pm 0.669 ^e
RMT-1	42.168 \pm 0.258 ^d	2.888 \pm 0.052 ^{fg}	0.402 \pm 0.003 ^c	26.373 \pm 0.634 ^{de}	55.167 \pm 8.792 ^{fg}	140.753 \pm 0.669 ^e
GM-2	41.048 \pm 0.193 ^{de}	2.979 \pm 0.170 ^{fg}	0.392 \pm 0.003 ^{cd}	16.263 \pm 0.507 ^g	90.700 \pm 1.439 ^{de}	234.666 \pm 8.032 ^d
Pant ragini	42.346 \pm 0.544 ^d	3.130 \pm 0.052 ^{ef}	0.360 \pm 0.003 ^e	34.175 \pm 0.190 ^b	34.862 \pm 2.930 ^{sh}	422.492 \pm 17.693 ^a

*The values with same superscript are not ($p \leq 0.05$) significantly different (Duncan Post Hoc Analysis)

4.1.2 Total flavonoid content

Total flavonoid content was expressed as mg quercetin equivalents g^{-1} extract. Highest flavonoid content was observed in RMt-303 and UM-222, *i.e.*, 4.682 ± 0.105 and 3.952 ± 0.070 mg QE g^{-1} extract, respectively. Lowest phenol content was observed in AM-316, *i.e.*, 2.736 ± 0.035 mg QE g^{-1} extract and it was 1.71 fold lower than that of RMt-303. No significant difference was observed in IC-143847 and IC-066843, *i.e.*, 3.800 ± 0.157 and 3.679 ± 0.087 mg QE g^{-1} extract. Similarly, the genotype GM-2 and RMt-1 showed no significant difference ($p \leq 0.05$) in flavonoids content, *i.e.*, 2.979 ± 0.170 and 2.888 ± 0.052 mg QE g^{-1} extract (Table 1). In general, there was a significant difference among the genotypes w.r.t flavonoids content.

4.1.3 Tannin content

Tannin content was expressed as mg tannic acid equivalents g^{-1} extract. Highest tannin content was observed in AM-316 and IC-143847, *i.e.*, 0.749 ± 0.009 and 0.419 ± 0.003 mg TAE g^{-1} extract (Table 1). Lowest tannin content was observed in DM-267 and Pant ragini, *i.e.*, 0.362 ± 0.003 and 0.360 ± 0.003 mg TAE g^{-1} extract. No significant difference ($p \leq 0.05$) was observed in RMT-1 and IC-066843, *i.e.*, 0.402 ± 0.003 and 0.398 ± 0.003 mg TAE g^{-1} extract. Similarly, no significant difference observed in between GM-2 and UM-222 (*i.e.*, 0.392 ± 0.003 and 0.391 ± 0.001 mg TAE g^{-1} extract), RMt-303 and Pusa early bunching (*i.e.*, 0.384 ± 0.002 mg TAE g^{-1} extract), UM-265 and Pant ragini (*i.e.*, 0.362 ± 0.003 and 0.360 ± 0.003 mg TAE g^{-1} extract).

4.1.4 Total carotenoids

Carotenoid content within different genotypes was expressed as mg β -carotene equivalents g^{-1} seeds. Highest carotenoid content was observed in UM-222, followed by Pant ragini, *i.e.*, 85.384 ± 0.317 and 34.175 ± 0.190 mg β -carotene equivalent g^{-1} seeds, respectively. The carotenoid content between the genotypes was varied by 2.49 folds. Similarly, GH-2 showed the lowest carotenoid content, *i.e.*, 16.263 ± 0.507 mg β -carotene equivalents g^{-1} seeds and it varied by 5.20 folds w.r.t UM-222. No significant difference in carotene content was observed in RMt-1 and IC-143847, *i.e.*, 26.373 ± 0.634 and 27.272 ± 0.915 mg β -carotene equivalents g^{-1} seeds, respectively. In general, the genotypes differed ($p \leq 0.05$) significantly w.r.t carotenoid content (Table 1).

4.1.5 Total α -tocopherol content

The α -tocopherol content was expressed as μg α -tocopherol equivalents g^{-1} seeds. Highest α -tocopherol content was observed in RMt-303 (*i.e.*, 171.918 ± 11.722 μg α -tocopherol equivalents g^{-1} seeds), followed by Pusa early bunching (*i.e.*, 146.538 ± 20.514 μg α -tocopherol equivalents g^{-1} seeds) and α -tocopherol varied by 1.17 folds between the two genotypes. Lowest α -tocopherol content was observed in AM-316 (*i.e.*, 19.634 ± 5.861 μg α -tocopherol equivalents g^{-1} seeds) and it varied by 8.75 folds w.r.t RMt-303. No significant difference ($p \leq 0.05$) in α -tocopherol content was observed between GM-2 and UM-265 (*i.e.*, 90.007 ± 1.438 and 90.700 ± 5.861 μg α -tocopherol equivalents g^{-1} seeds), respectively. In general, the genotypes differed significantly ($p \leq 0.05$) w.r.t α -tocopherol content (Table 1).

4.1.6 Ascorbate content

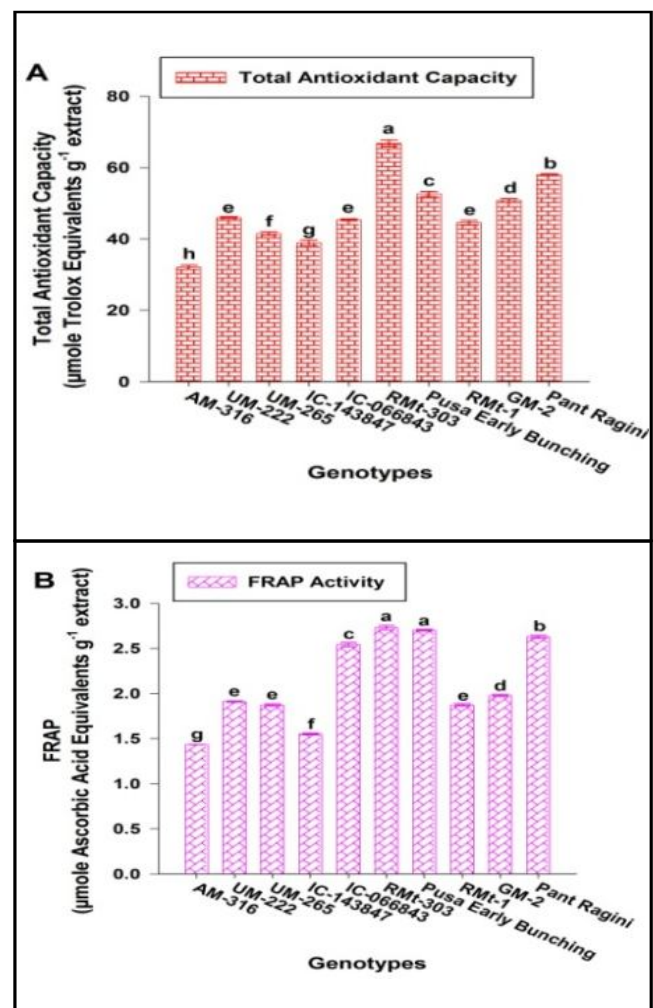
Total ascorbate content within the genotypes was expressed as μg ascorbic acid equivalents (AAE) g^{-1} seeds. Highest ascorbate content

was observed in Pant ragini, followed by RMt-303, *i.e.*, 422.942 ± 17.693 and 359.884 ± 30.886 μg AAE/g seed (Table 1). The genotypes varied in ascorbate content by 1.17 folds. Lowest ascorbate content was observed in IC-066843, *i.e.*, 49.159 ± 1.338 μg AAE/g seeds and it varied by 8.59 folds w.r.t Pant ragini. No significant difference ($p \leq 0.05$) in ascorbate content was observed between UM-222 and UM-267, *i.e.*, 290.318 ± 4.016 and 293.797 ± 14.057 μg AAE g^{-1} seeds. Similarly, there was no significant difference ($p \leq 0.05$) in ascorbate content between RMt-1 and Pusa early bunching. The genotypes IC-066843 and IC-143847 showed the lowest ascorbate and there was no significant difference ($p \leq 0.05$) between the genotypes w.r.t ascorbate content.

4.2 Antioxidant activity

4.2.1 Total antioxidant capacity

Total antioxidant activity was expressed as μ mole trolox equivalents g^{-1} extract. Highest antioxidant activity was observed in RMt-303 and Pant ragini, *i.e.*, 66.80 ± 0.95 and 38.00 ± 0.27 μ mole TE g^{-1} extract (Figure 1 (A)). It was 2.07 fold lower when compared with RMt-303. No significant difference ($p \leq 0.05$) in antioxidant activity was observed in UM-222, IC-066843 and RMt-1, *i.e.*, 46.04 ± 0.21 , 45.40 ± 0.25 and 44.55 ± 0.66 μ mole TE g^{-1} extract. Other genotypes differed significantly ($p \leq 0.05$) w.r.t antioxidant activity.



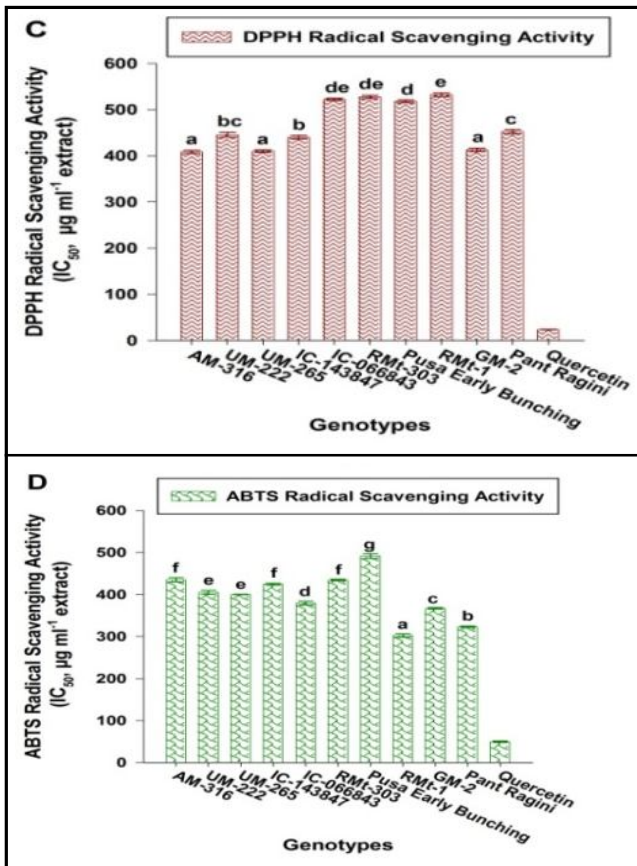


Figure 1: Antioxidant activities in different genotypes of *T. foenum graecum* L. (A) Total antioxidant activity, (B) FRAP, (C) DPPH radical scavenging activity, (D) ABTS radical scavenging activity, (*Note: The values with same superscript are not significantly ($p \leq 0.05$) different according to Duncan Post Hoc analysis.)

4.2.2 Ferric reducing antioxidant power assay

FRAP was used as a measure of antioxidant activity in methanolic extract of different genotypes. FRAP assay was expressed as Ascorbic acid equivalents/ g extract. Highest antioxidant activity was observed in RMt-303 and Pusa early bunching, *i.e.*, 2.730 ± 0.0236 and 2.704 ± 0.0078 $\mu\text{mole AAE g}^{-1}$ extract. No significant difference in their antioxidant activity was observed in RMt-303 and Pusa early bunching. Similarly, UM-222, UM-265 and RMt-1 showed no significant difference in their antioxidant activity, *i.e.*, 1.912 ± 0.0069 , 1.874 ± 0.0121 and 1.874 ± 0.0135 $\mu\text{mole AAE g}^{-1}$ extract (Figure 1 (B)).

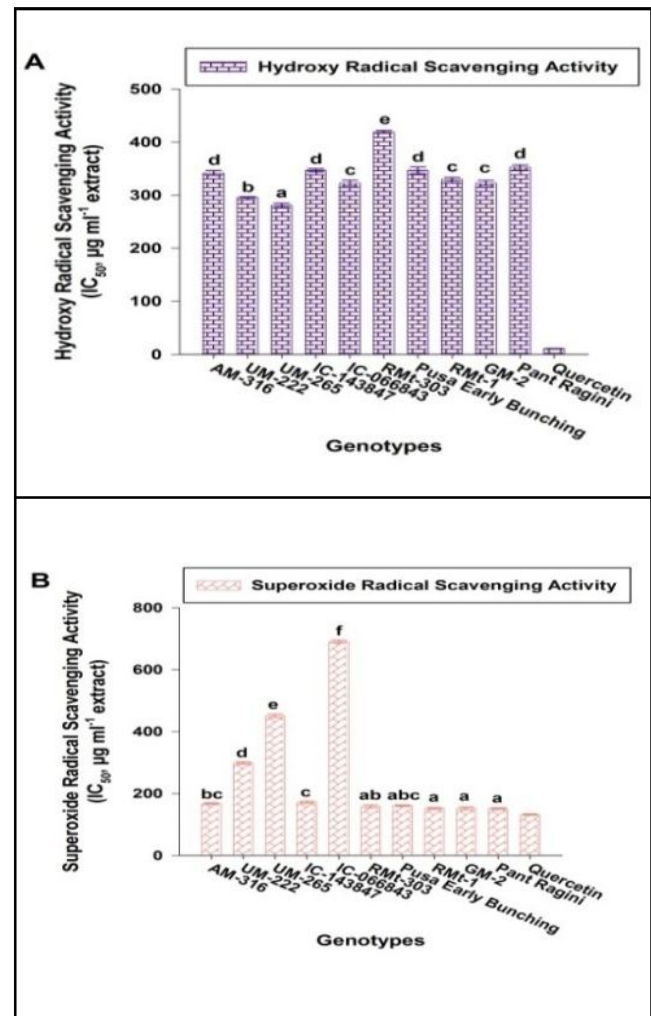
4.2.3 DPPH radical scavenging activity

DPPH radical scavenging activity was used as a measure of antioxidant activity in methanolic extract of different genotypes. The scavenging activity was expressed as IC₅₀ values of the extracts, *i.e.*, the inhibitory concentration which was able to scavenge 50% of DPPH radicals. Highest scavenging activity was observed in AM-316, UM-265 and GM-2, *i.e.*, 408.43 ± 3.12 , 410.14 ± 2.63 and 412.86 ± 3.74 $\mu\text{g ml}^{-1}$ extract (Figure 1 (C)). Lowest radical scavenging was observed in RMt-1, *i.e.*, 532.02 ± 4.17 $\mu\text{g ml}^{-1}$ extract. It was 1.3 folds lower in comparison to AM-316. No significant difference

($p \leq 0.05$) in DPPH radical scavenging (IC₅₀) was observed in AM-316, UM-265 and GM-2, respectively. Similarly, IC-066843 and RMt-303 showed no significant difference in DPPH-radical scavenging, *i.e.*, 522.30 ± 2.56 and 327.42 ± 3.46 $\mu\text{g ml}^{-1}$ extract, respectively. All genotypes differed significantly w.r.t positive control, *i.e.*, quercetin (IC₅₀: 23.70 ± 0.64 $\mu\text{g ml}^{-1}$).

4.2.4 ABTS radical scavenging assay

ABTS radical scavenging activity of the methanolic extracts was used as a measure of antioxidant activity of different genotypes. ABTS radical scavenging was expressed as IC₅₀ values of the extracts. Highest ABTS radical scavenging was observed in RMt-1, *i.e.*, 302.38 ± 4.17 $\mu\text{g ml}^{-1}$ extract. Pusa early bunching showed lowest ABTS radical scavenging, *i.e.*, 491.47 ± 5.96 $\mu\text{g ml}^{-1}$ extract and was 1.62 folds lower w.r.t RMt-1. No significant difference ($p \leq 0.05$) in ABTS radical scavenging was observed in UM-265 and UM-222, *i.e.*, 4.00 ± 0.50 and 404.94 ± 4.72 $\mu\text{g ml}^{-1}$ extract, respectively. Similarly, IC-143847, RMt-303 and AM-316 showed no significant difference in ABTS radical scavenging, *i.e.*, 424.44 ± 5.06 $\mu\text{g ml}^{-1}$ extract. All genotypes showed significantly, *i.e.*, 424.44 ± 2.32 , 434.42 ± 2.00 and 434.82 ± 5.06 $\mu\text{g ml}^{-1}$ extract (Figure 1 (D)). All genotypes showed significantly ABTS scavenging activity w.r.t Quercetin which was used as a positive control.



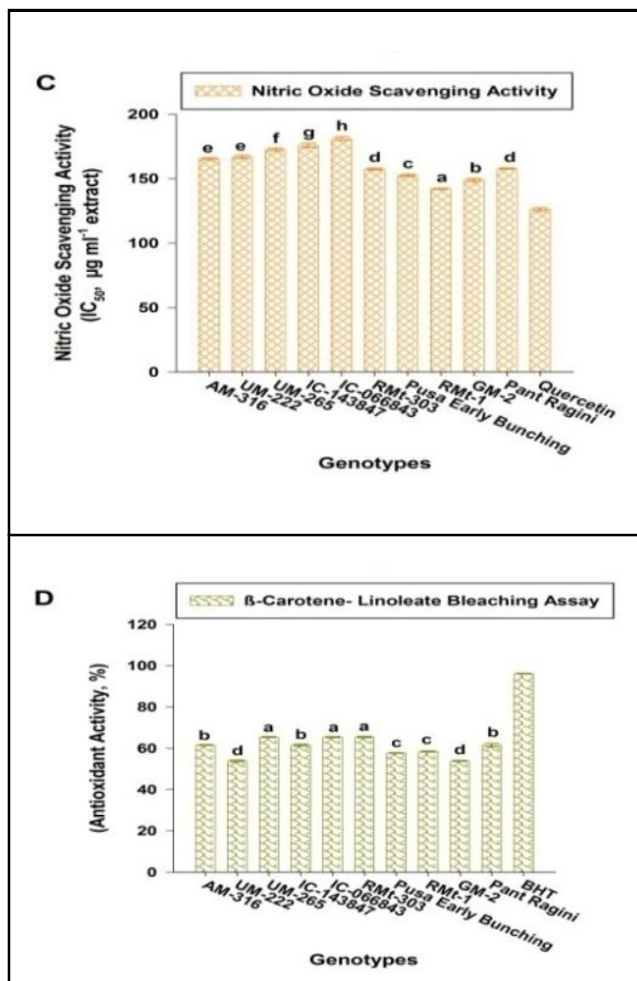


Figure 2: Antioxidant activities in different genotypes of *T. foenum graecum* L. (A) Hydroxy radical scavenging activity, (B) Superoxide radical scavenging activity, (C) Nitric oxide radical scavenging activity, (D) β -carotene linoleate bleaching assay. (*Note: The values with same superscript are not significantly ($p \leq 0.05$) different according to Duncan Post Hoc analysis.)

4.2.5 Hydroxy radical scavenging

Activity hydroxyl radical scavenging activity of the methanolic extract was measured as a measure of antioxidant activity. The scavenging of hydroxy radical by different extracts increased with concentration of the activity was observed in UM-265, *i.e.*, $281.53 \pm 3.38 \mu\text{g ml}^{-1}$ extract. RMt-303 showed lowest hydroxyl radical scavenging activity, *i.e.*, $419.28 \pm 2.60 \mu\text{g ml}^{-1}$ extract and was 1.48 fold lower than that of UM-265. No significant difference ($p \leq 0.05$) in hydroxyl radical scavenging was observed in GM-2, IC-066843 and RMt-1, *i.e.*, 322.73 ± 4.94 , 322.73 ± 5.34 and $329.65 \pm 3.63 \mu\text{g ml}^{-1}$ extract (Figure 2 (A)). Similarly, no significant difference ($p \leq 0.05$) in hydroxyl radical scavenging activity was observed in AM-316, IC-143847, Pusa early bunching and Pant ragini *i.e.*, 341.82 ± 4.48 , 346.98 ± 2.91 , 346.98 ± 6.27 and $352.23 \pm 4.89 \mu\text{g ml}^{-1}$ extract, respectively. In general, all genotypes showed significant difference in hydroxyl radical activity w.r.t quercetin, which was used as a positive control.

4.2.6 Superoxide radical scavenging activity

Superoxide radical scavenging of the methanolic extract of different genotypes was used as a measure of superoxide radical scavenging ability (antioxidant activity) of different extracts. The scavenging of superoxide radical increased with the concentration of the extract and was expressed as IC₅₀ value of the extracts as shown in Figure 2 (B). Highest superoxide radical scavenging activity was observed in Pant ragini, RMt-1 and GM-2, *i.e.*, 151.17 ± 2.68 , 152.04 ± 3.29 and $152.53 \pm 4.66 \mu\text{g ml}^{-1}$ extract, respectively. There was no significant difference ($p \leq 0.05$) in the superoxide radical scavenging activity of these three genotypes. Lowest radical scavenging activity was observed in IC-066843, *i.e.*, $690.85 \pm 4.44 \mu\text{g ml}^{-1}$ extract. It was 4.57 folds lower than that of Pant ragini. All other genotypes differed significantly w.r.t superoxide radical scavenging ability. The genotypes Pant ragini, RMt-1 and GM-2 which showed highest superoxide radical scavenging activity, only showed slightly lower scavenging activity when compared with quercetin (positive control) *i.e.*, 1.14, 1.15 and 1.15 fold lower super oxide radical scavenging activity was observed in these genotypes, respectively.

4.2.7 Nitric oxide scavenging activity

Nitric oxide is an important mediator of various physiological processes. The nitric oxide scavenging activity of the methanolic extracts of different genotypes was used as a measure of nitric oxide scavenging (antioxidant activity) present in different extracts. Highest nitric oxide scavenging activity was observed is RMt-1, *i.e.*, $142.16 \pm 0.32 \mu\text{g ml}^{-1}$ extract, whereas lowest activity was observed in IC-066843, *i.e.*, $181.06 \pm 1.49 \mu\text{g ml}^{-1}$ extract. It was 1.27 fold lower in comparison to RMt-1. No significant difference ($p \leq 0.05$) w.r.t nitric oxide scavenging activity, *i.e.*, 165.37 ± 0.86 and $166.86 \pm 1.27 \mu\text{g ml}^{-1}$ extract, respectively. In generally, all the genotypes showed significant nitric oxide scavenging w.r.t quercetin ($126.07 \pm 1.37 \mu\text{g ml}^{-1}$) which was used as positive control (Figure 2 C).

4.2.8 β -carotene bleaching activity

β -carotene-linoleate bleaching assay was used as a measure of antioxidant activity of methanolic extracts of different genotypes. The antioxidant activity of the extracts was expressed as percentage. BHT was used as a positive control and showed the highest antioxidant activity, *i.e.*, $96.15 \pm 0.21\%$. Highest antioxidant activity was observed in UM-265, IC-066843 and RMt-303, *i.e.*, 65.38 ± 0.23 , ($p \leq 0.05$) in antioxidant activity (Figure 2 D). Similarly, no significant difference in antioxidant activity (%) was observed in AM-316, IC-143947 and Pant ragini (*i.e.*, 61.53 ± 0.23 , 61.53 ± 0.46 and $61.53 \pm 0.81\%$, respectively). In Pusa early bunching and RMt-1 also, no significant difference in antioxidant activity was observed (*i.e.*, 57.69 ± 0.17 , 53.84 ± 0.28 and 53.84 ± 0.48 , respectively). There was no significant difference ($p \leq 0.05$) in the antioxidant activity among the respective genotypes, *i.e.*, GM-2 and UM-222, respectively. In general, all the genotypes showed significant antioxidant activity (%) which varied from 53.84 ± 0.24 to $65.38 \pm 0.26\%$.

5. Discussion

Plant phenolics are ubiquitous in all plants and, therefore, are an integral part of human diet. They are partially responsible for organoleptic properties of plant based food products (Dai and Mumber, 2010). The genotypes differed significantly w.r.t phenol

content. RMt-303 and UM-222 showed higher phenol content than other genotypes. Higher antioxidant activities in these genotypes may be attributed to higher phenol content. Dietary polyphenols have come to the attention of nutritionists and food manufacturers due to their potent antioxidant properties, their abundance in diet and their pharmacological effects in prevention of oxidative stress associated diseases (Manach *et al.*, 2004).

Flavonoids are one of the main groups of polyphenols and are responsible to exert wide range of physiological effects of plants based formulations flavonoids are known for their antioxidant properties which provide protection against oxidative stress, *i.e.*, they are able to scavenge free radicals and reactive oxygen species (Unno *et al.*, 2000). High amount of flavonoids in RMt-303 and UM-222 correlates with high phenol content and higher antioxidant activities in these genotypes. The protective role of Fenugreek polyphenols have been investigated by different workers (Pandian *et al.*, 2002; Thirunvukkarasu *et al.*, 2003) and their physiological effects have been proven beyond doubt. The genotypes with higher polyphenol content may well be advocated as potential functional food.

Tannins an important group of polyphenols, occur in a wide variety of plants and are the compounds responsible for their known biological activity, besides antioxidant properties and other physiological properties, *i.e.*, anti-allergic, anti-inflammatory, antimicrobial, cardioprotective and antithrombotic activities of different medicinal plants may be attributed to high tannin content in them (Balasundram *et al.*, 2006). Higher tannin content was observed in UM-316 and IC-143897 and other genotypes also showed significant tannin content. The variation in polyphenolic content including tannins in genotype under investigation may be due to factors such as plant genetics, growth conditions, maturity and post harvest conditions (Faller and Fialho, 2009). The profile and quantities of polyphenols and tannins in food are affected by processing due to highly reactive nature of these compounds, which may adversely affect the antioxidant value of corresponding foods (Dlamini *et al.*, 2009).

Fenugreek is a rich source of various vitamins like A, B1, B2, C and Niacin, *etc.* In recent years, greater emphasis has been given by the types and concentration of different carotenoids present in foods. Structural studies have shown that only few carotenoids possess vitamin A (Banerfeind, 1972). Many functional roles may be attributed to carotenoids and are essential components of animal diets including humans (Dellapenna and Pogson, 2006). The genotypes varied significantly w.r.t carotenoids content with highest concentration observed in UM-222 and Pant ragini. Fenugreek has been shown to possess high amount of β -carotene (Srinivasan, 2006). The variation in carotenoid content as shown in present investigation, thus provide a good basis for the use of carotenoid rich genotypes as potential functional foods and may form an important constituent of human diet.

α -tocopherol (Vit-E) is an important lipid soluble vitamin, inhibitor of many human diseases, *i.e.*, certain types of cancer, neurodegenerative and cardiovascular diseases are associated with deficiency of vitamin E (Dellapenna and Pogson, 2006). The protective effect of vitamin E against atherosclerosis, cardiovascular diseases, cataracts, and various type of cancers variation in α -tocopherol content. Highest amount of α -tocopherol content was observed in the genotype RMt-303 also, correlates to higher antioxidant activity

observed in the genotypes. Tocopherols are known to occur antioxidant activities. α -tocopherol being a major lipophilic antioxidant serves as an genotypes with high vitamin E (α -tocopherol) and polyphenol content, *viz.*, RMt-303 and Pusa early bunching could be used as potential functional foods with high antioxidant properties.

Vitamin C (Ascorbic acid) a water soluble vitamin has been known to affect cardiovascular function, iron utilization, immune cell and connective tissue development (Chen *et al.*, 2003). It also acts as an antioxidant and plays an important role in scavenging of reactive oxygen species (ROS) associated with various diseases. Genotypes such as Pant ragini and RMt-303 showed highest amount of this vitamin which correlates with the earlier reports of presence of vitamin C in Fenugreek (Leela and Shafeekh, 2008; Srinivasan, 2006). Human beings cannot synthesize vitamin C and, thus it must be derived from dietary sources. Fenugreek, being an important functional food and many of its properties may be attributed to different antioxidants, *viz.*, vitamin C and α -tocopherol and polyphenols. The genotypes under investigation showed marked variation in vitamin C. The significant difference is ascorbic acid content together with other antioxidants, may be responsible for variation in antioxidant activity shown by the genotypes.

Total antioxidant activity/capacity assay has been widely used to determine the antioxidant activity in plant extracts (Prieto *et al.*, 1990). The genotypes showed significant difference in antioxidant activity w.r.t total antioxidant activity assay. Similar results were also reported by Subhishine *et al.* (2011). Presence of higher antioxidant activities in Fenugreek seed extracts have also been reported by Priya *et al.* (2011). The total antioxidant capacity among the genotypes was positively correlated to flavonoid ($r = 0.35$), α -tocopherol ($r = 0.437$) and ascorbic acid content ($r = 0.485$) among the genotypes (Figure 3B and 5A and B). Thus, the antioxidant activity in a given plant species may be attributed to polyphenolic content (Luo *et al.*, 2004), ascorbic acid, α -tocopherol and β -carotene content, *etc.*, and therefore may serve as a valuable source of natural antioxidant.

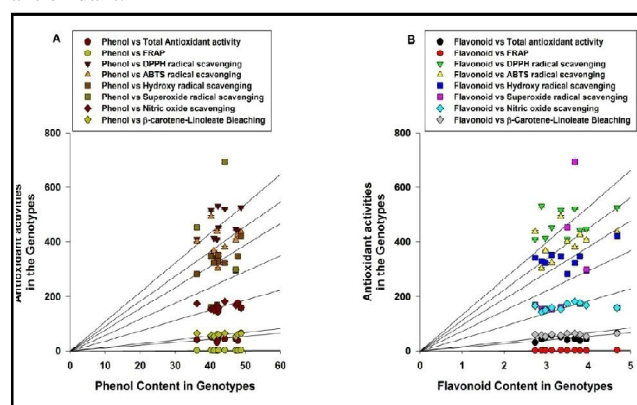


Figure 3: Correlation analysis of total phenol and flavonoids with antioxidant activities. (A) Correlation between phenol content with antioxidant activities, (B) Correlation between flavonoid content with antioxidant activities.

FRAP assay has been widely used for evaluating potential of polyphenols in various plant extracts (Luximan-Ramma *et al.*, 2002; Bhojar *et al.*, 2011). The genotypes showed significant difference

in antioxidant activity as measured by FRAP assay. The antioxidant activity (FRAP activity) showed a positive correlation w.r.t the flavonoid content ($r = 0.123$) among genotypes, ascorbic acid and α -tocopherol content also showed a positive correlation ($r = 0.057$ and 0.663), respectively with the antioxidant activity among the genotypes (Figure 3 B and 5 A and B). The presence of antioxidant activity in Fenugreek seeds have also been reported by other workers (Ravikumar and Anuradha, 1999; Kaviarasan *et al.*, 2007). The genotypic difference among different Fenugreek genotypes accounts for the difference in antioxidant activity as observed in the present investigation. The observed difference accounts for their efficiency as potential food.

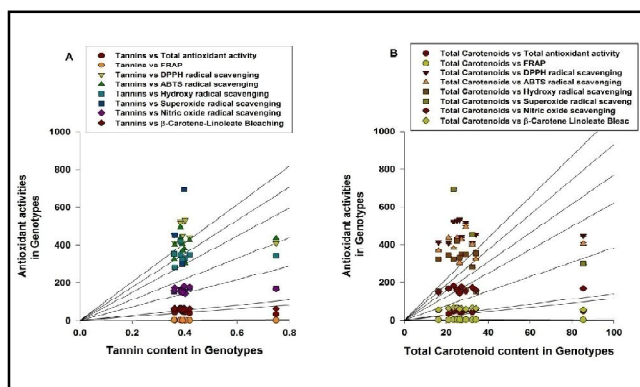


Figure 4: Correlation analysis of tannins and total carotenoids with antioxidant activities. (A) Correlation between tannin content with antioxidant activities, (B) Correlation between total carotenoids with antioxidant activities.

DPPH free radicals have been used for evaluation of antioxidant activities of different plant extracts. The free radical (DPPH radical) quenching ability of plant extract is used as a measure of their antioxidant activity (Leung *et al.*, 2006). The genotypes showed significant variation in DPPH radical scavenging activity. The results clearly support the earlier findings of high antioxidant activity in Fenugreek seed extracts (Subhasree *et al.*, 2009). DPPH radical scavenging among the genotypes showed a positive correlation with phenol content ($r = 0.537$), flavonoids ($r = 0.171$), α -tocopherol ($r = 0.151$) and ascorbic acid content ($r = 0.132$) in the genotypes (Figure 3 A and B and 5 A and B). The difference in antioxidant activity as shown in genotypes under investigation may be due to variation in phytochemical content among the genotypes (Blois, 1958). The genotypes with high phytochemical content and corresponding higher antioxidant activity may well be used as functional food, providing desired health benefits.

ABTS⁺ radical scavenging assay involves an electron transfer process and is rather more sensitive than DPPH radical scavenging and other antioxidant assays in determining the antioxidant activity of plant extracts. Due to solubility of ABTS⁺ in both aqueous and organic solvents, it can be used for determination of both hydrophobic and hydrophilic antioxidants in plant extracts (Prior *et al.*, 2005). The Fenugreek genotypes showed significant antioxidant properties (Subhasini *et al.*, 2011; Kaviarasan *et al.*, 2007). ABTS⁺ radical scavenging among the genotypes showed a positive correlation with phenol ($r = 0.353$), flavonoids ($r = 0.310$), total carotenoids ($r = 0.067$) and α -tocopherol ($r = 0.409$) content in the genotypes (Figure 3 A and B 4 B and 5 A). Thus, variation in the content of

these antioxidants may be responsible for difference in antioxidant properties observed in the present investigation.

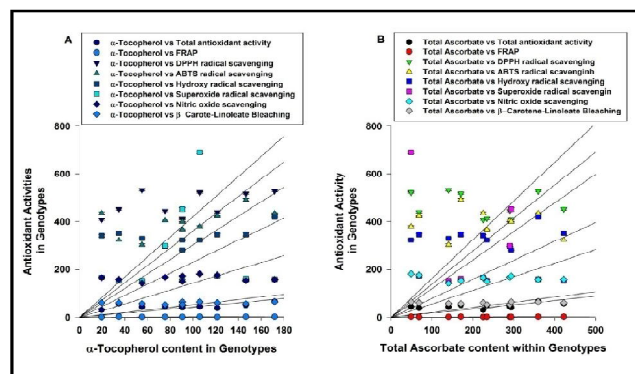


Figure 5: Correlation analysis of α -tocopherol and total ascorbate with antioxidant activities. (A) Correlation between α -tocopherol content with antioxidant activities, (B) Correlation between total ascorbate content with antioxidant activities.

Hydroxy radicals being highly electrophilic can release electrons from proteins and polyunsaturated fatty acids (Hollivell, 1996). Oxidation of these constituents of cell membrane leads to membrane damage and corresponding electrolyte leakage. Hydroxyl radical scavenging by Fenugreek genotypes was ascertained by measuring the inhibition of 2-deoxyribose degradation by hydroxyl radical. The Fenugreek genotypes showed significant hydroxyl radical scavenging activity. Presence of significant hydroxyl radical scavenging activity in Fenugreek has also been reported by Priya *et al.* (2011) and Kariarasan *et al.* (2007). Hydroxyl radical scavenging by different Fenugreek genotypes showed a positive correlation with flavonoids ($r = 0.304$), α -tocopherol ($r = 0.524$) and ascorbate ($r = 0.163$) content within the genotypes (Figure 3 B and 5 A and B). Significant correlation between hydroxyl radical scavenging and α -tocopherol content within Fenugreek genotypes clearly supports the earlier findings that α -tocopherol is a potent scavenger of hydroxyl radical (Thirunavakkarasu *et al.*, 2003). Thus, Fenugreek genotypes with high α -tocopherol and corresponding hydroxyl radical scavenging activity, *i.e.*, RMT-303 and Pusa early bunching can be utilized as potential functional food/neutraceutical.

Superoxide radicals cause oxidation of various cellular components (Steif, 2003). Superoxide radicals by itself are no strong oxidant but its ability to form H_2O_2 which serve as a substrate for generation of hydroxyl radicals and singlet oxygen (Halliwell, 1993) makes superoxide anion as one of the most damaging ROS, causing cellular oxidation and damage. The Fenugreek genotypes showed significant superoxide radical scavenging activity which was at par with the corresponding positive control (quercetin). Fenugreek seeds are rich source of polyphenols especially flavonoids and antioxidant properties of some flavonoids are due to their ability to scavenge superoxide radicals. The inhibition of superoxide radical formation provides valuable insights about free radical scavenging potential of Fenugreek genotypes. Superoxide radical scavenging by different genotypes showed a positive correlation with total carotenoids ($r = 0.086$) and α -tocopherol ($r = 0.165$) content (Figure 4 B and 5 A) within the genotypes. Thus, apart from polyphenols, carotenoids and α -tocopherol also serve as effective scavengers of superoxide

radical and may be responsible for high superoxide radical scavenging activity present in the fenugreek genotypes under investigation.

Nitric oxide is an important mediator of many physiological processes, *viz.*, smooth muscle relaxation, inhibition of platelet aggregation, nerve signalling. It also regulates cell mediated toxicity apart from having antitumor and antimicrobial effects (Hagerman *et al.*, 1998). The Fenugreek genotypes showed significant nitric oxide scavenging activity which was at par with quercetin (positive control). The flavonoid content within the genotypes under investigation showed a positive correlation ($r = 0.378$) with nitric oxide scavenging activity. Similarly, total carotenoid content among the genotypes also showed a positive correlation ($r = 0.220$) with NO scavenging activity of the genotypes (Figure 3 B and 4 B). A number of phenolic compounds (Kawada *et al.*, 1998) and α -tocopherol (Arroyo *et al.*, 1992) have known to inhibit the harmful effect of reactive nitrogen species, *i.e.*, nitric oxide, peroxynitrite (ONOO⁻) (Pannala *et al.*, 1998). Fenugreek seeds are rich source of their natural antioxidants, *i.e.*, flavonoids and carotenoids and presence of these compounds in Fenugreek may effectively prevent reactive nitrogen species (RNS) mediated injuries (Kim *et al.*, 1998).

The inhibition of linoleic acid oxidation in β -carotene-linoleate bleaching assay is a useful model to test antioxidant activity of plant extracts. The model mimics lipid peroxidation in biological membranes (Ferreira *et al.*, 2004). In multiphase systems, localization of antioxidants depends on their solubility as well as polarity. The natural antioxidants exhibiting apolar properties are indispensable because they are localized in the lipid water interface, thereby preventing the formation of lipid radicals and preventing membrane damage. The Fenugreek genotypes showed significant antioxidant activity under β -carotene-linoleate bleaching model. The antioxidant activity of Fenugreek genotypes showed positive correlation with flavonoids ($r = 0.230$) and α -tocopherol ($r = 0.072$) content (Figure 3 B and 5 A) in the genotypes. These having apolar characteristics may thus be effective in preventing formation of lipid peroxide and corresponding membrane damage. Due to significant amount of apolar antioxidants (*i.e.*, flavonoids, α -tocopherol and carotenoids), Fenugreek genotypes with high amount of these compounds (RMt-303) may be potential functional foods. Fenugreek seeds are rich source of flavonoids which have ability to decolorize and stabilize unpaired electrons (Rice-Evans *et al.*, 1997) and in turn help in reducing oxidized intermediates of lipid peroxidation, so they may act as efficient primary and secondary antioxidants (Yen and Chen, 1995). All Fenugreek peroxidation as seen by their antioxidant activity and the genotypes with high antioxidant activity (*i.e.*, RMt-303, IC-143847 and Pant ragini) may, thus be used as reliable functional food.

6. Conclusion

The results of the present investigation clearly showed, that the genotypes have a significant difference w.r.t various phytochemical content and their respective antioxidant activities. The genotypes with high phytochemicals, *viz.*, RMt-303, UM-222, Pant ragini, *etc.* may be used as potential functional foods. The phytochemicals content also showed positive correlation with different antioxidant activity assays. High level of polyphenols in RMt-303 and other genotypes is associated with higher antioxidant activities as shown in total antioxidant capacity assay, FRAP, superoxide radical scavenging and β -carotene-linoleate bleaching assay. Similarly, high

amount of ascorbic acid and total carotenoids in Pant Ragini may well be correlated with high antioxidant activity in the genotypes as shown by total antioxidant capacity assay. FRAP, ABTS radical scavenging, superoxide radical scavenging and β -carotene-linoleate bleaching assay, respectively: α -tocopherol content among the genotype RMt-303 and Pusa early bunching also correlates for higher antioxidant activity in these genotypes as shown by FRAP and superoxide radical scavenging assays. The present investigation may, thus be helpful in understanding the antioxidant properties associated with Fenugreek. It also clearly reveals that the genotypes differs significantly w.r.t their antioxidant properties and the respective properties depends upon variation in phytochemicals within the genotypes. The genotypes with high phytochemical content antioxidant activity may well be utilized as potential functional food.

Acknowledgements

The financial support provided by Council of Scientific and Industrial Research (CSIR) and the infrastructural grant provided by DST (Department of Science and Technology, Govt. of India) is duly acknowledged.

Conflict of interest

We declare that we have no conflict of interest.

References

- Adegako, G. O.; Vijay, Kumar. M.; Gopal Krishna, A. G.; Vardaraj, M. C.; Sambaiah, K., and Lokesh, B. R. (1998). Antioxidants and lipid oxidation in foods. *Journal of Food Science and Technology*, **35**:283-298.
- Arroyo, P.L.; Hatch-pigott, V.; Mower, H. F. and Cooney, R.V. (1992). Mutagenicity by nitric oxide and its inhibition by antioxidants. *Mutation Research*, **281**:193-202.
- Backer, H.; Frank, O.; Angells, B.D. and Feingold, S. (1980). Plasma tocopherol in man at various times after ingesting free or acetylated tocopherol. *Nutr. Rep. Int.*, **21**:531-536.
- Balasundram, N.; Sundram, K. and Samman, S. (2006). "Phenolic compounds in plant and agri-industrial by-products antioxidant activity occurrence and potential uses", *Food Chemistry*, **99**(1):191-203.
- Balmani, M.; Shirzad, H. and Mirhosseini, M. (2015). A review on ethanobotanical and therapeutic uses of fenugreek (*Tigonella foenum-graceum* L). *Journal of Evidence Based Complementary and Alternative Medicine*, **21**(1):53-62.
- Bauernfeind, J. C. (1972). Carotenoid vitamin A precursors and analogs in foods and feeds. *J. Agric. Food Chem.*, **20**(3):456-473.
- Benzie, I.E.F. and Strain, J.J. (1996). The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": The FRAP assay. *Anal. Biochem.*, **239**:70-76.
- Bhojar, M.; Mishra, G.; Naik, P. and Srivastava, R. (2011). Estimation of antioxidant activity and total phenolics among natural populations of Caper (*Capparis spinosa*) leaves collected from cold arid desert of trans-Himalayas. *Aust. J. Crop Sci.*, **5**(7):912-919.
- Biglari, F.; Alkarkhi Abbas, F. M. and Easa, A. M. (2008). Antioxidant activity and phenolic content of various date palm (*Phoenix dactylifera*) Fruits from Iran. *Food Chemistry*, **107**:1636-1641.
- Blois, M. S. (1958). Antioxidant determination by the use of stable free radical. *Nature*, **181**:1199-1200.
- Braca, A.; Tommasi, N.D.; Bari, L.D.; Pizza, C.; Politi, M. and Morelli, I. (2001). Natural anti-oxidants from plant material in phenolic compounds in food and their effects on health. *J. Nat. Prod.* **64**: 892-895.

- Chen, Z.; Young, T.; Ling, J.; Chang, Su-Chih. and Gallie, D. (2003). Increasing vitamin C content of plants through enhanced ascorbate recycling. Proceedings of the National Academy of Sciences, USA., **100**:3525-3530.
- Dai, J. and Mumper, R. (2010). Plant phenolics: extraction, analysis and their antioxidant and anticancer properties. *Molecules*, **15**:7313-7352.
- Decker, E.A. and Welch, B. (1990). Role of ferritin as a lipid oxidation catalyst in muscle food. *J. Agric. Food Chem.*, **38**:674-677.
- Dellapenna, D. and Pogson, B.J. (2006). Vitamin synthesis in plants: Tocopherols and carotenoids. *Annual Review of Plant Biology*, **57**:711-738.
- Dlamini, N.R.; Taylor, J.R. N. and Rooney, L. W. (2007). The effect of sorghum type and processing on the antioxidant properties of African sorghum-based foods. *Food Chemistry*, **105**:1412-1419.
- Faller, A. L. K. and Fialho, E. (2009). The antioxidant capacity and polyphenol content of organic and conventional retail vegetables after domestic cooking. *Food Research International*, **42**:210-215.
- Farzaneh, V. and Carvalho, I. S. (2015). A review of the health benefit potentials of herbal plant infusions and their mechanism of actions. *Industrial Crops and Products*, **65**:247-258.
- Ferreira, K.N.; Iverson, T. M.; Maghlaoui, K.; Barber, J. and Iwata, S. (2004). Architecture of the photosynthetic oxygen-evolving center. *Science*, **303**:1831-1838.
- Garrat, D.C. (1964). *The Quantitative analysis of Drugs*. Chapman and Hall ltd. Japan, 3:456-458.
- Hagerman, A.E.; Riedl, K.M.; Jones, G.A.; Sovik, K.N.; Ritchard, N.T. and Hartzfeld, P.W. (1998). High molecular weight plant polyphenolics (tannins) as biological antioxidants. *J. Agric. and Food Chem.*, **46**:1887-1892.
- Halliwell, B. (1993). The chemistry of free radicals. *Toxicology and Industrial Health*, **9**:1-21.
- Halliwell, B. (1996). Mechanisms involved in the generation of free radicals. *Pathol. Biol.*, **44**:6-13
- Halliwell, B.; Murcia, M.A.; Chirico, S. and Aruoma, O.I. (1995). Free radicals and antioxidants in food and *in vivo*: What they do and how they work. *Crit. Rev. Food Sci. Nutr.*, **35**:7-20.
- Hannan, J.M.A.; Rokeya, B.; Faruque, O.; Nahar, N.; Moshuazzaman, M.; Azad Khan A. K. and Ali, L. (2003). Effect of soluble dietary fibre fraction of *Trigonella foenum-graecum* regation status of type 2 diabetic model rats. *J. Ethnopharmacol.*, **88**:73-77.
- He, Y.; Ding, C.; Wang, X.; Wang, H. and Suo, Y. (2015). Using response surface methodology to optimize countercurrent chromatographic separation of polyphenol compounds from fenugreek (*Trigonella foenum-graecum* L.). *Seeds. J. Liq. Chromatogr. R.T.*, **38**(1):29-35.
- Jensen, A. (1978). Chlorophylls and carotenoids: In J. A. Hellebust and J. S. Carraig (Eds.), *Phycological methods: Physiological and biochemical methods*. Cambridge: Cambridge University Press, pp:59-70.
- Kaviarasan, S.; Naik, G. H.; Gangabhagirathi, R.; Anuradha, C.V. and Priyadarsini, K. I. (2007). *In vitro* studies on antiradical and antioxidant activities of fenugreek (*Trigonella foenum-graecum*) seeds. *Food Chem.*, **103**:31-37.
- Kawabata, T.; Cui, M. Y.; Hasegawa, T.; Takano, F. and Ohta, T. (2011). Anti-inflammatory and anti-melanogenic steroidal saponin glycosides from Fenugreek (*Trigonella foenum-graecum* L.) seeds. *Planta Med.*, **77**:705-710.
- Kawada, N. Seki, S. and Kuroki, T. (1998). Effect of Antioxidants resveratrol, quercetin and N-acetyl cystein, on the functions of cultured rat hepatic stellate cells and kupfer cells. *Hepatology.*, **27**:1265-1274.
- Kim, D.O.; Jeong, S.W. and Lee C.Y. (2003). Antioxidant capacity of phenolic phytochemicals from various cultivars of plums. *Food Chemistry*, **81**:321-326.
- Kim, O.K.; Murakami, A.; Nakamura, Y. and Oihigashi, H. (1998). Screening of edible Japanese plants for nitric oxide generation inhibitory activities in RAW 264.7 cells. *Cancer Letter.*, **125**:199-207.
- Law, M.Y, Charles, S.A. and Halliwell, B. (1983). Glutathione and ascorbic acid in spinach (*Spinacea oleraceu*) chloroplasts. The effect of hydrogen peroxide and of paraquat. *Biochem. J.*, **210**:899-903.
- Leela, N and Shafeekh K. (2008). CAB International; Pondicherry, India: Fenugreek, Chemistry of Spices.
- Leung, M.Y.K.; Liu, C.; Koon, J.C.M. and Fung, K.P. (2006). Polysaccharide biological response modifiers. *Immunol. Lett.*, **105**(2):101-114.
- Liu, J.; Edamatsu, R.; Kabuto, H. and Mori, A. (1990). Antioxidant action of Guilingji in the brain of rats with FeCl₃ induced epilepsy. *Free Rad. Biol. Med.*, **9**:451-454.
- Luo, Q.; Cai, Y.; Yan, J.; Sun, M. and Corke, H. (2004). Hypoglycemic and hypolipidemic effects and antioxidant activity of fruit extracts from *Lycium barbarum*. *Life Sciences.*, **76**:137-149.
- Lust, J.B. (1986). *The Herb Book*, Bantam Books Inc., New York.
- Luximon-Ramma, A.; Bahorun, T.; Crozier, A.; Zbarsky, V.; Datla, K.K.; Dexter, D.T. and Aruoma, O.I. (2005). Characterization of the antioxidant functions of flavonoids and proanthocyanidins in Mauritian black teas. *Food Res. Int.*, **38**:357-367.
- Manach, C.; Scalbert, A.; Morand, C.; Remesy, C. and Jimenez, L. (2004). Polyphenols: Food sources and bioavailability. *Am. J. Clin. Nutr.*, **79**:727-747.
- Mandegarya, A.; Pournamdari, M.; Shariûfar, F.; Pournourmohammadi, S.; Fardiar, R. and Shooli, S. (2012). Alkaloid and flavonoid rich fractions of fenugreek seeds (*Trigonella foenum-graecum* L.) with antinociceptive and anti-inflammatory effects. *Food and Chemical Toxicology*, **50**:2503-2507.
- Mensor, L. I.; Menezes, F. S.; Leitao, G. G.; Reis, A. S.; dos Santos, T.; Coube, C. S. and Leitao, S. G. (2001). Screening of Brazilian plant extracts for antioxidant activity by the use of DPPH free radical method. *Phytotherapy Research*, **15**:127-130.
- Niciforovic, N.; Mihailovic, V.; Maskovic, P.; Solujic, S.; Stojkovic, A. and Pavlovic, M. D. (2010). Antioxidant activity of selected plant species; potential new sources of natural antioxidants. *Food and Chemical Toxicology*, **48**:3125-3130.
- Nishikimi, M.; Appaji, N. and Yagi, K. (1972). The occurrence of superoxide anion in the reaction of reduced phenazine methosulfate and molecular oxygen. *Biochem. Biophys. Res. Communication*, **46**:849-854.
- Ohkawa, H.; Ohishi, N. and Yagi, K. (1979). Assay of lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal. Biochem.*, **95**:351-358.
- Pandian, R.S.; Anuradha, C.V. and Viswanathan, P. (2002). Gastroprotective effect of fenugreek seeds (*Trigonella foenum-graecum*) on experimental gastric ulcer in rats. *J. Ethnopharmacol.*, **81**:393-397.
- Pannala, A.S.; Raza, Q.R.; Halliwell, B.; Singh, H.S. and Rice-Evans, C.A. (1998). Inhibition of peroxynitrite dependent tyrosine nitration by hydroxyl cunamates, nitration or electron donation. *Free Radical Biol. Med.*, **24**:594-606.
- Preito, P.; Pineda, M. and Aguliar, M. (1999). Spectrophotometric quantitation of antioxidant capacity through the formation of a phosphomolybdenum complex: Specific application to the determination of vitamin E. *Annals of Biochemistry*, **269**:337-341.

- Prior, R.L.; Wu, X. and Schaich, K. (2005).** Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. *Journal of Agriculture and Food Chemistry*, **53**:4290-4302.
- Priya, V.; Jananie, R. and Vijayalakshmi, K. (2011).** GC/MS determination of bioactive components of *Trigonella foenum-graecum*. *J. Chem. Pharm. Res.*, **5**:35-40.
- Rani, V.; Deep, G.; Singh, R. K.; Palle, K. and Yadav, C. S. (2016).** Oxidative stress and metabolic disorders: Pathogenesis and therapeutic strategies. *Life Sciences*, **144**:183-193.
- Ravikumar, P. and Anuradha, C. V. (1999).** Effect of fenugreek seeds on blood lipid peroxidation and antioxidants in diabetic rats. *Phytotherapy Research*, **13**(3):197-201.
- Re, R.; Pellegrini, N.; Proteggente, A.; Pannala, A.; Yang, M. and Rice-Evans, C. (1999).** Antioxidant activity applying improved ABTS radical cation decolorization assay. *Free Radical Biology and Medicine*, **26**(9-10):1231-1237.
- Rice-Evans, C. A.; Miller N. J. and Paganga, G. (1997).** Antioxidant properties of phenolic compounds. *Trends Plant Sci.*, **2**:152-159.
- Srinivasan, K. (2006).** Fenugreek (*Trigonella foenum-graecum*): A review of health beneficial physiological effects. *Food Reviews International*, **22**:203-224.
- Stief, T.W. (2003).** The physiology and pharmacology of singlet oxygen. *Med. Hypotheses*, **60**:567-572.
- Subhashini, N., G. Nagarajan and S. Kavimani, (2011).** Anti-inflammatory and *in vitro* antioxidant property of *Trigonella foenum-graecum* seeds. *J. Pharmacol. Toxicol.*, **6**:371-380.
- Subhasree, B.; Baskar, R.; Keerthana, R.L.; Susan, R.L. and Rajasekaran, P. (2009).** Evaluation of antioxidant potential in selected green leafy vegetables. *Food Chem.*, **115**:1213-1220.
- Swain, T. and Hillis, W. E. (1959).** The phenolic constituents of *Prunus domestica*. I. The quantitative analysis of phenolic constituents. *J. Sci. Food. Agric.*, **10**:63-68.
- Tanou, G.; Molassiotis, A. and Diamantidis, G. (2009).** Introduction of reactive oxygen species and necrotic death like destruction in strawberry leaves by salinity. *Environmental and Experimental Botany*, **66**(2-3):270-281.
- Thirunavukkarasu, V. Anuradha, C.V. and Viswanathan, P. (2003).** Protective effect of fenugreek (*Trigonella foenum-graecum*) seeds in experimental ethanol toxicity. *Phytotherapy Research*, **17**:737-743.
- Traka, M.H. and Mithen, R.F. (2011).** Plant Science and Human Nutrition: Challenges in Assessing Health-Promoting Properties of Phytochemicals. *The Plant Cell*, **23**:2483-2497.
- Unno, K.; Asakura, H. and Shibuya, Y. (2000).** Increase in basal level of Hsp70, consisting chiefly of constitutively expressed Hsp70 (Hsc70) in aged rat brain. *J. Gerontol. A Biol. Sci. Med. Sci.*, **55**(7):329-335.
- Vyas, S.; Agrawal, R.P.; Solanki, P. and Trivedi, P. (2008).** Analgesic and anti-inflammatory activities of *Trigonella foenum-graecum* (seed) extract. *Acta. Pol. Pharm.*, **65**(4):473-6.
- Wani, S.A. and Kumar, P. (2016).** Fenugreek: A review on its nutraceutical properties and utilization in various food products. *Journal of the Saudi Society of Agricultural Sciences*, 10.1016/j.jssas.2016.01.007
- Wong, C.C.; Li, H.B.; Cheng, K.W. and Chen, F. (2006).** A systematic survey of antioxidant activity of 30 Chinese medicinal plants using the ferric reducing antioxidant power assay. *Food Chemistry*, **97**:705-711.
- Yeng, C. and Chen, Y. (1995).** Antioxidant activity of various tea extracts in relation to their antimutagenicity. *J. Agr. Food Chem.*, **43**:27-32.
-