

## Finger Print Analysis by HPTLC, Isolation, Evaluation of its *In-vitro* Antioxidant Activity and Spectroscopic Characterization of Bioactive Compounds of *Trigonella foenum-graecum* Seeds

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### ABSTRACT

The present study was aimed at post chromatographic identification, isolation, antioxidant activity and characterization of bioactive constituents obtained from *Trigonella foenum-graecum* seeds. HPTLC fingerprinting and *in-vitro* antioxidant activity was carried out for four isolated fractions given the trivial name fraction 1 (TF1), fraction 2 (TF2), fraction 3 (TF3) and fraction 4 (TF4) of methanolic seed extract of *T. foenum-graecum* (METFG). From the HPTLC fingerprint the fluorescent band (under 300 nm) at Rf: 0.66 and 0.69 (mobile phase n-butanol: acetic acid: water, v/v/v) was found in the fractions TF3 and TF4 of METFG. TF4 showed more effective antioxidant activity than TF3 as compared to other fractions TF1 and TF2 w.r.t. the standard substance used being ascorbic acid in all three models viz DPPH radical scavenging, nitric oxide radical scavenging and hydrogen peroxide scavenging assay may be due to the presence of more percentage of bioactive compounds TF-k1 (46.38%) in TF3 and TF-k2 (60.27%) in TF4. The GC-MS, <sup>1</sup>H-NMR and <sup>13</sup>C-NMR analysis of TF3 and TF4 revealed the presence of TF-k1 and TF-k2 as the major component in respectively. The present study indicated that *T. foenum-graecum* and its isolated bioactive compounds be a very useful antioxidant and may form the clinical agents with better efficacy.

**Keywords:** *T. foenum-graecum*, Column chromatography, HPTLC fingerprinting, Gas chromatography-Mass spectroscopy, Nuclear magnetic resonance spectroscopy.

### INTRODUCTION

The study of botanicals in the ayurvedic materia medica has been proven to be relatively safe and effective, from times immemorial. *T. foenum-graecum* L. (Leguminosae) has a record of safety and efficacy, spanning several centuries<sup>1</sup>. Traditional medicine preparations were made by traditional healers with herbal and non-herbal ingredients to act on various diseases which had different mechanism of actions. Nowadays phytoconstituents are isolated from the different species of plants which are having a probability to reduce diseases<sup>2</sup>. It is very difficult to identify marker compounds for all traditional medicines, because some medicines have unknown active constituents and others have multiple active constituents. The extraction methods used to process the herbal plants can affect the quantities of biologically active compounds in the extract. Thus, the quality control of the bioactive compounds in the herbal extract is of great importance in medicinals<sup>3</sup>. Herbal drugs are used to treat different diseases because of effectiveness, lesser side effects and relatively low cost<sup>4</sup>. Therefore, it is important to isolate the active constituent from traditional used antidiabetic plants; *T. foenum-graecum* is used as a spice and condiment in India and other tropical countries<sup>5</sup>. It has been reported to have carminative, gastric stimulant, antidiabetic and galactagogue (lactation-inducer) effects, hypocholesterolemic,

antioxidant, hepatoprotective, anti-inflammatory, antibacterial, antifungal, antiulcer and other miscellaneous<sup>6-8</sup>. Current research is now directed towards to isolate naturally occurring antioxidants from plant parts and to investigate remedies, researchers are keen interested to research in new natural antioxidants<sup>9-10</sup>. Hence the present study is aimed to identify, isolate compound in *T. foenum-graecum* along with evaluation of the antioxidant activity of the fractions.

### MATERIALS AND METHODS

#### Apparatus

Analytical thin-layer chromatography (TLC) was performed on Kieselgel 60F 254 (Merck, Darmstadt, Germany) glass plates. Column chromatography was carried out silica gel (60-120 mesh, Merck, Darmstadt, Germany). NMR spectra were recorded on Bruker DRX 400 NMR spectrometers with methanol (MeOD) as internal standard. GC-MS spectra were recorded on a model GC-MS-QP-2010- Plus1, Shimadzu and elemental analysis (CHNO) on (Elementar, Vario EL III).

#### Chemicals

1, 1-diphenyl-2-picryl-hydrazyl (DPPH) and ascorbic acid were obtained from Sigma Aldrich, USA. All other chemicals used were of analytical grade and obtained from Merck USA.

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Table 1: Percentage inhibition of fractions of METFG by DPPH assay.

S.no.	Concentration of solution ( $\mu\text{g/ml}$ )	DPPH free radical scavenging activity				
		Ascorbic acid (AA)	TF1	TF2	TF3	TF4
1	25	13.21 $\pm$ 1.4	03.26 $\pm$ 3.3	04.76 $\pm$ 2.4	08.03 $\pm$ 2.2	11.02 $\pm$ 5.2
2	50	19.27 $\pm$ 2.3	07.40 $\pm$ 2.4	09.29 $\pm$ 3.0	13.93 $\pm$ 1.5	22.75 $\pm$ 2.8
3	75	35.37 $\pm$ 4.7	11.98 $\pm$ 1.3	12.07 $\pm$ 4.5	20.05 $\pm$ 3.2	38.62 $\pm$ 3.2
4	100	47.82 $\pm$ 3.5	18.87 $\pm$ 4.6	17.85 $\pm$ 5.2	31.84 $\pm$ 5.3	46.51 $\pm$ 7.5
5	125	58.25 $\pm$ 2.7	23.00 $\pm$ 2.5	21.99 $\pm$ 4.9	40.07 $\pm$ 6.3	51.94 $\pm$ 5.6

Values are expressed as Mean  $\pm$  Standard deviation.

Table 2: Percentage inhibition of fractions of METFG by nitric oxide radical scavenging assay.

S.no.	Concentration of solution ( $\mu\text{g/ml}$ )	Nitric oxide radical scavenging activity				
		Ascorbic acid (AA)	TF1	TF2	TF3	TF4
1	25	16.92 $\pm$ 1.4	04.10 $\pm$ 2.1	02.82 $\pm$ 1.6	14.38 $\pm$ 1.6	12.95 $\pm$ 2.7
2	50	21.03 $\pm$ 4.2	08.39 $\pm$ 1.6	05.37 $\pm$ 1.5	19.26 $\pm$ 1.4	23.73 $\pm$ 4.6
3	75	38.29 $\pm$ 2.6	13.37 $\pm$ 1.7	11.29 $\pm$ 3.2	29.62 $\pm$ 2.6	35.32 $\pm$ 6.8
4	100	51.93 $\pm$ 3.8	16.28 $\pm$ 2.0	19.82 $\pm$ 2.8	41.58 $\pm$ 3.8	43.82 $\pm$ 8.3
5	125	62.21 $\pm$ 4.9	26.17 $\pm$ 3.1	27.27 $\pm$ 2.5	52.38 $\pm$ 2.7	58.36 $\pm$ 4.2

Values are expressed as Mean  $\pm$  Standard deviation.

Table 3: Percentage inhibition of fractions of METFG by nitric oxide radical scavenging method.

S.no.	Concentration of solution ( $\mu\text{g/ml}$ )	Hydrogen peroxide scavenging activity				
		Ascorbic acid (AA)	TF1	TF2	TF3	TF4
1	25	16.92 $\pm$ 2.4	9.45 $\pm$ 1.8	9.76 $\pm$ 1.1	11.00 $\pm$ 1.1	15.92 $\pm$ 1.4
2	50	21.03 $\pm$ 1.5	11.70 $\pm$ 1.2	13.62 $\pm$ 1.8	20.92 $\pm$ 1.3	22.08 $\pm$ 2.0
3	75	38.29 $\pm$ 2.9	18.94 $\pm$ 2.4	19.70 $\pm$ 2.0	31.34 $\pm$ 2.7	37.92 $\pm$ 4.2
4	100	51.93 $\pm$ 7.6	23.48 $\pm$ 1.8	25.07 $\pm$ 1.4	50.12 $\pm$ 7.4	56.37 $\pm$ 6.2
5	125	62.21 $\pm$ 3.7	35.66 $\pm$ 2.7	40.92 $\pm$ 6.3	65.80 $\pm$ 4.6	70.51 $\pm$ 8.6

Values are expressed as Mean  $\pm$  Standard deviation.

#### Plant Materials

The dried seeds of *T. foenum-graecum* were purchased from the local market of Mesra, Ranchi in the month of March, 2011. The plant material was taxonomically identified and authenticated by K. Karthikeyan, Scientist 'C', Botanical Survey of India (BSI), Central National Herbarium, Howrah, with ref. no. CNH/103/2011/Tech-II/620. The voucher specimen was deposited in the herbarium section of B.I.T., Mesra, Ranchi, Department of Pharmaceutical Sciences and Technology.

#### Preparation of extract and fractions

The dried seeds of *T. foenum-graecum* were cleaned and made into coarse powder with the help of mortar and pestle and stone blender. It was then passed through a 1-mm sieve and again dried in hot air oven below 50°C and stored at room temperature in an air tight container. The coarsely powdered material were subjected to successive soxhlet extraction using solvent petroleum ether, chloroform and methanol in the increasing order of their polarity for 48 hours with each solvent<sup>11</sup>. The obtained seed extracts were finally dried at low temperature under reduced pressure in a rotavapor (Buchi Labortechnik AG, CH-9230 Flawil 1/Switzerland) to obtain a semisolid mass and then finally lyophilized by freeze dryer (MPS-55, Korea) to yield solid residues. TLC was used for isolation of different compounds present in METFG<sup>12</sup>. The

METFG (10 g) was repeatedly separated by column using silica gel (60-120  $\mu$  size) as adsorbent and n-butanol: acetic acid: water in different ratio as the mobile phase. It led to the isolation of four fractions namely TF1, TF2, TF3 and TF4. Then the fractions were concentrated under reduced pressure on rotavapor to obtain a residue (TF1: 0.8 %, TF2: 2.6 %, TF3: 3.0 % and TF4: 4.6 % w/w).

#### HPTLC finger print profiles for extracts and fractions

##### HPTLC Conditions

Post-Chromatographic characterization of METFG, TF1, TF2, TF3 and TF4 was performed on precoated silica gel aluminium plate 60 GF254 (10 cm $\times$ 10 cm with 250  $\mu\text{m}$  thickness, E. Merck, Darmstadt, Germany supplied by Anchrome Technologies, Mumbai, India) using a Camag Linomat IV sample applicator (Camag, Muttenz Switzerland) and a 100- $\mu\text{l}$  syringe (Hamilton, Rena, Nevada, USA). The sample was spotted in the form of bands of 6 mm length and 10 mm higher from the bottom, 10 mm from the left margin, and 10 mm apart, at a constant application rate of 15  $\mu\text{L/s}$  using nitrogen aspirator. Plates were developed using a mobile phase consisting of n-butanol: acetic acid: water in the ratio of 4:5:1. Linear ascending development was carried out in 10 cm $\times$ 10 cm twin trough glass chamber (Camag Muttenz, Switzerland) equilibrated with the mobile phase. The optimized chamber saturation time for mobile phase was 30 min at room temperature. After development, the HPTLC plates were

dried with a hot air blower. The slit dimension settings of length 5 mm and width 0.45 mm and a scanning rate of 20 mm per second was employed. Densitometric scanning was performed on Camag TLC scanner II in the re-

flectance mode at  $\lambda_{\max}$  at 300 nm and operated by win CATS software version (1.1.3.0.). The source of radiation utilized was deuterium lamp. Evaluation was done via peak areas<sup>13</sup>.

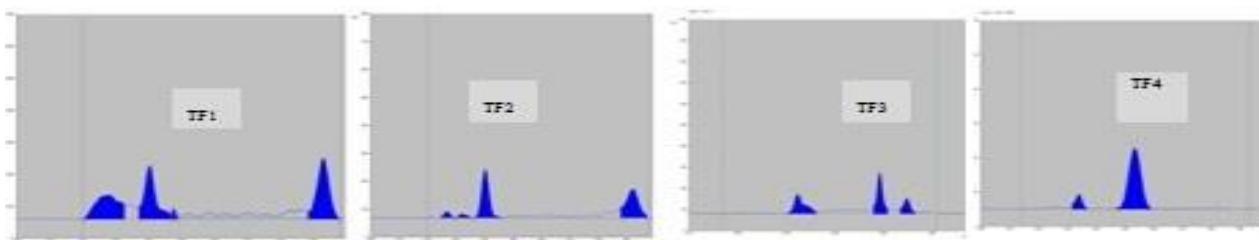


Figure 1: HPTLC chromatogram of fractions TF1, TF2, TF3 and TF4 of METFG and compound showing peaks of phytoconstituents

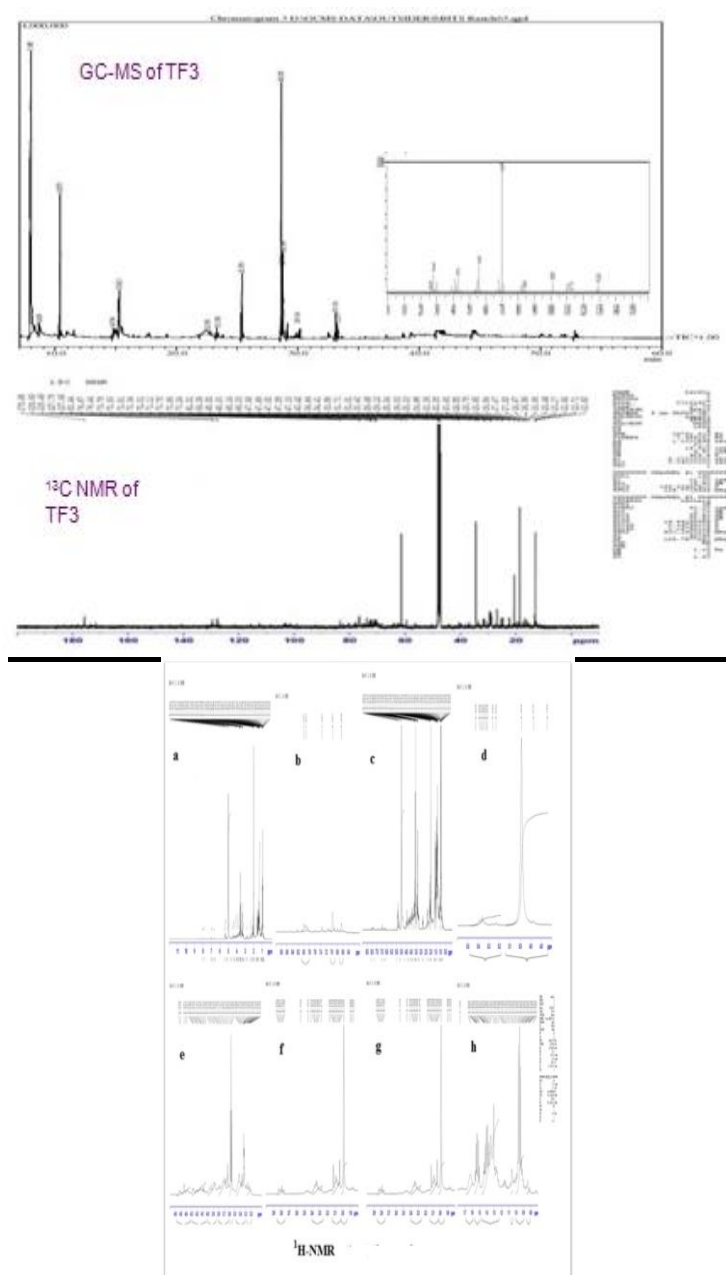


Figure 2: GC-MS, <sup>13</sup>C and <sup>1</sup>H NMR of isolated compound TF-k1 in TF3.

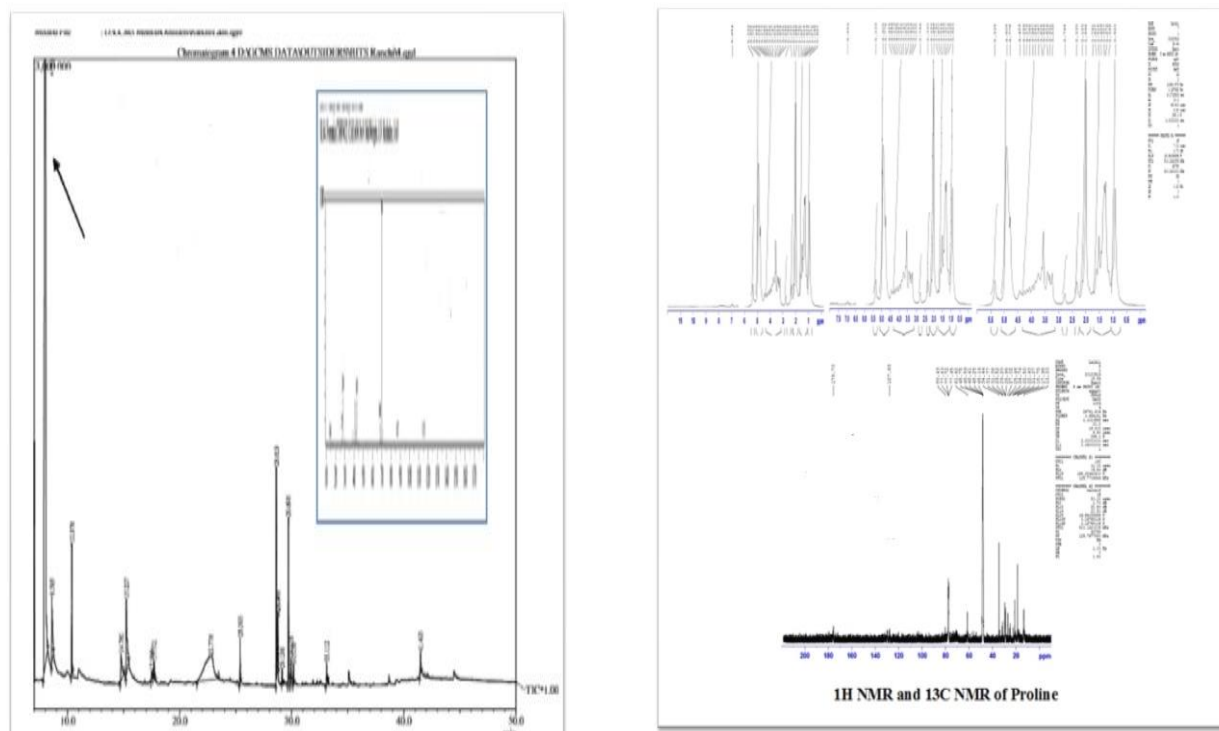


Figure 3: GC-MS,  $^{13}\text{C}$  and  $^1\text{H}$  NMR of isolated compound TF-k2 in TF4.

**Procedure:** Extracts and fractions of *T. foenum-graecum* were applied on HPTLC plate and the plate was developed in n-butanol: acetic acid: water v/v/v solvent system. The plates were dried at room temperature. The plate was scanned at 300 nm after spraying with detection reagent (Ninhydrin reagent) and plate was heated at  $105^\circ\text{C}$  for 5 minutes. The Rf values and color of the resolved bands were noted<sup>14</sup>.

#### *In vitro* antioxidant activity of fractions of METFG

The fractions TF1, TF2, TF3 and TF4 of METFG were dissolved in ethanol at the concentration 2 mg/ml to make a test and also standard solution of 25, 50, 75, 100 and 125  $\mu\text{g}/\text{mL}$  concentrations respectively.

#### DPPH radical scavenging assay

The DPPH free radical scavenging activity of fractions TF1, TF2, TF3 and TF4 of METFG was carried out according to the method of Deore *et al*<sup>15</sup>. 0.2 ml of test solution at different concentrations (25-125  $\mu\text{g ml}^{-1}$ ) were mixed with 0.8 ml of Tris- HCL buffer (100 mM, 7.4). 1 ml of DPPH (500 mM in ethanol) solution was added to the above mixture and this mixture was shaken vigorously and incubated for 20 min in the dark. The absorbance of the resulting solution was measured at 517 nm by UV-Visible spectrophotometer (Shimadzu UV-Visible spectrophotometer 1700, India). All the assays were carried out in triplicates against ascorbic acid (AA) as a positive control. Blank was prepared without the addition of DPPH and for control, 0.2 ml of ethanol (without test sample) was added. Decreased absorbance values indicated a higher free radical scavenging activity. The percentage of DPPH scavenging effect was calculated.

#### Nitric oxide radical scavenging assay

Nitric oxide radical scavenging capacity of fractions TF1, TF2, TF3 and TF4 of METFG was determined according

to the method of Chaurasia *et al*<sup>16</sup>. 3 ml of the ethanolic solution of the test samples at different concentrations (25-125  $\mu\text{g}/\text{ml}$ ) were mixed with 1 ml of sodium nitroprusside (10 mM) in standard phosphate buffer solution (0.02 M, pH 7.4). The mixture was shaken vigorously and incubated at  $25^\circ\text{C}$  for 150 min. Control experiments without the test compounds, but with an equivalent amount of buffer were prepared in the same manner as done for the test. Thereafter, 0.5 ml of the incubation solution was removed and diluted with 0.5 ml of Griess reagent (1% sulphanilamide, 2% ophosphoric acid and 0.1% naphthyl ethylenediamine dihydrochloride) and allowed to stand at room temperature for 30 min. The absorbance of the chromophore formed during the diazotization of nitrite with sulphanilamide and subsequent coupling with naphthyl ethylene diamine dihydrochloride was read spectrophotometrically by (Shimadzu UV-Visible spectrophotometer 1700, India) at 546 nm. The increased absorbance of the reaction mixture indicated higher scavenging effect. The percentage inhibition of nitric oxide radical generation was calculated.

#### Hydrogen peroxide scavenging assay

Measurement of hydrogen peroxide scavenging activity of fractions TF1, TF2, TF3 and TF4 of METFG was based on the method described by Deschner *et al*<sup>17</sup>. Hydrogen peroxide solution (2 mM) was prepared with standard buffer (pH 7.4). Test samples (25-125  $\mu\text{g}/\text{ml}$ ) in distilled water were added to hydrogen peroxide solution (0.6 ml). Then the absorbance was measured spectrophotometrically by (UV Visible spectrophotometer 1700, Shimadzu, India) at 230 nm after 10 min in triplicates against blank solution containing phosphate buffer without hydrogen peroxide solution as compared with ascorbic acid as positive control. Increased absorbance of the

reaction mixture indicated strong scavenging effect. The percentage scavenging of hydrogen peroxide was calculated.

#### Statistical analysis

The percentage of inhibition was calculated as:

% of inhibition =  $\{(\text{absorbance of control} - \text{absorbance of sample}) / \text{absorbance of control}\} \times 100$

Results were expressed as mean by triplicates measurement and IC<sub>50</sub> was graphically determined by a linear regression method using MS Windows based graph pad prism (Trial Version 5.01) software. Results were expressed as Mean  $\pm$  Standard deviation.

#### Isolation and characterization of bioactive compound

Concentrated TF3 and TF4 were subjected to repetitive preparative thin layer chromatography using silica gel as stationary phase (20 $\times$ 20 cm glass plates) and n-butanol: acetic acid: water 4:4:2 v/v/v as mobile phase<sup>18</sup>. It led to the isolation of a compound TF-k1 and TF-k2 from TF3 and TF4. The isolated compound was concentrated under reduced pressure to dryness and then the structure was established on the basis of spectroscopic evidences (UV, <sup>1</sup>H NMR, <sup>13</sup>C NMR and GC-MS).

## RESULTS

### Antioxidant activity

The antioxidant properties of the chromatographically separated fractions (TF1-TF4) of METFG were carried out by standard following methods.

#### DPPH (1,1-diphenyl-2-picrylhydrazyl) free radical scavenging assay

The effects of fractions of METFG (TF1, TF2, TF3 and TF4) on free radical scavenging activity by DPPH method have been summarized in Table 1. Higher the percentage of inhibition value, more active is the fraction in scavenging the DPPH free radical. Ascorbic acid was used as the standard. Fraction of TF4 showed potent DPPH free radical scavenging activity, whereas TF1, TF2, TF3 were found to be less active. *T. foenum-graecum* showed percentage of inhibition value: 23.00  $\pm$  2.5, 21.99  $\pm$  4.9, 40.07  $\pm$  6.3 and 51.94  $\pm$  5.6 in TF1, TF2, TF3 and TF4, respectively at 125 $\mu$ g/ml as compared to standard ascorbic acid (AA) which was having the inhibition value of 58.25  $\pm$  2.7  $\mu$ g/ml. The IC<sub>50</sub> (the inhibitory concentration at which there is 50% decrease of free radical) of TF1, TF2, TF3 and TF4 was found to be 234.29  $\pm$  2.6, 285.7  $\pm$  3.8, 167.8  $\pm$  2.4 and 119.21  $\pm$  1.9  $\mu$ g/ml, respectively.

#### Nitric oxide radical scavenging assay

The effects of TF1, TF2, TF3 and TF4 on nitric oxide radical scavenging activity have been summarized in Table 2. Higher percentage of inhibition in the concentration of nitric oxide radical was observed due to the scavenging ability of the fraction and standard used namely ascorbic acid. Fraction of the plant extracts TF4 showed potent nitric oxide radical scavenging activity, whereas TF1, TF2, TF3 were found to be less active. The percentage decrease in nitric oxide radical content at 125  $\mu$ g/ml of TF1, TF2, TF3 and TF4 was 26.17  $\pm$  3.1, 24.27  $\pm$  2.5, 52.38  $\pm$  2.7 and 58.36  $\pm$  4.2  $\mu$ g/ml, respectively. The radical scavenging of standard ascorbic acid at the same

concentration was 62.21  $\pm$  4.9  $\mu$ g/ml. The IC<sub>50</sub> of TF1, TF2, TF3 and TF4 was found to be 294.03  $\pm$  14, 289.37  $\pm$  20.4, 117.25  $\pm$  18.2 and 109.69  $\pm$  21.8  $\mu$ g/ml, respectively.

#### Hydrogen peroxide scavenging assay

The effects of TF1, TF2, TF3 and TF4 on hydrogen peroxide scavenging activity has been summarized for the plants in Table 3. Higher the value of percentage of inhibition, more active is the fraction in scavenging the hydrogen peroxide. Ascorbic acid was used as the standard. Fraction of the plants extracts TF4 showed potent hydrogen peroxide radical scavenging activity, whereas TF1, TF2, TF3 were found to be less active. Hydrogen peroxide itself is not very reactive, it can generate the highly reactive hydroxyl radical (OH) through fenton reaction. The scavenging of hydrogen peroxide is an important antioxidant defense mechanism<sup>21</sup>. The percentage decrease in hydrogen peroxide content at 125  $\mu$ g/ml of TF1, TF2, TF3 and TF4, respectively was 35.66  $\pm$  2.7, 40.92  $\pm$  6.3, 65.80  $\pm$  4.6 and 70.51  $\pm$  8.6  $\mu$ g/ml. The radical scavenging of standard ascorbic acid at the same concentration was 62.21  $\pm$  3.7  $\mu$ g/ml. The IC<sub>50</sub> value of TF1, TF2, TF3 and TF4 was found to be 183.15  $\pm$  21.6, 139.72  $\pm$  25.7, 99.26  $\pm$  17.5 and 192.79  $\pm$  18.8  $\mu$ g/ml, respectively.

#### Finger print analysis of fractions of METFG using HPTLC

Figure 1 represents the HPTLC finger print of fractions of METFG namely TF1-TF4. TF1 showed a mixture of three different components having the following R<sub>f</sub> values 0.26, 0.35 and 0.98. TF2 showed a mixture of two different components having the following R<sub>f</sub> values 0.36 and 0.92. TF3 showed a mixture of three different components with the following R<sub>f</sub> values 0.29, 0.66 and 0.74. TF4 showed a mixture of two different components with the R<sub>f</sub> values 0.33 and 0.69. These chromatograms were useful for the isolation, purification, characterization and identification of marker chemical compounds of the plant parts.

#### Characterization of isolated components from fractions TF3 and TF4 of methanol extract of *T. foenum-graecum* seeds

The isolated compound from the active fraction TF3 and TF4 of METFG was obtained as brownish solid. It gave positive Millon's and Ninhydrin test and confirmed the presence of amino acids.

Yield: TF3 = 2.5 % and TF4 = 4.6 %.

Analysis of isolated compound TF-k1 in fraction TF3 (Figure 2):

<sup>1</sup>H-NMR (400 MHz, MeOD):  $\delta$  0.953 (m, 3H, H-3), 1.347 (m, 1H, H-2), 1.542 (t, 2H, H-4), 1.988 (s, 3H, H-5), 3.568 (d, 1H, H-1,  $J$  = 7.0).

<sup>13</sup>C-NMR (100 MHz, MeOD):  $\delta$  12.92 (C-5), 18.65 (C-3), 20.83 (C-4), 34.41 (C-2), 63.41 (C-1), 175.68 (C-6).

GC-MS: RT 7.987 (46.38 %), MW: 129.01

Analysis of isolated compound TF-k2 in fraction TF4 (Figure 3):

<sup>13</sup>C-NMR (100 MHz, MeOD): 13.33, 17.98, 18.75, 21.07, 22.40, 22.50, 24.74, 25.46, 27.02, 28.95, 29.20, 29.52, 31.38, 31.77, 34.44, 61.62, 127.93, 175.70.

GC-MS: RT 8.023 (60.27 %), MW: 115.04. The GC-MS, <sup>1</sup>H-NMR and <sup>13</sup>C-NMR analysis of TF3 and TF4 of methanol extract of *T. foenum-graecum* revealed the presence of TF-k1 (46.38 %) and TF-k2 (60.27%) as the major component in fraction TF3 and TF4.

## DISCUSSION

*T. foenum-graecum* is used in folklore medicine for the treatment of various diseases in the different states of India and also by alternative medical practitioners for an array of diseases<sup>19</sup>.

The active extracts of METFG were subsequently subjected for HPTLC finger printing analysis in different solvent systems in order to do chromatographic characterizations and were targeted to isolate the active components from these extracts<sup>20</sup>. METFG were subjected for fractionation by column chromatography in order to get the active components in the fractions. Fractionation of METFG led to the separation of four fractions (TF1-TF4). The developed chromatogram of the fractions of methanol extracts of *T. foenum-graecum* (TF1-TF4) by HPTLC, helped in the identification of chemical components as per WHO guidelines<sup>21</sup>. These chromatograms were also useful for the isolation, purification, characterization and identification of marker chemical compounds of the plant parts. In the present study, HPTLC fingerprinting of fractions of METFG (TF1-TF4) determined the major active constituents present in them.

The antioxidant properties of the chromatographically separated fractions of METFG were carried out by different antioxidant assay. Higher the percentage of inhibition value, more active is the fraction in scavenging the DPPH free radical. Ascorbic acid was used as the standard. Free radical scavenging activity of the test was found to be concentration dependent. As the concentration of the test sample increased, the free radical scavenging activity also increased and lower IC<sub>50</sub> value reflected better protective action<sup>22</sup>. The TF4 fraction of *T. foenum-graecum* were observed to be potent scavengers with the least IC<sub>50</sub> value and the potency order of fractions of METFG was TF4 > TF3 > TF2 > TF1. Nitric oxide radical scavenging activity of the test is concentration dependent. As the concentration of the test sample increased, the nitric oxide radical scavenging activity also increased and the lower IC<sub>50</sub> value reflected better protective action<sup>23</sup>. From the present study of nitric oxide radical scavenging assay<sup>24</sup>, it was proved that TF4 exhibited more free radical scavenging activity and competes with oxygen leading to a reduced production of NO as compared to TF1, TF2 and TF3. The hydrogen peroxide scavenging effect of TF4 of *T. foenum-graecum* were observed to be more potent with least IC<sub>50</sub> value and the rank order potency of hydrogen peroxide scavenging of the potency order of fractions of METFG was TF4 > TF3 > TF2 > TF1.

From the above study, it has been concluded that based on *in-vitro* antioxidant activity of fractions of methanol extracts of *T. foenum-graecum* (TF1-TF4). As per WHO guidelines for HPTLC finger print of active fractions TF3 and TF4 showed the presence of major active constituents

as compared to other fractions and thus showed significant antioxidant activity as has been represented in the above results. The active fractions TF3 and TF4 of METFG was characterized by GC-MS, <sup>1</sup>H NMR and <sup>13</sup>C NMR spectra. It was further confirmed by the spectral analysis that TF3 contained bioactive compound TF-k1 (46.38%), having the molecular formula C<sub>6</sub>H<sub>11</sub>NO<sub>2</sub>, TF4 contained bioactive compound TF-k2 (60.27%) with the molecular formula of C<sub>5</sub>H<sub>9</sub>NO<sub>2</sub>.

HPTLC analysis of *T. foenum-graecum* extracts can provide standard fingerprints and fast alternative for quantitative determination of bioactive compound and quality control of the drug. On the basis of present study, fractions TF3 and TF4 of seeds of *T. foenum-graecum* were selected as active fractions for further *in-vivo* pharmacological studies in animal model.

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## REFERENCES

1. Kokate C.K., Purohit A.P., Gokhale S.B. Practical pharmacognosy. Nirali Prakashan, Pune, India, 2<sup>nd</sup> edn, 1994: 50-69.
2. Mazumder P.M., Farswan M., Parcha V., Singh V. Hypoglycemic and antioxidant activity of an isolated compound from *Ficus arnottiana* bark. *Pharmacologyonline*, 2008, (3): 509-19.
3. Mukherjee P.K. Quality control of herb drugs. 2002. pp. 426-35.
4. Muhson I. and Mashkor A.L. Phenolic content and antioxidant activity of fenugreek seeds extract. *International Journal of Pharmacognosy and Phytochemical Research*, 2014, 6(4): 841-44.
5. Gupta A., Gupta R., Lal B. Effect of *Trigonella foenum-graecum* (fenugreek) seeds on glycaemic control and insulin resistance in type 2 diabetes mellitus: a double blind placebo controlled study. *J Association Physician India*, 2001, 49(2): 1057-61.
6. Haeri M.R., Izaddoost M., Ardekani M.R., Nobar M.R., White K.N. The effect of fenugreek 4-hydroxyisoleucine on liver function biomarkers and glucose in diabetic and fructose fed rats. *Phytotherapy Research*, 2009, 23(1): 61-64.
7. Khosla P., Gupta D.D., Nagpal R.K. Effect of *Trigonella foenum-graecum* (fenugreek) seeds on blood glucose in normal and diabetic rats. *Indian Journal of physiology and pharmacology*, 1995, 32(2): 173-74.
8. Mazumder P.M., Farswan M., Parcha V., Singh V. Hypoglycemic and antioxidant activity of an isolated

- compound from *Ficus arnottiana* bark. *Pharmacologyonline*, 2008, (3): 509-19.
9. Suralkar A.A., Jadhav A.S., Vaidya G.S., Ashish S., Chaudary M., Bhora V. Anti-allergic, antianaphylactic and mast cell stabilizing, activity of *Embelia ribes* brum. *Research Journal of Pharmaceutical Technology*, 2013, 6(2): 1231-34.
  10. Schaible A.M., Traber H., Temml V., Noha S.M., Filosa R., Peduto A. Potent inhibition of human 5-lipoxygenase and microsomal prostaglandin E2 synthase-1 by the anticarcinogenic and anti-inflammatory agent embelin. *Biochemical Pharmacology*, 2013; 86: 476-86.
  11. Mukherjee P.K. Quality control of herb drugs. 2002. pp. 426-35.
  12. Kumari L., Mazumder, P.M., Lal, U.R. Activity of proline and its analogs isolated from *Murraya koenigii* against hyperglycemia, oxidative stress and renal insufficiency in diabetic nephropathy. *International Journal of Pharmacognosy and Phytochemical Research*, 2016, 8(1): 71-79.
  13. Kumari L., Mazumder, P.M., Manik., Lal, U.R., Dubey R. High performance thin layer chromatography fingerprint profile, isolation, its antioxidant activity and spectroscopic characterization of marker compound of *Murraya koenigii*. *Journal of Pharmacognosy and Phytochemistry* 2014, 3(4): 86-92.
  14. V. Bentina. Bioautography in paper and thin layer chromatography and its scope in the antidiabetic field. *Journal of Chromatography*, 1973, 78 (2): 41-51.
  15. S.L. Deore S.S., Khadabadi B.A., Baviskar R.A., Khangenbam U.S.K. *In vitro* antioxidant activity and phenolic content of *Croton caudatum*. *International Journal of Chemical and Technical Research*. 2009, 5(1), 174-76.
  16. Chaurasia S and Tripathi B. Antioxidant and anti-inflammatory property of sandhika: A compound herbal drug. *Journal of Experimental Biology*, 1995, 33 (3): 428-32.
  17. Deschner E.E., Ruperto J., Wong G., Newmark H.L. Quercetin and rutin as inhibitors of azoxy methanol induced colonic neoplasia. *Carcinogenesis*, 1991, 12(7): 1193-96.
  18. Jeong A.K., Jeong H.S., Seok B.S., Seo Y.Y., Young H.K. Sterols isolated from seeds of *Panax ginseng* and their antiinflammatory activities. *Pharmacognosy Magazine*, 2013, 34 (9): 182-85.
  19. Petit P., Sauvair Y., Broca C., Manteghetti M., Baissac Y., Gross R. Hydroxyisoleucine: a novel amino acid potentiator of insulin secretion. *Laboratoire de Recherche sur les Substances Naturelles Végétales, Université Montpellier II, France*. 1998, 47(2): 206-10.
  20. Scartezzini P and Sproni E. Review on some plants of Indian traditional medicine with antioxidant activity. *Journal of Ethnopharmacology*, 2000, 71(1-2): 23-43.
  21. Sherifa H.S., Halima S.A., Amal S., Abdel A. The antioxidative effects of some medicinal plants as hypoglycemic agents on chromosomal aberration and abnormal nucleic acids metabolism produced by diabetes stress in male adult albino rats. *Journal of Diabetes Mellitus*, 2011 (1): 6 -14.
  22. Nilufer O., Mustafa A., Didem D.O., Fatma E., Erden Y. *In vivo* assessment of antidiabetic and antioxidant activities of grapevine leaves (*Vitis vinifera*) in diabetic rats. *Journal of Ethnopharmacology*, 2006, 108(2): 280 -86.
  23. Soon Y.Y. and Tan B.K. Evaluation of the hypoglycemic and antioxidant activities of *Morinda officinalis* in streptozotocin induced diabetic rats. *Singapore Journal of Medicine*, 2002, 43(2): 77-85.
  24. Syeda B.B., Muhammad I.B., Memon S. Antioxidant activity of extracts from fenugreek seeds. *Pakistan Journal of Analytical and Environmental Chemistry*, 2008, 9(2): 78-83.