International Journal of Herbal Medicine
Available online at www.florajournal.com

I J H M International Journal of Herbal Medicine

ISSN 2321-2187
IJHM 2014; 2 (1): 53-57
Received: 28-02-2014
Accepted: 30-03-2014

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Antioxidant activity of *Trigonella foenum graecum* L. using various *in vitro* models

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ABSTRACT
With changes in lifestyle, eating habits and exposure to hazardous substances, the risk of oxidative damage is increasing which ultimately leads to various ailments. One way to safeguard oneself from these hazards is incorporation of naturally occurring antioxidants in daily diet that provide nutrition besides being a reservoir of numerous health promoting phytochemicals. Methanolic Extract of fenugreek seeds were evaluated for total phenolics content, antioxidant activity using various assay systems such as iron(III) reduction, inhibition of hydroxyl radical-mediated 2-deoxy-D-ribose degradation, metal ion (Fe^{2+}) chelation assay and DPPH radical scavenging assay. The results indicate that fenugreek is a very efficient antioxidant.

Keywords: *Trigonella foenum graecum,* Free radicals, Antioxidant

1. Introduction

Fenugreek is an annual herbaceous legume. It is consumed as a spice as well as a vegetable in different parts of the world. Fenugreek has been recognized and utilized for its medicinal attributes since times immemorial as evidenced by its use in traditional ayurvedic and Chinese medicines. It has been used in alleviating high cholesterol plasma levels [1] diabetes [2] oxidative damage [3]. It has also been used as an appetite stimulant [4] and as a laxative [5]. Now days there is an increased risk of oxidative damage as a result of increased exposure to hazardous substances, changes in diet and lifestyle. The oxidative damage gives rise to numerous ailments. So there is an increasing demand of antioxidants of natural origin that could curb oxidative damage without side effects. Keeping in view the above challenges Fenugreek extracts were tested, in the present study, as a source of antioxidants. Therefore the aim of the present study was to determine the antioxidant activity of methanolic extracts of *Trigonella foenum-graecum* L.

2. Material and Method

The seeds of *Trigonella foenum graecum* L. were collected from SKN College of Agriculture, Jobner, Swami Keshwanand Rajasthan Agricultural University, Rajasthan, India. All the genotypes that have been analyzed were under coordinated varietal trial. All reagents and solvents were of analytical grade and were obtained from Sigma Chemical Co. (St. Louis, MQ, USA) and Himedia, Mumbai, India.

2.1. Preparation of the extract

Five gram finely powdered seed powder was mixed with twenty five mL 80% methanol and kept for 72 hours. The extract was filtered through Whatman, No.1 filter paper and evaporated to dryness in rotary evaporator. The residue was redissolved in 80% methanol to obtain the methanolic extracts. For performing various asays different concentrations of the methanolic extracts were used (200,400,600,800 and 1000 µg).

2.2. Determination of total phenolics content

The method of Wolfe [6] was followed for total phenolics quantification.0.5 ml water was added to 0.125 ml of the extracts prepared as described above. Folin – Ciocalteu reagent (0.125 ml) was added and the reaction mixture was incubated for 6 minutes. Sodium carbonate (7%) was added and the volume of the resulting solution was made up to 3 ml with double distilled water. Absorbance was recorded at 760 nm after 90 minutes incubation. Gallic acid was taken as standard.

2.3. Determination of DPPH free-radical scavenging activity
For determination of DPPH scavenging activity method of Shimada [7] was used 0.1 mM solution of DPPH was prepared and 1 ml of the DPPH solution was added to 3 ml of the extracts. Five different concentrations of the methanolic extracts were used. The resulting solution was mixed by shaking and absorbance was recorded after 30 minutes incubation at 517 nm. Ascorbic acid was used as positive control.

2.4. Measurement of reducing power
For determination of reducing power the method of Oyaizu [8] was used. 2.5 ml of methanolic extracts were mixed with 2.5 ml sodium phosphate buffer (200 mM pH 6.6) and 2.5 ml of 1% potassium ferricyanide. 20 min incubation was given at 50 ºC followed by addition of 2.5 ml of 10% trichloroacetic acid (w/v). Aliquot (5 mL) from the above solution was mixed with 1 ml of 0.1% of ferric chloride, and the absorbance was measured at 700 nm. BHT was used as positive control.

2.5. Estimation of metal ion (Fe2+) chelating activity
Chelating activity of the extracts was measured as reported by Liyana- Pathirana and Shahidi [9]. Different concentrations of methanolic extracts were taken and volume was made up to 0.4 ml followed by 0.285 ml of double distilled water, 0.275 ml of FeCl2.4H2O (0.2 mM) and 0.04 ml of ferrozine (5 mM). Absorbance was measured at 562 nm after 10 minutes. EDTA was used as positive control. Fe2+ chelating activity (%) was calculated as follows

Chelating activity % = Absorbance control – Absorbance sample / Absorbance control x 100

2.6. Hydroxyl radical scavenging activity assay
This assay was performed by following the protocol given by Monogolkolsilp [10]. Briefly 100 µl of 28 mM 2-deoxy 2-ribose dissolved in phosphate buffer, pH 7.4, 500 µl of the plant extract of various concentrations in buffer, 200 µl of 200 mM ferric chloride (1:1 v/v) and 1.04 mM EDTA and 100µl of 1.0 mM hydrogen peroxide and 100 µl of 1.0 µM ascorbic acid. The total volume was made up to 1 ml. After incubation of the test sample at 37 ºC for one hour colour was developed using TBA reaction. Ascorbate was used as positive control. Percentage inhibition of deoxyribose degradation was calculated.

2.7. Statistical analysis
All the results have been presented as ± standard deviation. The statistical analysis was done using Anova and the results. The IC 50 values have been determined graphically.

3 Results and Discussion
3.1 Total phenolics content
The total phenolics content was estimated quantitatively (Table 1). It ranged from 1.51 ± .03 to 1.92 ± .06. The total phenolics content in fenugreek genotypes has also been reported by Singh [11]. They reported polyphenolic content in fenugreek genotypes ranging from 0 .9947 mg/gm to 1.12 mg/ gm. The phenolics present in plants are considered as antioxidants. The presence of phenolics in appreciable amount make the substance suitable as a neutraceutical. The correlation between the total phenolics content and IC 50 value for DPPH radical scavenging activity was calculated. A weak positive correlation was found with (r = 0.25).

3.2 DPPH free-radical scavenging activity
DPPH is a nitrogen centered free radical. Methanolic solution of DPPH radical gives a violet color which is discolored by addition of antioxidants. The greater the antioxidant capacity greater will be the discoloration of DPPH solution [12]. All the extracts tested showed DPPH radical scavenging. The scavenging shown by all the genotypes is good and is comparable with the positive control which is ascorbic acid in this case. The ability of the extracts to scavenge free radicals and that too significantly comparable with a purified compound indicates the efficacy of the test material as an antioxidant. The scavenging of DPPH radical by fenugreek seed extracts have been given in table 2. The IC 50 values of all the genotypes were calculated (Table 6). The IC_{50} values ranged from 167 to 467 µg. Lower IC_{50} indicates a higher DPPH radical scavenging activity. Genotype HM 348 showed the lowest IC_{50} value of 167 µg. Earlier Madhava [13] have reported IC_{50} value of 156 µg in fenugreek seed extract. The methanolic extracts have show significant and concentration dependent DPPH scavenging indicating the antioxidant potential of fenugreek as reported earlier [14].

3.3. Iron (III) to iron (II)-reducing activity
The reducing capacity is indicative of the electron donation potential of the antioxidant [15]; Arabshahi-Delouee and Urooj, 2007 [16]. The antioxidants reduce the the Fe3+/ferricyanide complex to the ferrous form. The resulting Perl's Prussian blue color can be observed at 700 nm. The extracts showed an increase in reducing power with increase in concentration (Table 3). The readings obtained from the methanolic extracts of fenugreek seeds were lower as compared to the positive control taken (BHT).

3.4. Iron (II) chelating activity assay
Ferrous ions have the ability to generate ROS in the cellular system. They can react with hydrogen peroxide present in the system to generate hydroxyl radicals [17]. They can also give rise to lipid peroxidation and act as food deteriorating agents [18]. A substance having the potential of chelating the ferrous ions can prevent the damage caused by these ions, hence can be considered as antioxidants. The chelating ability of the methanolic extracts of fenugreek (Table 4) was compared with EDTA which was taken as positive control. All the extracts tested showed ferrous ion chelating capacity that ranged from 24 to 60%.

3.5. Hydroxyl radical scavenging activity
Hydroxyl radicals are generated by reaction between hydrogen peroxide and iron ions. They give rise to lipid peroxidation and these are amongst the most reactive radicals that can damage a cellular system. All the genotypes showed a concentration dependent increase in hydroxyl radical scavenging ability, proving their efficacy as antioxidants. The IC_{50} values were determined and the lowest was shown by LFC 103 (110 µg) while the highest was shown by AFG 3 (500 µg) (Table 6). All the extracts showed appreciable hydroxyl radical scavenging (Table 5) that showed a concentration dependent increase.

Table 1: Quantitative estimation of total phenolics in fenugreek genotypes

Genotype	Total phenolics content (mg/gram)
PRM 45	1.51 ± .03
AFG 3	1.56 ± .02
AFG 4	1.86 ± .02
LFC 105	1.76 ± .03
LFC 103	1.92 ± .06
HM 348	1.52 ± .02
HM 355	1.75 ± .07
UM 330	1.86 ± .01

Values are reported as ±SD (n=3). Results were found to be significant (p≤.01)

Table 2: Scavenging of DPPH radical by fenugreek genotypes

Concentration of methanolic extracts (μg)					
Sample	**200**	**400**	**600**	**800**	**1000**
Ascorbic acid	82.53±.003	85.86±.006	87.52±.002	88.53±.002	89.32±.004
PRM 45	31.39±.01	65.48±.006	66.73±.01	87.52±.002	88.35±.002
AFG 3	26.4±.002	33.05±.009	36.79±.02	36.79±.009	75.88±.002
AFG 4	35.55±.02	53.84±.03	55.92±.01	74.63±.002	76.71±.008
LFC 105	17.25±.001	32.22±.02	54.26±.03	58. 41±.002	75.46±.02
LFC 103	21.41±.02	45.43±.009	57.17±.01	60.91±.02	77.13±.02
HM 348	40.12±.01	75.46±.03	76.71±.005	79.62±.008	81.70±.005
HM 355	33.47±.03	49.68±.03	60.08±.006	72.97±.004	75.05±.002
UM 330	20.99±.002	41.78±.002	47.60±.005	56.75±.01	58.41±.002

Values are reported as ±SD (n=3). Results were found to be significant (p≤.05)

Table 3: Ferric ion reducing activity of fenugreek genotypes

Concentration of methanolic extracts (μg)					
Sample	**200**	**400**	**600**	**800**	**1000**
BHT	0.726±.01	1.302±.02	1.55±.01	1.837±.02	1.883±.002
PRM 45	0.041±.003	0.075±.004	0.103±.01	0.144±.01	0.243±.06
AFG 3	0.052±.006	.064±.01	00.105±.004	0.184±.03	0.242±.03
AFG 4	0.057±.02	0.094±.03	0.130±.03	0.153±.005	0.178±.04
LFC 105	0.055±.01	0.07±.01	0.074±.005	0.111 ±.01	0.12±.01
LFC 103	0.057±.001	0.116±.003	0.139±.008	0.247±.006	0.267±.002
HM 348	0.046±.01	0.078±.01	0.152±.004	0.156 ±.004	0.275±.01
HM 355	0.011±.05	0.097±.002	0.156±.03	0.173±.01	0.221±.01
UM 330	0.044±.004	0.073±.01	0.099±.01	0.137±.01	0.181±.03

Values are reported as ±SD (n=3). Results were found to be significant (p≤.05)

Table 4: Metal ion chelating activity of fenugreek genotypes

Concentration of methanolic extracts (μg)					
Sample	**200**	**400**	**600**	**800**	**1000**
EDTA	98.11±.001	99.31±.0005	99.58±.001	99.73±.005	99.89±.001
PRM 45	37.44 ± .01	40.17± .06	45.24± .06	53.29± .14	57.59±.01
AFG 3	31.72± .08	38.38± .04	39.68± .01	46.06± . 04	52.92±.06
AFG 4	39.85± .01	47.03± .05	50.12± .15	54.63±.14	60.56± .01
LFC 105	36.75±.01	37.4±.01	38.58± .03	40.82± .11	44.27± .14
LFC 103	24.28± .08	30.99±.01	34.93± .004	35.49± .01	44.23± .01
HM 348	24.49± .17	29.04± .01	36.36± .05	45.65± .15	57.67± .002
HM 355	29.56± .08	32.41± .07	37.12± .02	43.82± .05	45.49± .04
UM 330	23.43± .02	31.15± .05	32.37± .01	44.76± .01	50.56± .11

Values are reported as ±SD (n=3). Results were found to be significant (p≤.05).

Table 5: Hydroxyl ion scavenging activity of fenugreek genotypes

	Concentration of methanolic extracts (µg)				
Sample	**200**	**400**	**600**	**800**	**1000**
Ascorbic acid	92± .001	93.78± .003	94.18± .002	94.50± .001	94.91± .0005
PRM 45	57.43± .03	62.84± .006	73.99± .001	77.38± .008	89.01± .008
AFG 3	29.32± .001	34.81± .001	66.39± .05	78.59± .005	81.27± .01
AFG 4	56.38± .01	64.94± .009	73.34± .002	81.74± .02	88.61± .009
LFC 105	74.55± .05	75.92± .01	83.60±.05	86.51±.05	87.64± .05
LFC 103	83.27±.01	83.6±.05	86.83± .01	88.44± .001	92.64± .007
HM 348	65.78± .01	70.75±.01	79.8± .01	87.64± .02	93.13± .03
HM 355	59.77± .01	74.23± .01	75.76± .02	85.70± .01	90.79± .01
UM 330	54.28± .07	67.93± .05	72.85± .02	79.96± .01	82.39± .01

Values are reported as ±SD (n=3). Results were found to be significant (p≤.05)

Table 6: IC_{50} values (µg)

Sample	DPPH radical scavenging assay	Hydroxy radical scavenging assay
PRM 45	200	160
AFG 3	467	500
AFG 4	227	170
LFC 105	367	120
LFC 103	300	110
HM 348	167	140
HM 355	267	160
UM 330	434	180

4. Conclusion

The objective of the in vitro study is a preliminary examination of whether the selected plant could be considered as a potential antioxidant. For this purpose methanolic extracts have been taken. Results show a significant antioxidant activity in the methanolic extracts. Overall, the plant can be considered a potent natural antioxidant that can be important in safeguarding the system against the harmful effect of free radical induced damage hence can be regarded promoter of longevity. Fenugreek is also a well-known and promising hypoglycemic, hypocholesterolemic agent that is also rich in nutrition, courtesy its chemical composition. Fenugreek is rich in proteins, carbohydrates, minerals particularly calcium, magnesium etc. and a significant source of fiber. This composition of fenugreek seeds makes it a naturally occurring pharmacological agent. It can be concluded that fenugreek consumption is a good means to ensure availability of nutritional and medicinal phytochemicals to the system. In near future, in vivo experiments with aqueous and ethanolic extracts can be performed to determine the antioxidant potential of the plant in the living system.

5. Acknowledgments

Author N.P. acknowledges the financial grant provided by Department of Science and Technology (DST), New Delhi in the form of fellowship. The financial assistance and infrastructural grant provided by DST FIST is also duly acknowledged.

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