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Free radical scavenging activity, phenolic contents and Phytochemical analysis of seeds of *trigonella foenum graecum*

LEENA SEASOTIYA[1, 2], PRIYANKA SIWACH[2], SHEEMA BAI[1], ANUPMA MALIK[1], POOJA BHARTI[1], SUNITA DALAL[1*]

[1]Department of Biotechnology, Kurukshetra University, Kurukshetra, Haryana, India

[2]Department of Biotechnology, Chaudhary Devi Lal University, Sirsa, Haryana, India

ABSTRACT

The search for sources of potent antioxidants of natural origin derived from plants is very important in the wake of decreasing resistance of human beings to various diseases. In the present study, antioxidant activity, phenolic contents and phytochemical profile of various extracts (methanol, chloroform, ethyl acetate and hexane) of seeds of *Trigonella foenum graecum (*fenugreek) were investigated. Ethyl acetate extract (100 µg/ml) showed highest inhibitory potential with application of DPPH scavenging (69.70%) and chelating power assay (63.44%). The differences in antioxidant activity of extracts are attributed to the presence of various phytochemicals. The IC_{50} values of different extracts were also calculated. There was found a positive correlation ($R \geq 0.895$) between the total phenolic content and the antioxidant activity of extracts. The phytochemical analysis revealed the presence of alkaloids, flavonoids, saponins, phenols and tannins. Results from different parameters were in agreement with each other. The results reveal that all extracts of the fenugreek exhibit antioxidant activity. These findings suggest that the fenugreek extracts could act as potent source of antioxidants.

Keywords: Antioxidant activity, Free radical, Phenolic content, Phytochemical profile.

Introduction

Plant phenolics have potential health benefits mainly due to their antioxidant properties such as reactive oxygen species (ROS) scavenging and inhibition, electrophile scavenging and metal chelation [1]. Epidemiological studies support a relationship between the consumption of phenolic rich food products and a low incidence of coronary heart disease [2], atherosclerosis [3], certain forms of cancer [4] and stroke [5]. They have also been reported to exhibit pharmacological properties such as antitumor, antiviral, antimicrobial, anti-inflammatory, hypotensive and antioxidant activity [6-7]. Nevertheless, all aerobic organisms, including humans, have antioxidant defences that protect against oxidative harm and repair damaged molecules.

**Correspondence*
Dr. Sunita Dalal
Department of Biotechnology,
KurukshetraUniversity,Kurukshetra, Haryana**,** India**.**
Email:sdalal@kuk.ac.in

However, as natural antioxidant mechanisms can be inadequate, the supply of antioxidants through dietary ingredients is of great interest for a healthy life. A number of plants have been documented for their health promoting benefits [8]. The use of plant materials as a source of natural antioxidants and for other applications is important not only for food safety reasons, but also because they are natural and are more readily acceptable to consumers.

Trigonella foenum graecum belongs to the Family Leguminosae. Fenugreek is an annual, leguminous plant. The seeds of the plant are used as a spice and leaves are edible and used as vegetable. Seed is reported to have antidiabetic, antimicrobial, anticancer, antifertility, antiparasitic, lactation stimulant and hypocholesterolemic effects. Ethanol extract of Fenugreek leaf is an important source for antibacterial components and phenolic antioxidants [9]. As the antioxidant activity had been reported in the leaves of *T. foenum graecum*, the same activity may be found in the seeds of *T. foenum graecum* [10]. Therefore, the objectives of the present investigation were to evaluate

the antioxidant activity of various extracts of the seeds of *T. foenum graecum* by employing antioxidant assays and solvent systems in addition to provide data on total phenolics and phytochemical profile of the extracts.

MATERIALS AND METHODS

Collection and Identification of Plant Material

T. foenum graecum seeds were collected from Nakhrola village, District Gurgaon, Haryana, India, during the period 2011-2012. Further identification of the specimens was done from Department of Botany, Kurukshetra University, Kurukshetra. Authentication of plant material was done from Wild Life Institute of India, Dehradun with specimen number GS-413.

Preparation of Plant Extract

The seeds were carefully washed under running tap water followed by sterile water and shade dried for 4-5 days. The dried seeds were ground to powder and stored in airtight containers. Plants secondary metabolites possess various biological activities and methanol is capable of extracting a wide range of polar and rather non-polar compounds such as alkaloids, sterols, flavonoids and carbohydrates due to its high polarity therefore it was used for extraction. 10g of powdered leaves was soaked in conical flask containing 100ml of methanol for 24 hrs. Conical flask was allowed to stand for 30 mins in a water bath (at 100°C) with occasional shaking followed by keeping all the flasks on rotary shaker at 200 rpm for 24h [11]. Each preparation was filtered through a sterilized Whatman No. 1 filter paper and finally concentrated to dryness under vacuum at 40°C using a rotary vaccum evaporator. The dried extract, thus, obtained was sterilized by overnight UV- irradiation, checked for sterility on nutrient agar plates and stored at 4°C in refrigerator for further use [12].

Preparation of sub fractions

Sub fractions of the methanol extracts of *T. foenum graecum* were prepared in three solvents on the basis of increasing order of their polarity *i.e.* n-hexane, chloroform and ethyl acetate [13]. To prepare the sub fractions the methanol extracts of the plants were dissolved in hot water. The aqueous solution of methanol extract was transferred into a separating funnel for partitioning with n- hexane, chloroform and ethyl acetate successively. Each sub fraction was dried in rotary vaccum evaporator and stored in refrigerator for further use. Phytochemical investigation, total phenolic content and antioxidant potential of various extracts of *T. foenum graecum* seeds were determined.

Qualitative Phytochemical Analysis

The extracts were tested for the presence of bioactive compounds by using standard methods [14,15].

Flavonoids

Extract mixed with few fragments of magnesium turnings. Concentrated HCl was added drop wise. Appearnce of pink scarlet colour after few minutes indicates the presence of flavonoids.

Phenols and Tannins

The sample mixed with 2ml of 2% solution of $FeCl_3$. A blue-green or black coloration indicates the presence of phenols and tannins.

Saponins

5ml of distilled water mixed with extract in a test tube shaken vigorously. The formation of stable foam is taken as an indication for the presence of saponins.

Alkaloids

2ml of 1% HCl mixed with crude extract and heated gently. Mayer's and Wagner's reagent was added to the mixture. Turbidity of the resulting precipitate is taken as evidence for the presence of alkaloids.

Antioxidant assays

DPPH scavenging assay

The extracts were measured in terms of hydrogen donating or radical scavenging ability using the stable radical DPPH scavenging method [16]. Scavenging of 2,2-diphenyl-1-picryl-hydrazyl (DPPH) represents the free radical reducing activity of extracts based on a one-electron reduction. The reaction mixture contained 300 µl of extract of varying concentrations (1-100 µg/ml) and 2 ml of DPPH (0.1 mM in methanol). The reaction mixture was then placed in the cuvette holder of the spectrophotometer and the absorbance was measured at 517 nm against the blank (containing water instead of extract). The L-ascorbic acid was used as a positive control. The percent DPPH decolorization of the sample was calculated by the equation:

% inhibition = B_0-B_1/B_0×100

Where, B_0 is the absorbance of negative control and B_1 is the absorbance of reaction mixture.

The measurements of DPPH scavenging were carried out for three sample replication, and values were an average of three replicates. The decolorization was plotted against the concentration of sample extract in order to calculate the IC_{50} values, which is the amount of sample necessary to decrease the absorbance of DPPH by 50 %.

The VCEAC (Vitamin-C equivalent antioxidant capacity) was also calculated according to the formula given below:

ΔA = A (reference) – A (sample) in 600 sec.

$\Delta c = \Delta A / (\varepsilon * L)$

VCEAC = $\Delta c * V$ cuvette/ (2* mass of sample)

VCEAC = mmol/g

Where,

εDPPH = 12.5 dm3/(mmol*cm)

L (Length of cuvette) = 1 cm, A = Absorbance, V = Volume of cuvette

The per cent inhibition was also correlated with total phenolic content.

Chelating effect on ferrous ions

The chelating effect on ferrous ions was determined according to the method of Dinis *et al* [17]. Ferrozine quantitatively form complexes with Fe^{2+}. In the presence of chelating agents, the complex formation was disrupted, resulting in decrease of the red colored complex. Measurement of color reduction was the measure of the metal chelating activity. In brief, the extracts (0.25 ml) were mixed with 1.75 ml of methanol and 0.25 ml of 250 mM $FeCl_2$. This was followed by the addition of 0.25 ml of 2 mM ferrozine, which was left to react at room temperature for 10 minutes before determining the absorbance of the mixture at 562 nm. The chelating effect (%) was calculated from the formula as given for DPPH scavenging assay. The IC_{50} value was also calculated.

Determination of total phenolics

The total phenolic content (TPC) of the extracts was determined using Folin-Ciocalteu reaction [18]. Under alkaline conditions, Folin–Ciocalteu's (FC) phenol reagent (yellow color) reacts with phenolic compounds and, consequently, a phenolate anion is formed by dissociation of a phenolic hydrogen atom. This sequence of reversible one or two electron reduction reactions leads to blue-colored chromophores being formed between phenolate and the FC reagent. In brief, to 100 µl of extract (20 µg/ml) added 500 µl of (50 %) Folin-Ciocalteu reagent followed by the addition of 1 ml of 20 % Na_2CO_3 solution. After 20 minutes incubation at room temperature the absorbance was measured at 730 nm. The total phenolic content was expressed as gallic acid equivalents (GAE) in mg/g of samples.

Statistical analysis and correlations

The mean values and the standard deviations were calculated from the data obtained from three independent experiments. Statistical differences at $p < 0.05$ were considered to be significant coefficient of determination (r^2) to determine the relationship between two variables were calculated using Microcal Origin 5.0 and Microsoft Excel.

Results

Phytochemical Screening

The Phytochemical tests on various extracts of *T. foenum graecum* seeds showed the presence of various phytoconstituents like alkaloids, saponins, flavonoids, phenols and tannins (Table 1).

Table 1: Preliminary phytochemical screening of different extracts of *T. foenum graecum* seeds

Phytoconstituents	Ethyl acetate	Methanol	Chloroform	Hexane
Flavonoids	+	+	+	–
Phenols &Tannins	+	+	–	–
Saponins	+	+	+	+
Alkaloids	+	+	+	+

M: Methanol extract, EA: Ethyl acetate extract, "+" Present," – "Absent.

Antioxidant Assays

DPPH scavenging assay

The DPPH is a stable free radical at room temperature and accepts an electron or hydrogen radical to become a stable diamagnetic molecule [19]. Methanolic DPPH (0.1 mM) solution gives a violet color which shows maximum wavelength at 517 nm. When an antioxidant is mixed with this solution, the concentration of the stable free radical 2,2-diphenyl-1-picryl-hydrazyl or DPPH is reduced which can be detected by the decrease in the

optical absorbance at 517nm. DPPH radical has been widely used to test the free radical scavenging ability of various natural products and has been accepted as a model compound for free radicals originating in lipids. When the *T. foenum graecum* seeds extracts were tested for the DPPH free radical scavenging ability, the ethyl acetate extract at 100µg/ml showed highest radical scavenging activity (69.7%) followed by methanol (67.9%), chloroform (57.5%) and hexane extracts (50.9%) (Figure 1). The DPPH radical scavenging capacities were expressed as Vitamin-C Equivalent Antioxidant Capacity (VCEAC) in µmol/g of extract as Vitamin-C is a potent antioxidant and as used as standard in the present study. The VCEAC value of ethyl acetate extract was found to be maximum with a value of 0.00466 µmol/g (Table 2). Table 3 shows the IC_{50} values (µg/ml) of various extracts of *T. foenum graecum* extracts in the antioxidant activity evaluation assays. The lower the IC_{50} value the higher the free radical scavenging activity of the extract.

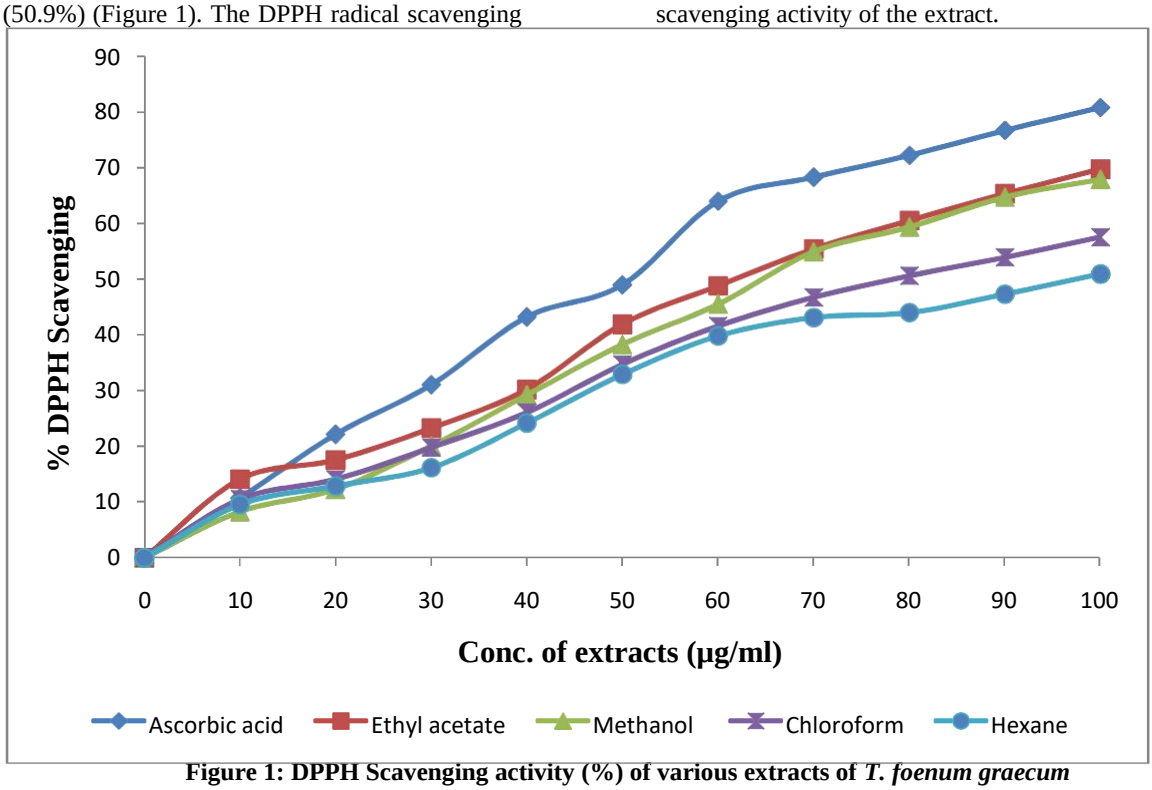

Figure 1: DPPH Scavenging activity (%) of various extracts of *T. foenum graecum*

Table 2: Vitamin C equivalent antioxidant capacity (VCEAC) of various seed extracts of *T. foenum graecum* of DPPH radical

	Extract VCEAC (µmol/g)			
Extracts (Conc.µg/ml)	Ethyl Acetate	Methanol	Chloroform	Hexane
10	0.00094	0.00056	0.0007	0.00064
20	0.00118	0.00082	0.00094	0.00086
30	0.00156	0.00134	0.00132	0.00108
40	0.00202	0.0018	0.00174	0.00162
50	0.0028	0.00256	0.00232	0.0022
60	0.00326	0.00304	0.00278	0.00266
70	0.0037	0.00358	0.00312	0.00287
80	0.00404	0.00396	0.00338	0.00294
90	0.00436	0.00432	0.0036	0.00316
100	0.00466	0.00454	0.00384	0.0034

Chelating effect on ferrous ions

The chelating effect of ferrous ions by various seed extracts of *T. foenum graecum* is shown in Figure 2. In this assay all the extracts interfered with the formation of ferrous and ferrozine complex, suggesting that they have chelating activity and are able to capture ferrous ion before the formation of ferrrozine. The per cent inhibition was found to be maximum (63.44%) in ethyl acetate extract whereas for hexane extract it was minimum (33.69%) at 100 µg/ml concentration. The IC_{50} values ranged from 67.429 to 135.455µg/ml at 100 µg/ml concentration (Table 3). The antioxidant potential evaluated by chelating power assay is also highly correlated to the total phenolic content showing a positive correlation in most of the extracts of the tested plant as represented by r^2 values (Table 5).

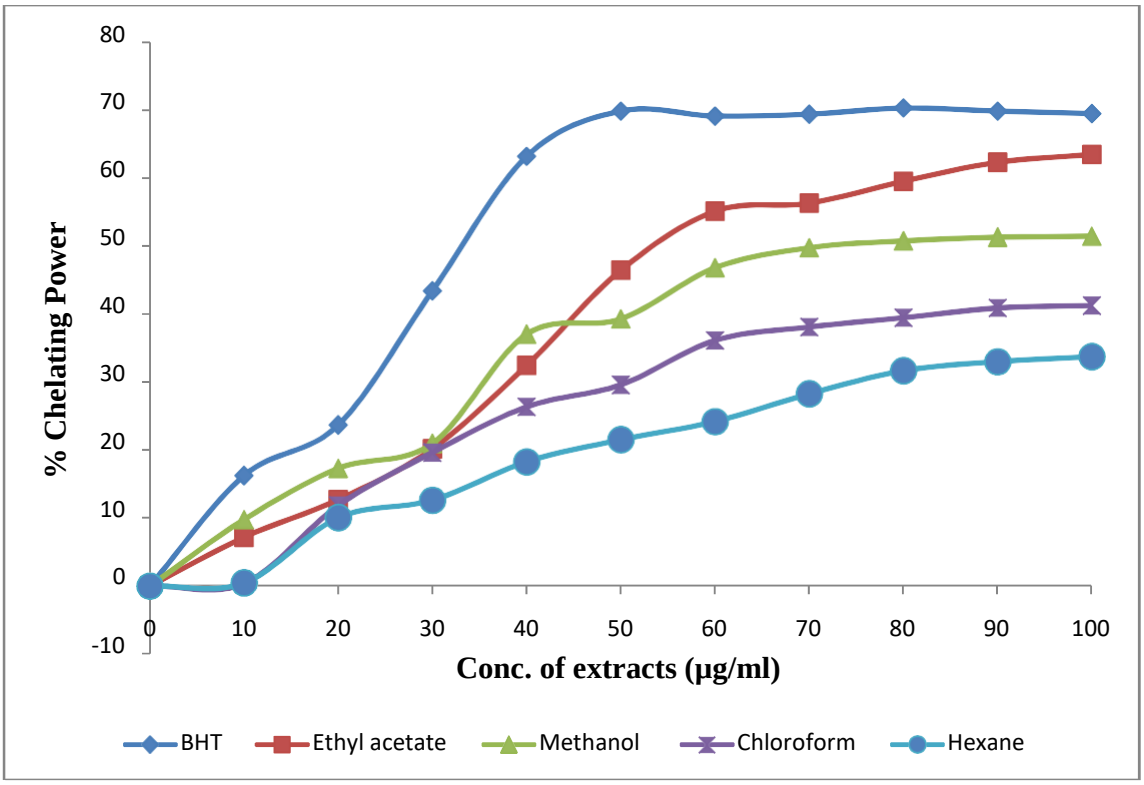

Figure 2: Ferrous ion chelating activity (%) of various extracts of *T. foenum graecum*

Table 3: IC_{50} values (µg/ml) of *T. foenum graecum* extracts in the antioxidant activity evaluation assays

T. foenum graecum extracts	DPPH (IC_{50}^{a})	Chelating effect on ferrous ion (IC_{50}^{b})
Ethyl Acetate	65.98	67.429
Methanol	68.96	80.756
Chloroform	79.02	105.315
Hexane	93.7	135.455
Positive control (Ascorbic acid)	62.877	-
Positive control (BHT)	-	43.944

a IC_{50} (µg/ml): effective concentration at which 50% of DPPH radicals are scavenged.
b IC_{50} (µg/ml): effective concentration at which 50% of ferrous ions are chelated.

Determination of total phenolics

In the present investigation, total phenolic content (TPC) was expressed as gallic acid equivalent. The highest total phenolic content was observed in ethyl acetate extract of *T. foenum graecum* (198 mg GAE/g dry weight of extract) followed by methanol (186 mg GAE/g dry weight of extract) (Table 4).

Table 4:Total phenolic content (TPC) of different extracts of the seeds of *T. foenum graecum* mg/g, as gallic acid equivalent (GAE)

	TPC			
Extracts (Conc.µg/ml)	**Ethyl acetate**	**Methanol**	**Chloroform**	**Hexane**
10	36	31	22	14
20	47	39	31	18
30	61	52	43	26
40	73	69	62	40
50	98	88	80	59
60	121	120	97	81
70	139	143	112	102
80	157	161	123	110
90	171	177	131	121
100	198	186	139	129

All the values are an average of three determinations and expressed as mean.

Correlation between total phenolic content antioxidant activity evaluation assays

There was a strong positive correlation (r^2 value ≥ 0.895) between the total phenolic content and antioxidant activity in the plant extracts (Table 5). The data of present study have shown that high total phenol content increases antioxidant activity and there is a linear correlation between phenolic content and antioxidant activity. There is a need to characterize phenolic compounds present within each plant extract to assign different antioxidant activities and to ascertain whether the phenolic structure affects antioxidant activity.

Table 5: r^2 value representing correlation between total phenolic content and per cent inhibition of *T. foenum graecum* extracts in the antioxidant activity evaluation assays

***T. foenum graecum* extracts**	**r^2 value**	
	DPPH	**Chelating effect on ferrous ion**
Ethyl Acetate	0.984	0.954
Methanol	0.985	0.975
Chloroform	0.999	0.924
Hexane	0.968	0.895

The results of present study revealed that ethyl acetate, methanol, chloroform and hexane extracts exhibited good antioxidant activity. Among the different solvent extracts used, ethyl acetate extract showed maximum antioxidant activity complemented with impressive total phenolic content.

Discussion

Keeping in mind the adverse effects of synthetic antioxidants, researchers have focused their interest in isolating antioxidants from nature directly. There is increasing evidence that indigenous antioxidants may be useful in preventing the deleterious consequences of oxidative stress and there is increasing interest in the protective biochemical functions of natural antioxidants contained in spices, herbs and medicinal plants [20].

There are numerous published methods for measuring total antioxidant capacity *in vitro*. No single method is sufficient; more than one type of antioxidant capacity measurement needs to be performed to take into account the different modes of action of antioxidants [21].

In an ethanol toxicity rat study, an aqueous extract of fenugreek seeds prevented the rise in lipid peroxidation and enhanced antioxidant potential [22]. These results are supported by *in vitro* evidence in diabetic human erythrocytes, that polyphenol acids from fenugreek seeds showed a concentration-dependent inhibition of lipid peroxidation [23]. In another study it was proved that higher the amount of the phenolic compounds and reducing power, higher the percent DPPH scavenging activity [24]. The results of the present study are in accordance with these findings.

Some publications reported that there is no correlation between the phenolic content and radical scavenging capacity [25]. The results obtained in present study do not support these claims. We found a strong correlation between total phenolic content and antioxidant activity. The data of present study are in accordance with others who have shown that high total phenol content increases antioxidant activity [26] and there is a linear correlation between phenolic content and antioxidant activity. The difference in the antioxidant activity of various extracts as evaluated by all the above antioxidant assays may be ascribed to the difference in the TPC as well as the phenolic compositions.

Conclusion

From the present work, it could be concluded that the solvent play a vital role in the extraction of the plant constituents. Significant variations were found in total phenolic content, antioxidant activity and release of phytochemicals depending on the solvent. As methanol and ethyl acetate are highly polar among the solvents used therefore, they contain high yield of phenolic compounds as compared to the other solvents. The ethyl acetate extract of fenugreek seeds was shown highest antioxidant activity (% DPPH scavenging activity). The antioxidant activity could be correlated with the polyphenolic components present in the extract. The results obtained from these methods provide some important factors responsible for the antioxidant potential of fenugreek seeds. Further studies will be focused on cytotoxic effects, fractionation and purification of active components in extracts of fenugreek seeds. This will most likely improve the antioxidant activity and other potential health benefits, promoting their use as natural antioxidant source.

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