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Antioxidative Activity of Extracts from Fenugreek Seeds (*Trigonella foenum-graecum)*

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Abstract

Spices and herbs possess antioxidant activity and can be applied for preservation of lipid peroxidation in biological systems. Fenugreek **(***Trigonella foenum-graecum)* is an important spice; its dried seeds have wide application in food and beverages as a flavoring additive as well as in medicines. Crude extracts of fenugreek were prepared by soxhelt extraction method with different solvents such as methanol, ethanol, dichloromethane, acetone, hexane and ethyl acetate. Extracts were subjected for the measurement of total phenolic content (TPC) by Folin-Ciocalteu method as well as flavonoid content, chelating activity, reducing power and antioxidant/radical scavenging activity [1,1-diphenyl-2-picryl-hydrazyl (DPPH°) free radical scavenging activity]. 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: Fenugreek (*Trigonella foenum-graecum*), Antioxidant activity, Phenolic contents, Flavonoids.

Introduction

Herbs and spices have been extensively used as food additives for natural antioxidants. Spices and aromatic herbs are considered to be essential in diets or medical therapies for delaying aging and biological tissue deterioration [1]. The search for synthetic antioxidants as alternatives to naturally occurring antioxidants is of great interest both in industry as well as in scientific research [2]. The antioxidant property of the plant material is due to the presence of many active phytochemicals including vitamins, flavonoids, terpenoids, carotenoids, cumarins, curcumins, lignin, saponin, plant sterol and etc [2-6].

Fenugreek (Trigonella foenum graecum) is an annual herb that belongs to the family Leguminosae widely grown in Pakistan, India, Egypt, and Middle Eastern countries [7]. Due to its strong flavor and aroma fenugreek in one of such plants whose leaves and seeds are widely consumed in Indo-Pak subcontinent as well as in other oriental countries as a spice in food preparations, and as an ingredient in traditional medicine. It is rich source of calcium, iron, β-carotene and other vitamins [8]. Both leaves and seeds should be included in normal diet of family, especially diet of growing kids, pregnant ladies, puberty reaching girls and elder members of family because they have haematinic (i.e. blood formation) value [9]. Fenugreek seed is widely used as a galactagogue (milk producing agent) by nursing mothers to increase inadequate breast milk supply [10]. The seeds of fenugreek contain lysine and L-tryptophan rich proteins, mucilaginous fiber and other rare chemical constituents such as saponins, coumarin, fenugreekine, nicotinic acid, sapogenins, phytic acid, scopoletin and trigonelline, which are thought to account for many of its presumed therapeutic effects, may inhibit cholesterol absorption and thought to help lower sugar levels [11-13]. Therefore, fenugreek seeds are used as a traditional remedy for the treatment of diabetes and hypercholesterolemia in Indian and Chinese medicines [14,15]. It's reported to have restorative and nutritive properties and to stimulate digestive processes, useful in healing of different ulcers in digestive tract [16]. Fenugreek has also been reported to exhibit pharmacological properties such as antitumor, antiviral, antimicrobial, anti-inflammatory, hypotensive and antioxidant activity [17, 18].

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The purpose of this study was to evaluate fenugreek as new potential source of natural antioxidants. In this study, the extracts of fenugreek were prepared in methanol, ethanol, dichloromethane, acetone, hexane and ethyl acetate by soxhelt continuous extraction; because organic solvents have different polarity and therefore have different nature to extract the compounds. The antioxidant activity of the extracts was assessed by modification of established assays, such as total phenolic content by Folin-Ciocalteu reagent; total flavonoids content, chelating activity by 2, 2' bipyridyl competition assay; antioxidant activity as free radical scavenging by DPPH˚ and reducing power.

Material and Methods

Chemicals and Reagents

Folin-Ciocalteu reagent, methanol, ethanol, dichloromethane, acetone, hexane and ethyl acetate were purchased from (E. Merck). Ferrous sulphate, Disodium ethylenediaminetetraacetate (Na_2EDTA), and butylated hydroxyanisole (BHA), were purchased from (Fluka Riedel-de Haën). Quercetin, Gallic acid, 2, 2′-bipyridyl, HCl and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were purchased from (Sigma-Aldrich GmbH, Germany). All other chemicals and solvents were of the highest analytical grade and used as supplied.

Plant Material and Extraction procedures

A fenugreek seed sample was collected from the local market of Hyderabad, Pakistan. Fenugreek seed subjected to various treatments for investigation of antioxidant potential. Extraction was carried out by the reported procedure [19]. Dry fenugreek seed (10 g) was cleaned and ground into small pieces by a waring blender and passed through a 1-mm sieve. Methanol, ethanol, dichloromethane, acetone, hexane and ethyl acetate (each 150 ml) were used for extraction by soxhelt extraction method for six hours. The extracts were filtered. The residue was re-extracted twice under the same condition to ensure complete extraction. The extracts were combined, filtered and evaporated to dryness under reduced pressure at 60 ^{o}C by a rotary evaporator. Extracts were placed in dark bottle, and stored at -8 °C until further analysis.

Yield Estimation

Yield was estimated by reported method [20]. Each extract (10 ml) was measured into a pre-weighed aluminum dish. The samples were kept in an oven at 85 °C for 24 hours then followed by placing in desiccator for 12 hours. The weight difference was used to calculate percentage yield as well as expressed in mg/10 ml.

Determination of Total Phenolic Content (TPC)

TPC in different solvent extracts of fenugreek seeds was determined spectrophotometrically following Folin-Ciocalteu method described previously [21, 22] with minor modification. The appropriate dilution of extract 200 µl oxidized with 1 ml of Folin-Ciocalteu reagent, and then the reaction mixture was neutralized with saturated 2 ml of 7.5 % sodium carbonate (w/v). The final mixture volume was brought up to 7 ml with deionized water. The absorbance of the resulting blue color was measured at 765 nm on UV-Vis. spectrophotometer with a 1 cm cell after incubation for 2 hours in dark at room temperature. Gallic acid was used as a standard for the calibration curve. The phenolic compound content was determined as gallic acid equivalents using the following linear equation based on the calibration curve.

$$A = 0.1786\ C - 0.1739,\ R^2 = 0.999$$

A is the absorbance, and C is gallic acid equivalents (mg).

Determination of Total Flavonoid Content

The total flavonoid content was measured by using previously reported colorimetric assay [23] with minor modifications. Briefly 1ml of appropriately dilute sample was added to a 10ml of volumetric flask containing 4 ml of distilled water followed by immediate addition of 0.6 ml of 5% $NaNO_2$, 0.5 ml of 10% $AlCl_3$ after 5 min, and 2 ml of 1 M NaOH after 1 min. Furthermore, each reaction flask was then immediately diluted with 2.4 ml of distilled water and mixed. The absorbance of pink colored solution was noted at 510 nm. The quercetin (µg/g) was used as a standard for the calibration curve. The total flavonoid content of the samples was calculated by using the following linear equation based on calibration curve.

$$Y = 0.0205X - 1494, \qquad r = 0.9992$$

Y is the absorbance, and X is the flavonoid content in µg g^{-1}.

Chelating Activity

Chelating activity (Fe^{2+}) was measured by 2, 2′-bipyridyl competition assay [24]. The reaction mixture containing 0.25 ml of of $FeSO_4$ solution (1 mM), 0.25 ml of antioxidant solution, 1 ml of Tris-HCl buffer (pH 7.4), 1 ml 2,2′-bipyridyl solution (0.1% in 0.2 M HCl) and 2.5 ml of ethanol. The final volume was made up 6.0 ml with distilled water. The absorbance was measure at 522 nm and used to evaluate Fe^{+2} chelating activity using disodium ethylenediamine-tetracetate (Na_2EDTA) as a standard**.**

Measurement of Antioxidant Properties

Reducing Power Ability (RPA)

The reducing power of fenugreek extracts was quantified by the method described previously [25] with minor modification. Fenugreek extract (0, 1.0, 2.0, 3.0, 5.0, 7.0, 9.0, 11.0 mg) in 1 ml of 80% methanol were mixed with phosphate buffer (5.0 ml, 2.0 M, pH 6.6) and potassium ferricyanide (5.0 ml, 1.0%) ; the mixtures were incubated at 50 ˚C for 20 min. A portion (5.0 ml) of trichloroacetic acid (10%) was added and the mixture was centrifuged at 3000 rpm for 10 min. The upper layer of the solution (5.0 ml) was mixed with distilled water (5.0 ml) and ferric chloride (1.0 ml, 0.1%), and than absorbance of the pink color mixture was measured spectrophotometrically at 700 nm. Increased absorbance of the mixture indicates increased reducing power. The experiment was conducted in triplicate and results were averaged.

Free Radical Scavenging (FRS) Activity

Free radical scavenging capacity of fenugreek extracts was determined according to the previous reported procedure using the stable 2, 2-diphenyl-1-picrylhydrazyl radical ($DPPH^o$) [26, 27]. Briefly, a freshly prepared DPPH° solution in ethanol (0.5 ml) was added to 3 ml of diluted each fenugreek extract to start the radical antioxidant reaction. The final concentration was 100 µM for DPPH°. The decrease in absorbance was measured at different intervals, i.e. 0, 0.5, 1, 2, 5, 10 and 15 min. up to 50% at 517 nm. The remaining concentration of $DPPH^o$ in the reaction mixture was calculated from a standard calibration curve. The absorbance measured at 5min of the antioxidant-$DPPH^o$ radical reaction was used to compare the $DPPH^o$ radical scavenging capacity of each fenugreek extract.

$$\% \text{ of DPPH remaining} = [DPPH]_T / [DPPH]_{T=0} \times 100$$

Where T is the time interval.

Statistical Analysis

Three replicates of each sample were used for statistical analysis. Data were reported as means ± S.D. Analysis of variance and least significant difference tests were conducted to identify differences among means. Statistical significance was declared at $P<0.05$.

Results and Discussion

The yields of the extracts obtained by the soxhelt method were calculated as percent by weight of the fenugreek seed. According to the chemical composition and polar nature of phenolic compounds, fenugreek contains a relatively high percentage yield in ethanol and methanol while lower in hexane. Percentage yields in ethanol and methanol are comparable but have slight difference as shown in Table 1**.**

Table 1. **Percentage yield of fenugreek extract in different organic solvents as well as in mg/10ml.**

Organic solvents	Yield (mg/10ml)	%yield of fenugreek extract
Methanol	64.72	25.89
Ethanol	63.3	25.32
Dichloro methane	32.4	12.96
Acetone	44.1	17.65
Hexane	24.2	9.68
Ethyl acetate	40.3	16.13

Data are means (n = 3) ± SD (n = 3), ($p<0.05$)

The phenolic compounds may contribute directly to the antioxidant action [28]; therefore, it is necessary to investigate total phenolic content. The total phenolic content was determined by following a modified Folin-Ciocalteu reagent method. In Table 2 the results were expressed as gallic acid equivalent**.** TPC was in the range of 1.35-6.85 mg/g of the fenugreek extract. The amounts of total phenolic compounds were higher in ethanol extract 6.85 mg/g while lowest for hexane 1.35 mg/g. Using a standard curve of gallic acid (R^2= 0.999). All results coincide with those of total antioxidant capacity. In other words, the spice extract sample shows a tendency to have high phenolic content.

Table 2. **Total phenolic content (TPC), flavonoid content (FC) and chelating activity of organic solvent extracts of fenugreek expressed as gallic acid, quercetin and Na_2EDTA equivalent, respectively.**

Sample	TPC Gallic Acid eq. (mg/g of fenugreek)	FC Quercetin eq. (µg/g of fenugreek)	Chelating Activity EDTA eq (µg/g of f enugreek)
Methanol	5.75 ± 0.002	607. ± 3.6	1021 ± 1.7
Ethanol	6.85 ± 0.002	653 ± 4.3	1098 ± 2.4
Dichloromethane	2.27 ± 0.003	234 ± 3.5	633 ± 2.3
Acetone	4.04 ± 0.004	416 ± 2.7	982 ± 2.1
Hexane	1.35 ± 0.002	208 ± 4.2	557 ± 3.2
Ethylacetat	3.32± 0.004	251 ± 3.3	838 ± 2.8

Data are mean (n = 3) ± Standard deviation (n=3), ($p<0.05$), TPC = Total phenolic content.

Using the $AlCl_3$ reagent and quercetin as standard (R_2 = 0.9996), the total flavonoids are in the range from 208-653 µg/g of quercetin equivalent (Table 2). The highest value for the ethanol was 653 µg/g and the lowest was 208 µg/g of the fenugreek with the following decreasing order of the extract *ethanol> methanol> acetone> ethyl acetate> dichloromethane> hexane*. Flavonoids are not easily detectable therefore, in the extract $AlCl_3$ was used as complexing reagent.

The chelating activity was measured against Fe^{2+} and reported as EDTA equivalents as shown in Table 2. The difference in chelating activity was observed among the extract. The highest chelating activity was observed in ethanol. The EDTA equivalent was in the range of 1098-557 µg/g of fenugreek extract. According to Ilhami et al. [29] metal chelating capacity is significant since it reduces the concentration of catalyzing transition metal in lipid peroxidation. Moreover, the chelating agents, which form σ-bonds with a metal, are effective as secondary antioxidants, because they reduce the redox potential thereby stabilizing the oxidized form of the metal ion, therefore it is an important parameter. The results from this parameter were in agreement with total phenolic contents, the highest chelating activity were found in ethanol (1098 µg/g of extract) while lowest in hexane (557 µg/g of extract).

Antioxidant capacity

The antioxidant capacity of the fenugreek extracts were analyzed by using the free radical scavenging ($DPPH^o$) (Fig. 1) and the ferric reducing antioxidant power (FRAP) methods (Fig. 2).

The $DPPH^o$ test is the oldest indirect method for determining the antioxidant activity, which is based on the ability of the stable free radical 2, 2-diphenyl-1-picrylhydrazyl to react with hydrogen donors including phenols [30].

Radical scavengers may directly react and quench with peroxide radicals to terminate the peroxidation chain reaction and improve the quality and stability of food product. The stable DPPH radical has been used to evaluate antioxidants for their radical quenching capacity [31,32] and to better understand their antioxidant mechanism(s) each fenugreek extract was evaluated for radical scavenging activity against $DPPH^o$. The decrease in absorbance of DPPH radical is caused by antioxidant through the reaction between antioxidant molecule and radical results in the scavenging of the radical by hydrogen donation. As Fig. 1a illustrates a significant ($p<0.05$) decrease in the concentration of $DPPH^o$ due to scavenging activity of fenugreek extract.

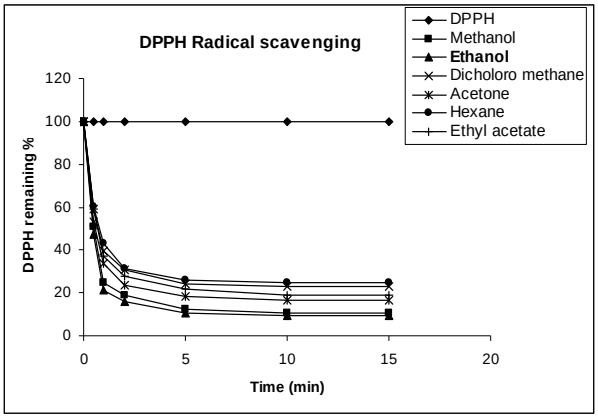

Figure 1 a. **Kinetic behavior of radical scavenging activity of cumin extracts as assayed by the $DPPH^\circ$ method. The final DPPH concentration was kept 100 µM in all reaction mixtures. Values are mean (n =3), ($P < 0.05$).**

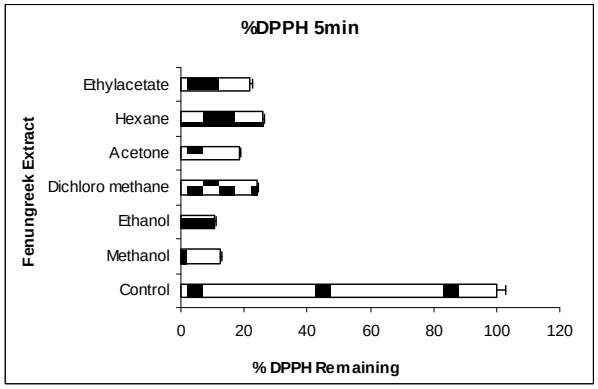

Figure 1 II. **DPPH radical scavenging activity of fenugreek extract at 5 min. Vertical bars represents the standard deviation of each data point. Values are mean (n = 3), ($P < 0.05$).**

Kinetic studies of $DPPH^o$-extract reaction were carried out to estimate scavenging activity as a function of time. Scavenging activity was nearly the same at first minute of reaction and diverges with the increase in time. Maximum difference among the extract was observed at 5 min of the reaction and the remaining amount (%) of $DPPH^\circ$ radical at 5 min after initiation of reaction as shown in Figure 1b was 10.88, 12.42, 24.04, 18.38, 25.77 and 21.97 for ethanol, methanol, dichloromethane, acetone, hexane and ethyl acetate respectively. The high amount of the phenolic compounds and reducing power having the highest percent $DPPH^\circ$ scavenging activity was shown by the ethanol extract, and the second highest activity was determined in the methanol while lowest in hexane.

It has been reported by Yildirim et al and Siddhuraju et al [33, 34] that the reducing power of bioactive compounds is associated with antioxidant activity. Thus, it is necessary to determine the reducing power of phenolic constituents to elucidate the relationship between their antioxidant effects and there reducing power [35]. The reducing power of the extracts increases with an increase in the amount of the extract as shown in Fig. 2. The amount of the phenolic compounds was high in ethanol extract of fenugreek, therefore; similar results were obtained in reducing power activities. Hence, by correlating these results; we can suggest that there may be relationship between the amount of total phenolic content and reducing power.

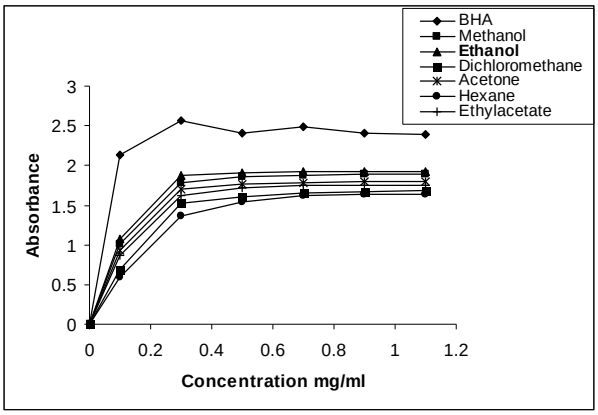

Figure 2. **Reducing power of ethanol, methanol, dichloromethane, acetone, hexane and ethyl acetate extract of fenugreek. All data is reported as mean ± S.D (n = 3) statistically significant as P < 0.01.**

The reducing power of the extracts was compared with a known reducing agent BHA. The reducing power of the extract was markedly lower than that of BHA. However, among these extracts the ethanol extract of fenugreek has shown the highest reducing power. According to Shimon et al. [36] the fenugreek has volatile oil, phenolic acids and flavonoids; therefore it is a potent source of antioxidants.

Conclusion

From the present work, it could be concluded that the solvent play a vital role in the extraction of the plant constituents. As methanol and ethanol are highly polar among the solvents used therefore, they contain high yield of phenolic compounds as compared to the other solvents. An ethanolic 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.

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