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

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Abstract

Spices and herbs posses' antioxidant activity can be applied for preservation of lipid peroxidation in biological systems. Fenugreek **(***Trigonella foenum-graecum)* is an important spice and aromatic crop its dried seeds having the wide application in a food, a flavoring, beverages and a medicine. methanol, ethanol, dichloromethane, acetone, hexane and ethyl acetate crude extracts of Fenugreek were prepared by soxhelt extraction method. Extracts were investigated for there antioxidant and radical scavenging activities by different methods such as measurement of total phenolic content (TPC) by Folin-Ciocalteu method, flavonoid content, chelating activity, reducing power and 1, 1-diphenyl-2-picryl-hydrazyl (DPPH°) free radical scavenging activity. All extract of the fenugreek exhibited antioxidant activity. These finding suggest that the fenugreek extract could act as a potent source of antioxidants. Results from different parameter were in agreement with one another.

Keywords: Fenugreek, antioxidant activity, phenolic contents, flavonoids, radical scavenging activity.

Introduction

Herbs and spices have been extensively used as natural food additives. Since then the search for antioxidants naturally occurring in plant as alternatives to synthetic antioxidant is of great interest both in the industry and in scientific research by Ford et al. [1]. These properties are due to many active phytochemicals including vitamins, flavonoids, terpenoids, carotenoids, cumarins, curcumins, lignin, saponin, plant sterol etc by Lucia et al, Madsen and Bertelsen, Nielsen et al, Marja, et al, Zheng and Wang [2-6]. Spices and aromatic herbs are considered to be essential in diets or medical therapies for delaying aging and biological tissue deterioration due to free radicals Lucia et al. [2].

Fenugreek (Trigonella foenum graecum) is an annual herb that belongs to the family Leguminosae widely grown in Pakistan, India, Egypt, and Middle Eastern countries by Alarcon-Aguilara et al. [7]. Fenugreek in one such plant whose leaves and seeds are widely consumed in Pakistan, India and other oriental countries as a spice in food preparations due to their strong flavor and aroma, and as an ingredient in traditional medicine. It is rich source of calcium, iron, β-carotene and other vitamins by Sharma et al. [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 by Oday [9]. Fenugreek seed is widely used as a galactagogue (milk producing agent) by nursing mothers to increase inadequate breast milk supply by Fleiss [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 levers by Billaud, Sauvaire et al and Sauvaire et al. [11-13]. Therefore, fenugreek seeds are used as a traditional remedy for the treatment of diabetes and hypercholesterolemia in Indian and Chinese medicine by Basch et al and Miraldi et al. [14,15]. Its reported to have restorative and nutritive properties and to stimulate digestive processes, useful in healing of different ulcers in digestive tract by Khosla et al. [16]. Fenugreek have also been reported to exhibit

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pharmacological properties such as antitumor, antiviral, antimicrobial, anti-inflamatory, hypotensive and antioxidant activity by Cowan and Shetty [17, 18].

The purpose of this study was to evaluate fenugreek as new potential source of natural antioxidant. 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 were assessed by modification of established assays, such as total phenolic content by Folin- ciocaltue reagent; total flavonoids content, chelating activity by 2, 2ʾ 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 form (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, 2, 2-diphenyl-1-picrylhydrazyl (DPPH), were purchased from (Sigma-Aldrich GmbH, Germany). All other chemicals and solvents were of the highest commercial grade and used after further purification.

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 Lim et al. [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 over whatman no: 5 and residue was re-extracted twice under the same condition to ensure complete extraction. The extract were combined, filtered through whatman no: 5, 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 estimated by Prashani et al. [20]. Each extract (10ml) 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 /10ml.

Determination of Total Phenolic Content

TPC in different solvent extracts of fenugreek seed was determined spectrophotometrically following Folin-Ciocalteu method describe previously Iqbal et al and Liangili et al. [21, 22] with minor modification. The appropriate dilution of extract 200µl oxidized with 1ml of Folin-Ciocalteu reagent, and then the reaction mixture was neutralized with saturated 2ml, 7.5% sodium carbonate (w/v). The final mixture volume was brought up to 7ml with deionized water. The absorbance of the resulting blue color was measured at 765 nm on the Perkin-Elmer Lambda-2 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 Zhishen et al. [23] with minor modifications. Briefly 1ml of appropriately dilute sample was added to a 10ml of volumetric flask containing 4ml of DDH_2O followed by immediate addition of 0.6ml of 5% $NaNO_2$, 0.5ml of 10%$AlCl_3$ after 5min, and 2ml of 1M NaOH after 1min. Further each reaction flask was then immediately diluted with 2.4ml of DDH_2O and mixed. Absorbance of resulted pink colored solution was noted with Perkin-Elmer Lambda- 2 Spectrophotometer, with a 1cm cell, at 510nm. The Quercetin (µg/g) was used as a standard for the calibration curve. The total flavonoid content of the samples was calculated using the following linear equation based on calibration curve.

$$Y = 0.0205X - 1494,\ R^2 = 0.9992$$

Y is the absorbance, and X is the flavonoid content in µg/g.

Chelating Activity

Fe^{+2} chelating activity was measured by 2, 2´-bipyridyl competition assay Re at al. [24]. The reaction

mixture contain 0.25 ml of 1mM of $FeSO_4$ solution, 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 ethylenediaminetetracetate (Na_2EDTA) as a standard.

Measurement of Antioxidant Properties

Reducing Power Ability (RPA)

The reducing power of fenugreek extracts was quantifies by the method described previously by Perumal and Klaus. [25] with minor modification. Fenugreek extract (0, 1.0, 2.0, 3.0, 5.0, 7.0, 9.0, 11.0 mg) in 1ml 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 read 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 fnugreek extracts was determined according to the previous reported procedure using the stable 2, 2-diphenyl-1-picrylhydrazyl radical (DPPH˚) Ali et al and Marina et al. [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 100µM 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˚ in the reaction mixture was calculated from a standard calibration curve. The absorbance measured at 5min of the antioxidant-DPPH radical reaction was used to compare the DPPH radical scavenging capacity of each fenugreek extract.

% of DPPH remaining= $[DPPH]_T / [DPPH]_{T=0} \times 100$ 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 at among means. Statistical significance was declared at $P<0.05$.

Results and Discussion

The yields of the extracts obtained by the Soxhelt were calculated as percent by weight of the fenugreek seed. According to the chemical composition, fenugreek contains a relatively high percentage yield in ethanol and methanol because of the polar nature of phenolic compound while lower in hexane. Ethanol and methanol are comparable there is slightly 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	64.72mg	25.89%
Ethanol	63.3mg	25.32%
Dichloro methane	32.4mg	12.96%
Acetone	44.1mg	17.65%
Hexane	24.2mg	9.68%
Ethyl acetate	40.3mg	16.13%

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

The phenolic compounds may contribute directly to the antioxidant action Awika et al. [28] therefor it is necessary to investigate total phenolic contents. The total phenolic content was determined by following a modified method Folin-Ciocalteu reagent method. In Table 2 the results were expressed as gallic acid equivalent**.**

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.

TPC was in the range of 1.35-6.85 mg/g of fenugreek extract. The amounts of total phenolic compounds were higher in ethanol extract 6.85mg/g while lowest for hexane 1.35mg/g of fenugreek. Using a standard curve of gallic acid (R^2= 0.999). All results coincided with those of total antioxidant capacity. In other words, the spice extract sample showed a tendency to have high phenolic content.

Using the AlCl3 reagent and quercetin as standard (R2 = 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 653µg/g and the lowest was 208µg/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 extract using $AlCl_3$ 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 1098-557µg/g of fenugreek extract. According to Ilhami et al. [29] Metal chelating capacity was significant since it reduced the concentration of the catalyzing transition metal in lipid peroxidation. It was reported that chelating agents, which form σ-bonds with a metal, are effective as secondary antioxidant because they reduce the redox potential thereby stabilizing the oxidized from the metal ion, therefore it is an important parameter. The result from this parameter were in agreement with total phenolic contents, the highest chelating activity were found in ethanol (1098µg/g of extract) while lowest in hexane (557µg/g of extract).

Antioxidant capacity

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

The DPPH 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 Roginsky and Lissi [30]

Radical scavengers may directly react with and quench 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 capacities by Brand-Williams et al, Hong and Chi-Tang, [31,32] to better understand their antioxidant mechanism(s) each fenugreek extract was evaluated for radical scavenging activities against DPPH˚. The decrease in absorbance of DPPH radical caused by antioxidants, because of the reaction between antioxidant molecule and radical progresses, which results in the scavenging of the radical by hydrogen donating. As **Figure 1a** illustrates a significant ($p<0.05$) decrease the concentration of DPPH˚ due to scavenging activity of fenugreek extract.

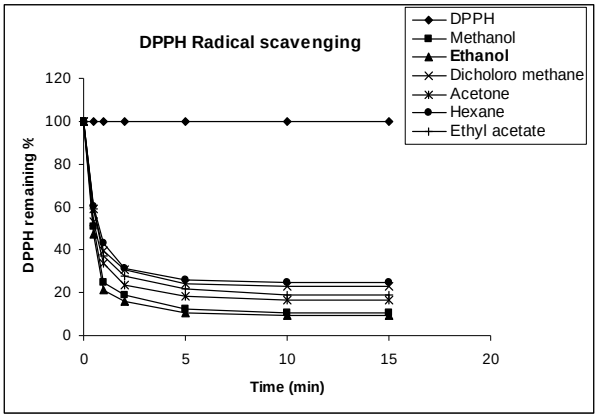

Figure 1 a. **Kinetic behavior of radical scavenging activity of cumin extracts as assayed by the DPPH˚ method. The final DPPH concentration was kept 100 µM in all reaction mixtures. Values are mean (n =3), (P < 0.05)**

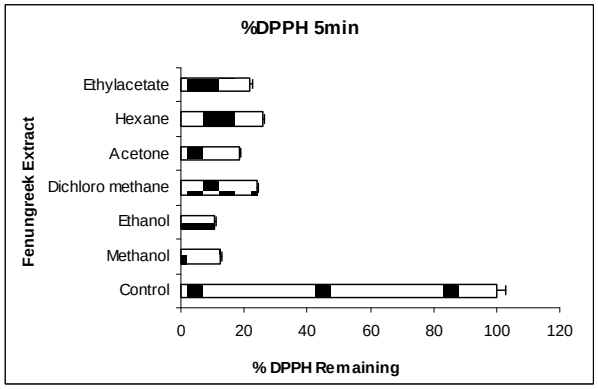

Figure 1 II. **DPPH radical scavenging activity of fenugreek extract at 5 min. Vertical bars represents the standard deviation of each data point. Values are mean (n = 3), (P < 0.05).**

Kinetic studies of DPPH˚- 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° 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. The high amount of the phenolic compounds and reducing power having the highest percent DPPH° 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 by Shan et al. [35]. The reducing power of the extracts increased with an increase the amount of the extract. As in **Fig. 2**. The amount of the phenolic compounds was high in ethanol extract of fenugreek; similar results were obtained in reducing power activities. Hence correlate these results; we can suggest that there may be relation ship between the amount of total phenolic content and reducing power.

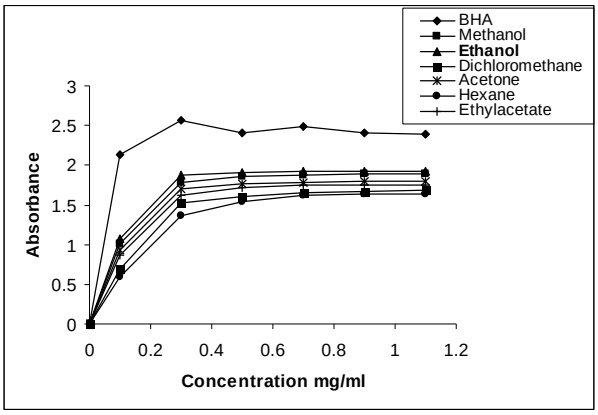

Figure 2. **Reducing power of ethanol, methanol, dichloromethane, acetone, hexane and ethyl acetate extract of fenugreek. All data is reported as mean ± S.D (n = 3) statistically significant as P < 0.01.**

The reducing power of there extracts was compared with a known reducing agent BHA. The reducing powers of the extract were 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. It reported as a potent source of antioxidant.

Conclusion

From the present work, it could be concluded that the solvent play a vital role in the extraction of the constituents. As methanol and ethanol are highly polar among the solvent therefore they contain the high yield of phenolic as compared to the other solvents. An ethanolic extract of fenugreek seeds was examined for its antioxidant activity. The antioxidant activity could be correlated with the polyphenolic components present in the extract. The results gained by these methods provide some important factors responsible for the antioxidant potential of fenugreek seeds.

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