Original Article

Phytochemical Screening, Antioxidant Activity, and Chromatographic Fractionation of Fenugreek (Trigonella Foenum Graecum) Seeds Extract

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Abstract

Medicinal plants are an excellent therapy option since they contain bioactive ingredients that have been utilized for centuries. India, the Mediterranean, France, Russia, North Africa, Germany, Yemen, and Russia all cultivate fenugreek (Trigonella foenum graecum) for human use. Many nations utilize fenugreek leaves and seeds medicinally. This research aimed to investigate the phytochemistry, antioxidant capabilities, and fractionation of fenugreek seed extract by employing a silica-gel-based glass column. The High-Performance Liquid Chromatography (HPLC) technique investigated each fraction. Polyphenolic (33.58 mg GAE/g), flavonoid (16.11 mg QE/g), flavonol (21.81 mg RE/g), and anthocyanin (1.87 mg cyanidin-3-glucoside/kg dry extract) concentrations were high. The extract exhibited ferric reduction and chelating metal properties, including 11.07 mg $FeSO_4$/g and 76.35 mg Fe^{2+}/g dry weight. Fenugreek seed had a high antioxidant capacity and inhibited α-amylase activity with high efficiency (84.21%). Column chromatography was run using silica gel and ethyl acetate-toluene-formic acid (100:36:36) as the solvent system, separating four fractions. Each collected fraction was tested using thin-layer chromatography (TLC). The crude extract and fractions were evaluated using HPLC, revealing that fenugreek seed extract and fractions are highly natural antioxidants.

Index Terms— antioxidant activity, chromatographic fractionation, fenugreek, phenolics.

INTRODUCTION

Plant-based systems have been pivotal in primary health care since antiquity in 80% of the world's poor and developing countries [1]. Compared to synthetic medications, herbal ones are generally considered less harmful and have fewer adverse effects [2]. Plants have long been utilized as traditional natural remedies to treat a wide range of illnesses, and they provide a safe alternative to modern pharmaceuticals [3].

The fenugreek plant, also known as Trigonella foenum-graecum L., is a member of the Fabaceae family and is most often cultivated in Germany, France, India, northern Africa, and Russia. Its original home was in the Near East and Mediterranean areas. Despite the limited opportunities for breeding and testing in the southern hemisphere, at least two cultivars have been made available in Canada. Multiple samples from the Midwest have been analyzed [4].

Asian, African, and Mediterranean people eat the seeds of this plant as part of their everyday diet. It is employed in various areas, including medicine, nutrition, drinks, perfumes, cosmetics, and more [5]. An excellent source of protein, the seeds also include inaccessible carbohydrates, mucilage, and saponins. Compared to other legumes, the seed is richer in minerals (Ca, P, Fe, Zn, and Mn) [3]. The seeds include flavonoids, carotenoids, and coumarins, among other compounds [6]. Bioactive phytochemicals such as flavonoids are linked to fenugreek's antioxidant and anti-inflammatory properties. The antioxidant and anti-inflammatory properties of flavonoids are the most well-recognized benefits of this class of compounds [7].

For medicinal purposes, the seeds may be employed in cooking or as a flavour enhancer for other treatments (Fig. 1) [8]. Fenugreek seeds are widely used in folk medicine to cure various ailments, including paralysis, diabetes, epilepsy, and fever [9]. Traditionally, fenugreek was suggested to breastfeeding mothers to boost milk production and, curiously, to Moroccan Saharawi women to enhance their physical beauty by increasing their appetite [10].

Fig. 1: Seeds of Trigonella foenum-graecum with leaves

There are sapogenin, galactomannans, antioxidants, and amino acids like 4-hydroxy isoleucine in fenugreek seeds. These ingredients possess sugar-lowering, cholesterol-lowering, fever-reducing, anti-inflammatory, cytotoxic, apoptosis-activating, antifertility properties [11], antifungal, antibacterial, and anticarcinogenic [10]. The phytochemical screening and antioxidant activity of fenugreek seed extract were examined in this work after the isolation of bioactive components. The first investigation employed TLC to find an acceptable mobile phase, followed by column chromatography to generate four fractions, and finally HPLC to analyze the fractions that had been separated.

EXPERIMENTAL SECTION

A. Chemicals

In this investigation, chemicals and solvents of analytical grade were utilized. Both quercetin and EtOAc were purchased from Sigma-Aldrich (Germany). The sodium dihydrogen phosphate and hexane were obtained from Carlo Erba, Sabadell (Spain). ISOLAB GmbH obtained gallic acid hydrochloride. Rutin trihydrate was bought from (Dr. EhrenstorferTM). Thin-layer chromatography (TLC) aluminum sheets 20 x 20 cm (60 F254), ferrous sulfate heptahydrate, aluminum chloride, iodine, Silica gel for column chromatography (70–230 mesh ASTM), phenanthroline monohydrate, trichloroacetic acid,potassium ferricyanide, sodium acetate trihydrate, Folin-Ciocalteu's phenol reagent, sodium carbonate, dipotassium hydrogen phosphate, sodium hydroxide, ferric chloride trihydrate, potassium hydroxide, and formic acid were purchased from Merck (Germany).

B. Plant material

In northern Iraq's Koy Sanjaq/Erbil area, they bought a fenugreek plant and cleaned it by hand. Used a dry grinder to ground dried plant materials into a fine powder. Before usage, it was kept in a container at ambient temperature and total darkness.

C. Plant extract preparation

Maceration was a successful extraction method for plant constituents. It took three extractions of MeOH (75 ml) with 10 g of powder at room temperature and for 24 hours to get MeOH extract from the powdered material. After passing through Whatman filter paper No. 1, the filtrate was concentrated at a temperature of 40 °C while the pressure was lowered. Hexane was used to remove the fat after the evaporated extract. Before further analysis, the methanolic extract will be dried to 1.16 g and kept in tubes at 4-6 °C.

D. Total phenolic content (TPC)

A modified Folin-Ciocalteu method was used to determine the extract's TPC [12]. After combining 0.1 mL of extract (3 mg/mL MeOH) with 1.5 mL of distilled water and agitating the mixture for 5 minutes, 0.1 mL of Folin-Ciocalteu reagents and 1.5 mL of sodium carbonate (10%) were added to the mixture. A measurement of the absorbance at 765 nm was obtained after the combination had been kept in the dark for an hour. Use a Thermo Scientific GENESYS 10S UV-VIS spectrophotometer from the United States of America. Using a regression equation established from a gallic acid standard calibration curve, a TPC measured in (mg GAE/g of dry extract) was determined and reported.

E. Total flavonoid content (TFC)

The quantity of flavonoids in a crude extract with minimal development was evaluated using the aluminium chloride colourimetric technique [13]. In brief, 0.2 mL of extract (3 mg/mL) was mixed with 1 mL of a 5% $AlCl_3$ solution and then 0.1 mL of a 1.0 M CH_3COOK solution. Finally, it was put to the test after letting 2.7 mL of MeOH stand for 60 minutes. Then the 420 nm absorbance value was given. We calculated the total flavonoid concentration in mg QE/g of dry weight using a calibration curve.

F. Total flavonol content (TF)

They calculated the TF using a modified colourimetric method based on aluminium chloride [14]. This approach required the use of rutin to generate a calibration curve A 0.5 mL extract solution of (3 mg/mL) was poured into a test tube, followed by equal volumes of 2% aluminium chloride and (6 mL) 5% sodium acetate. After allowing the tubes to rest for two and a half hours at room temperature and without light, they constantly swirled all of the tubes. Absorbance readings at 440 nm were taken from both samples and standards; the results were then converted to mg RE/g dry weight using a calibration curve.

G. Total anthocyanin content (TAC)

Used a spectrophotometer and a pH differential method to determine anthocyanin contents [15]. The concentration of anthocyanins in fenugreek seeds was measured by diluting the extract with KCl (0.025 M) and CH_3COONa (0.40 M) and changing the pH with HCl to 1.0 and 4.5, correspondingly. At 520 and 700 nm, the absorbance (A) was recorded and reported concerning pure water. The cyanidin-3-glucoside equivalents of this TAC were determined by using the following equations:

$$A=(A_{520}-A_{700})_{pH=1.0} - (A_{520}-A_{700})_{pH=4.5}$$

Equation 1 was used in order to provide an accurate reading on the monomeric anthocyanin content:

$$[\text{anthocyanins monomeric}]\ (mg/L) = (A \times M.wt \times FD \times 1000)/ (\varepsilon \times l) \quad (1)$$

Calculated the total anthocyanin concentration using equation 2:

$$[\text{anthocyanins in total}]\ (mg/L) = (A^* \times M.wt \times FD \times 1000)/ (\varepsilon \times l) \quad (2)$$

Where: $A^*=(A_{520}-A_{700})_{pH=1.0}$; M.wt = molecular weight (449.2 g/mol cyanidin-3-glucoside); FD = factor of diluting; ε = coefficient of molar extinction, (26900 L/ mol cm cyanidin-3-glucoside); l = path length (1 cm).

H. Ferric reducing antioxidant power (FRAP)

Potassium ferricyanide evaluated the FRAP to make a coloured complex containing antioxidant activities [16]. The standard and the sample were combined with 2.5 mL of 0.2 M phosphate buffer with a pH of 6.6 and 2.5 mL of 1% $K_3Fe(CN)_6$. The concentration of the sample was 3 mg/mL. After incubation for 30 mins at 50 ° C., the mixture was centrifuged for 10 min at 3000 rpm per minute, and then it was treated with 2.5 mL of a 10% TCA solution. In a second tube containing 2.5 mL of water and 0.5 mL of freshly made 0.1% $FeCl_3$, a supernatant of 2.5 mL was pipetted in. Absorbance at 700 nm was used to generate the quercetin standard calibration curve. It quantified fenugreek seed extract by its Quercetin equivalents (mg QE/g dry weight).

I. Metal chelating ability (MCA)

The chelating capacity of fenugreek seed extract for metals was determined using the 1,10-phenanthroline technique [17]. Pipette 0.1 mL of standard solution and the sample (3 mg/mL) into the tubes. Then they added 1.5 mL of water to the mixture, 1 mL of 0.20% $FeCl_3$, 0.20% phenanthroline, and 1.4 mL of distilled water, respectively. Afterwards, they were placed in a dark place and incubated for around 20 minutes. At a 510 nm wavelength, the absorbance was determined. Utilizing the calibration curve and regression equation, determine the equivalent iron II sulfate concentrations in mg Fe^{2+}/g of dry extract.

J. α-amylase inhibition (AAI)

The percentage of alpha inhibition was determined using a modified starch iodine method [18]. In brief, a 0.25 mL of 3 mg/mL plant extract and 0.25 mL of α-amylase solution was incubated at 37 °C for 15 minutes. Then, a 0.25 mL of starch was added, re-incubating the mixture for another 30 minutes. Put in 0.1 mL of HCl (1.0 M) to stop the reaction. Once the vortexing was complete, 1.0 mL of iodine and 3.0 mL of water were added. A spectrophotometer was used to get the absorbance value at 580 nm. We normalized the absorbance of the experiment by preparing blanks and substituting 0.25 volumes of distilled water for the plant extracts in the controls. Acarbose, a commonly prescribed medication for diabetes, served as a positive control. The formula used to calculate the percentage of inhibition is as follows:

$$\%\ \alpha\text{-amylase inhibition} = [\ (A_c - A_S) / A_c] \times 100$$

A_c = Absorption of control; A_s = Absorbance of standard

K. Select a plant fractionation solvent

TLC was used to validate the successful isolation of bioactive components from the methanolic extract.They tried a variety of solvents to find the one that worked best for them. Following the creation of the chromatogram, spots were identified using UV light (254 nm and 365 nm) and the iodine chamber. EtOAc-toluene-FA was used as the mobile phase because it provided the required separation resolution. Using EtOAc-toluene-FA (100:36:36) as a column chromatography solvent proved to be a good choice because of its higher R_f value.

L. Column chromatographic fractionation of the extract

Column chromatography is one of the most efficient methods for isolating phytocomponents. In the course of this research, a novel mobile solvent system was uncovered for the very first time. The chromatographic separation was performed using a wide glass column (450 mm x 40 mm). This column was packed with activated silica gel ranging in mesh size from 70 to 230 and was eluted using the mobile phase. In order to separate the desired substance from the column, a mobile phase solvent solution was used to make the slurry (Fig. 2). The 58 obtained fractions were carefully tracked on TLC plates, and fractions of a similar kind were combined and concentrated at low pressure. Finally, we discovered four different fenugreek seeds extract fractions.

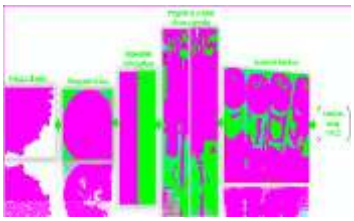

Fig. 2: Fractions obtained by column chromatography after selecting a suitable mobile phase and confirmed by TLC

M. HPLC analysis of phenolic components

The Shimadzu HPLC system with a Shimadzu DGU-20A5 vacuum degasser and a Shimadzu 20 ADXR solvent pump was used to conduct the polyphenol analysis. The Clipeus C18 5 m reversed-phase column had dimensions of 250 mm x 4.6 mm, and all separations were carried out. For reliable results, we used a photodiode array detector (a Shimadzu SPD-M20A, to be precise). Following the dissolution of 0.01 g of polyphenols in deionized water, we added 10 mL of a mixture of MeOH and water (1:1 v/v). This allowed us to produce 1000 mg/L stock solutions to prepare analytically pure polyphenol standards. Using these stock solutions as a starting point, solution dilutions of each polyphenol were created. For the determination of the phenolic chemicals, the HPLC-DAD technique was used [19]. For this investigation, the concentration was measured by injecting 20 µL at a flow rate of 1.0 mL/min into a separation column filled with a solution of 4.5% acetic acid (solvent A) and acetonitrile (solvent B). We evaluated the diluted extract and the fractions by injecting them into the HPLC system and analyzing the results using a photodiode array detector.

RESULTS AND DISCUSSION

A. Quantities of polyphenols, flavonoids, flavonols, and anthocyanins

The information presented in Table 1 provides a comprehensive summary of the phenolic, flavonoid, flavonol and anthocyanin content found in fenugreek seeds extract. Using the Folin-Ciocalteu method, which involves the transfer of electrons from phenolic compounds to the Folin-Ciocalteu reagent in alkaline, the total phenolic content of an extract was determined. This approach is a straightforward strategy that is widely used [20]. The dry extract had the most significant TPC, measuring 33.58 mg GAE/g dry extract.

Several essential polyphenols, such as flavonoids, aid in the body's defence against disease [21]. Environmental, Genetic diversity and biological, annual fluctuations and seasonal exerted significant effects on flavonoid concentrations [22]. With a TFC of 16.11 mg of QE/g dry extract, this extract ranks as having the highest quality flavonoid content possible.

Table 1: Analyses of the phytochemical content of fenugreek seeds extract

Phytoconstituents	Total quantity
TPC (mg GAE/g dry extract)	33.58 ± 1.39
TFC (mg QE/g dry extract)	16.11 ± 0.71
TF (mg RE/g dry extract)	21.81 ± 0.59
TAC (mg cy-3-glu/kg dry extract)	1.87 ± 1.69

Gallic acid equivalents, quercetin equivalents, and rutin equivalents (GAE, QE, and RE, respectively) are presented as the means ±SD of three individual measurements

Plant foods high in flavonoids and polyphenols, such flavonols, are necessary to protect against coronary heart disease and other age-related conditions like dementia [23]. Among flavonol's many names are rutin, also called quercetin-3-rutinoside, rutoside and sophorin. It is a powerful plant-based antioxidant that is often used to cure many human ailments, including diabetes, oxidative stress, microbial contamination, cancer recurrence, and cardiovascular disease [24]. Most flavonols were found in fenugreek seeds extract, which yielded 21.81 mg of R/g dry extract of all flavonols.

Based on a study of the pertinent literature conducted by Bhanger et al., TPC was determined to be 5.75 mg GAE/g dry extract, and TFC was determined to be 0.607 mg QE/g dry extract [25]. Priya et al., determined that the fenugreek extract seeds contained 0.876 mg GA and 0.489 mg QE per gram dry weight of TPC and TFC, respectively [26]. According to Al-Maamari et al., the fenugreek seeds extract contained TPC at a concentration of 2.16 mg GAE/g dry extract and TFC at a concentration of 0.328 mg QE/g dry extract [27].

Naturally occurring pigments called phytochemicals, including anthocyanins, are what give fruits, vegetables, and plants their vibrant hues. Outside of chlorophyll, they make up the largest class of plant pigments [28]. The antioxidant, anti-inflammatory, anti-viral, and anti-allergic properties of anthocyanins have been shown in vitro and in vivo. Additionally, the fact that they can function as reducing agents, hydrogen donors, and singlet oxygen quenchers lends further weight to this notion [29]. Essential criteria for triggering this phenomenon include the presence of OH groups, the degree to which the structure is conjugated, and the availability or absence of electron-donating and electron-drawing substituents in the ring system [30].

pH differential analysis is the most used technique for determining anthocyanin concentration because of its quickness and ease of use. The fenugreek seed extract was tested for its TA concentration, and 1.87 mg cy-3-glu/kg dry extract was found (Table 1).

B. Ferric reducing antioxidant power (FRAP)

The FRAP test is often used to quantify antioxidant capability. Testing the ability of phenolic antioxidants to give electrons or hydrogen is a common way to assess their antioxidant activity [31]. In this study, the ability of a material to reduce Fe $[(CN)_6]^{3+}$ into Fe $[(CN)_6]^{2+}$ is being probed. The complex formation results in a rise in absorbance, demonstrating the enhanced reduction capacity. Measurements of absorbance at 700 nm were used to determine the FRAP content in fenugreek seeds extract, which came out to 11.07 mg/g dry extract (Table 2). According to Al-Maamari et al., an earlier investigation determined the FRAP to be 0.259 mMoles/L dry extract [27].

Table 2. Amount of FRAP, MCA, and AAI extracted from fenugreek seeds

Antioxidants	Total quantity
FRAP (mg $FeSO_4$/g dry extract)	11.07 ± 0.81
MCA (mg Fe^{2+}/g dry extract)	76.35 ± 0.25
AAI %	84.21 ± 0.99

The mean ±SD of three separate measurements is shown

C. Metal chelation capability.

Cofactors for metallic iron enzymes are essential for cellular processes, including oxygen transport and respiration, for which iron is required [32]. 1,10-phenanthroline is expected to bind iron primarily as $[Fe(phenanthroline)]^{2+}$ when used as a chelating agent. In pH 2.5 to 8.0, the concentration of Fe^{2+} in a sample may be determined by measuring the absorbance at 510 nm, which is proportional to the concentration of phenanthroline. Hence, the spectrophotometric analysis may be used to distinguish between Fe^{2+} and Fe^{3+} [33]. At a dry extract concentration of 76.35 mg/g, fenugreek seeds were shown to have the potential to chelate metals (Table 2). Bhanger et al., previously reported a chelating activity of 1.02 mg/g dry weight [25].

D. α -amylase inhibition assay

Inhibitors of α-amylase have been found in many plants, making them potentially effective in the treatment of diabetes [34]. One of the essential enzymes in the human body is α-amylase, which breaks down starch into simpler sugars [35]. In inhibition studies, fenugreek seed extract inhibited α-amylase. Test samples with varying concentrations of the plant extract were compared to corresponding control samples with no extract. As a reference point for quality, acarbose was used. The α-amylase inhibiting properties of the plant extract were measured using the quantitative starch-iodine technique. Amylase inhibition impacts a plant extract, as shown in Table 2. An examination of the available literature indicated that Keskes et al., reported inhibition of α-amylase at a 52.1 µg/mL concentration [36].

E. HPLC analysis of extract and fractions

The use of column chromatography obtained a total of 58 fractions. Due to the similarity of TLC, they were separated into four fractions. As a result, determining the structure and biological characteristics of active plant components is very challenging. Consequently, high-resolution chromatographic technologies like HPLC detect and characterize several bioactive compounds discovered in the extract and fractions as a consequence [37]. Four fenugreek seed fractions (F_1 to F_4) and crude extract were tested. The crude methanolic extract had at least one major component, whereas three fractions contained the resveratrol substance (Table 3). The chromatograms indicated other peaks linked to additional phenolics, but they could not be identified due to inadequate standards.

Table 3. Phenolic compound concentrations (mg/kg dry matter) in fenugreek seed extract and fractions

Phenolic compounds		Fractions of fenugreek seeds extract			
	Extract	F_1	F_2	F_3	F^4
Gallic acid	n.d	n.d	n.d	n.d	n.d
Catechin	8.77	n.d	n.d	n.d	n.d
p-Coumaric acid	n.d	n.d	n.d	n.d	n.d
Epicatechin	n.d	n.d	n.d	n.d	n.d
Rutin	n.d	n.d	n.d	n.d	n.d
Resveratrol	n.d	0.135	0.065	0.092	n.d
Naringin	n.d	n.d	n.d	n.d	n.d

n.d, Not detected.

CONCLUSION

This study aims to analyze the extract's antioxidant capacity and separate its components using column chromatography. Antioxidant properties need a phytochemical analysis of the material. For instance, the extract included a high concentration of polyphenolics, flavonoids, flavonols, and anthocyanins, which all possess antioxidant qualities. Furthermore, FRAP and MCA analyses of the natural extract's antioxidant activity confirmed the plant's remarkable free radical scavenging potential. The most significant level of inhibition was seen for the α-amylase inhibitory capacity. In addition, a suitable mobile phase was discovered for the first time, and four fractions of fenugreek seed extract were isolated by column chromatography.

Polyphenols, including catechin and resveratrol, were found in an HPLC analysis. Future studies will benefit from this study's findings when trying to isolate, identify, and identify the unique molecule responsible for improved antioxidant activity.

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