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FENUGREEK SEED PROTEIN ISOLATE: AMINO ACID COMPOSITION AND FUNCTIONAL PROPERTIES

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ABSTRACT: With increasing demand for new protein sources, research on plant protein extraction and evaluation of the functional properties of protein isolates is necessary. Proteins were obtained from fenugreek seeds. Protein solubility, emulsifying capacity and emulsion stability, foaming capacity, and foam stability were used to determine the fenugreek a chemical composition and functional properties of proteins. The profiles of the solubility curves corresponding to the participating fenugreek protein indicate a minimum solubility at pH2. The emulsifying capacity of fenugreek participated fenugreek protein was low (3.2%) but it has high emulsifying stability (98.9%). Fenugreek proteins have show a high foaming capacity at pH 2 and the suitable time to form the foam is 30 min. the high stability of foam was 78.8%. The result of this study emphasized that fenugreek protein has a greater ability to absorb water than oil. Thus, the experiments suggest that fenugreek protein can be successfully used as a food ingredient due to its nutritional value, chemical composition, and functional properties.

Key words: Fenugreek, Seed protein, extraction, functional properties.

INTRODUCTION

Plant proteins play a significant role in the human diet, especially in developing countries where the average protein consumption is less than the recommended protein consumption allowance. There is much interest in providing new sources of plant proteins, due to the inadequate resources of animal proteins with equal nutritional and functional benefits in food systems **(Amza *et al.*, 2004**).

Legumes are excellent foods, with several nutritional and functional advantages and alow price. Among legumes, fenugreek, belonging to the Fabaceae family, is extensively cultivated and produced in Mediterranean countries and Asia. Fenugreek seed is widely used as a herbal medicine with antidiabetic effects in India and Iran. Furthermore, the seeds are an excellent source of plant proteins with a total protein content of approximately 250.000–350.000 g/kg. The main protein fractions include albumins (438.00 g kg^{-1}), globulins (272.00 g kg^{-1}), glutelins (172.00 g kg^{-1}) and prolamines (74.00 g kg^{-1}). Partial replacement of wheat flour with fenugreek flour in the range 50–200 g kg^{-1} showed significant improvement of protein content, sensory and rheological properties of products in bread, biscuits, noodles and macaroni (**Leela and Shafeekh, 2008**).

Studies also showed that replacing 150 g kg^{-1} of wheat flour with germinated fenugreek seed flour resulted in significantly higher protein digestibility, crude fiber and lysine levels; however there are concerns regarding bread volume and consistency due to fenugreek seed flour supplementation. To address this matter, further investigation on the functional and nutritional properties of fenugreek protein isolate is needed to provide practical information on the utilization of fenugreek seed flour in food formulations (**Kanu *et al.*, 2007**).

Legumes are important crops that play a major role in human and animal nutrition.

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Furthermore, they are considered a good source of complex carbohydrates and dietary fiber as well as proteins and minerals. One of the oldest crops used for human and animal consumption in fenugreek (**Masood and Batool, 2010**).

These kinds of crops can resistance a cold weather in all stages of grow, and it could be an exceptional alternative to soybean in cold region of the world. Field fenugreek is identified as a main source of nutritional constituents and can be fractionized into many ingredients and foods products enriched in protein, starch, fiber etc. In general, fenugreek seeds contain 20-25% protein, 40-50% starch and 10-20% fiber. Many features that made a fenugreek protein popular in worldwide such as its low cost, availability, and health benefits (**Hegazy and Ibrahim, 2009**).

Legumin is composed of two subunits an α-chain which contains significant amounts acidic of amino acids, and a β-chain which contains significant quantities of basic amino acids, with molecular weights of about 40 kDa and 20 kDa respectively. The two subunits of the legume are linked together by a disulfide bond and have total molecular weight of 330 kDa. On the other hand, fenugreek vicilin contains three chains α (19 kDa), β (13.5 kDa) and γ (16 kDa) (**Cai *et al.*, 2001; Samira *et al.*, 2014**)

Functional properties are defined as physical and chemical properties, that affect protein behaviour in food systems during processing, storage, preparation and consumption. Protein functional properties could be altered due to the protein source and procedures used for flour preparation, protein concentrate and isolate extraction and production.

Physiochemical parameters such as pH, temperature, salt and ionic strength can also highly affect proteins' functional properties (**Hussein *et al.*, 2018**).

In the present study, therefore, fenugreek seed protein was extracted under optimized conditions in terms of pH and salt concentration of the solvent. The experimental design for optimization of protein extraction conditions (salt concentration and pH) was performed using response surface methodology. Functional and thermal properties of the optimized protein isolate and its electrophoresis pattern have also been investigated. To evaluate the nutritional properties of the extracted protein, amino acid composition was also evaluated (**Feyzi *et al.*, 2014**).

Fenugreek (*Trigonella foenum-graecum* L.,) belongs to the family *Fabaceae* (*Leguminosa*e). It is an annual crop mainly grown for use as a spice in many parts of the world. Herbs and spices have been extensively used as food additives for their natural antioxidants. Spices and aromatic herbs are considered to be essential in diets or medical therapies for delaying ageing and biological tissue deterioration. Fenugreek leaves and seeds have been used extensively to prepare extracts and powders for medicinal uses. The medicinal value of plants lies in some chemicals active substances that produce a definite physiological action on the human body (**Sajad and Pradyuman, 2018**).

Fenugreek is an annual plant that belongs to the family Leguminosae. It is an of the most the famous spices in human food. The seeds and green leaves of fenugreek are used in food as well as in medicinal applications which is an old practise of human history. It has been used toenhance the flavour and colour of foods as well as to change the texture of food materials. The seeds of fenugreek have medicinal properties such as hypocholesterolemic, lactation aid, antibacterial, gastric stimulant, for anorexia, antidiabetic agent, galactogogue, hepatoprotective effect and anticancer. These beneficial physiological effects including the antidiabetic and hypocholesterolemic effects of fenugreek are mainly attributable to the intrinsic dietary fiber constituent which has promising nutraceutical value (**Srinivasan, 2006**). It is well known for its fiber, gum, other chemical constituents and volatile contents. Dietary fiber of fenugreek seed is about 25% which changes the texture of food. These days it is used as food stabilizer, adhesive and emulsifying agent due to its high fiber, protein and gum content. The protein of fenugreek is found to be more soluble at an alkaline pH (**Meghwal and Goswami, 2012**).

In the present study, the chemical composition of fenugreek seeds was estimated crude extracts (petroleum ether and methanol were prepared from fenugreek seeds to estimated total phenolic, total flavonoids, and antioxidant activity. On the

other hand, protein was isolated from defatted fenugreek flour to evaluate the functional properties such as solubility, foaming properties and emulsifying properties.

MATERIALS AND METHODS

Materials

The mature seeds of Fenugreek were purchased from the local market in Zagazig, Sharkia. The seeds were washed with tap water and milled using an electric miller (M_{20}; IKA, Germany) after drying. Fenugreek seed flour was defatted using hexane by mixing at a ratio of 1:4 (w/v) and continuously stirring for 3-h at room temperature. Hexane was removed using a Buchner vacuum funnel and the flour was air dried at room temperature. All chemicals were of analytical grade, and were purchased from Sigma Chemical Co. (St Louis, MO, USA), and Merck KGaA (Darmstadt, Germany).

Methods

Proximate Analysis

Proximate composition of fenugreek seed was determined from samples were determined as described in AOAC official methods (A**OAC, 2000**). The total carbohydrate content (on a dry weight basis) was calculated by the difference (100 - (% protein + % lipid + % ash + fiber). A nitrogen conversion factor of 6.25 was considered for protein content.

Preparation of Plant Crude Extract

Twenty gm of dry fenugreek seeds were cleaned and ground using a mortar and pestle. The extraction was carried out by the soxhlet method. The fine powder was packed tightly in a soxhlet extractor and petroleum ether (200 ml) was used as solvent for extraction. The process was carried out for 6 hrs. The fine powder was re-extracted under the same conditions by using methanol (90%v/v). The obtained extracts were evaporated by an arotory evaporater under reduced pressure at 60°C to get a dried solid product then stored in dried bottles

Determination of Total Phenolic Content of Fenugreek Extracts

The total phenolic content was estimated according to **Wolfe *et al.* (2008)**. 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 of Fenugreek Extracts

Total flavonoid content was determined by the aluminum chloride colorimetric method of **Singleton *et al.* (1999)**. The total flavonoid content of the samples was calculated by using the following linear equation based on calibration curve.

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

Y is the absorbance, and X is the flavonoid content in $\mu g\ g^{-1}$

Antioxidant Activity

The antioxidant activity of the seeds extracts and the standard (Ascorbic acid) were assessed on the basis of the radical scavenging effect of the stable1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical activity by a modified method of **Braca *et al.* (2002)**.

Antioxidant Activity

The radical scavenging activity of the extractions was determined with the DPPH method as described Braca et al., (2002). with modifications. A solution of 0.3 mM DPPH was freshly prepared with 96% ethanol. The extracts (2.5 mL) with varying concentrations (0.05 to 0.25 mg dry residue of the extract/mL analysis solution) were mixed with 1.0 mL of DPPH solution in a test tube. The mixtures were left to stand for 30 min at room temperature under dark conditions. Absorption was measured at 520 nm using a UV-VIS spectrophotometer (Cary 50 Bio; Varian, Australia). Blank solution was 96% ethanol. DPPH solution (1.0 mL, 0.3 mM) mixed with 96% ethanol (2.5 mL) served as a negative control. Ascorbic acid was employed as a positive control. The half-maximal inhibitory concentration (IC_{50}) was calculated as the amount of antioxidant required to decrease the initial DPPH concentration by 50%. The IC_{50} was obtained using linear regression analysis.

Isolation of Protein from Defatted Fenugreek Powder

The extraction of proteins was carried out using the standardized method of **Mir *et al.* (2019)** with some modification. The defatted fenugreek seed powder was blended with water (deionized) (1:20, 10 g of defatted fenugreek in 20 mL of water, w/v). The pH of the solution was kept in the range of 10 to 13 by using a 2.5 N NaOH solution. The mix was then shaken while heating in a water bath for 2 h at a temperature of 45°C followed by centrifugation. The variables used for the extraction of proteins were pH, extraction time, and extraction rpm. The extraction pH was set at 10, 11, 12, and 13 (**Lawal *et al*., 2007**), at two different extraction rpm (7500×g and 8000×g) and at two different extraction times (15 min and 20 min). The supernatant was accumulated and the sediment was disposed off. The pH of the supernatant was maintained at 2 by using 2.5 N HCl dropwise followed by isoelectric precipitation. Centrifugation was again performed on the precipitated protein at 8452 rpm for 20 min. The pH of the collected sediment was neutralized followed by drying at 50°C in a hot air oven for 24 h. The dried protein was stored in refrigerated conditions for further analysis.

Amino Acid Composition

To determine amino acid composition, 30 mg FPI was hydrolyzed under nitrogen in 4 mL of 6 mol L^{-1} HCl at 110°C for 22 h. Derivatization was carried out using isothiocyanate. Chromatography was performed using a Knauer high-performance liquid chromatography (HPLC) system (Berlin, Germany) which consisted of a gradient controller (Manager 5000), UV detector (UV 2500) set at 254 nm and a Eurospher C18 column (250 mm × 4.6 mm, with a 5 μm particle size) maintained at 25 °C. The solvent system consisted of two eluents. Solvent A was a solution of 50 mmol L^{-1} sodium acetate containing 0.4 mL L^{-1} triethylamine. The pH was adjusted to 6.8 using glacial acetic acid. Solvent B consisted of 60 mL acetonitrile and 40 mL water (**Suzuki and Early, 1989**). Furthermore, essential amino acids of both samples were compared with the FAO recommended allowance (**FAO, 2007**).

Functional Properties of Isolated Fenugreek Protein

Protein solubility

Protein solubility was determined in water (0.02g/10ml) at various pH (2,4,8 and 10) levels after 30 min of stirring at room temperature. After that, they centrifuged the solution for 30 min and, then the protein concentration was determined in the supernatant using the biuret method (**Wang and Ismail, 2012**).

PS was evaluated as:

Protein solubility in (%) = SP / PT × 100

Where, PT = total protein proportion in the sample calculated prior centrifugation, and SP is the protein proportion after centrifugation in the supernatant

Foam capacity and foam stability:

Each sample (0.19) was mixed with 10 ml of distilled water, with varying of pH (2- 4- 8- 10) and shock for 5 min. the foam value of formed at different times (0- 30- 60- 90- 120) min. the method of foam capacity (FC) was calculated .

$$FC\ (\%) = \frac{\text{foam volume}}{\text{initial sample volume}} \times 100$$

The foam was tested for foam stability at different interval times (0 – 30 – 60 – 90 – 120) min, then the foam volume remaning was measured and foam stability was calculated as follows.

$$FS\ (\%) = \frac{\text{Foam volume after the tested times}}{\text{Initial foam volume}} \times 100$$

Emulsifying Properties

The emulsification between distilled water and commercialized corn oil was determined (**Stone and Nickerson, 2012**) when 0.07 g of isolate fenugreek proteins were added. With pH 7 the emulsion is measured by centimeters and calculated by **Lu *et al.* (2020).** Furthermore, the stability of the emulsion is determined by heating it in a water bath at 90 °C for 30 min, then cooling it to room temperature.

$$ES\ (\%) = \frac{V_b - V_{ad}}{V_b} \times 100$$

V_b and V_a refer to the aqueous phase prior to heat and after heating at 90 °C for 30 min.

RESULTS AND DISCUSSION

Chemical Composition Fenugreek's Seeds

The Fig. 1 below shows the chemical composition of fenugreek**.** The protein contents of the fenugreek seeds powder reached up to 19%. The ash and fat contents ranged from 3.95% to 5.71%. The fat and ash contents obtained in this study were within the ranges of different cultivars investigated in other studies (**Arteaga *et al.*, 2021**). The reported protein content of seed fenugreek powder was 19%. The protein contents were not in the same range as in other studies (**Stonea *et al.*, 2015**). The great variability of the protein content and composition has been reported by many authors, both between genotypes and also due to environmental effects within genotypes (**Gueguen and Barbot, 1988**). **Klein and Raidl 1986**), explained that environmental factors that affect the protein content of field fenugreek include nitrogen fertiliser, maturation, soil P and K content and temperature.

Functional Properties of Participated Fenugreek Protein

Solubility

The pH solubility curve of EPI is presented in Fig. 2. The minimum sobuility for FPI (0.3%) was at pH 2 which corresponding to isolelectric point. The highest protein solubility (0.62%) was observed at pH 8, this result agreed with the results recorded by **Damodaran *et al.* (1996)**.

Foaming capacity and foam stability

The effects of pH and time on the foam capacity and stability of FPI is shown in Figs. 3 and 4 respectively. At pH2, the FPI had the highest FC as well as the highest FS (Fig. 3).

Foaming stability and capacity are influenced by time and pH. According to the results shown in fig.4 foaming capacitywas highly formed at pH2 (78.8%) the lowest reduction in the foaming capacity was recorded at pH10 (6.5%). This is consistent with what was mentioned by **Sathe *et al.* (1982)** that the foam formed from the protein isolated from lupine seeds increases at high acidic media, and he explained (**Ragab *et al.*, 2004**) that the ability to form foam is affected by the pH value. Also, the stability of the foam is affected by the time factor, where the highest percentage of stability of the foam was recorded in a time of 30 minutes at pH2, and the percentage of foam capacity was 78.8%, while at pH (4,8,10) it was noticed that the decrease in the percentage of foam capacity reached (38, 22.8 and 6.5%), respectively, and therefore it can be seen that alkalinity affects the results of foam formation as it reduces the amount of foam formed as well as its stability. And the increase in the foam capacity is due to the increase in the electrical charge of the protein and then the increase in the solubility and flexibility of the protein, which results in the diffusion of the protein at the water-air interface and surrounding air bubbles, increasing the formation of foam. The lowest foam stabilities at pH 4,8,10 may be due to the presence of polysaccharides or due to aggregation or denaturation.

Emulsifying property

The result illustrated in Fig. 5 clarified that FPI has low emulsification activity (3.2%) but it has higher emulsification stability (98.9%). The decrease in the emulsifying activity of the protein may be due to the protein reaching the electrolyte point, which causes it to precipitate and reduce its emulsifying activity. The emulsifying property of protein is important to forms a gel structure with water by imbibing it and binding to it, Protein prevents slow melting. Thus reducing the free water in the mixture. As for the stability of the emulsification, it was clear from the results that FPI has a high percentage of stability, and the percentage of stability of the emulsifier was high in the medium at pH=7. It was explained by **Keerati *et al.* (2012)** that the stability of the emulsion depends on the formation of a charged layer through the dissolved proteins around the oil droplets, leading to the repulsion of the droplets with each other. Which has an effective surface with the ability to emulsify and its stability through the stable electrostatic force on the surface of the fat drop (**Yin *et al.*, 2015**).

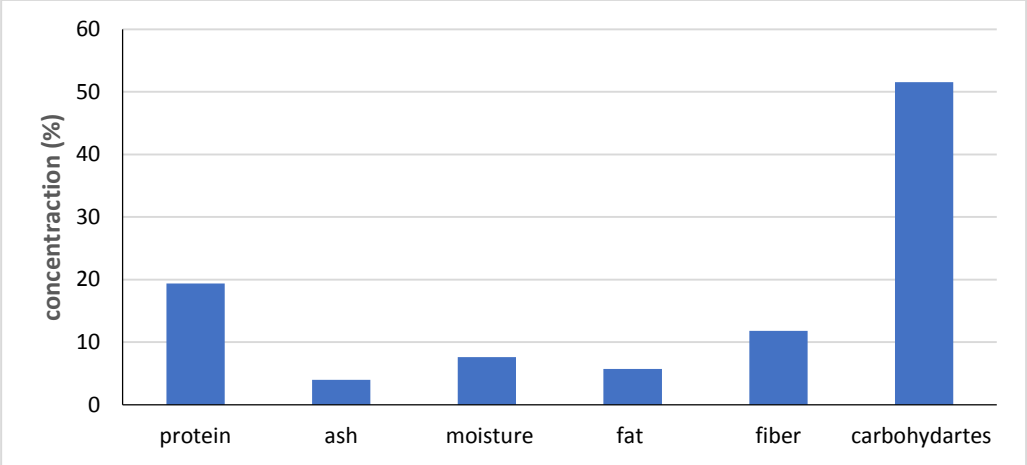

Fig. 1. Chemical composition of fenugreek seeds

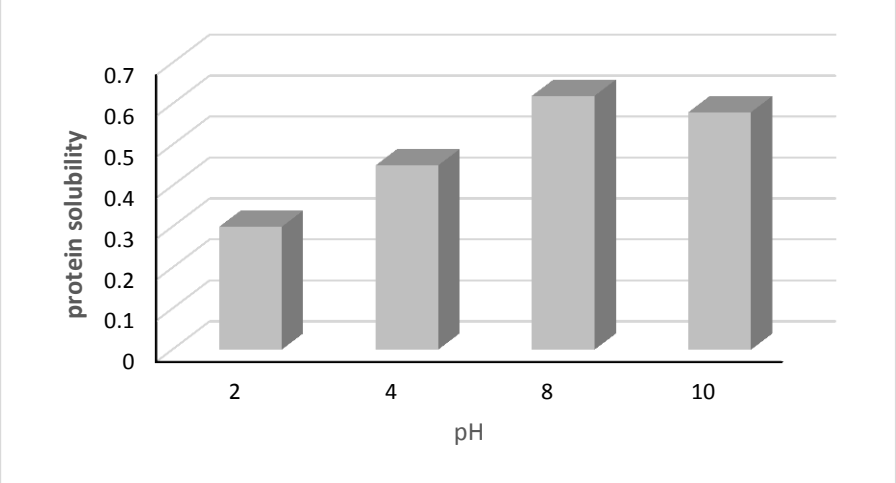

Fig.2. Effect of different pH on protein solubility

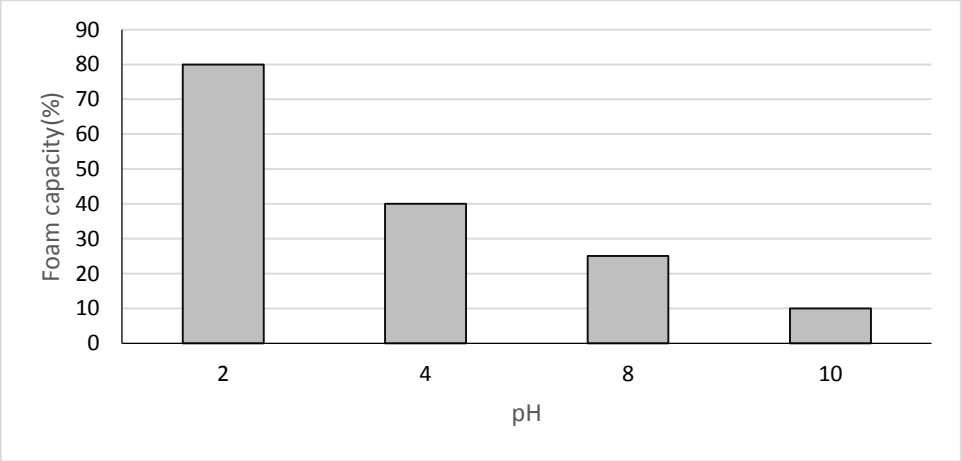

Fig. 3. Foam capacity of fenugreek protein isolate

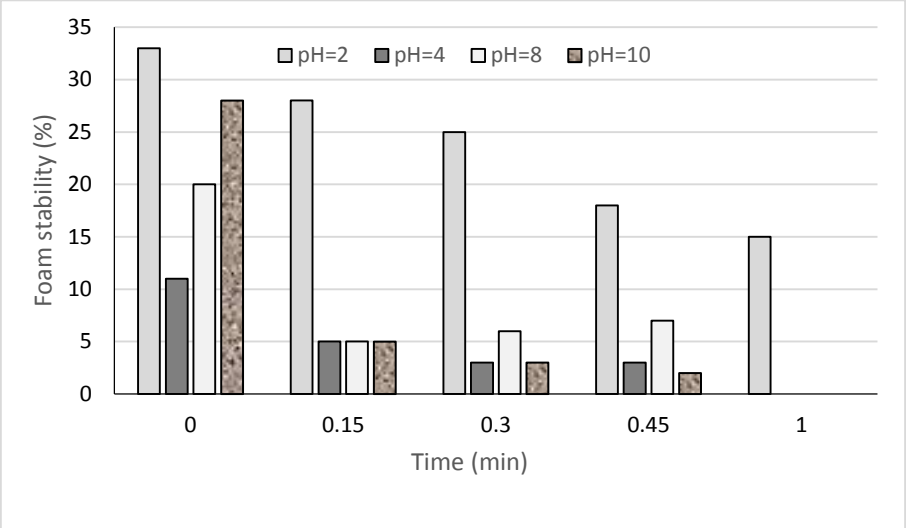

Fig. 4. Foam stability of fenugreek protein isolate

Free Radical Scavenging Activity (DPPH)

The methanolic and petroleum ether extracts of Fenugreek seeds and methanolic extracts of callus derived of hypocotyls and cotyledons segments were evaluated for their *in vitro* antioxidant activity using DPPH method and compared with standard ascorbic acid. Percentage inhibition of DPPH free radical scavenging activity of *Fenugreek* callus and plant seeds extracts were presented in Fig. 6a. DPPH radical scavenging activity of the tested extracts were concentration dependent. Maximum antioxidant effect was observed in highest concentration (2 mg/ml) of all tested samples. These results suggested that the methanolic extracts contain compounds that are capable of donating hydrogen to a free radical in order to remove odd electron which is responsible for radical's reactivity. However, the petroleum ether extract of seeds demonstrated weak antioxidant activity with inhibition percentage 24.56±0.12%. The IC_{50} values were also calculated for extracts of seeds and callus and compared with that of ascorbic acid. A lower value of IC_{50} indicates a higher antioxidant activity. The IC_{50} values of DPPH scavenging capacity of methanolic extracts were evaluated in descending order, plant seeds (1.1185 mg/g) > hypocotyls derived callus (0.7159 mg/g) > cotyledons derived callus (0.4914 mg/g) > ascorbic acid (0.1874mg/g) (Fig. 6b).

Total phenol and flavonoid contents of fenugreek extracts

Total phenolic, total flavonoid at petroleum ether and methanol extracts were estimated and found be varied (Fig. 7). The highest amount of TPC was observed in methanol extract 400 mg/l). The highest amount of TFC was observed with petroleum ether (120 mg/l). The amounts of total phenolic compounds were calculated as mg/l gallic acid equivalent of phenols. These results revealed that there were 2.3 and 0.8 folds increase of phenolic contents in the cotyledons and hypocotyls callus tissues respectively when compared with that of the plant seeds. Previous study carried out by **Kaur and Kapoor (2007)** on the total phenolic content of some Asian vegetables, categorized Fenugreek as high phenolic contents vegetables with very high antioxidant activity **Seasotiya *et al.*** reported high value of phenolic contents 186 mg gallic acid/g dry weight in methanolic extracts of Fenugreek seeds, whereas **Taj Al-Deen (2010)** recorded low value of total phenolic content 128.67 mg/g of dry weight in alcoholic extract of callus derived from hypocotyls of Fenugreek. The highest level of flavonoid content were detected in methanolic extract of seeds 424.951 mg/l followed by 217.285 mg/l in methanolic extract of cotyledons derived callus and 95.92 mg/l in methanolic extract of hypocotyls derived callus. The amounts of flavonoid content were calculated as mg/l quercetin equivalent of

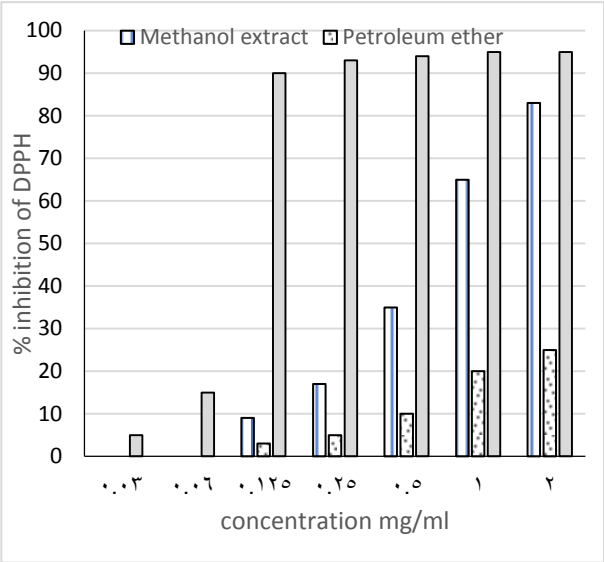

Fig. 6a. DPPH radical-scavenging activity

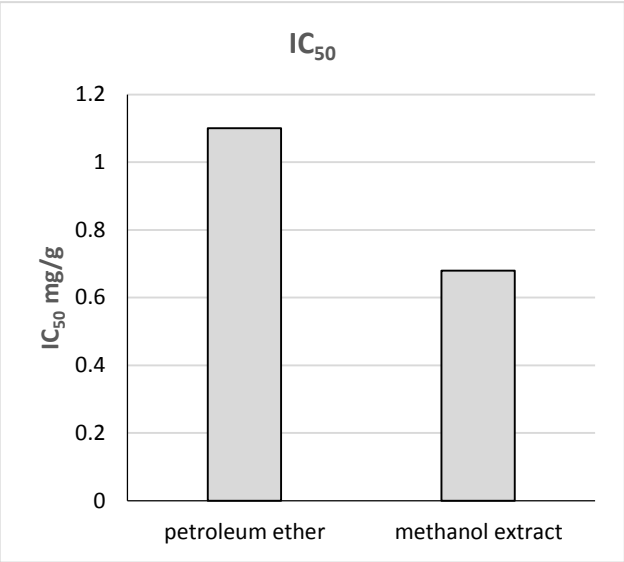

Fig. 6b. IC_{50} of extracts of fenugreek

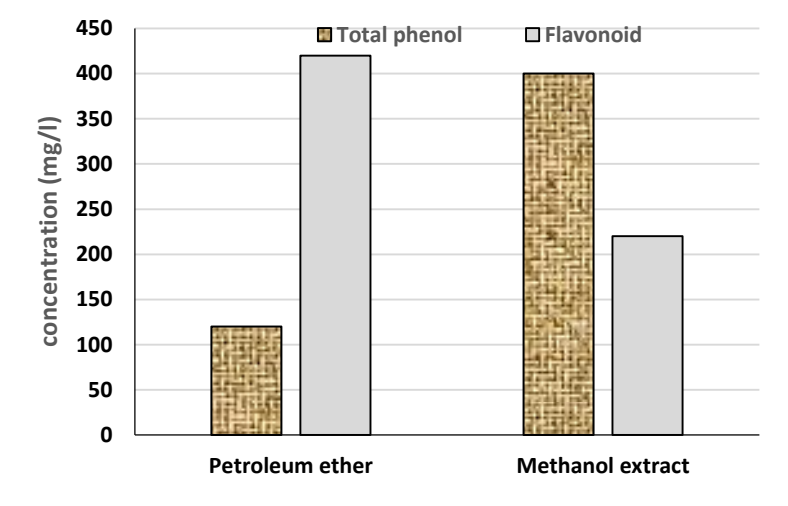

Fig.7. Total phenol and flavonoid contents in extract of fenugreek

flavonoids. **Aljawfi *et al*. (2013)** found in their results the total flavonoid contents in *Fenugreek* seeds were 4.99 g/100g dry weight. This variation in the phenolic and flavonoid contents may be due to the variety of Fenugreek or the differences in environmental conditions. Moreover, the antioxidant effect of this valuable medicinal plant is strongly supported by the presence of phenolic compounds like quercetin. However, the higher antioxidant activity of callus tissues observed in this study might be correlated with the increase in their phenolic composition and it was in accordance with previous studies that demonstrated a significant correlation between phenolic composition and antioxidant activity.

Conclusion

The functional properties of fenugreek seeds obtained in this study suggest their potential for the development of different food products. The findings of this study show that isolated fenugreek proteins (IPP) are rich in proteins. The results obtained for fenugreek protein isolate included protein solubility, foam stability and capacity, emulsion stability and capacity.

The FPT exhibit a good foaming and emulsion stability, which could be exploited for nutrition and food formulation

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فصـــل بـروتيـــن بـذور الحلبـــة: تكـــوين الأحمـاض الأمينيـــة، الخصـائص الحـرارية والوظيفية

سلامة عبدالهادي مصطفي - رجب عبدالفتاح المصري - احمد محمد أبو عيطة - عبدالعليم اسماعيل ابراهيم

قسم الكيمياء الحيوية - كلية الزراعة - جامعة الزقازيق - الزقازيق - مصر

في هذه الدراسة تم فصل المعزول البروتيني لبذور الحلبة وتقدير محتواها من الاحماض الامينية ودراسة خصائصها الوظيفية (الذوبانية – الخصائص الرغوية – الخصائص الاستحلابية) علي الجانب الاخر تم عمل مستخلص ايثر بترولي وميثانولي من بذور الحلبة منزوعة الدهن وتقدير محتواها من المركبات الفينولية والفلافونيدية وتقدير نشاطها المضاد للاكسدة. أظهرت النتائج أعلي تركيز لمركبات الفينولية في حالة المستخلص الميثانولي ومركبات الفلافونيدية في حالة مستخلص الايثر البترولي. أظهرت نتائج الذوبانية ان أقل درجة ذوبان عند درجة حموضة (2) واعلاها عند درجة حموضة (8) وانعكس ذلك علي الخصائص الرغوية والقدرة الاستحلابية تشير النتائج انه يمكن استخدام المعزول البروتيني للحلبة كمكون غذائي بسبب قيمته الغذائية وخصائصة الوظيفية.

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