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Effect of extraction solvents on antioxidant and antimicrobial properties of fenugreek seeds (*Trigonella foenum-graecum* L.)

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Abstract

The present study was aimed to investigate the efficacy of fenugreek seeds as a potential natural source of antioxidants and antimicrobials. Fenugreek seed (FS) extracts were prepared using ethanol (75%), methanol (75%) and water as extraction solvents. Ethanol (E-FSP), methanol (M-FSP), water (W-FSP) and hot water (HW-FSP) extracts were obtained from ground FS, whilst water extract (W-GeFS) was obtained from germinated FS. The results revealed that all extracts of the ground FS exhibited antioxidant and antimicrobial activities and the extractability of bioactive compounds in the presence of water was higher in germinated seeds (W-GeFS). Highest phenolic (156.3 mg GAE/ g) and flavonoid (38.5 mg CE/ g) contents were found in W-GeFS. It also showed the strongest DPPH radical-scavenging activity of 68 % inhibition at a lower concentration (0.06 mg/ ml). In addition, highest vitamin C equivalent antioxidant capacity (143.28 mg vitamin C/ g) with an IC_{50} value of 42.1 µg/ ml were found in W-GeFS. Based on disc diffusion method, W-GeFS exhibited highest antimicrobial activity against all tested bacterial pathogens (*Bacillus subtilis*, *Staphylococcus aureus*, and *Escherichia coli*). Thus, it can be concluded from the results that W-GeFS extract from germinating fenugreek seeds (W-GeFS) has the potential to be used as a natural source of bioactive compounds with varied applications in food industry especially, for active film packaging purposes to prolong the shelf-life of food products.

Introduction

Lipid oxidation and microbial activity are two major factors causing food spoilage which poses great challenges to the food industry in preserving food. Oxidation is one of the major factors of chemical spoilage in food products, resulting in rancidity and deterioration of the nutritional quality, color, flavor, texture and safety of the products (Antolovich *et al*., 2002; Suhaj, 2006). On the other hand, microbial activity is a primary mode of deterioration of many foods and is often responsible for the loss of quality and safety (Negi, 2012). Presently, the food industry uses chemical preservatives to improve oxidative stability of lipids and to prevent microbial growth in order to prolong the shelf-life of packaged food products. The use of chemical preservatives and additives may result in health hazards when consumed regularly. However, there is a growing awareness as well as increasing demand by consumers for high quality and good shelf-life stable packaged foods containing natural preservatives. Natural preservatives may be more effective in protecting food products from chemical deterioration and microbial spoilage in addition to having less toxic effects compared to chemical or synthetic additives (Giner *et al*. 2012).

Medicinal plants, herbs and spices have received much attention as the most important source of biologically active compounds. The most important of these bioactive compounds include polyphenolic compounds, alkaloids, tannins, vitamins, essential oils and terpenoids. The bioactive compounds could be extracted and isolated from plant raw materials using water or organic solvents. Numerous studies have shown that these bioactive substances extracted from medicinal plants have a wide range of biological effects including antioxidant and antimicrobial properties (Wojdylo *et al*., 2007; Khoobchandani *et al*., 2010; Goncalves *et al*., 2013). Studies have shown that plant extracts from green tea, grape seed, cinnamon bark, and clove buds have potential as alternative sources of synthetic antioxidants (Rababah *et al*., 2004; Chan *et al*., 2011).

Fenugreek (*Trigonella foenum-graecum* L) is a member of the leguminous family (Board, 2010) which is an annual plant found primarily in Mediterranean countries, the Middle East, and India. Fenugreek is an important spice and its dried seeds have wide application in food and beverages. The leaves of fenugreek plant are edible and often used as a vegetable dish in many parts of India. Fenugreek has been reported to be an important

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medicinal plant with a large number of medicinal properties such as restorative and nutritive properties (appetite stimulant) with hypocholesterolemic, antidiabetic, antileukemic and antimicrobial effects (Acharya *et al*., 2008; Meghwal and Goswami, 2012). The chemical composition of fenugreek seed (FS) has been thoroughly studied and its medicinal properties are associated with its phytochemicals such as galactomannans, phenolic compounds, alkaloids, proteins, vitamins (A, B1, C and nicotinic acid) and volatile oils (Acharya *et al*., 2008). Germinated fenugreek seeds rich in bioactive antioxidant substances are also used extensively as an important ingredient in daily food preparations and herbal formulations (Khole *et al*., 2014). There have been some studies on the antioxidant properties of crude fenugreek seeds extracts (Liu *et al*., 2012; Kenny *et al*., 2013) but fewer reports about the antimicrobial activity in extracts of fenugreek seed and germinating fenugreek seed extracts. There is still little information on the antioxidant capacity and antimicrobial activity of fenugreek seed extracts in different solvents especially in water. Fenugreek seeds are gummy, fibrous and sticky in nature. It is difficult to obtain water extracts of FS due to its high viscosity contributed by the high content in carbohydrates and mucilaginous fiber (galactomannans).

Therefore, the aim of this study was to evaluate both whole fenugreek seed and germinating fenugreek seed extracts as potential sources of natural antioxidants and antimicrobials. In this study, the effects of extraction solvents (methanol, ethanol and water) and solids-to-liquid ratio on the extract yield, antioxidant capacity and antibacterial activity of fenugreek seed extracts were investigated. Information on the total phenolic content, antioxidant capacity and antibacterial activity of these FS extracts could be helpful in the selection of suitable solvents for extracting bioactive compounds from other similar plant materials.

Materials and Methods

Materials

Fenugreek seeds (semi-dried) were purchased from a local supermarket in Penang, Malaysia. Ascorbic acid was purchased from BDH (Poole, England). DPPH (2,2-Diphenyl-1-picrylhydrazyl) and catechin were purchased from Sigma Aldrich. Gentamicin and 0.5 McFarland standard were purchased from OXOID (Oxoid Ltd., Basingstoke, UK). Nutrient agar, Muller Hinton agar and Muller Hinton broth were purchased from Himedia (Himedia Laboratories Pvt. Ltd. India). All chemicals used were analytical grade.

Preparation of crude extracts from fenugreek seeds

Preparation of crude extracts from ground fenugreek seeds

The moisture content of semi-dried and oven-dried whole fenugreek seeds (FS) was determined using a moisture analyzer (Denver Instrument IR-30 Moisture Analyzer). Prior to extraction, whole fenugreek seeds were washed with distilled water to remove dirt and other foreign matter and then dried in an oven (Binder, model VD53) at 40°C for 24 h. After that, the moisture content of dried seeds was determined. Dried seeds were ground using a lab grinder and the fenugreek seeds powder (FSP) obtained was passed through a 0.5 mm sieve. The FSP was then used as the raw material for preparation of crude extracts using 75% ethanol, 75% methanol and distilled water as the extraction solvents. The extractions were carried out following method described by Khanra *et al.* (2010) with slight modification. For extraction using water at room temperature, a prior disinfection treatment using sodium hypochlorite (2%) for 30 min was performed on fenugreek seeds according to the method described by Haouala *et al*. (2008). Different ratios of solids to solvent were used as shown in Table 1. For extraction with ethanol and methanol, solids: solvent ratios used were 1:5, 1:8 and 1:10. However, for water extraction, due to the high viscosity of FSP in water mixture, higher solids: solvent ratios of 1:40, 1:50 and 1:60 were used. Each extraction process was performed under continuous stirring with a magnetic stirrer hot plate (Model: 11-102-50SH, Fisher Scientific, USA) at ambient temperature (23 ± 2°C), in the dark for 24 hr. The mixture was filtered under vacuum with Whatman No. 1 filter paper (Whatman International Ltd., Maidstone, England) followed by centrifugation at 48,384 x g for 20 min at 20°C using a refrigerated centrifuge (Model: Avanti J-26XPI, Beckman Coulter, Inc., California, USA). Due to the high viscosity of FSP in water mixture, filtration was carried out with a sintered glass funnel instead. After centrifugation, the supernatant was decanted and collected. A second extraction cycle (another 24 hr) at the same solids: solvent ratio was conducted by adding solvent to the residues from the first extraction. The supernatant obtained from both extraction cycles was combined and the solvent was evaporated off using a rotary evaporator (Model: N-1100, Eyela, Tokyo, Japan) operated under reduced pressure at 30°C. The residue was collected, oven-dried at 30°C for 24 hr and ground to obtain a crude extract powder. The crude extract powder samples

from extraction with ethanol (E-FSP), with methanol (M-FSP) and with water (W-FSP) were then kept in airtight bottles, in the dark at 4°C until further use.

Another extraction method was also carried out using hot water (80°C) at solids: solvent ratio of 1:20 with only one extraction cycle performed under continuous stirring on a magnetic stirrer hot plate for 15 min. The hot water extract powder (HW-FSP) was obtained in the same manner as described for water extraction at ambient temperature, and the sample was kept for further analysis.

Preparation of crude water extracts from germinated fenugreek seeds

Fenugreek seeds were harvested after being soaked in water at 30°C for 32 hr, to obtain crude water extract from germinated fenugreek seeds (GeFS) during the sprouting process. Similar water extraction method as described in the previous section was used to obtain the crude water extract from GeFS, except that the extraction was performed using water heated to 30°C to enhance the germination of fenugreek seeds, and in solids: water ratio of 1:10. Finally, a crude water extract powder (W-GeFS) was obtained. All crude extracts of FS prepared were kept for analysis of total phenols and flavonoids including antioxidant and antimicrobial activities. Recovery of the extract was calculated as yield (%) using the following equation:

$$\text{Yield (\%)} = [W_f / W_i] \times 100,$$

where W_f is the final weight of the crude extract powder and W_i is the initial weight of the raw material.

Determination of total phenolic content

The Folin-Ciocalteu assay method described by Sakanaka *et al.* (2005) was used to quantify the total phenolic content in the crude extracts. Briefly, 125 µl of Folin–Ciocalteu phenol reagent was added to 625 µl of crude extract solution (of appropriate concentration) and mixed well to allow for reaction (6 min) at 25°C. The mixture was neutralized with 1.25 ml of 7% Na_2CO_3 solution and diluted to 3 ml with deionized water. The mixture was shaken and left for 90 min at 25°C for color development and the absorbance at 760 nm was measured using a UV-Vis spectrophotometer (model UV-160A, Shimadzu, Japan). Total phenolic content was determined according to the standard calibration curve of gallic acid solutions. The results were expressed as milligram of gallic acid equivalents per gram sample (mg GAE/ g) on dry weight basis.

Determination of total flavonoid content

A colorimetric method described by Sakanaka *et al*. (2005) was used to determine the total flavonoid content in the crude extracts. Firstly, 0.25 ml of crude extract (of appropriate concentration) was diluted with 1.25 ml of distilled water, followed by the addition of 75 µl of $NaNO_2$ solution (5%). After the mixture was left to stand for 6 min, 150 µl of $AlCl_3$ (10%) was added. The mixture was again left to stand for another 5 min at 25°C before addition of 0.5 ml of 1M NaOH. Finally, the mixture was brought to a total volume of 2.5 ml with distilled water and mixed well. The absorbance was measured at 510 nm with a spectrophotometer (Model UV-160A, Shimadzu, Japan). The results were expressed on dry weight basis as a milligram of catechin equivalents per gram sample (mg CE/ g) based on the standard curve of catechin solutions.

DPPH free radical scavenging activity

The DPPH (2,2-diphenyl-1-picrylhydrazyl) assay was used to determine the free radical scavenging activity of crude extracts following the method described by Kaviarasan *et al*. (2007). Firstly, 1 ml of freshly prepared 100 µM DPPH in methanol was mixed with 1 ml of the extract and the mixture was shaken vigorously. The decrease in DPPH absorbance at 515 nm was measured in the presence of different concentrations of extract using a micro-plate reader (Model: EL800, BioTek Instrument, USA). Standard solutions, ascorbic acid and catechin and control (using distilled water) readings were also taken in a similar manner. The antioxidant activity of fenugreek seed extracts was evaluated based on the following parameters:

(a) DPPH radical scavenging activity was calculated on the basis of the observed decrease in absorbance of the free radical. It was expressed as inhibition (%) of DPPH free radical according to the following equation:

$$\text{Inhibition (\%) of DPPH} = [(\text{A control} - \text{A sample}) / \text{A control}] \times 100,$$

where A is the absorbance.

(b) Results of DPPH inhibition (%) were converted to vitamin C equivalent antioxidant capacity (VCEAC) expressed as mg of vitamin C per g extract. This was done by relating the absorbance decrease of the resultant oxidized solution to the vitamin C standard curve. A vitamin C standard plot of DPPH inhibition (%) against concentration of ascorbic acid was

made with at least five different concentrations of ascorbic acid solution (0.002–0.01 mg/ ml) under the same assay conditions as previously described. The antioxidant capacity in VCEAC was then correlated with total phenolic and flavonoid compounds, and the statistical analysis was performed to evaluate the significance of the relationship. The higher the VCEAC value of test sample, the more effective the antioxidant.

(c) The half-inhibition concentration (IC_{50}) is the concentration of antioxidants required to reduce the absorbance of DPPH free radical to half of its initial value. The IC_{50} values of each sample extract were calculated from plots of inhibition (%) vs concentration of the test sample to establish the dose-response curves. At least five different concentrations of each sample extract were prepared: E-FSP and M-FSP (0.1–0.5 mg/ ml), W-FSP (0.5–2.5 mg/ ml), HW-FSP (0.25–0.45 mg/ ml), W-GeFS (0.02–0.06 mg/ ml). The inhibition (%) was then assayed at the same conditions as described above. The test samples assayed in this study included catechin and vitamin C (0.002–0.01 mg/ ml).

Study on the reaction kinetics

The decrease in absorbance of DPPH radical is caused by the reaction between DPPH free radical and antioxidants resulting in the scavenging of the free radical by hydrogen donation. The reaction kinetic behavior of the FS extracts was investigated and the kinetic rate of each extract was determined through reaction kinetic experiments to determine the percentage of remaining DPPH as a function of time. Sample extracts, E-FSP, M-FSP, W-FSP, HW-FSP and W-GeFS were used at concentrations of 0.5, 0.5, 0.5, 0.45 and 0.06 mg/ ml, respectively.

Evaluation of antimicrobial activity

Test Microorganisms

The test microorganisms used were: *Bacillus subtilis, Staphylococcus aureus,* and *Escherichia coli* O157:H7 obtained from stock cultures maintained in the Food Microbiological Laboratory (School of Industrial Technology, Universiti Sains Malaysia). The bacterial stock cultures were maintained on nutrient agar slants, which were stored at 4°C. Subculturing was carried out every month to maintain bacterial viability. Strains were grown in Mueller-Hinton broth at 37°C for 24 hr before being used for the antimicrobial activity test.

Disc diffusion method

The antimicrobial activity of the crude extracts against the bacterial cultures was determined using agar disc diffusion assay method according to the method described by Hussain *et al*. (2008). A crude extract solution of concentration, 30 mg/ ml was prepared. The bacterial strains were cultured in a Mueller-Hinton broth for 24 hr at 37°C, and diluted with Mueller-Hinton broth before being used. Then, 100 µl of suspension of each test bacteria containing approximately 10^6–10^8 CFU/ ml of bacteria cells (the bacterial suspension was standardized using a commercial 0.5 McFarland standard) was spread on the surface of Mueller–Hinton agar using cotton swabs to create a bacterial culture. Sterile filter paper discs (6 mm in diameter) were individually soaked with 20 µl of each sample extract and then left to dry for 1 hr (under the laminar flow cabinet). Then the dried discs were placed on the agar plates which had previously been inoculated with the test microorganisms. Filter paper discs wetted with distilled water were used as a negative control. Gentamicin (10 µg/ disc) was used as a positive reference to compare the antimicrobial activity. The agar plates were kept at 4°C for 2 hr and then were incubated at 37°C for 24 hr. Antimicrobial activity was evaluated by measuring the area of growth inhibition zones. The area of the whole zone was calculated and then subtracted from the disc area and this difference in area was reported as the zone of inhibition in mm^2 (Maizura *et al*., 2007).

Statistical analysis

Data obtained was analyzed using SPSS software. Analysis of variance (ANOVA) was performed and significant differences between mean values were determined by Duncan's test. The results obtained (samples run in triplicates or more) were expressed as means ± standard deviation. Differences were considered significant at $P < 0.05$.

Results and Discussion

Extraction yield

The moisture content of semi-dried and oven-dried FS was 4.03 ± 0.02% and 2.4 ± 0.7%, respectively. The oven-dried FS were finely powdered and the resulting powder was directly subjected to the extraction process using different solvents. The recovery of bioactive compounds (such as phenolic compounds) from plant materials can be influenced by many factors such as the nature of these compounds, extracting solvents, solids-solvent ratio and extraction conditions (Wijekoon *et al*., 2011). Thus, this study attempted to maximize the extraction method in order to obtain crude ethanol, methanol and water extracts with high concentration

Table 1. Yield of crude fenugreek seed extracts

Crude extracts	Ratio (solids: solvent)	Yield (%)
Extracts from FSP		
Ethanol extract (E-FSP)	1:5	12.87 ± 2.8[b]
	1:8	16.54 ± 1.1[b]
	1:10	14.77 ± 2.3[b]
Methanol extract (M-FSP)	1:5	14.45 ± 1.0[b]
	1:8	14.47 ± 1.4[b]
	1:10	13.60 ±1.0[b]
Water extract (W-FSP)	1:40	26.93 ± 3.3[c]
	1:50	31.80 ± 3.7[d]
	1:60	38.16 ± 2.4[e]
Hot water extract (HW-FSP)	1:20	15.96 ± 1.9[b]
Extract from germinated FS		
Water extract (W-GeFS)	1:10	2.59 ± 0.1[a]

Values are mean ± standard deviation (n = 3). [a–e] Values with different letters significantly different at $P < 0.05$. FSP: Fenugreek seeds powder; FS: Fenugreek seeds

of bioactive compounds. The factors investigated were the types and amount of solvent used and solids-to-liquid ratio. Table 1 shows that the highest extraction yield of 38.16% was obtained in W-FSP extract from ground FS at 1:60, solid: water ratio. It was observed that yields for E-FSP, M-FSP and HW-FSP extracts from ground FS were about 50% less than that of W-FSP extract. No significant differences ($P > 0.05$) in yields among E-FSP, M-FSP and HW-FSP extracts, at all solids to solvent ratios were observed. W-FSP extracts from ground FS at all solids: water ratios (1:40, 1:50 and 1:60) were observed to give significantly higher yields than the other extracts. Also, the yield of W-FSP extracts prepared from FSP significantly increased as the water ratio increased in the extraction medium. These results could be due to the presence of considerable amounts of the galactomannan (water-soluble gum) in FS (Işıklı and Karababa, 2005; Youssef *et al.*, 2009; Chang *et al.*, 2011), and thus an increase in the water ratio can easily facilitate the extraction of more galactomannan, resulting in higher extraction yield. However, further increase in water ratio did not produce any significant increase in the yield (data not shown). This might be due to a saturation effect produced by the increased water ratio without increasing the yield. The addition of increased water ratio (1:60) increases the local density around the bioactive molecules (Bulgarevich *et al.*, 2002). This causes the intermolecular interaction between bioactive compounds and water molecules. Depending on the molecules characteristics, it leads to the formation of specific interactions such as hydrogen bonding. The combination of an increase in density with the development of physical and chemical interactions has an important role on the formation of the solvation complex and consequently on the solubility

Table 2. Total phenolic and flavonoid contents of fenugreek seed extracts (on dry weight basis)

Crude extracts	Total phenolic (mg GAE/ g)	Total flavonoid (mg CE/ g)
Extracts from FSP		
Ethanol extract (E-FSP)	44.96 ± 2.8[c]	14.2 ± 0.5[d]
Methanol extract (M-FSP)	43.15 ± 3.6[c]	9.48 ± 0.1[c]
Water extract (W-FSP)	19.31 ± 0.2[a]	3.76 ± 0.4[a]
Hot water extract (HW-FSP)	25.60 ± 0.2[b]	7.30 ± 0.4[b]
Extract from germinated FS		
Water extract (W-GeFS)	156.3 ± 2.8[d]	38.5 ± 0.9[e]

Values are mean ± standard deviation (n= 3). [a–e] Values with different letters in the same column are significantly different ($P < 0.05$). GAE: gallic acid equivalents; CE: catechin equivalents. FSP: Fenugreek seeds powder; FS: Fenugreek seeds

(Ke *et al.*, 1996). In contrast, the lowest extraction yield (2.6%) was observed in W-GeFS extract of germinated seeds. During the process of germination, increase in protein and starch digestibility occurred and causes subsequent decrease in total weight loss in FS. The decrease in the total fat content along with the loss of free fatty acids, monoglycerides and polar lipids in germinating seeds is coincident with the decrease in the extraction yield (Mansour and El-Adawy, 1994). It is interesting to note that the water and hot water extracts were very viscous due to the presence of water soluble gum, galactomannan in these extracts, thus making extraction and filtration processes difficult.

Total phenolic and flavonoid contents

Phenolic compounds have been confirmed to possess diverse bioactivities beneficial to human health. Table 2 shows the total phenolic and flavonoid contents in FS extracts. Amongst the extracts from ground FS, E-FSP had the highest flavonoids (14.2 mg CE/ g). This suggested that ethanol was a better solvent than methanol and water in extracting flavonoids from ground FS. The results obtained indicated that the quantity of phenolic compounds extracted from FS depended highly on the polarity of extraction solvent. E-FSP and M-FSP extracts contained significantly higher quantities of phenolic compounds compared to W-FSP and HW-FSP extracts from ground FS. However, W-GeFS extract obtained from germinated seeds had significantly highest phenolics (156.3 mg GAE/ g) and flavonoids (38.5 mg CE/ g) compared to all other extracts. Upon germination, fenugreek sprouts have shown to be rich in polyphenols, reducing sugars and minerals (K, Zn and Fe) (Randhir *et al.*, 2004; Shakuntala *et al.*, 2011). Generally, solvent system denatures the cell membranes, simultaneously dissolves the anthocyanins, and stabilizes them. However,

Table 3. Antioxidant capacity and half-inhibition concentration (IC_{50}) of fenugreek seed extracts

Test samples	Conc. (mg/ ml)	Inhibition (%) of DPPH	VCEAC (mg vit C/g)	IC_{50} (µg/ ml)
Extracts from FSP				
Ethanolic extract (E-FSP)	0.5	68.60 ± 0.6^{d}	17.35 ± 0.2^{d}	345.70 ± 6.5^{c}
Methanolic extract (M-FSP)	0.5	64.04 ± 2.0^{c}	16.21 ± 0.5^{c}	361.09 ± 6.5^{d}
Water extract (W-FSP)	0.5	10.31 ± 0.8^{a}	2.61 ± 0.2^{a}	ND
Hot water extract (HW-FSP)	0.45	50.80 ± 0.8^{b}	14.27 ± 0.2^{b}	432.03 ± 1.4^{e}
Water extract from germinated FS (W-GeFS)	0.06	67.95 ± 0.2^{d}	143.28 ± 0.4^{e}	42.10 ± 0.1^{b}
Standard antioxidants				
Catechin	0.01			7.31 ± 0.1^{a}
Vitamin C	0.01			6.32 ± 0.1^{a}

Values are mean ± standard deviation (n= 3). $^{a-e}$ Values with different letters in the same column are significantly different ($P < 0.05$). DPPH: 2,2-Diphenyl-1-picryl-hydrazyl; VCEAC: vitamin C equivalent antioxidant capacity; IC_{50}: Half-inhibition concentration; ND: not detected;. FSP: Fenugreek seeds powder; FS: Fenugreek seeds. Conc.: concentration.

methanol and ethanol has been generally found to be more efficient in extraction of lower molecular weight polyphenols, while the higher molecular weight flavonoids are better extracted with aqueous water. In our study, total phenolics were higher than total flavonoids in all the extracts obtained ($P < 0.05$). Similar trend was also reported by other related studies on other medicinal plants for example in extracts obtained from Japanese persimmon leaf tea (Sakanaka *et al*., 2005) and from loquat flowers (Zhou *et al*., 2011).

Chan *et al*. (2011) reported a yield of about 23% from water extraction of ground fenugreek seeds at 1: 20, solids to water ratio and the phenolic content was about 10 mg GAE/ g. Dixit *et al*. (2005) found that the phenolic content in water and boiled water extracts from germinated FS was 64.6 and 47.6 mg GAE/ g, respectively, whereas the flavonoid content in mg of quercetin/ g was 19.2 (in water extract) and 17.5 (in hot water extract). Bukhari *et al*. (2008) reported total phenolic content of ethanolic and methanolic extracts of ground FS was 6.85 and 5.75 mg GAE/ g, respectively. Other studies found phenolic content of 15.2 mg GAE/ g in the acetone extract of ground FS (Yacoubi *et al.*, 2011) and 7.3 mg GAE/ g in the methanol extract of FS (Alzoreky and Nakahara, 2001). These differences in the total contents of phenolic and flavonoid of the extracts obtained from fenugreek seeds are dependent on the polarity of the solvents used, extraction method and extraction time (Wijekoon *et al*., 2011; Kowalczyk *et al*., 2013). Furthermore, it may be due to the chemical diversity of phenolic and flavonoid compounds and the complexity of composition in plant sources.

Despite these results, plant extracts obtained using organic solvents have limitations. The use of water as the extracting solvent is more desirable than the use of organic solvents due its environmentally friendly and non-toxic characteristics. From the results shown in Table 2, it is evident that water is a good solvent in extracting a sizable quantity of phenolic and flavonoid compounds from germinated fenugreek seeds. Water extracts containing phenols and flavonoids with high activities can safely be exploited in numerous food applications.

Antioxidant activity

Free radical scavenging assay by using DPPH radical as a substrate is widely used to evaluate the antioxidant capacity of extracts produced from the medicinal plants in which higher inhibition level is an indicator of a strong antioxidant. The antioxidant activity is measured based on the reduction of DPPH free radical by an antioxidant and was calculated on the basis of the observed decrease in absorbance of the radical. It was expressed as percent inhibition of DPPH free radical. All FS extracts obtained in this study exhibited antioxidant activity. Preliminary experiments performed to determine the DPPH radical scavenging activity of all crude FS extracts at varying concentrations ranging from 0.02 to 2.5 mg/ ml, showed that as the concentration of extracts increased, DPPH radical scavenging activity also increased (data not shown). Table 3 shows that among extracts (0.45-0.5 mg/ml), E-FSP exhibited the highest inhibition activity (68.6%), followed by M-FSP (64.0%), HW-FSP (50.8%) and W-FSP (10.3%). High DPPH inhibition activity in E-FSP could be attributed to its higher contents of flavonoid and phenolic compared to other extracts obtained from ground FS (Table 2). However, W-GeFS extract from germinated seeds at a much lower concentration

Table 4. Antimicrobial activity of fenugreek seed extracts by measuring zone of inhibition (mm^2) using disc diffusion method

Crude fenugreek seed (FS) extracts	Zone of inhibition (mm^2)		
	B. subtilis	*S. aureus*	*E. coli*
Extracts from FSP			
Ethanolic extract (E-FSP)	51.8 ± 3.6^{bB}	38.2 ± 3.9^{aB}	70.3 ± 4.8^{cC}
Methanolic extract (M-FSP)	55.1 ± 4.4^{cB}	25.9 ± 3.5^{aA}	44.1 ± 3.4^{bB}
Water extract (W-FSP)	51.9 ± 3.6^{bB}	44.1 ± 3.4^{abC}	70.3 ± 4.8^{cC}
Hot water extract (HW-FSP)	19.5 ± 3.3^{aA}	45.6 ± 4.2^{bC}	23.3 ± 2.9^{aA}
Water extract from germinated FS (W-GeFS)	79.2 ± 5.1^{aC}	72.0 ± 4.8^{aD}	119.1 ± 5.9^{bD}
Gentamicin (positive reference)	470.3 ± 10.8^{cD}	348.3 ± 7.6^{bE}	170.2 ± 5.5^{aE}
Blank (negative control)	ND	ND	ND

Values are mean ± standard deviation (n= 5). [a–c] Values with different small letters within the same row are significantly different ($P < 0.05$). [A–E] Values with different capital letters within the same column are significantly different ($P < 0.05$). ND: not detected; FSP: Fenugreek seeds powder; FS: Fenugreek seeds.

range (0.02–0.06 mg/ ml) than the other extracts, had to be used in order to obtain similar results for % DPPH inhibition as the other extracts. As seen in Table 3, the W-GeFS extract from germinated seeds used at a much lower concentration (0.06 mg/ ml) than all other extracts, exhibited a very strong scavenging activity of 67.95%, and similar to that of E-FSP extract which is at a higher concentration (0.5 mg/ ml). From these results, it can be concluded that W-GeFS extract from germinated seeds exhibited stronger antioxidant activity compared to all extracts prepared, which could be attributed to its high phenolic (156.5 mg GAE/g) and flavonoid (38.5 mg CE/g) contents. These results highlighted that higher contents of phenolic and/or flavonoid led to higher antioxidant activity.

It is known that phytochemicals such as phenolic and flavonoid compounds were mostly responsible for such antioxidant activity. Ethanolic extract of ground FS tested at 1 mg/ml exhibited 82.05% DPPH scavenging activity (Priya *et al*., 2011) whilst methanolic extract at the same concentration showed 74.16% DPPH scavenging activity (Jha and Srivastava, 2012) which are lower than the results shown by all the extracts obtained (with the exception of W-FSP) in our study. Total phenolics and flavonoids were then correlated to vitamin C equivalent antioxidant capacity (VCEAC, mg vitamin C/ g) obtained from the vitamin C standard curve (data not shown). From these plots, a linear relationship was observed between antioxidant capacity and total phenolic or flavonoid content in FS extracts ($r^2 = 0.9698–0.9751$). This indicates that the higher the total phenolic/ flavonoid contents in the extracts, the greater is the antioxidant capacity. The VCEAC values of the FS extracts are given in Table 3. Among all the extracts obtained, W-GeFS extract from germinated seeds exhibited a very high VCEAC value of 143.28 mg vitamin C/ g which is attributed to its high contents of phenolic and flavonoid, which in turn led to strong antioxidant activity. It was observed that as the contents of phenolic and flavonoid increased in the FS extracts, the VCEAC values also increased, indicating a positive relationship between antioxidant capacity and total phenolics and flavonoids in FS extracts. In this context, many studies have similarly shown that phenolics and flavonoids were positively correlated to antioxidant capacities in plant extracts (Bouayed *et al*., 2007; Zhou *et al*., 2011). These results revealed that phenolics and flavonoids play an important role in free radical scavenging.

In order to further quantify the antioxidant activity, the results of antioxidant activity were also expressed as the half-inhibition concentration (IC_{50}). The lower the IC_{50} value, the stronger is the antioxidant activity. Table 3 presents the IC_{50} values as another parameter in order to evaluate antioxidant properties of FS extracts in comparison to standard antioxidants such as ascorbic acid and catechin. Among the extracts obtained from ground FS, E-FSP had the lowest IC_{50} value (345.70 µg/ ml), indicating strong antioxidant activity as compared to other extracts obtained from ground FS. The IC_{50} value of W-FSP from ground FS could not be determined because even at high extract concentrations, it was unable to inhibit 50%

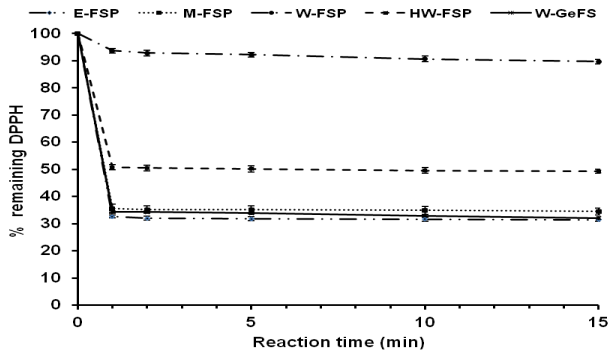

Figure 1. Kinetics of the reaction of fenugreek seed extracts with DPPH free radical.
E-FSP: ethanol extract, 0.5 mg/ml; M-FSP: methanol extract, 0.5 mg/ ml; W-FSP: water extract, 0.5 mg/ ml; HW-FSP: hot water extract, 0.45 mg/ ml; W-GeFS: water extract from germinated fenugreek seeds, 0.06 mg/ ml. DPPH: 2,2-Diphenyl-1-picryl-hydrazyl.

of the initial value of DPPH free radical indicating that W-FSP extract exhibited a very weak antioxidant capacity compared to other extracts. At a very high W-FSP extract concentration of 2.5 mg/ ml in the reaction medium, DPPH radical was observed to lose only about 38% from its initial activity. Among all the extracts obtained from FS, W-GeFS had the lowest IC_{50} value (42.1 µg/ ml), indicating stronger antioxidant activity compared to all extracts obtained from FS. However, when compared to catechin and ascorbic acid, the antioxidant activity in W-GeFS extract was found to be about 6–7 times lower than catechin and ascorbic acid. Chan *et al.* (2011) reported that the hot water extract of ground FS exhibited nearly 60 times lower scavenging activity than ascorbic acid. No significant difference in IC_{50} values was observed between ascorbic acid and catechin. Other studies have reported IC_{50} values: 350 µg/ ml in methanol extract of ground FS (Kaviarasan *et al*., 2007); 366.52 µg/ ml in methanol extract of non-irradiated FS (Chatterjee *et al*., 2011); 350 µg/ ml in ethanol extract of ground FS (Priya *et al*., 2011); and 156 µg/ ml in whole FS extract, 138 µg/ ml in husk extract and 178 µg/ ml in endosperm extract (Madhava Naidu *et al.*, 2011), and these values indicated much lower antioxidant activity present in these extracts compared to the water extract of germinated fenugreek seeds (W-GeFS) obtained in this study.

Reaction kinetics

The kinetics of the reaction between FS extracts and DPPH free radical were studied. E-FSP, M-FSP and W-FSP extracts of ground FS were tested at a concentration of 0.5 mg/ ml whilst HW-FSP and W-GeFS extracts were tested at 0.45 mg/ ml and 0.06 mg/ ml, respectively. The remaining DPPH free radical (which corresponded inversely to the radical scavenging activity) was measured during a reaction time of 15 min. A plot of percentage DDPH remaining as a function of time are given in Figure 1. It was found that the reaction between FS extracts and DPPH free radical reached a steady state after 2 min. The rates of reaction for the extracts (in %/ min) were observed to be in the following order: E-FSP (67.4) > W-GeFS (65.6) > M-FSP (64.5) > HW-FSP (49.3) > W-FSP (6.4). W-FSP water extract from ground FS showed the slowest kinetic behavior (6.4%/ min). It can be concluded that W-GeFS showed a very fast reaction kinetic even at very low concentration (0.06 mg/ ml) with very small difference from E-FSP extract at 0.5 mg/ ml. Generally, reaction kinetic behavior can be classified into three types with ascorbic acid as an example of a very good antioxidant showing rapid kinetic behavior, reacting very rapidly with DPPH free radical and reaching a steady state in less than 1 min (Brand-Williams *et al*., 1995). However, most compounds were intermediate in behavior, reaching a steady state after about 5 to 30 min. The activity of natural antioxidants often decreases during their isolation and purification due to their decomposition (Bandoniene and Murkovic, 2002). In conclusion, W-GeFS extract obtained during germination process of FS had high levels of phenolic and flavonoid compounds with good antioxidant properties.

Antimicrobial activity

Disc diffusion techniques are extensively used to evaluate the antimicrobial activity of the antibiotics and plant extracts against different types of the microorganisms. These techniques are based on the use of paper discs saturated with solutions of substances to be tested against the microbes. In the present study, the antimicrobial activity of FS extracts was evaluated against three types of the microorganisms on the basis of disc-diffusion technique, by quantitatively measuring the zone of growth inhibition which is the clear zone surrounding the circular disc. In these experiments, gentamicin was used as a positive reference due to its bactericidal properties against a broad spectrum of Gram-positive and Gram-negative pathogens such as *Staphylococcus aureus* and *Escherichia coli.* Filter paper disc saturated with distilled water was used as a negative control.

The results shown in Table 4 indicate that all FS extracts have the potential to inhibit the test microorganisms (*B. subtilis, S. aureus* and *E. coli*). The differences in antimicrobial activity of the FS extracts could be linked to their different

composition of bioactive compounds. Amongst the prepared FS extracts, W-GeFS extract of germinated seeds exhibited the highest antimicrobial activity, as observed from the area of the zone of inhibition, against all test microorganisms. This could be due to the presence of high content of phenols and other bioactive compounds in this water extract as presented in Table 2. In addition, W-GeFS extract showed significantly stronger antimicrobial activity (zone of inhibition, 119.1 mm^2) against *E. coli* than against *B. subtilis* and *S. aureus*. However, the antimicrobial activity of the W-GeFS extract was lower than the standard antibiotic (gentamicin). These results are in agreement with the studies reported by Randhir *et al*. (2004) and Haouala *et al*. (2008) who evaluated the antimicrobial activity of germinated FS extracts against different types of the microorganisms. Khanra *et al.* (2010) found that the ethanolic extract of FS had antibacterial activity against *B. subtilis, S. aureus* and *E. coli*. Das *et al*. (2012) reported that aqueous and ethanol extracts of FS showed antimicrobial activity against *S. aureus* and *E. coli*. In another study, Asimi *et al*. (2013) found that water and ethyl acetate extracts of FS exhibited antimicrobial activity against two bacterial strains (*Vibrio vulnificus* and *Micrococcus luteus*).

Conclusions

The various fenugreek seed extracts from different solvents obtained in this study exhibited antioxidant and antimicrobial activity. However, water extract of germinated fenugreek seed (W-GeFS) showed the highest antioxidant and antimicrobial activities compared to the other extracts. This study reveals strong antioxidant potential with significant antimicrobial activity in germinated fenugreek seeds which may be due partly to the presence of flavonoids and polyphenols. Finally we conclude that water extract of germinated fenugreek seeds shows great potential to be used as a natural antioxidant and antimicrobial source and to be further explored for use in the food industry. The present study provides additional information for supporting the use of germination process as economic, easy and safe method to extract the bioactive compounds from medicinal plant seeds with significant levels of their activities.

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References

Acharya, S. N., Thomas, J. E. and Basu, S. K. 2008. Fenugreek, an alternative crop for semiarid regions of North America. Crop Science 48 (3): 841–853.

Alzoreky, N. and Nakahara, K. 2001. Antioxidant activity of some edible Yemeni plants evaluated by Ferrylmyoglobin/ ABTS $^+$ assay. Food Science and Technology Research 7 (2): 141–144.

Antolovich, M., Prenzler, P. D., Patsalides, E., Mcdonald, S. and Robards, K. 2002. Methods for testing antioxidant activity. Analyst 127 (1): 183–198.

Asimi, O. A., Sahu, N. P. and Pal, A. K. 2013. Antioxidant capacity of crude water and ethyl acetate extracts of some Indian spices and their antimicrobial activity against *Vibrio vulnificus* and *Micrococcus luteus.* Journal of Medicinal Plants Research 7 (26): 1907–1915.

Bandoniene, D. and Murkovic, M. 2002. On-Line HPLC-DPPH screening method for evaluation of radical scavenging phenols extracted from apples (*Malus domestica* L.). Journal of Agricultural and Food Chemistry 50 (9): 2482–2487.

Board, N. 2010. Hand book on spices. Asia Pacific Business Press Inc, Delhi. 137-138.

Bouayed, J., Piri, K., Rammal, H., Dicko, A., Desor, F., Younos, C. and Soulimani, R. 2007. Comparative evaluation of the antioxidant potential of some Iranian medicinal plants. Food Chemistry 104 (1): 364–368.

Brand-Williams, W., Cuvelier, M. E. and Berset C. 1995. Use of a free radical method to evaluate antioxidant activity. LWT – Food Science and Technology 28 (1): 25–30.

Bukhari, S. B., Bhanger, M. I. and Memon, S. 2008. Antioxidative activity of extracts from fenugreek seeds *(Trigonella foenum-graecum)*. Pakistan Journal of Analytical and Environmental Chemistry 9 (2): 78–83.

Bulgarevich, D. S., Sako, T., Sugeta, T., Otake, K., Takebayashi, Y., Kamizawa, C., Horikawa, Y. and Kato, M. 2002. The role of general and hydrogen-bonding interactions in the solvation processes of organic compounds by supercritical CO_2/n-alcohol mixtures. Industrial and Engineering Chemistry Research 41(9): 2074–2081.

Chan, K. W., Iqbal, S., Khong, N. M. H. and Babji, A. S. 2011. Preparation of deodorized antioxidant rich extracts from 15 selected spices through optimized aqueous extraction. Journal of Medicinal Plants Research 5 (25): 6067–6075.

Chang, Y., Cui, S., Roberts, K. T., Ng, P. K. W. and Wang, Q. 2011. Evaluation of extrusion-modified fenugreek gum. Food Hydrocolloids 25 (5): 1296–1301.

Chatterjee, S., Variyar, P. S. and Sharma, A. 2011. Effect of γ-Irradiation on the antioxidant activity of fenugreek *(Trigonella foenum graecum)* seed extracts. Electronic

Journal of Environmental, Agricultural and Food chemistry 10 (8): 2798–2805.

Das, S., Anjeza, C. and Mandal, S. 2012. Synergistic or additive antimicrobial activities of Indian spice and herbal extracts against pathogenic, probiotic and food-spoiler micro-organisms. International Food Research Journal 19 (3): 1185–1191.

Dixit, P., Ghaskadbi, S., Mohan, H. and Devasagayam, T. P. A. 2005. Antioxidant properties of germinated fenugreek seeds. Phytotherapy Research 19 (11): 977–983.

Giner, M. J., Vegara, S., Funes, L., Marti, N., Saura, D., Micol, V. and Valero, M. 2012. Antimicrobial activity of food-compatible plant extracts and chitosan against naturally occurring micro-organisms in tomato juice. Journal of the Science of Food and Agriculture 92 (9): 1917–1923.

Goncalves, S., Gomes, D., Costa, P. and Romano, A. 2013. The phenolic content and antioxidant activity of infusions from Mediterranean medicinal plants. Industrial Crops and Products 43, 465–471.

Haouala, R., Hawala, S., El-Ayeb, A., Khanfir, R. and Boughanmi, N. 2008. Aqueous and organic extracts of *Trigonella foenum- graecum* L. inhibit the mycelia growth of fungi. Journal of Environmental Sciences 20 (12): 1453–1457.

Hussain, A. I, Anwar, F., Hussain Sherazi, S. T. and Przybylski, R. 2008. Chemical composition, antioxidant and antimicrobial activities of basil *(Ocimum basilicum)* essential oils depend on seasonal variations. Food Chemistry 108 (3): 986–995.

Işıklı N. D. and Karababa, E. 2005. Rheological characterization of fenugreek paste (çemen). Journal of Food Engineering 69 (2): 185–190.

Jha, S. K. and Srivastava, S. K. (2012). Determination of antioxidant activity of *Trigonella foenum-graecum* Linn. in mice. Annals Phytomedicin 1(1): 69–74.

Kaviarasan, S., Naik, G. H., Gangabhagirathi, R., Anuradha, C. V. and Priyadarsini, K.I. 2007. In vitro studies on antiradical and antioxidant activities of fenugreek *(Trigonella foenum graecum)* seeds. Food Chemistry 103 (1): 31–37.

Ke, J., Mao, C., Zhong, M., Han, B. and Yan, H. 1996. Solubilities of salicylic acid in supercritical carbon dioxide with ethanol cosolvent. Journal of Supercritical Fluids 9 (2): 82–87.

Kenny, O., Smyth, T. J., Hewage, C. M. and Brunton, N. P. 2013. Antioxidant properties and quantitative UPLC-MS analysis of phenolic compounds from extracts of fenugreek *(Trigonella foenum-graecum)* seeds and bitter melon *(Momordica charantia)* fruit. Food Chemistry 141 (4): 4295–4302.

Khanra, R., Atanu, C., Pal S. B., Ashutosh G. and Kalyan, S. 2010. Antimicrobial activity of ethanolic extract of *Trigonella foenum-graecum* Linn. International Research Journal of Pharmacy 1 (1): 181–183.

Khole, S., Chatterjee, S., Variyar, P., Sharma, A., Devasagayam, T. P. A. and Ghaskadbi, S. 2014. Bioactive constituents of germinated fenugreek seeds with strong antioxidant potential. Journal of Functional Foods 6: 270–279.

Khoobchandani, M., Ojeswi, B. K., Ganesh, N., Srivastava, M. M., Gabbanini, S., Matera, R., Iori, R. and Valgimigli, L. 2010. Antimicrobial properties and analytical profile of traditional *Eruca sativa* seed oil: Comparison with various aerial and root plant extracts. Food Chemistry 120 (1): 217–224.

Kowalczyk, D., Swieca, M., Cichocka, J. and Gawlik-Dziki, U. 2013. The phenolic content and antioxidant activity of the aqueous and hydroalcoholic extracts of hops and their pellets. Journal of the Institute of Brewing 119 (3): 103–110.

Liu, Y., Kakani, R. and Nair. M. G. 2012. Compounds in functional food fenugreek spice exhibit anti-inflammatory and antioxidant activities. Food Chemistry 131 (4): 1187–1192.

Madhava Naidu, M., Shyamala, B. N., Pura Naik, J., Sulochanamma, G. and Srinivas, P. 2011. Chemical composition and antioxidant activity of the husk and endosperm of fenugreek seeds. LWT- Food Science and Technology 44 (2): 451–456.

Maizura, M., Fazilah, A. Norziah, M. H. And Karim, A. A. 2007. Antibacterial activity and mechanical properties of partially hydrolyzed sago starch-alginate edible film containing lemongrass oil. Journal of Food Science 72 (6): 324–330.

Mansour, E. H. and El-Adawy, T. A. 1994. Nutritional potential and functional properties of heat-treated and germinated fenugreek seeds. LWT-Food Science and Technology 27 (6): 568–572.

Meghwal, M. and Goswami, T. K. 2012. A review on the functional properties, nutritional content, medicinal utilization and potential application of fenugreek. Journal of Food Processing and Technology 3 (9): 1–10.

Negi, P.S. 2012. Plant extracts for the control of bacterial growth: Efficacy, stability and safety issues for food application. International Journal of Food Microbiology 156 (1): 7–17.

Priya, V., Jananie, R. K. and Vijayalakshmi, K. 2011. Studies on anti-oxidant activity of *Trigonella foenum graecum* seed using *in vitro* models. International Journal of Pharmaceutical Science and Research 2 (10): 2704–2708.

Rababah, T. M., Hettiarachchy, N. S. and Horax, R. 2004. Total phenolics and antioxidant activities of fenugreek, green tea, black tea, grape seed, ginger, rosemary, gotu kola, and *Ginkgo* extracts, Vitamin E, and tert-Butylhydroquinone. Journal of Agricultural and Food Chemistry 52 (16): 5183–5186.

Randhir, R., Lin, Y.-T. and Shetty, K. 2004. Phenolics, their antioxidant and antimicrobial activity in dark germinated fenugreek sprouts in response to peptide and phytochemical elicitors. Asia Pacific Journal of Clinical Nutrition 13 (3): 295–307.

Sakanaka, S., Tachibana, Y. and Okada, Y. 2005. Preparation and antioxidant properties of extracts of Japanese persimmon leaf tea (kakinoha-cha). Food Chemistry 89 (4): 569–575.

Shakuntala, S., Naik, J. P., Jeyarani, T., Naidu, M. M. and

Srinivas, P. (2011). Characterisation of germinated fenugreek (*Trigonella foenum-graecum* L.) seed fractions. International Journal of Food Science & Technology, 46 (11): 2337–2343.

Suhaj, M. 2006. Spice antioxidants isolation and their antiradical activity: a review. Journal of Food Composition and Analysis 19 (6): 531–537.

Wijekoon, M. M. J. O., Bhat, R. and Karim, A. A. 2011. Effect of extraction solvents on the phenolic compounds and antioxidant activities of bunga kantan (*Etlingera elatior* Jack) inflorescence. Journal of Food Composition and Analysis 24 (4): 615–619.

Wojdylo, A., Oszmianski, J. and Czemerys, R. 2007. Antioxidant activity and phenolic compounds in 32 selected herbs. Food Chemistry 105 (3): 940–949.

Yacoubi, L., Rabaoui, L., Hamdaoui, M. H., Fattouch, S., Serairi, R., Kourda, N. and Khamsa, S.B. 2011. Anti oxidative and anti inflammatory effects of *Trigonella foenum graecum* Linnaeus, 1753 (Fenugreek) seed extract in experimental pulmonary fibrosis. Journal of Medicinal Plants Research 5 (17): 4315–4325.

Youssef, M. K., Wang, Q., Cui, S. W. and Barbut, S. 2009. Purification and partial physicochemical characteristics of protein free fenugreek gums. Food Hydrocolloids 23 (8): 2049–2053.

Zhou, C., Sun, C., Chen, K. and Li, X. 2011. Flavonoids, phenolics, and antioxidant capacity in the flower of *Eriobotrya japonica* Lindl. International Journal of Molecular Sciences 12 (5): 2935–2945.