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Phytochemical screening, antimicrobial and antioxidant activity determination of *Trigonella foenum-graecum* seeds

Abstract

To evaluate the phytochemical constituents by maceration and Soxhlet extract, antimicrobial and antioxidant scavenging activity in the seed of Trigonella foenum-graecum. In the present research, maceration and Soxhlet extraction were performed to the seed of Trigonella foenum-graecum by using 95% ethanol. Phytochemical analysis for the important chemical constituents from ethanolic extract was carried out. Antimicrobial activity of Trigonella foenum-graecum extract was carried out using agar well diffusion method by the zone of inhibition on nutrient agar. MIC and MBC tests were carried out to determine the relatively antimicrobial properties of the extract. Antioxidant activity of Trigonella foenum-graecum was investigated using DPPH free radical scavenging activity assay and total phenolic content test to determine the total amount of phenolic compound of the seed extract. For phytochemical analysis, test for alkaloids, test for glycosides, test for saponin, test for Salkowski test, test for anthraquinone, test for tannins, test for phenol, test for carbohydrates that showed positive results for Trigonella foenum-graecum extract while the extract showed negative results towards the flavonoid test and reducing sugar test. For agar well diffusion method of antimicrobial activity screening, all the concentrations of Trigonella foenum-graecum showed negative results in all the bacteria except for Bacillus subtilis. The concentrations of extract should increase in order to obtain better results. The antioxidant analysis, total phenolic contents and DPPH radical scavenging showed positive antioxidant results.

Keywords: Trigonella foenum-graecum, agar well diffusion method, bacterial strains, inhibition, anticancer

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Abbreviations: DPPH, 2,2-diphenyl-1-picrylhydrazyl; TPC, total phenolic contents; MIC, minimum inhibitory concentration; MBC, minimum bactericidal concentration; BHT, butylated hydroxytoluene

Introduction

Trigonella foenum-graecum L. also known as Fenugreek which is herb that looks similar to clover. It is a legume and its leaves consist of three small oblong or obovate leaflets. It belongs to the plant family of Fabaceae and grows natively in an area of Iran and Northern India. However, it is now cultivated universally in the area of North and East Africa, China, Ukraine and Greece. Fenugreek seeds are commonly used as spices in the cooking for seasoning worldwide, while its leaves can be eaten as green leafy vegetables in the daily diet. Fenugreek seeds have been in use for over 2500 years. Its seeds taste bitter, aromatic and are recognized for their therapeutic properties in some diseases such as diabetes mellitus and hypertension. India is the major producer of fenugreek and main consumer for culinary and medicinal uses.[1] The plant and seeds are shown in Figures 1 and 2 respectively. Taxonomical classification of Fenugreek is mentioned in Table 1.[2] Seeds of *T. foenum-graecum* have been used extensively to prepare extracts and powders for the use for therapeutic purpose. According to many researchers, the various medicinal properties of *T. foenum-graecum*, has been found as antimicrobial, antidiabetic and anticancer. The bioactive constituents of seeds of *T. foenum-graecum* such as volatile oils and alkaloid, flavonoid and phenol were reported to have the tendency to exert effect on the bacteria, fungi and parasites (Table 1).[3]

Figure 1 & Figure 2 *Trigonella foenum-graecum* plant with flower and seeds.

Table 1 Taxonomical classification of Fenugreek[2]

Taxanomy	Classification
Kingdom	Plantae
Subkingdom	Tracheobionta
Superdivison	Spermatophyta
Division	Magnoliophyta
Class	Magnoliopsida
Subclass	Rosidae
Order	Fabales

Table Continues...

Taxanomy	Classification
Family	Fabaceae
Genus	*Trigonella* L.
Species	*Trigonella foenum-graecum* L.

The name fenugreek comes from *foenum-graecum*, meaning Greek hay, as the plant was traditionally used to scent inferior hay. The name, *Trigonella*, is derived from the ancient Greek name, which denotes the meaning of 'three-angled' and refers to the triangular shape of the flowers. The first recorded use of fenugreek is described in an ancient Egyptian Papyrus dated to 1500 B.C. The plant is known in different names based upon the geographical region as shown in Table 2.

Table 2 Common names of Fenugreek[4]

Languages	Names
Malay	Halba
Chinese	胡芦巴 [hú lú bā], Ku Dou
Tamil	Meti, Vendayam
Urdu	Methi
Arabic	Hulba, Hilbeh
Italian	Fieno Greco
Dutch	Fenegriek
Norwegian	Bukkehonrkler
Portuguese	Feno-grego, Alforva
Farsi	Sambelil
Swedish	Bockshornklee
Indonesian	Kelabet, Klabat, Kelabat
German	Bockshornsamen
Russian	Sambala, Pazhitnik
Finnish	Sarviapila
French	Fenugrec, Trigonelle
Spanish	Alholva, Fenogrec
Sanskrit	Methika
Japanese	Fenu-guriku
Korean	Horopa, Penigurik

Description of the plant

It is a rigidly upright hairy annual legume which can reach a height of 30 to 60cm. The plant grows to a height of about three feet, has three parts leaves, the long, slender stems possess tripartite, toothed, grey-green obovate leaves which normally 20-25mm long. *Trigonella foenum-graecum* has long stalked leaves up to 5cm long stipules triangular, lanceolate, leaflets about 2.5 cm long, obovate to oblanceolate. The root is a mass of fingery structures. The colour of sessile axillary flowers is white or yellowish. The thin, sword-shaped pods are 10-15cm long, and have a curved braided tip, each with 10-20 seeds. The plant emits a spicy scent that will remain on the hands after touching. Wild and cultivated varieties exist. Flowers are 1 to 2, axillary, sessile, raceme, whitish or lemon yellow that bloom from June to July. Mild Mediterranean climates are most suitable for the growth of the plant. The plant matures in about duration of four months. The flowering season for the herb fenugreek is usually midsummer. Fenugreek seeds are about 5mm long and are hard. These are brownish yellow in colour although the colour may vary and angular in shape but some are oblong, some are diamond- shaped, some are almost cubic, and the sides are about 3mm long. A deep furrow divides them into two parts. They are available in whole and dried, or as a dull yellow powder, ground from the roasted seeds. According to the report, the life zone of fenugreek is 8 to 27°C, while the annual precipitation is 0.4 to 1.5 meters and a soil pH of 5.3 to 8.2. The plant thrives in rich, well-drained soils under plenty of sunlight. It grows slowly and weakly in cold temperatures and moist soils. As a leguminous plant, fenugreek requires almost no nitrogen fertilizer, and the plant can use nitrogen to enrich the soils. As native of the Mediterranean area, the seeds have been used for medicinal purposes for thousands of years. It is undeniable that fenugreek is one of the oldest cultivated medicinal plants, and now fenugreek is widely grown today in the Mediterranean countries, Pakistan, Argentina, France, India, North Africa, and the United States as a food, condiment, medicinal, dye, and forage plant.[5]

Experimental

Materials and methods

500g of seeds of *Trigonella foenum-graecum* (Fenugreek) were weighed and allowed to dry under room temperature for duration of one week. The seeds were placed at shaded area without exposing to the sunlight to avoid any evaporation of active constituent such as phenol. The seeds were crushed into powder by using the crusher and collected in the conical flasks.

Maceration extraction of seeds powder using ethanol 95%

250g of seeds powder were weighed and added in 500ml of ethanol 95%. The mouth of the conical flask was filmed with para-film and the flask was covered with aluminium foil to avoid any evaporation of the ethanol and the active components of the extract. The flask was then kept in the dark in the cupboard for one week. The mixture was filtered by using muslin cloth. The filtrate was collected in a conical flask and kept covered with parafilm and aluminium foil.

Soxhlet extraction of seeds powder using ethanol 95%

The weighed seeds powder was transferred to the porous extraction thimble of the Soxhlet apparatus and 350ml of ethanol was transferred into the 500ml round bottom flask. The temperature was maintained throughout the extraction process. The duration of Soxhlet extraction process was completed in 24 hours. Decolorization of yellow colour of the seeds powder at the extraction chamber could be seen in the end of the extraction process. The Soxhlet extract was collected from the round bottom flask and was filtrated using muslin cloth. The filtrate was transferred to a conical flask.

Concentration of extracts using rotary evaporator

Both the maceration extract and Soxhlet extract were underwent evaporation by using rotary evaporator. Both the extracts were concentrated separately. The temperature of the heating water bath

was set up to 63°C and the speed of the rotator was set to 100rpm. This evaporation process required approximately 4 hours to be completed. The same procedure was adopted with Soxhlet extract. Both the maceration extract and Soxhlet extracts were transferred to the conical flasks separately. The mouth of conical flasks was sealed with parafilm.

Methodolgy (phytochemical analysis)3,6–15

The results of phytochemical analysis are tabulated in Table 3.

Table 3 Results of phytochemical screening

S.No	Test	Ethanolic soxhlet extract	Ethanolic maceration extract
1	Alkaloid Test	+	+
2	Reducing Sugar Test	-	-
3	Saponin Test	++	+
4	Salkowski Test	+	+
5	Anthraquinone Test	+	+
6	Glycoside Test	+	+
7	Tannins Test	+	+
8	Flavonoid Test	-	-
9	Carbohydrate Test	+	+
10	Phenol Test	+	+

+ =Indicates positive result, ++ =Indicates more positive reaction towards the test, - =Indicates negative result

Alkaloids

1.0ml of the extract warmed with 2.0ml of 2.0 % Sulfuric Acid for 2 minutes. It was filtered and few drops of Dragendorff's reagent were added. Orange red precipitate was observed in all the extracts. This indicated the presence of alkaloids.

Reducing Sugars

1.0ml of each extract were shaken with 1ml of distilled water and filtered. Filtrate boiled with few drops of Fehling's solution A and B (equal volume of Fehling's A and Fehling's B was mixed) for few minutes. Orange red precipitate indicated reducing sugars.

Saponins

1.0ml of each extract added with 5.0ml of distilled water and heated to boil. Frothing appearance of creamy mist of small bubbles indicated presence of saponins.

Terpenoids or Steroids (Salkowski Test)

1.0ml of each extract was mixed with 2.0ml of chloroform ($CHC1_3$) and 3.0ml concentrated sulphuric acid (H_2SO_4) was carefully added to form a layer. Reddish brown coloration at the interface slowed the presence of terpenoids.

Anthraquinones

1.0ml of each extract was boiled with 2.0ml of 10.0 % Hydrochloric Acid (HCl) solution for few minutes in a water bath. It was filtered and allowed to cool. Equal volume of chloroform ($CHCl_3$) was added to filtrate. Few drops of 10.0 % NH_3 were added to mixture and heated. A rose-pink color indicated anthraquinones.

Glycosides

1.0ml of each extract was hydrolyzed with 1.0ml of 10.0% Hydrochloric Acid (HCl) solution and neutralized with 1.0ml of 0.2M Sodium Hydroxide (NaOH) solution. A few drops of mixture of Fehling's solution A and B were added. Red precipitate showed the presence of glycosides.

Tannins

1.0ml of each extract was mixed with 2.0ml of distilled water and heated on water bath. The heated mixture filtered and 1ml of ferric chloride added to filtrate. Dark green solution indicated presence of tannins.

Flavonoids

1.0ml of each extract was dissolved in 1.0ml of diluted 0.2M Sodium Hydroxide (NaOH) and 1.0ml of 10% Hydrochloric Acid (HCl) solution was added. Yellow solution that turned colourless indicated flavonoids.

Carbohydrates

0.2ml of Molisch's Reagent was added to 1.0ml of each extract in a small test tube. After mixing, the tube was tilted. Without stirring, 0.5ml of concentrated Sulfuric Acid (H_2SO_4) was carefully added by pouring down the side of the test tube. A red violet ring at the interface between acid (bottom) and aqueous (upper) layer showed the presence of carbohydrates.

Phenols

Few drops of 10.0% Lead Acetate solution was added to 1.0ml of each extract in a small test tube. White precipitate showed the presence of phenols.

Antimicrobial activity screening

For the antimicrobial activity of the plant extract, agar well diffusion method was used in this study. MIC (minimum inhibitory concentration) was further carried out with the extract of *T. foenum-graecum* and MBC was carried out also to reconfirm the MIC (minimum inhibitory concentration). Minimum inhibitory concentration (MIC) is the lowest concentration of an antimicrobial agent required to inhibit the visible growth of a microorganism after overnight incubation. The antimicrobial activity screening procedures were performed based on the protocol as stated by Iqbal,[16] Baydoun et al.,[17] with slight modification.

Agar well diffusion method

Agar well diffusion method is widely used to evaluate the antibacterial activity of plants or microbial extracts. The agar plate surface was inoculated by spreading a volume of the microbial inoculum over the entire agar surface. Then, a hole with a diameter of 6 to 8mm was punched aseptically with a sterile metallic borer, and a volume (20–100µL) of the antimicrobial agent or extract solution at desired concentration was introduced into the well. Then, agar plates were incubated under suitable conditions depending upon the test microorganism.[18] The standard microorganisms used in this study were: *Bacillus subtilis* (ATCC 6633), *Staphylococcus aureus* (ATCC

29737), *Escherichia coli* (ATCC 8739), *Pseudomonas aeruginosa* (ATCC 27853). All of the process of antimicrobial screening activity was done in laminar air flow cabinet to avoid any contamination or cross contamination occur especially during the process nutrient agar preparation and well diffusion test.

Serial dilution of ethanolic macerated extract

4g of the gummy macerated extract and 40ml of distilled water was measured and distilled water was used for serial dilution and sent to autoclave prior to use for sterilization. This gave a stock solution which contained a concentration of 100mg/ml. The solution was then transferred into a test tube and mixed well by using vortex mixer and water bath sonicator. Six dilutions were prepared from the extract as 10mg/ml, 20mg/ml, 40mg/ml, 60mg/ml, 80mg/ml and 100mg/ml.

Preparation of bacterial strains

0.65g of nutrient broth was weighed and dissolved in 50ml of distilled water. Magnetic stirrer was used to dissolve the nutrient broth completely. Total of four nutrient broths were prepared by following in the same way. The mouth of the four conical flasks were fitted with cottons and covered with aluminium foil. Then four conical flasks containing the nutrient broth were subjected to autoclave for 2 hours at 121°C for sterilization and were allowed to cool down. Inoculation of bacteria strains into the nutrient broth was done in the laminar air flow cabinet. The 3 to 4 loops of bacterial culture of *Staphylococcus aureus* were inoculated into the nutrient broth. The nutrient broth with bacterial strain was incubated in an incubator at temperature of 37°C for 24 hours. The same process was carried out with *Bacillus subtilis*, *Escherichia coli*, and *Pseudomonas aeruginosa*.

Preparation of nutrient agar plate

Twenty one gram of nutrient agar powder was weighed. The nutrient agar powder was dissolved in 750ml of distilled water in 1000ml conical flask. Magnetic stirrer was used to stir the mixture to ensure proper mixing. After stirring, nutrient agar solution was sent into autoclave for sterilization at temperature of 121°C for 2 hours. After autoclave, the hot sterilized nutrient agar solution was poured into the petri plates in the laminar air flow cabinet. Each of the petri plate contained approximately 25ml of nutrient agar solution which can only occupy 60-70% of the petri plate. The nutrient agar solution allowed to solidifying in the cabinet and the UV of the laminar air flow cabinet was on for 15 mins to achieve sterilization.

Agar well diffusion test

70% of ethanol was sprayed to sterilize both hands before the start of test. Total six concentrations of extract were used as 10mg/ml, 20mg/ml, 40mg/ml, 60mg/ml, 80mg/ml and 100mg/ml. Two plates were needed for each bacterial strain. 0.1ml of nutrient broth containing bacterial strain was transferred on the two plates by using micropipette. The sterilized L- shaped spreader was used to spread the bacterial strain on the plate evenly. 3 wells were made on one of the two plates by using sterilized borer. The 3 wells were separated with each other evenly forming an equal triangular distance. The well was filled until around 60 to 70% of the well's height. Next, the lid was showed to the flame a while and closed the plate. The process was repeated for each bacterial strain and duplicates sets were made for the purpose of comparison. The plates with bacterial strain and extract were incubated in the incubator at temperature of 37°C for 24 hours. The zone of inhibition of each plate was observed, measured and recorded. For each bacterial strain, four plates were needed for the duplicate sets. One plate was used as a positive control plate for each bacterial strain and another extra one plate was used for negative control plate.

Preparation of standard McFarland bacterial culture

Four to five loops of the bacterial strain were cultured on sterile nutrient broth and incubated for 24 hours at temperature of 37°C. This step was done for four bacterial strains. After incubation of 24 hours, the nutrient broth, the bacterial culture was centrifuged at 5000 rpm for 10 minutes. Cell mass was obtained at the bottom of the centrifuge tube. The supernatant was discarded and the cell mass collected was re-suspended into another new sterile nutrient broth. The bacterial cell mass suspension was standardized to value of 0.08 to 0.1 of the absorbance according to McFarland standard by using a UV-Visible spectrophotometer at a range of 625 nm.

Serial dilution procedure for MIC test

30mg of the dried gummy macerated extract was dissolved in of distilled water to obtain concentration of 1000µg/ml. Two-fold serial dilutions were made to get 5 different concentration which was 1000, 500, 250, 125, and 62.5µg/ml. The process was repeated for each bacterial strain and duplicate sets were made. Four positive tubes containing only the bacterial culture suspension were prepared respectively. One negative tube containing only the sterilized nutrient broth was also prepared. The tubes were sealed with para-film and covered with aluminum foil. The tubes were then incubated at 37°C for 24 hours and MIC results were observed and determined.

MBC (minimum bactericidal concentration)

The minimum bactericidal concentration (MBC) is the lowest concentration of an antibacterial agent required to kill a bacterium over a fixed, somewhat extended period, such as 18 hours or 24 hours, under a specific set of conditions. It can be determined from the broth dilution of MIC tests by sub-culturing to agar plates that do not contain the test agent. The MBC is identified by determining the lowest concentration of antibacterial agent that reduces the viability of the initial bacterial inoculum by a pre-determined reduction such as larger than or equal to 99.9%.[19] After 24 hours of incubation, two tubes which showed clear appearance of solution and were least turbid chosen as test sample for each bacterial strain. 0.1ml of the selected incubated tubes was transferred onto the agar plates and spread evenly for each bacterial strain. Duplicate sets of agar plates were made. 0.1ml from each positive tube which contains the bacterial strain was transferred onto an agar plate and spread evenly. The plates were incubated at 37°C for 24 hours and the results were observed and recorded.

Antioxidant activity screening

DPPH free radical scavenging activity assay

Free radical scavenging activity of the extracts was measured by using the DPPH stable radical. The reduction of the DPPH radical was measured by continuous monitoring of the decrease in absorbance at 518nm until a stable value was obtained. The purple colour of DPPH will turn to yellow colour when it get reduced. This assay is based on the measurement of the loss of DPPH colour at 518nm after reaction with the extract. Thus, the lower the absorbance value shows the better antioxidant activity. The percentage inhibition which is the percentage of DPPH scavenging activity was determined based on the following equation:

$$\%\ radical\ scavenging\ activity\ /\ \%\ inhibition\ =\ \left[\left(absorbance\ of\ blank - absorbance\ of\ sample\right) / absorbance\ of\ blank\right] \times 100$$

The antioxidant activity of the extract was expressed as IC_{50}, which the concentration (mg/ml) of extract inhibits formation of DPPH radicals by 50%.[20] The DPPH free radical scavenging activity assay was conducted by referencing to the procedure as stated by Soni et al.,[7] with slight modifications.

Preparation of extracts and standard

The Soxhlet extract was used in antioxidant activity screening. Six different concentrations of extracts were prepared at 10, 20, 40, 60, 80 and 100µg/ml. Butylated hydroxyl toluene (BHT) was used as standard. 3ml of 0.1mM of alcoholic solution of DPPH was added to 2.5ml of different concentration of plant extracts. The tubes containing the mixtures were covered with para-film and aluminum foil. The mixture was kept in the dark place at room temperature for 30 minutes. Then, the absorbance was measured at 518nm using a spectrophotometer. The same procedure was repeated by replacing the extracts with different concentration of BHT to determine the standard graph. The control absorbance value was determined by repeating the same procedure with replacement of the extracts with ml of ethanol. Sample measurement was done once and the result was calculated. The anti-oxidant activity was determined by following formula:

$$Percentage\ of\ Antioxidant\ Activity\ (\%)\ =\ \left[\left(Control\ absorbance\ -\ Sample\ absorbance\right) / Control\ absorbance\right] x\ 100\%$$

Determination of total phenolic content

The Folin-Ciocalteu (FC) assay is a method to determine the total phenolic content in plant. The FC reagent uses a mixture containing sodium molybdate, sodium tungstate, and other reagents. In the presence of phenols, it produces a blue color which absorbs at 750nm. It is believed that the blue color is due to a complexed molybdate species. Therefore, the higher the absorbance produced, higher the total phenolic content. It also called as Gallic Acid Equivalent Method (GAE) as gallic acid is used as the standard in this method.[20] Total phenolic assay was performed according to the procedure described in standard protocol with slight modification to identify the phenolic content of fenugreek.[21]

Preparation of the extracts and standard

The extracts were obtained from Soxhlet extraction method using ethanol. Four different concentrations of extracts were prepared which were 25µg/ml, 50µg/ml, 100µg/ml, and 200µg/ml. Gallic acid was used as the standard for this assay. For every 0.2ml of extract, about 0.2ml of Folin-Ciocalteu reagent and 4ml of 2.5% sodium carbonate were added. The mixture was mixed completely and allowed to stand for 2hours. The absorbance of the solution at 750nm was measured. The same procedure was repeated by replacing the extract with the gallic acid. Quantification of total phenolic content was done using standard curve of gallic acid as a standard phenolic compound (1, 2, 4, 6, 8 and 10µg/ml), which was dissolved in ethanol and expressed as mg gallic acid per gram of plant material.

Results

Qualitative phytochemical screening

The results for alkaloid test, saponin test (lipids), salkowski test (terpenoids) ,anthraquinone test, glycoside test, tannins test, , carbohydrate test and phenol test were present. Flavonoid test and reducing sugar test were absent as mentioned in Table 4.

Table 4 Zone of inhibition for *Bacillus subtilis* (Set 2)

Concentration (mg/ml)	Diameter Reading of Zone of Inhibition (mm)								
	1st	2nd	3rd	4th	5th	6th	7th	8th	Mean
10	-	-	-	-	-	-	-	-	-
20	10	6	8	6	8	6	7	8	7.375
40	10	10	10	10	10	9	9	10	9.75
60	20	10	20	10	10	10	20	20	15
80	22	22	28	18	10	20	20	20	20
100	24	16	36	30	38	36	20	20	27.5

Antimicrobial activity

Agar well diffusion method

In this antimicrobial activity study, ethanolic maceration extract was used to examine the antimicrobial properties of *Trigonella foenum-graecum*. The extract concentrations which used in agar well diffusion method are 10mg/ml, 20mg/ml, 40mg/ml, 60mg/ml, 80mg/ml and 100mg/ml. Among the four bacteria strains, only *Bacillus subtilis* showed zone of inhibition which means that the extract of *T. foenum-graecum* only inhibit the growth of *Bacillus subtilis* in this study. In the case of Set 2 agar plate of *Bacillus subtilis*, extract started to inhibit the growth at concentration of 20mg/ml with a little zone of inhibition and showed greatest activity at concentration 100mg/ml which obtained the highest diameter of zone of inhibition which was 27.5mm among all the extract concentrations. This showed that higher concentrations of extract will lead to higher zone of inhibition.[22] The antimicrobial properties of the plant extract may due to the presence of phenolic compounds in the extract. Phenol acted as an active compound which could destroyed the cell membrane causing cell death. There is a close relationship between antimicrobial activity and antioxidant activity since the active components which contribute to these two activities are phenolic compounds Zone of inhibition of set 2 for *Bacillus subtilis* is shown in the Table 4.

However, in the case of Set 1 agar plate of *Bacillus subtilis*, the extract only showed its antimicrobial activity starting from concentration of 60mg/ml and reached its highest activity at 100mg/ml with a diameter of zone of inhibition of 8.75mm. From the table of diameter of zone of inhibition as shown in results, the diameter of zone of inhibition showed in the Set 1 agar plates are relatively small compared to Set 2 agar plates. The reasons which may associated to this phenomenon are the insufficient concentration of the extract in the agar well, improper spreading as well as improper diffusion of extract. The irregular shape of the zone of inhibition around the wells may due to the improper diffusion of the extract and also the improper spreading of the bacteria inoculum which may cause the abundant or uneven bacterial growth on particular part area of the agar. For *Staphylococcus aureus, Escherichia coli* and *Pseudomonas aeruginosa*, there are no zone of inhibition showed in the agar plates. This may due to the low concentration of extract and improper diffusion of the extract. Zone of inhibition of set 1 for *Bacillus subtilis* is shown in the Table 5.

Table 5 Zone of inhibition for *Bacillus subtilis* (Set 1)

Concentration (mg/ml)	Diameter Reading of Zone of Inhibition (mm)								
	1st	2nd	3rd	4th	5th	6th	7th	8th	Mean
10	-	-	-	-	-	-	-	-	-
20	-	-	-	-	-	-	-	-	-
40	-	-	-	-	-	-	-	-	-
60	10	4	6	4	4	6	6	6	5.75
80	10	10	10	6	6	4	8	8	7.75
100	10	10	8	8	8	10	8	8	8.75

The ethanolic seed extracts were further subjected to broth dilution method to determine the MIC of *Trigonella foenum-graecum* seed extract with concentrations ranging from 62.5µg/ml, 125µg/ml, 250µg/ml, 500µg/ml, to 1000µg/ml. Turbidity can be seen in almost all of the assay tubes which indicated the presence of bacterial growth. Hence, the two tubes showed the least turbidity appearance which were 1000µg/ml and 500µg/ml were selected for tested in MBC test to further confirm the presence of bacterial growth. In the MBC test, all the nutrient agar plates for the four bacteria strains showed the growth of the bacteria and negative results were obtained after incubated the plates for 24 hours. The negative results for both MIC and MBC test might be due to the very low concentrations of extract as the highest concentrations used in this MIC test was 1000µg/ml, compared to the concentrations used in well agar diffusion test which was 100mg/ml. The low concentrations of extract were insufficient to show the antibacterial effect towards the four bacteria strains. As referring to the MIC values proposed by other study,[23] it was found that minimum inhibitory concentration of ethanolic seed extract of *Trigonella foenum-graecum* was 400mg/ml. This statement suggested that a higher concentration should be used in the determination of MIC of the extract. The zones of inhibition of sets 1 and 2 for *Staphylococcus aureus* are shown in Table 6 & 7 respectively. The zones of inhibition of sets 1 and 2 for *Escherichia coli* are shown in Tables 8 & 9 respectively. The zones of inhibition of sets 1 and 2 for *Pseudomonas aeruginosa* are shown in Tables 10 & 11 respectively.

Table 6 Zone of inhibition for *Staphylococcus aureus* (Set 1)

Concentration (mg/ml)	Diameter Reading of Zone of Inhibition (mm)								
	1st	2nd	3rd	4th	5th	6th	7th	8th	Mean
10	-	-	-	-	-	-	-	-	-
20	-	-	-	-	-	-	-	-	-
40	-	-	-	-	-	-	-	-	-
60	-	-	-	-	-	-	-	-	-
80	-	-	-	-	-	-	-	-	-
100	-	-	-	-	-	-	-	-	-

Table 7 Zone of inhibition for *Staphylococcus aureus* (Set 2)

Concentration (mg/ml)	Diameter Reading of Zone of Inhibition (mm)								
	1st	2nd	3rd	4th	5th	6th	7th	8th	Mean
10	-	-	-	-	-	-	-	-	-
20	-	-	-	-	-	-	-	-	-

Table Continues...

Concentration (mg/ml)	Diameter Reading of Zone of Inhibition (mm)								
	1st	2nd	3rd	4th	5th	6th	7th	8th	Mean
40	-	-	-	-	-	-	-	-	-
60	-	-	-	-	-	-	-	-	-
80	-	-	-	-	-	-	-	-	-
100	-	-	-	-	-	-	-	-	-

Table 8 Zone of inhibition for *Escherichia coli* (Set 1)

Concentration (mg/ml)	Diameter Reading of Zone of Inhibition (mm)								
	1st	2nd	3rd	4th	5th	6th	7th	8th	Mean
10	-	-	-	-	-	-	-	-	-
20	-	-	-	-	-	-	-	-	-
40	-	-	-	-	-	-	-	-	-
60	-	-	-	-	-	-	-	-	-
80	-	-	-	-	-	-	-	-	-
100	-	-	-	-	-	-	-	-	-

Table 9 Zone of inhibition for *Escherichia coli* (Set 2)

Concentration (mg/ml)	Diameter Reading of Zone of Inhibition (mm)								
	1st	2nd	3rd	4th	5th	6th	7th	8th	Mean
10	-	-	-	-	-	-	-	-	-
20	-	-	-	-	-	-	-	-	-
40	-	-	-	-	-	-	-	-	-
60	-	-	-	-	-	-	-	-	-
80	-	-	-	-	-	-	-	-	-
100	-	-	-	-	-	-	-	-	-

Table 10 Zone of inhibition for *Pseudomonas aeruginosa* (Set 1)

Concentration (mg/ml)	Diameter Reading of Zone of Inhibition (mm)								
	1st	2nd	3rd	4th	5th	6th	7th	8th	Mean
10	-	-	-	-	-	-	-	-	-
20	-	-	-	-	-	-	-	-	-
40	-	-	-	-	-	-	-	-	-
60	-	-	-	-	-	-	-	-	-
80	-	-	-	-	-	-	-	-	-
100	-	-	-	-	-	-	-	-	-

Table 11 Zone of inhibition for *Pseudomonas aeruginosa* (Set 2)

Concentration (mg/ml)	Diameter Reading of Zone of Inhibition (mm)								
	1st	2nd	3rd	4th	5th	6th	7th	8th	Mean
10	-	-	-	-	-	-	-	-	-
20	-	-	-	-	-	-	-	-	-
40	-	-	-	-	-	-	-	-	-
60	-	-	-	-	-	-	-	-	-
80	-	-	-	-	-	-	-	-	-
100	-	-	-	-	-	-	-	-	-

MBC (Minimum Bactericidal Concentration) test

All the MBC tests for all the individual sets (sets 1 and 2) are shown in Figures 3-11, respectively.

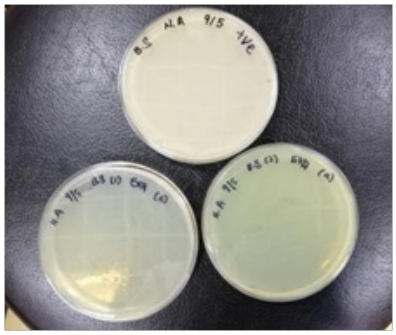


Figure 3 Results of Set 1 *Bacillus subtilis* and the positive control plate.

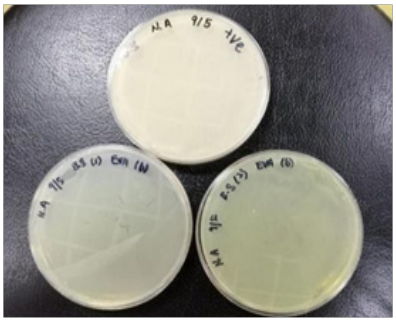


Figure 4 Results of Set 2 *Bacillus subtilis* and the positive control plate

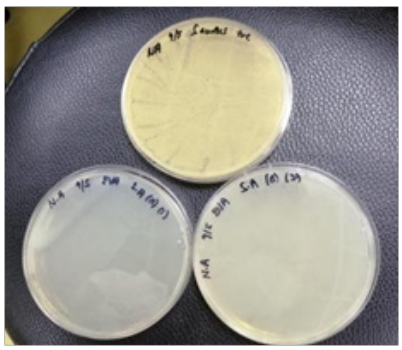


Figure 5 Results of Set 1 *Staphylococcus aureus* and the positive control plate.

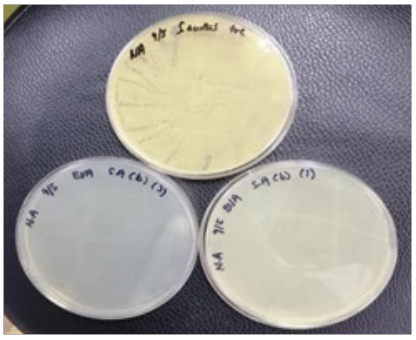


Figure 6 Results of Set 2 *Staphylococcus aureus* and the positive control plate.

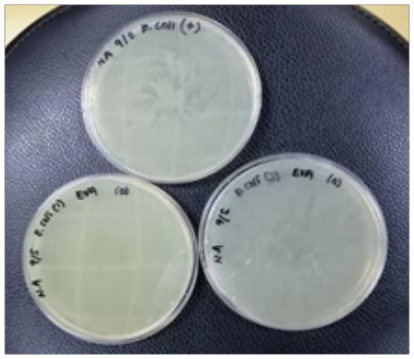


Figure 7 Results of Set 1 *Escherichia coli* and the positive control plate.

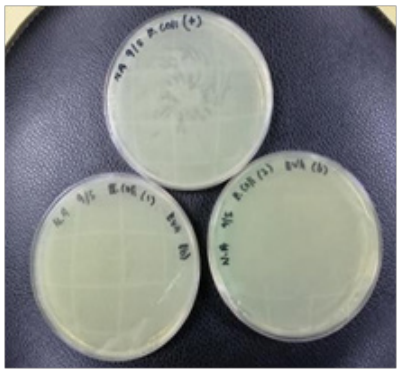


Figure 8 Results of Set 2 *Escherichia coli* and the positive control plate.

Citation: Hwa CY, Perveen N, Paliwal N, et al. Phytochemical screening, antimicrobial and antioxidant activity determination of *Trigonella foenum-graecum seeds*. *Pharm Pharmacol Int J.* 2019;7(4):175–186. DOI: 10.15406/ppij.2019.07.00249

Figure 9 Results of Set 1 *Pseudomonas aeruginosa* and the positive control plate.

Figure 10 Results of Set 2 *Pseudomonas aeruginosa* and the positive control

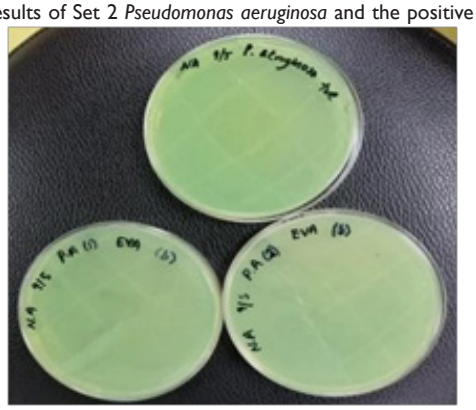


plate.

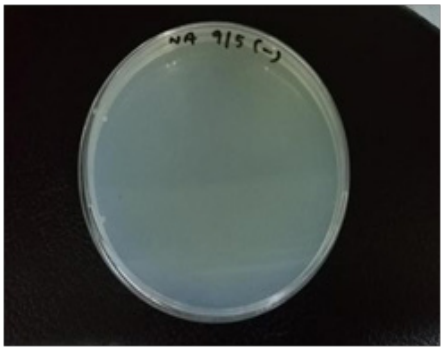


Figure 11 Negative control plate.

Antioxidant activity

DPPH free radical scavenging activity

Free radical scavenging activity of the extracts was measured by using the DPPH stable radical. The free radical DPPH will get reduced by antioxidant which results in the discoloration of the DPPH solution. Discoloration can be seen when the purple color solution of DPPH turns into yellow color when the DPPH get reduced. The absorbance value of the reduced DPPH mixture decreases, the free radical scavenging activity increases. Percentage inhibition of DPPH free radical was calculated based on the control reading. The antioxidant activity of the extract was expressed as IC_{50}, which the concentration (mg/ml) of extract inhibits formation of DPPH radicals by 50%.[24] The graph of DPPH radical scavenging activity of crude extracts of plant was observed, when compared with standard BHT and shown in results. Table 12 shows the concentration of BHT and extract of *Trigonella foenum-graecum* and the corresponding UV Absorbance at 518nm and Table 13 shows the percentage scavenging activity of standard BHT and extract of *Trigonella foenum-graecum.*

Table 12 Concentration of BHT and extract of *Trigonella foenum-graecum* and the corresponding UV absorbance at 518 nm

Concentration (µg/ml)	UV Absorbance	
	BHT	Extract of *Trigonella foenum-graecum*
10	0.488	0.564
20	0.461	0.549
40	0.418	0.538
60	0.375	0.52
80	0.332	0.509
100	0.268	0.493

Control Absorbance= 0.582

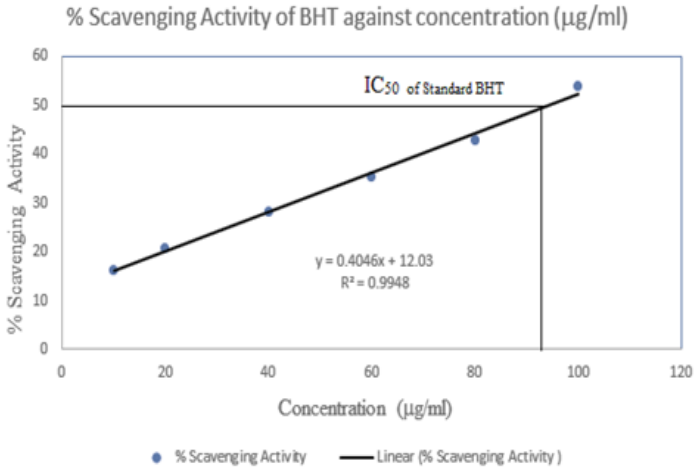
Table 13 Percentage scavenging activity of standard BHT and extract of *Trigonella foenum-graecum*

Concentration (µg/ml)	Scavenging Activity (%)	
	BHT	Extract of *Trigonella foenum-graecum*
10	16.15	3.1
20	20.79	5.67
40	28.18	7.56
60	35.57	10.65
80	42.96	12.54
100	53.95	15.29

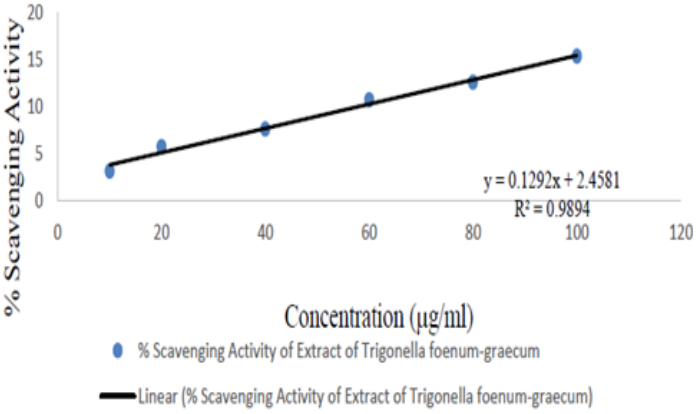
In the present study study, the IC_{50} values of the BHT standard was found to be 93.85µg/ml while for the IC_{50} value for ethanolic extract of *T. foenum-graecum* cannot be found. This is because the DPPH free radical scavenging activity of the extract did not exceed 50 % of the values of the graph. When the *T. foenum graecum* seeds extracts were tested for DPPH free radical scavenging activity, the extracts showed only 15.29 % even at the highest extract concentration of 100µg/ml which was significantly less than 50% of the graph. While for the standard BHT, the value was found to be 53.95%. Hence the IC_{50} value of standard BHT can be found. As reported by other study,[25] the DPPH scavenging activity of methanolic seed extract had been reported as 67.9%. Thus, the IC_{50} of the extract can be found since the value already exceeded 50% of the graph as shown in the Graph 1. Graphs 2 & 3 are shown for percentage and comparison study of BHT and *Trigonella foenum-graecum.*

The reading of DPPH scavenging activity percentage was higher than the value found in this study which indicates methanolic seed extract have higher antioxidant activity compared to ethanolic seed extract. The smaller the IC_{50}, the lower concentration required to scavenge 50% of the free radical DPPH, which also means higher

the antioxidant properties. The difference of the values may due to the solvent system used to extract the bioactive phytochemical of the seeds. Some phytochemicals which contribute to the antioxidant activity such as polyphenols and flavonoids might absorb and extracted well in methanol solvent system compared to ethanol due the high polarity of the methanol.[26] From this DPPH test, the ethanolic extract of the seeds of *Trigonella foenum-graecum* was suggested as having a very weak antioxidant properties and the reason contributed to the weak antioxidant showed might because of its active components might not be well extracted through ethanol solvent.



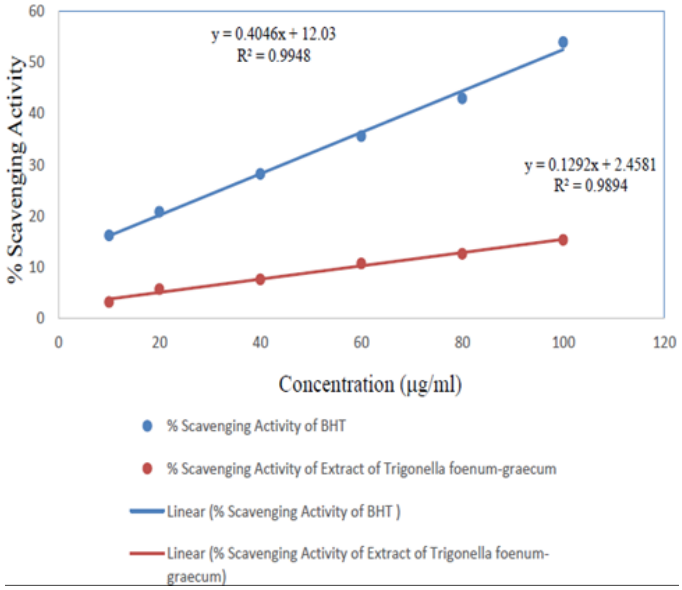
Graph 1 % Scavenging activity of standard BHT against concentration (µg/ml).



Graph 2 Percentage scavenging activity of extract of *Trigonella foenum-graecum* against concentration (µg/ml).

Determination of total phenolic content (TPC)

The total phenolic content in the extract was determined spectrophotometrically according to the Folin–Ciocalteu procedure and expressed as mg or mM Gallic acid equivalents. The absorbance with different concentrations was taken by using a spectrophotometer after 2 hours at 750nm of wavelength. The calibration curve of Gallic acid (GA) was used for the estimation of sample activity capacity. The results indicated that the extract of fenugreek seeds contains antioxidants and protects cellular structures from oxidative damage. The total phenolic content for extract was calculated based on the formula which is C=(A/B) x dilution factor, where C is the total phenolic content expressed as mg GAE/g dry weight of the extract, A is the equivalent concentration of Gallic acid established from calibration curve while B is concentration of the extract. It was expressed as Gallic acid equivalents (GAE) in mg/g of samples. The TPC values found in this study were 3.597 GAE/g, 3.839 GAE/g and 4.807 mg GAE/g for concentrations of 25µg/ml, 50µg/ml, 100µg/ml respectively. The total phenolic content for concentration of 200µg/ml could not be determined due to the absorbance value did not fall within the range of the calibration of the standard graph of Gallic acid. In other related studies by Seasotiya[25] and Bukhari,[27] the TPC have been reported as 6.85mg GAE/g and 186mg GAE/g from methanolic seed extract which are higher than the value obtained in this study. Therefore, other solvent systems can be used to obtain better antioxidant activity as well as the extraction yield of that particular phytochemical. The variations between the results may be due to differences in environmental conditions, for example, temperature, location, climate, diseases, assay method, selection of test components, sampling time and pest exposure within the species.[28] Concentration and corresponding absorbance of Gallic Acid obtained from UV-Visible Spectrophotometer is shown in Table 14. Table 15 shows the concentration and corresponding absorbance of extract of *Trigonella foenum-graecum.* Graph of concentration and corresponding absorbance of standard Gallic acid at 750nm is shown in Graph 4.[29–37]



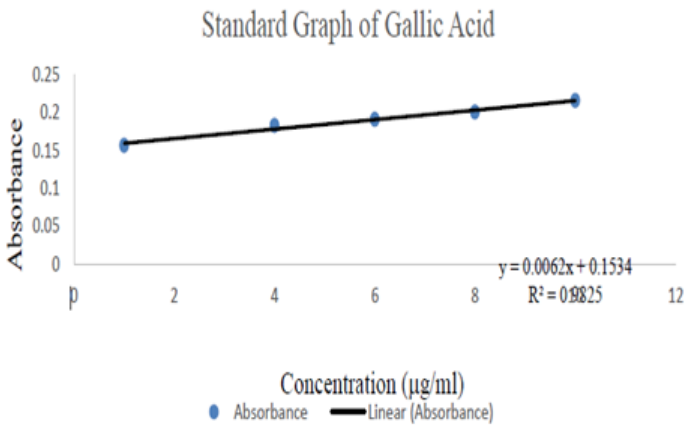
Graph 3 Comparison of % scavenging activity of standard BHT and *Trigonella foenum-graecum*

Table 14 Concentration and corresponding absorbance of gallic acid obtained from UV-Visible Spectrophotometer

Concentration (µg/ml)	Absorbance
1	0.157
2	0.176
4	0.183
6	0.191
8	0.201
10	0.216

Table 15 Concentration and corresponding absorbance of extract of *Trigonella foenum-graecum*

Concentration (µg/ml)	Absorbance
25	0.198
50	0.201
100	0.213
200	0.256



Graph 4 Concentration and corresponding absorbance of standard gallic acid at 750 nm.

Conclusion

Phytochemical screening of ethanolic extract of *Trigonella foenum-graecum* through maceration and Soxhlet method determined the presence of alkaloids, saponins, terpenoids, anthraquinone, glycosides, tannins, carbohydrate and phenol in the seeds. In the other hand, it also revealed the absence of reducing sugars and flavonoids in the seeds of *Trigonella foenum-graecum*. The antimicrobial activity of the plant extract was determined by agar well diffusion method. The antimicrobial activity was only shown by *Bacillus subtilis*, while antimicrobial activity was not seen in the case of *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*. Higher concentration of the plant extract is required to inhibit the growth of the bacteria in agar well diffusion method and to obtain better result for MIC test. The antioxidant activity of the plant extract was assessed through the method of DPPH test while total amount of phenolic compounds in the extract can be determined through TPC test. It can be concluded that *Trigonella foenum-graecum* possess weak antioxidant activity which contributed by the presence of polyphenols or phenolic compound in the seeds. Positive correlation was seen whereby the antioxidant activity and the total phenolic content increase with the increase of concentration. It was suggested that seeds of *Trigonella foenum-graecum* can be a potential source as a natural significant antioxidant and marked antimicrobial agent. Further isolation, purification and investigations of the bioactive constituents of this plant are required to reveal more health benefits for the public and therapeutics uses in the medical field.

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Conflicts of interest

Authors declare that there is no conflict of interest.

References

1. Srinivasan K. Fenugreek (Trigonella foenum-graecum): A review of health beneficial physiological effects. *Food Rev Int*. 2006;22(2):203–224.
2. Lim TK. Trigonella foenum-graecum. *Edible Med Non-Medicinal Plants*. 2012;2(4):906–924.
3. Elnour MEM, Ali AMA, Saeed BEAE. Antimicrobial Activities and Phytochemical Screening of Callus and Seeds Extracts of Fenugreek (Trigonella foenum-graecum). *Int J Curr Microbiol App Sci*. 2015;4(2):147–157.
4. Gernot Katzer. Spice Pages: Fenugreek Seeds (Trigonella foenum-graecum) [Internet].
5. Fenugreek Seed - Whole, Ground, Roasted & Dry Fenugreek Seed. Altrafine Gums [Internet].
6. Nandagopal S, Dhanalakshmi DP, Ganesh Kumar A, et al. Phytochemical and Antibacterial studies of Fenugreek Trigonella foenum-graecum L.-A Multipurpose Medicinal Plant. *Journal of Pharmacy Research*. 2012;5(1):413–415.
7. Soni A, Sosa S. Phytochemical Analysis and Free Radical Scavenging Potential of Herbal and Medicinal Plant Extracts. *Journal of Pharmacognosy and Phytochemistry*. 2013;2(4):22–29.
8. Adel Amer. How does the Dragendorff reagent work for detecting Alkaloids? [Internet].
9. Fehling H. Die quantitative Bestimmung von Zucker und Stärkmehl mittelst Kupfervitriol. *Ann der Chemie und Pharm*. 2013;72(1):106–113.
10. William Hanz. *Test for Saponins*. Hunker [Internet].
11. Raiza Almirah. Salkowski reaction is the reaction of cholesterol with concentrated sulphuric [Internet]. Ateneo da Davao University: Philippines.
12. Manpreet Kaur. Anthraquinones Glycosides (3 Chemical Tests) [Internet].
13. Cam AJT, Traditional AJ. Extract In Alloxan-Induced Diabetic Rats And Its Use in Diabetes Mellitus. 2009;6:255–261.
14. Manpreet Kaur. Tannins: Classification, Properties and Chemical Tests [Internet].
15. *Carbohydrates-The Molisch Test*. Haper College: USA.
16. Abdel-massih R, Abdou E, Baydoun E, Daoud Z. Antibacterial Activity of the Extracts Obtained from Rosmarinus officinalis, Origanum majorana, and Trigonella foenum-graecum on Highly Drug-Resistant Gram Negative Bacilli. *Journal of Botany*. 2010;3(80):52–59.
17. Aqil F, Ahmad I. Broad-spectrum antibacterial and antifungal properties of certain traditionally used Indian medicinal plants. *World Journal of Microbiology and Biotechnology*. 2003;19(6):653–657.
18. Balouiri M, Sadiki M, Ibnsouda SK. Methods for in vitro evaluating antimicrobial activity: A review. *J Pharm Anal*. 2016;6(2):71–79.
19. Mark Goins. *Minimum Inhibitory (MIC) and Minimum Bactericidal Concentration (MBC) Evaluations as R&D Tools*. Q Laboratories [Internet]; 2017.
20. Abeed Al Mashkor IM. Phenolic Content and Antioxidant Activity of Fenugreek Seeds Extract. *International Journal of Pharmacognosy and Phytochemical Research*. 2014;6(4):841–844.

21. Bukhari SB, Iqbal Bhanger M, Memon S. Antioxidative Activity of Extracts from Fenugreek Seeds (Trigonella foenum-graecum). *Pak J Anal Environ Chem*. 2008;9(2):78–83.

22. Randhir R, Ms YL, Shetty K. Phenolics, their antioxidant and antimicrobial activity in dark germinated fenugreek sprouts in response to peptide and phytochemical elicitors. *Asia Pac J Clin Nutr*. 2004;13(3):295–307.

23. Khanra R. Atanu C, Singh BP, et al. Antimicrobial activity of Ethanolic extract of Trigonella foenum graecum. *IRJP*. 2010;1(1):181–183.

24. Kumar GP, Anand T, Singsit D, et al. Evaluation of antioxidant and anti-fatigue properties of Trigonella foenum-graecum L. in rats subjected to weight loaded forced swim test. *Pharmacognosy J*. 2013;5(2):66–71.

25. Seasotiya L, Siwach P, Bai S, et al. Free Radical Scavenging Activity, Phenolic Contents and Phytochemical Analysis of Seeds of Trigonella foenum-graecum. *Asian Pac J Health Sci*. 2014;1(3):219–226.

26. Deepti Agrawal. Why is methanol generally used as a first solvent for extraction purpose to look for bioactives in medicinal plants?; 2014.

27. Bukhari SB, Bhanger MI, Memon S. Antioxidative Activity of Extracts from Fenugreek. *Pak J Anal Environ Chem*. 2008;9(2):78–83.

28. Singh P, Vishwakarma SP, Singh RL. Antioxidant, oxidative DNA damage protective and antimicrobial activities of the plant Trigonella foenum-graecum. *J Sci Food Agric*. 2014;1(3):25–28.

29. Thirunavukkarasu V, Anuradha CV, Viswanathan P. Protective Effect of Fenugreek (Trigonella foenum graecum) Seeds in Experimental. *Phytother Res*. 2003;17(7):737–743.

30. Fenugreek. Flora Health – International [Internet].

31. Fenugreek, Medicinal Properties of Fenugreek, Methi, Trigonella Foenum Graecum [Internet].

32. Jawairia Zafar. Health Benefits of Fenugreek [Internet].

33. Mateen A, Ahmed P, Ahmed M, et al. A Study of Antimicrobial Activity of Few Medicinally Important Herbal Single Drugs Extracted in Ethanol, Methanol and Aqueous Solvents. *Nat Prod Chem Res*. 2010;2(10):351–356.

34. Wani SA, Kumar P. Fenugreek: A review on its nutraceutical properties and utilization in various food products. *J Saudi Soc Agric Sci*. 2018;17(2):97–106.

35. Gul R, Jan SU, Faridullah S, Sherani S, Jahan N. Preliminary Phytochemical Screening, Quantitative Analysis of Alkaloids, and Antioxidant Activity of Crude Plant Extracts from Ephedra intermedia Indigenous to Balochistan. *Sci World J*. 2017;5873648:1–7.

36. Khandelwal KR. Practical Pharmacognosy Techniques & Experiments [Internet].

37. Raaman N. *Phytochemical Techniques*. New India Publishing Agency: India; 2006.

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