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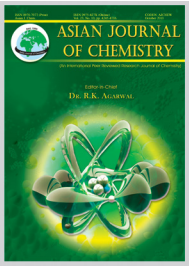

In vitro Antioxidant and Antimicrobial Activities of Crude Extracts of *Trigonella foenum-graecum* Seeds

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Trigonella foenum-graecum (fenugreek) is an annual herb that belongs to the family *Leguminosae*. Seeds of *Trigonella* are used as antidiabetic, inhibit cholesterol absorption (hyper cholesterolemia), antiatherosclerotic, mild antiseptic and acts as an emollient. In the present study, *Trigonella* seeds were investigated for the presence of total antioxidant activity, catalase, peroxidase, ascorbate oxidase, total phenolic compounds and antimicrobial activity. It was observed that crude extract of *Trigonella* seeds germinated in curd showed the highest activities of total antioxidants, catalase, peroxidase, ascorbate oxidase and total phenolics when compared with other samples tested. Antimicrobial activity is done on different bacteria and fungi using agar well diffusion method with ampicillin and fucanazole as the positive antimicrobial standards to determine the sensitivity of the microbial strains. It was found that ethyl acetate extract of *Trigonella foenum-graecum* seeds showed zone of inhibition ranging from 11-25 mm with bacteria and 35-40 mm with fungi and crude extract of *Trigonella* seed germinated in curd showed zone of inhibition ranging from 11-15 mm with bacteria and 38-42 mm with fungi.

Key Words: *Trigonella foenum-graecum*, Antioxidant activity, Antimicrobial activity.

INTRODUCTION

Fenugreek (*Trigonella foenum-graecum*), a member of the pea family *Leguminosae*, is an annual plant that grows from 4 to 20 inches in height and has pale-yellow flowers. The pods are *ca.* 4 inches long and contain up to 20 seeds per pod. The flattened seeds are mostly yellowish brown to reddish-brown or grayish in colour. The seeds of fenugreek are commonly used in India and in oriental countries as a spice in food preparations due to their strong flavour and aroma.

The seeds of fenugreek contain lysine and L-tryptophan rich proteins, mucilaginous fiber and other rare chemical constituents such as saponins, coumarin, nicotinic acid, sapogenins, phytic acid, scopoletin and trigonelline, which are thought to account for many of its presumed therapeutic effects, may inhibit cholesterol absorption and lowers sugar levels therefore, fenugreek seeds are used as a traditional remedy for the treatment of diabetes[1,2] and as hypercholesterolemia in Indian and Chinese medicines. It has a long history of use as a gastrointestinal remedy, as a mild laxative, for dyspepsia and for the loss of appetite, to treat catarrh of the upper respiratory tract. Fenugreek also has a mild antiseptic action and acts as an emollient.

The seeds are restorative and have nutritive properties and stimulate digestive processes[3]. Clinical and experimental studies have documented antidiabetic and antiatherosclerotic effects[4]. Ravikumar and Anuradha[5] reported that supplementation of fenugreek seed powder in the diet leads to a reduction in biomarkers of oxidative damage in alloxan-diabetic rats. The presence of seed polyphenols in fenugreek prevented oxidative hemolysis and lipid peroxidation induced by H_2O_2 *in vitro* in human erythorcytes and in rat liver[6,7]. Plant phenolics have potential health benefits mainly due to their antioxidant properties such as reactive oxygen species (ROS) scavenging and inhibition and pharmacological properties such as antitumor, antiviral, antimicrobial, antiinflammatory, hypotensive and antioxidant activity. Subhashini *et al.*[8] reported that *Trigonella foenum-graecum* posses strong antioxidant activity, which may be due to the presence of phenolic components in the extract. The action of fenugreek and other trace elements in lowering blood glucose levels is almost comparable to the effect of insulin in diabetic tissues[9]. Premanath *et al.*[10] reported that ethanol extract of fenugreek leaf is an important source for antibacterial components and also a potential source of phenolic antioxidants.

The promotion of antioxidants as food additives or as therapeutic agents requires proof on their efficacy in various *in vitro* systems besides their pharmacological, toxicological properties and *in vivo* benefits. The antioxidant activity of the

seeds *in vivo* and the traditional medicinal uses has prompted us to investigate the *in vitro* antioxidant activity of the seeds.

Defensins are small cysteine-rich peptides with a size of 5-10 kDa and some of them exhibit antifungal activity. The antifungal activity of the steroidal saponin is associated with their aglycone moieties and has biological effects associated to their action on permeability of cell membranes. The saponins show the antimicrobial activity by inhibiting the growth of Gram +ve or Gram -ve microorganism and has prompted us to investigate the *in vitro* antimicrobial activity of the seeds.

EXPERIMENTAL

Preparation of seed extract: 10 g of *T. foenumgr-aecum* seeds were powdered and subjected to Soxhlet extraction with hexane, ethyl acetate and ethanol (in the order of polarity) for 12 h with each solvent used. The extracts are concentrated and made to final concentration of 1 mg/mL with DMSO and used for further studies.

10 g of *Trigonella* seeds are germinated in distilled water and curd separately for 24 h. The germinated seeds are ground to a fine paste and the supernatant is collected by centrifuging at 5000 rpm for 10 min at 4 ºC. All the extracts collected were used for antioxidant and antimicrobial studies.

Total antioxidant activity: The total antioxidant activity of the sample was evaluated by the method of Prieto *et al.*[11]. An aliquot of each sample (0.05 mL) was mixed with 0.5 mL of reagent (0.6 M H_2SO_4, 28 mM sodium phosphate and 4 mM ammonium molybdate) in 1.5 mL eppendorf tube. The tubes were capped and boiled in a boiling water bath at 95 ºC for 90 min and cooled. The absorbance of each sample was measured at 695 nm against blank in a spectrophotometer. A typical blank contained 0.5 mL of reagent solution and 0.05 mL of buffer and treated in the same manner as test. The antioxidant capacity was expressed as micromoles of ascorbic acid equivalents of antioxidant capacity.

Assay of catalase: The catalase activity was assayed by the titrimetric method based on the procedure described by Radhakrishnan and Sarma[12]. 2.5 mL of 0.9 % hydrogen peroxide (v/v) in the same buffer were taken and 0.5 mL of enzyme extract was added and incubated at 28 ºC for 3 min. The reaction was then arrested by adding 5 mL of 2 N H_2SO_4 and the residual H_2O_2 was titrated with 0.1 N $KMnO_4$ solution. Blank was carried out without extract. Units of catalase activity was expressed as mL of 0.1 N $KMnO_4$ equivalents of hydrogen peroxide decomposed per min per mg protein.

Assay of peroxidase: Peroxidase activity was assayed according to the method described by Seevers *et al.*[13]. The reaction mixture taken in a 3 mL cuvette containing 0.5 mL of 20 mM sodium acetate buffer (pH-5.0), 0.5 mL of 1.3 mM benzidine, 0.1 mL of enzyme extracts. The enzyme reaction was initiated by the addition of hydrogen peroxide. The reference cuvette contained all the components of the reaction mixture except hydrogen peroxide, which was substituted by an equal volume of the buffer, the increase in the optical density at 420 nm was recorded for 5 min at 1 min intervals in a UV visible spectrophotometer. The units of peroxidase activity were expressed as an increase in optical density at 420 nm per min per mg protein.

Assay of ascorbate oxidase: Ascorbate oxidase activity was assayed according to the method of Vines and Oberbacher[14]. To 3 mL of ascorbate solution (18.8 mg ascorbic acid dissolved in 300 mL of 0.1 M phosphate buffer, pH-5.6), 0.1 mL of enzyme extract was added and the change in the absorbance at 265 nm is measured at an interval of 30 sec of a period of 5 min. One enzyme unit is equivalent to 0.01 O.D. change per min per mg of protein.

Total phenolic compound analysis: The total phenolics present in the sample were determined by the method of McDonald *et al*[15]. To 0.5 of each sample, 2.5 mL of diluted Folin-Ciocalteau reagent and 2 mL of 7.5 % sodium carbonate were added and incubated at 45 ºC for 15 min. The absorbance of sample measured in a UV visible spectrophotometer at 765 nm. Gallic acid was used as standard and the results were expressed as mg of gallic acid equivalent/g tissue.

Antimicrobial studies

Test microorganisms: The following test microorganisms used in this study are: Bacteria - *Escherichia coli* (NCIM 2067), *Bacillus subtilis* (NCIM 2063), *Klebseilla pneumonia* (NCIM 2957), *Proteus vulgaris* (NCIM 2027), *Staphylococcus aureus* (NCIM 5021) and fungi- *Aspergillus niger* (NCIM 1054), *Trichophyton rubrum* (MTCC 1123). All the bacterial strains are obtained from National Collection of Industrial Microorganism (NCIM), National Chemical Laboratory (NCL), Pune and fungal strains were obtained from IMTECH, Chandigarh.

Maintenance of microorganisms: The bacterial cultures were maintained on *Luria bertani agar* (LB) and fungal cultures were maintained on *Sabouraud dextrose agar* (SDA) at 4 ºC temperature until used for the study.

Pour plate method: Inoculum of test organisms was prepared by growing pure isolate in nutrient broth at 37 ºC for overnight. The overnight broth cultures was subcultured in fresh nutrient broth and grown for 3 h to obtain log phase culture. The agar plates were prepared by pour plate method using 20 mL nutrient agar medium for bacteria and SB medium for fungi. The molten sterile medium is cooled to 45 ºC and mixed thoroughly with 10 µL of growth culture of concerned test organism (1×10^8 cells) and then poured into sterile petriplates and allowed to solidify. Fungal cultures are grown on *Sabourand dextrose agar* and suspension in one fourth strength ringers solution was used to prepare the seeded *Sabourand dextrose agar* plates.

Wells of 6 mm size were made with sterile gel puncture and 10 µL of plant extract was added to each well aseptically. Ampicillin (10 µL/mL) and fucanazole (10 µL/mL) were added as standard positive antimicrobial agents for bacteria and fungi respectively and tested along with plant extract samples. Nutrient agar plates were incubated at 37 ºC for 24 h for bacterial culture, while *Sabourand dextrose agar* plates at 28 ºC for 48 h for fungal growth. The diameter of zones of inhibition was measured in mm using Himedia zone reader.

RESULTS AND DISCUSSION

The antioxidant, catalase, peroxidase ascorbate oxidase and total phenolic compound activities of *Trigonella* seeds germinated in water, curd and Soxhlet extracted ethyl acetate

TABLE-1
TOTAL ANTIOXIDANT, CATALASE, PEROXIDASE, ASCORBATE OXIDASE AND PHENOLIC CONTENT IN SEEDS OF *Trigonella.*

Plant extract (10 %)	Total antioxidant	Catalase	Peroxidase	Ascorbate oxidase	Total phenolics
Seed in water	1.812	1.0	0.02	58	0.855
Seed in curd	2.156	1.3	0.02	73	0.894
Seed in ethyl acetate	0.544	0.8	0.01	50	0.041

TABLE-2
ANTIMICROBIAL ACTIVITY OF *Trigonella* EXTRACTS TOWARDS CERTAIN BACTERIA AND FUNGI

Microorganism	Zone of growth inhibition (mm)			
	Amp/Fuc (10 µL/well)	Germinated seed in water	Germinated seed in curd	Ethyl acetate fraction
Bacteria:				
Escherichia coli	10	11	14	25
Bacillus subtilis	10	11	14	11
Staphylococcus aureus	9	10	14	16
Klebsiella pneumonia	18	13	15	19
Proteus vulgaris	10	13	12	13
Serretia marcescence	10	11	13	16
Fungi:				
Aspergillus niger	20	32	42	40
Trichophyton rubrum	13	30	38	35

extract were tested and are tabulated in Table-1. The antioxidant activity of samples was expressed as equivalents of α-tocopherol (µg/gm wt), which was found to be 2.156, 1.812 and 0.544 in seeds germinated in curd, water and ethyl acetate extracts respectively. Units of catalase activity was measured by decomposing time of hydrogen peroxide of seed extracts, which is highest in seeds germinated in curd (1.3) followed by seeds in water (1.0) and ethyl acetate extracts (0.8). Peroxidase activity was expressed as an increase in optical density at 420 nm per min per mg protein and was found to be almost similar in all the three samples (0.02 in seeds germinated in curd and water and 0.01 in ethyl acetate extracts). Maximum ascorbate oxidase activity in terms of enzyme equivalents to 0.01 OD change per min at 420 nm is shown maximum in sample of seeds germinated in curd with 73 enzyme units followed by 58 enzyme units with seeds germinated in water and 50 with ethyl acetate extract. The units of total phenolic compound analysis were expressed as increase in optical density at 765 nm and the result was expressed as mg of gallic acid equivalents/g weight. It was found to be high in seed germinated in curd (0.894) followed by seeds in water (0.855) and ethyl acetate extract (0.041).

Antimicrobial activity: The antimicrobial activity of Soxhlet extracted ethyl acetate fraction of fenugreek and extracts of fenugreek in combination with curd and water are presented in Table-2. 10 µL (1 µg/µL) each of ampicillin for bacteria and fucanazole for fungi are added as positive standards. It was found that ethyl acetate extract and extract of seeds germinated in curd showed good antimicrobial activity. Most significant inhibitory activity was observed with all the strains used showing maximum with fungal strains with fraction collected from seeds germinated in curd. Significant inhibitory activity was also found with ethyl acetate fraction of seeds towards *E. coli* (25 mm), *Staphylococcus aureus* (16 mm), *Serretia marcescence* (16 mm), *A. niger* (40 mm), *T. rubrum* (35 mm). Activity was also seen with seeds germinated in water but not significant with bacteria but seen with fungi. All the samples showed higher inhibitory activity towards fungi than towards bacteria.

The reactive oxygen species such as super oxide, hydrogen peroxide and hydroxyl radicals are molecules that contain oxygen and have higher reactivity than ground state molecular oxygen. In our body, reactive oxygen species play an important role in cell death and signal transduction by ionizing radiation. When water, the most copious intracellular material is exposed to ionizing radiation, decomposition of reactions occur, which form a variety of free radicals and molecular products. These products can peroxide membrane lipids and attack proteins or DNA. In order to avoid these unnecessary reactions there is a requirement of enzymatic and non-enzymatic defense systems that deal with reactive oxygen species produced as a consequence of aerobic respiration.

In this study, it was observed that extract of *Trigonella* seed in curd showed higher activities of total antioxidant activity, catalase, peroxidase, ascorbate oxidase and total phenolics when compared with other samples. Total phenolics present are responsible for the antioxidant activity and defensins, small cysteine rich peptides exhibit antifungal activity assist in killing by binding to the microbial cell membrane and form pore-like membrane that allows efflux of essential ions and nutrients. The free radicals formed are scavenged by the increase in the amount of enzymes. Seeds germinated in curd showed higher activities with all the enzymes, which may be due to the proteins present in the seeds get activated to increase the protein content which in turn showed elevated levels of antioxidant and antimicrobial activity.

Phenolics obtained from fenugreek sprouts germinated in dark showed high antioxidant and antimicrobial activities by Randhir *et al.*[16]. Premanath *et al.*[10] reported higher antioxidant activity with leaves of *Trigonella* but we are reporting on seeds germinated in water and curd which showed high activity. Dixit *et al.*[17] worked with different fractions of germinated *Trigonella* seeds to determine their antioxidant potential at different time intervals which were considered to be more beneficial than dried seeds. Their study revealed significant antioxidant activity, which may be partly due to the presence of flavonoids and polyphenols.

The ethyl acetate extract of *Trigonella* found to have antimicrobial activity exhibiting a broad spectrum of micro organisms ranging from Gram positive to a few Gram negative and antifungal activities. The *Trigonella* seeds soaked in curd is used as a home remedy to decrease dandruff; hence activity of *T. rubrum*, which is dandruff causing fungus was checked. This may be attributed due to the presence of secondary metabolites (saponins) and defensins (antimicrobial peptides). Olli and Kirti[18] purified peptide from *E. coli* expression displayed inhibitory activity against broad spectrum fungal pathogens, *Rhizoctonia solani* and *Fusarium moniliforme*.

Bukhari *et al*.[19] reported that crude extracts of *Trigonella* seeds of methanol, ethanol, dichloromethane, acetone, hexane and ethyl acetate were subjected for the measurement of total phenolic content (TPC) by Folin-Ciocalteau method as well as flavonoid content, chelating activity. The results revealed that the *Trigonella* extracts could act as potent source of anti-oxidants and as antifungal agents.

Conclusion

Fenugreek seeds are good to health as they are rich in proteins and have good antioxidant and antimicrobial capacity, which is essential in fighting against diseases. Identification of plant extracts with known antimicrobial and antioxidant properties can be great significance in food and medicine.

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