ORIGINAL ARTICLE

In vitro study of Antibacterial Property and Cytotoxic Effects of Aqueous, Ethanolic, Methanolic, and Hydroalcoholic Extracts of Fenugreek seed

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ABSTRACT

Background: Fenugreek (*Trigonella Foenum-graecum*) seed is reported to have anti-diabetic, anti-microbial, anti-parasitic, and hypocholesterolemic effects.
Aim: To evaluate the antibacterial activity and cytotoxic effect of aqueous, ethanolic, methanolic, and hydroalcoholic extracts of fenugreek seed *in-vitro*.
Methods: Aqueous, ethanolic, methanolic, and hydroalcoholic extracts of fenugreek seed were prepared by maceration method. In this study, six standard bacterial strains were selected including; *Salmonella* Typhi, *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Staphylococcus aureus* and *Streptococcus pyogenes*. The antibacterial effect of fenugreek extracts was determined, using well diffusion agar and broth micro-dilution method. Cytotoxicity was determined on Vero cells by MTT (thiazolyl blue tetrazolium dye) assay.
Results: In well diffusion agar method, none of the extracts showed inhibition zone on the bacterial strains. In the broth micro-dilution method, the MIC of the hydroalcoholic extract was determined 100, 100, 50, 100, and 100mg/mL for *Escherichia coli, Pseudomonas aeruginosa, Salmonella* Typhi*, Streptococcus pyogenes* and *Staphylococcus aureus*, respectively. Also, MIC of the methanolic extract on *Streptococcus pyogenes* was 100mg/mL. Minimum inhibitory concentration (MIC) could not be determined for other extracts.
Conclusion: Our results showed that although fenugreek seed extracts might have antibacterial activity in concentration of 50-100 mg/mL, in safe and non-toxic concentrations have no *in-vitro* antibacterial effect on the studied bacteria.
Keywords: Trigonella, anti-bacterial, cytotoxic effect, MTT, in vitro.

INTRODUCTION

Iranian traditional and herbal medicine has many recommendations for treatment of disease[1,2]. Bacteria are among the most common and important causes of infectious diseases. Bacterial infections can lead to many complications, even death. Currently, the main method to treat bacterial diseases is to use antibiotics. Although broad-spectrum antibiotics are widely used to treat infections, these antibiotics can increase drug resistance[3]. Medicinal herbs are widely used and some have antibiotic properties. Their consumption can eliminate pathogenic bacteria. Also, daily consumption of these herbs can change the amount and type of normal flora in different organs[4,5].

Fenugreek (*Trigonella foenum-graecum*) is a common herb, used for its anti-diabetic, anti-hyperlipidemic, breast milk increasing properties[6]. In previous studies, fenugreek showed to have anti-bacterial and antiplasmodial effect[7,8,9].

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This study was designed to assess the *in-vitro* effect of aqueous, ethanolic, methanolic, and hydroalcoholic extracts of fenugreek seed on some gram-negative and gram-positive bacteria, and to determine the cytotoxicity of the extracts on Vero cells.

MATERIALS AND METHODS

Fenugreek seed were collected from the fields around Shiraz in September 2014. The spices were botanically identified and confirmed by a botanist.
Preparation of extracts and storage: Fenugreek seed were dried after being cleaned and washed with tap water, then powdered with a blender. The extracts were prepared using Alade and Irobl methods[10] with minor modifications. One hundred gram of fenugreek powder was separately soaked in 100 mL sterilized distilled water, 70% ethanol (as hydroalcoholic extract), ethanol (100%) and methanol (100%) for 72 hours. Each mixture was stirred in an Erlenmeyer flask for 24 hours by a shaker. At the end of the extraction, each extract was filtered (atman filter paper No. 1, Whatman Limited, UK). Finally, at 30°C, the solvent evaporated, and 11, 7, 2.4 and 1 g of dried aqueous, hydroalcoholic, ethanolic and methanolic extracts were obtained, respectively. The extracts were stored at 4°C until being used.
Bacterial strains: This study was conducted in the department of bacteriology and virology, school of medicine, Shiraz University of Medical Sciences, Shiraz, Iran.

Antibacterial activity of extracts was investigated against four gram-negative and two gram-positive bacteria.

These included *Escherichia coli* (*E.coli*) (ATCC: 25922), *Salmonella* Typhi (*S.* Typhi) (PTCC: 1609), *Pseudomonas aeruginosa* (*P. aeruginosa*) (ATCC: 27853), *Klebsiella pneumoniae* (*K. pneumoniae*) (ATCC: 700603), *Staphylococcus aureus* (*S. aureus*) (ATCC: 25923), and *Streptococcus pyogenes* (*S. pyogenes*) (ATCC: 8668).

Antibacterial susceptibility testing: The sensitivity of the bacteria was determined by well diffusion agar and broth micro-dilution methods.

Well diffusion agar method: Initially, the bacteria were cultured overnight (24 hours) at 37°C in 5% sheep blood agar to prepare cell suspension. The bacteria were homogenized and adjusted to 0.5 McFarland standards (1.5×10^8 CFU/mL) using spectrophotometry method. Then, *E. coli*, *S. Typhi, K. pneumoniae, P. aeruginosa, and S. aureus* strains were cultured on Mueller-Hinton agar medium, and *S. pyogenes* were cultured on Mueller-Hinton agar medium enriched with sheep blood using a sterile swab.

Then, by glass Pasteur pipette on the medium some wells were made in the diameter of 6 mm and a depth of 4 mm. Next, 100 µL of 25, 50, 100, and 200 mg/mL dilution of herbal extracts was poured in the wells and the plates were incubated at 37°C for 18-24 hour. Finally, effectiveness of the extracts was evaluated by comparing the diameter of the inhibition zone of them with antibiotics.

Antibiotic disk control for *P. aeruginosa, E. coli, S. pyogenes* and *K. pneumoniae* was ciprofloxacin. Also, antibiotic disc used for S. Typhi was ampicillin and for *S. aureus* was Synercid.

Determining minimum inhibitory concentration (MIC): For this purpose, first 100 µL of Mueller-Hinton Broth medium was inoculated in each well of 96-well (12×8) plates, and then 100 mg/mL of fenugreek extracts to the 1st well and up to 10th wells were serially diluted. The 11th well was considered as the negative control (Mueller-Hinton Broth only) and the 12th well containing the culture medium and a bacterium was considered as the positive control. From each bacterium, a suspension was prepared at 0.5 McFarland standard turbidity, and after dilution of the microbial suspension up to 1/20 ratios, 10µL was added to each well. The final inoculation of bacteria per well became 5×10^4 CFU/mL. Lastly, the microplates were incubated at 37°C for 18-24 hours (11). MIC was determined by the naked eye. This experiment was repeated three times for each bacterium, and their mean was reported.

Cytotoxicity assays: The cytotoxicity of extracts was examined through MTT (thiazolyl blue tetrazolium dye, Sigma, USA) assay. Initially, Vero cells were cultured in 96-well (Nunc, Denmark) microplates containing Dulbecco's modified Eagle's Medium (DMEM, Sigma Chemical Company) for 24 hours at 37ºC in the presence of 5% CO_2. Next, the extract was diluted serially and added to the wells. After incubation for 24-48 hours, DMEM was washed by PBS and substituted with MTT solution. After incubation at 37ºC for 3-4 hours, the contents were centrifuged for 10 minutes at 300 rpm. Next, the supernatant was removed, and the cells were suspended in 100 µL DMSO. Finally, the optical absorption was read at a wavelength of 405-450 nm, using an ELISA reader. The percentage of survived cells was calculated through dividing the optical absorption of the samples by the optical absorption of the control against the concentration of the extracts. The concentration that reduced the cell growth to less than 50% (CC50) was determined, using regression chart.

RESULTS

In well diffusion agar method, in all concentrations (25, 50, 100, and 200 mg/mL) of fenugreek extracts, no inhibition zone was observed. On the other hand, the controlled antibiotics inhibited bacteria growth (Fig. 1, Table 1). MIC of the hydroalcoholic extract was determined 100, 100, 50, 100, and 100 mg/mL for *E. coli, P. aeruginosa, S.* Typhi*, S. pyogenes* and *S. aureus*, respectively. Also, MIC of the methanolic extract on *S. pyogenes* was 100 mg/mL. MIC was not detectable for other extracts at the studied concentrations (Table 2). Cytotoxic concentrations of aqueous, ethanolic, methanolic, and hydroalcoholic extract of fenugreek were 10, 25, 25 and 10 mg/mL on Vero cell cytotoxicity, respectively.

Table 1. The diameters of inhibition zone (mm) of the studied extracts and antibiotic agents.

Bacteria	Aqueous extract	Ethanolic extract	Methanolic extract	Hydroalcoholic extract	Ciprofloxacin	Synercid	Ampicillin
Escherichia coli	0	0	0	0	34	ND	ND
Klebsiella pneumonia	0	0	0	0	28	ND	ND
Pseudomonas aeruginosa	0	0	0	0	27	ND	ND
Salmonella Typhi	0	0	0	0	ND	ND	16
Staphylococcus aureus	0	0	0	0	ND	21	ND
Streptococcus pyogenes	0	0	0	0	27	ND	ND

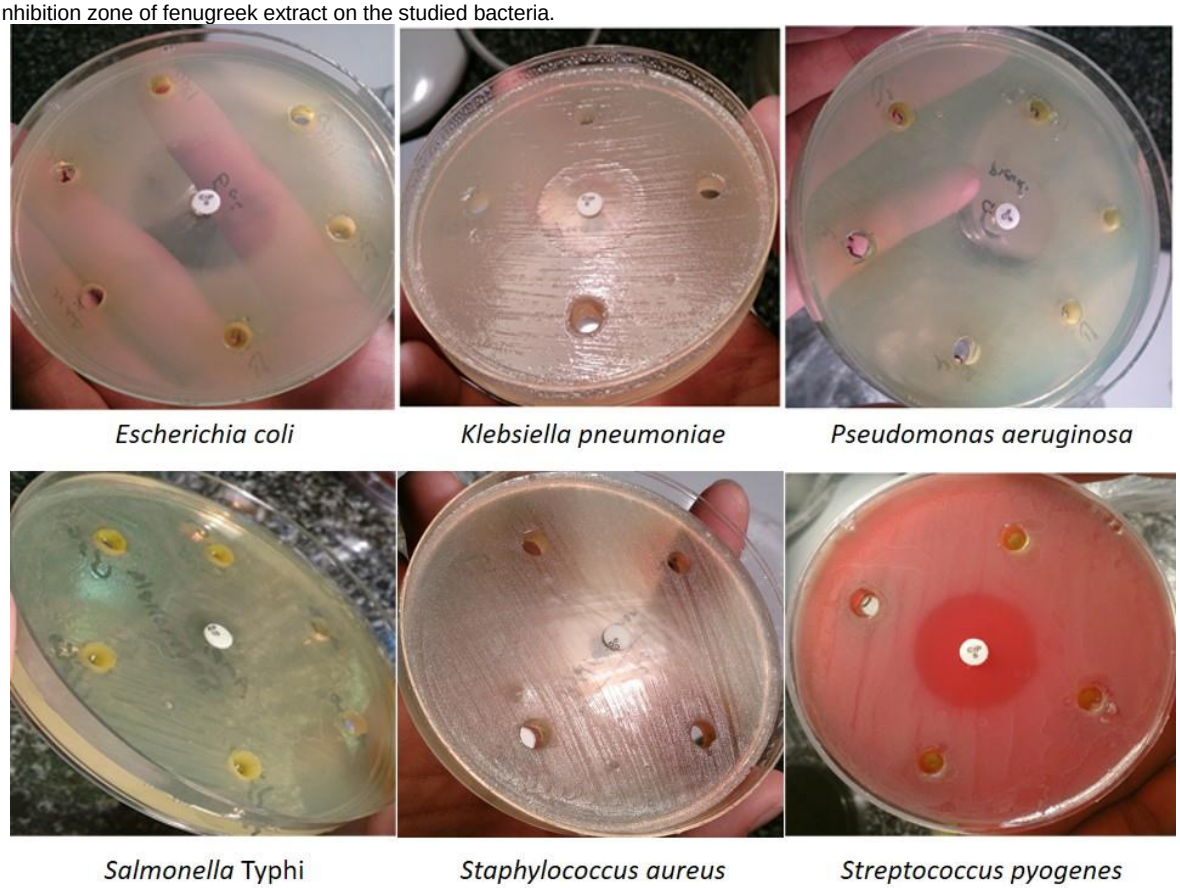
ND: not determined

Table 2: MIC of ethanolic, methanolic, and hydroalcoholic extracts of fenugreek seed.

Extract	MIC (mg/mL)
Escherichia coli	
Ethanolic	ND
Methanolic	ND
Hydroalcoholic	100
Klebsiella pneumoniae	
Ethanolic	ND
Methanolic	ND
Hydroalcoholic	ND
Pseudomonas aeruginosa	
Ethanolic	ND
Methanolic	ND
Hydroalcoholic	100
Salmonella Typhi	
Ethanolic	ND
Methanolic	ND
Hydroalcoholic	50
Staphylococcus aureus	
Ethanolic	ND
Methanolic	ND
Hydroalcoholic	100
Streptococcus pyogenes	
Ethanolic	ND
Methanolic	100
Hydroalcoholic	100

ND= MIC was “Not Determinable”.

Fig. 1: Inhibition zone of fenugreek extract on the studied bacteria.



DISCUSSION

In this study, the antibacterial and cytotoxic properties of aqueous, ethanolic, methanolic, and hydroalcoholic extract of fenugreek seed were investigated *in-vitro*. These extracts did not reveal any inhibitory effect at the non-toxic concentrations for Vero cells. Fenugreek is a common herb in Persian medicine with many pharmacological effects[12,13].

Previous studies examined the antimicrobial effects of fenugreek. In a study by Norziah et al., ethanolic extract of fenugreek seed showed an inhibition zone of 70.3 mm on *E. coli*. The inhibition zone of methanolic extract was 44.1 mm. The inhibition zone of ethanolic and methanolic extracts for *S. aureus* were 38.2 and 25.9 mm, respectively[14].

In another study, fenugreek methanolic extract showed more significant effect in comparison with aqueous, methanolic and acetonic extracts on the *E. coli* and *S. aureus*. Also, fenugreek leaves extract showed more antimicrobial effects than its seed[15].

In a study by El Nour et al., fenugreek seed methanolic extract in the concentration of 250 mg/mL did not inhibit the growth of *Pseudomonas spp., S. aureus,* and *E. coli*[16].

In a study by Nandagopal et al., aqueous and ethanolic extract of fenugreek seed at a concentration of 100 µL resulted in an inhibition zone of 9.2 and 16.3 mm on *P. aeruginosa*, respectively. This value for *S. aureus* was 6.45 and 13.9 mm, respectively[17].

In a study by Majnoni et al., MIC of hydroalcoholic extract of fenugreek seed on *K. pneumoniae, E. coli, P. aeruginosa* and *S. aureus* were 512, 512, 512 and 64 µg/mL, respectively. In this study, the inhibition zone of *K. pneumoniae, E. coli, and P. aeruginosa* was reported 10, 15 and 0 mm, respectively. They concluded that the antimicrobial activity of fenugreek extract on gram-positive bacteria was more than the gram-negative ones, and this might be due to the presence of a peptidoglycans enriched cell wall and a lack of outer membrane layer in gram-positive bacteria[18].

Investigation of the anti-bacterial effect of aqueous extract (cold, hot & boiling) and methanol fenugreek seed extract revealed that boiling water extract showed anti-*S. aureus* effect. The researchers concluded that only boiling water extract contains the antimicrobial active ingredients of fenugreek, responsible for the antimicrobial effect[19].

In another study, fenugreek methanolic extract showed the inhibition zone of 10, 8 and 7 mm on *P. aeruginosa, S. Typhi* and *E. coli*, respectively. MIC was also reported 32, 64 and 64 µg/mL, respectively[20].

The results of the study by Al-Oqail et al., using MTT and neutral red uptake (NRU) methods showed that fenugreek seed oil at a concentration of 1 mg/mL was cytotoxic for 14% of Vero cells[21]. The aqueous extract of fenugreek leaves was cytotoxic at a concentration of 2.5mg/mL on NIH/3T fibroblast cells by MTT method[22].

We investigated the effect of fenugreek extracts on both bacteria and Vero cells, but none of the mentioned studies had simultaneously investigated the effect of fenugreek extract on bacteria and eukaryotic cells.

According to the results of this study, it is possible that problem might occur at extraction or diffusion of extract in the culture medium. Also, the plants of different geographical regions have different pharmacological effects, and this factor plays an important role in their antimicrobial properties.

Therefore, we suggest that further studies determine antibacterial properties of the separated fractions of these extracts by high-performance liquid chromatography (HPLC) or gas chromatography (GC) techniques.

CONCLUSION

In this study, aqueous, ethanolic, methanolic, and hydroalcoholic extracts of fenugreek seed showed no antimicrobial property against *E. coli, K. pneumoniae, P. aeruginosa, S.* Typhi*, S. aureus* and *S. pyogenes* at non-toxic concentrations for Vero cells. However antibacterial concentrations of the mentioned extracts were toxic for the Vero cells.

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Competing interest: The authors declare no competing interest.

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