Antibacterial Activity of Fenugreek Seeds (Trigonella foenum-graecum) Crude Extracts Against a Rabbit Escherichia coli Isolate

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ABSTRACT

Post-weaned rabbits are sensitive to digestive disorders, some of which may be aggravated by enteric bacteria such as Escherichia coli. Fenugreek (Trigonella foenum-graecum) is a medicinal plant known for its various pharmacological properties, including its anti-bacterial activity. The purpose of this study was to screen phytochemical compounds present in crude fenugreek seeds extracts and evaluate their potential activity against an E. coli strain isolated from a morbid rabbit. Aqueous and organic (hexane, chloroform, acetone, ethanol and methanol) extracts were prepared from powdered fenugreek seeds. Bacterial growth inhibitory effects were evaluated at three concentrations (2.5, 5 and 10 mg/ml) by measuring the diameter of the inhibition zone (IZ) using an agar-well diffusion method. No anti-bacterial effect was demonstrated by the aqueous extract of fenugreek seeds; however, the organic extracts prepared with chloroform, acetone or methanol showed low to moderately high growth inhibitory effect (8.33 mm ≤ IZ ≤ 20 mm) when tested at a concentration equal to or above 5 mg/ml. Phytochemical screening of the extracts revealed the presence of the major compounds known to have anti-bacterial activity such as tannins and flavonoids in the aqueous and methanolic extracts. The results indicate that fenugreek seeds crude extracts may have anti-bacterial potential against E. coli depending on the solvent used for the extraction. Quantitative analysis of phytochemical compounds screened in the extracts is needed to more fully explain these results.

Key words: Fenugreek seeds, solvents extracts, growth inhibitory effect, inhibition zone, Escherichia coli.

INTRODUCTION

In rabbit farming, digestive pathologies play a major role in increasing production costs as they constitute the main cause of morbidity and mortality in post-weaned rabbits (Gidenne and Licois, 2012). Coccidia and bacterial species such as enteropathogenic Escherichia coli, Clostridium spiroforme and exceptionally Clostridium piliforme) and Klebsiella pneumonia are among the specific causes of enteric diseases in post-weaned rabbits (Licois, 2010).

E. coli is a normal inhabitant of the rabbit intestinal flora. Usually, its population is limited to less than 10^4 to 10^5 cfu/g of caecal content and some rabbits may not host the bacterium. Many environmental conditions (cold, heat, loud atmosphere and impaired ventilation) or feeding factors (insufficient watering, insufficient cellulose rate or excessive protein intake and use of antibiotics) can disrupt the digestive physiology of the rabbit. This may occur by a simple change in its eating behavior (no more caecotrophy), or sometimes by causing more permanent alterations of transit, or by changing the normal intestinal flora. Digestive stasis, followed by chemical changes in the caecal contents (imbalance of fatty acids and pH increase) promotes abnormal bacterial overgrowth very often with an
excessive development of colibacilli flora up to 10^8 to 10^9 cfu/g of caecal content. This severe colibacillosis can be fatal in young rabbits, particularly in the case of enteropathogenic *E. coli* strains (Licois, 2010; Bivolarsk et al., 2011).

Prevention or control of post-weaning enteric diseases was achieved by the incorporation of antibiotics in the feed of the young animals (Berge et al.; 2005; Kritas and Morrison, 2005). However, the misuse of antibiotics has led to the emergence and spread of antibiotic-resistant microorganisms in human and veterinary medicine (Seal et al., 2013). Therefore, antibiotic use in animal feeds has been banned in the European Union since 2006 (Regulation 1831/2003/EC). Consequently, studies are being done to develop new strategies exploring new sources of active antibacterial compounds of plant origin as alternatives for antibiotics, particularly on a prophylaxis level to reduce the incidence of enteric pathogens in farm animals (Seal et al., 2013).

Fenugreek (*Trigonella foenum-graecum*) is one of the medicinal plants originating in Northern Africa (Altuntas et al., 2005). A review of the literature indicates that many investigators have reported anti-bacterial effect of fenugreek seeds extracts (Upadhay et al., 2008; Alwhibi and Soliman, 2014; Majumdar and Alluri, 2014). Such effect seems to be related to the presence of molecular compounds, usually in the form of secondary metabolites, such as alkaloids, steroids, tannins and phenol compounds, flavonoids etc (Erdogru, 2002).

In the present study, fenugreek seeds extracts were screened for the presence of phytochemical active constituents and their anti-bacterial activity was evaluated against an *E. coli* strain isolated from the caecal content of a morbid rabbit.

**MATERIALS AND METHODS**

**Preparation of fenugreek seeds extracts**

Fenugreek seeds were purchased in bulk at a medicinal herbs grocery store. The method described by Nandagopal et al. (2012) was used for the preparation of fenugreek seeds extracts with some modifications. Five grams of powdered fenugreek seeds were soaked separately in 25 ml distilled water, acetone, chloroform, ethanol, methanol and hexane for 24 h under continuous stirring at room temperature and away from the light. At the end of soaking time, each extract was passed through Whatman No.1 filter paper. The resulting filtrate was collected in previously tared sterilized Petri plates and reduced to dryness by removing the solvent in an air-dried oven at 40°C. After the complete removal of the solvent, the Petri plates were weighed and the net weight of each dried extract determined and used to determine the extraction yield. The extraction was carried out in triplicate and results were expressed as mean value ± standard error of the mean (SEM).

Dried extracts were then individually dissolved in sterilized distilled water to reach a concentration of 10 mg/ml and stored in opaque sterile Falcon tubes at 4°C to serve as starting material for anti-bacterial assessment.

**Phytochemical screening of fenugreek seeds extracts**

The extracts were subjected to qualitative analysis for secondary metabolites such as coumarins, tannins, steroids, terpenoids, saponins, flavonoids and alkaloids. The analyses were carried out using standard methods as described by Jansi et al. (2013).

**Antibacterial activity assessment of fenugreek seeds extracts**

**Bacterial strain**

An *E. coli* strain was selectively isolated from the caecal content of a morbid rabbit (that is, a rabbit that suffered from diarrhea and showed low weight gain or feed intake compared to rabbits raised in the same group during the same rearing period). The *E. coli* count in the caecal content was 2.7×10^8 cfu *E. coli*/g. The isolate was morphologically characterized and examined for biochemical properties of *E. coli* (Goodridge et al., 2012) before running the anti-bacterial activity assay.

**Anti-bacterial activity assay**

The anti-bacterial activity of the fenugreek seeds crude extracts was tested using an agar-well diffusion method (NCCLS, 2002). A standardized bacterial inoculum was prepared, adjusted to 0.5 McFarland (±10^8 cfu/ml) and then diluted to 10^6 cfu/ml. Within 15 min, nutrient agar (Biokar Diagnostics, France) plates (Ø 120 mm) were seeded by flooding their surfaces with the prepared inoculum. Excess inoculum was removed using a sterile pipette. Once the plates were dried, 6 mm wells were prepared using a sterile cork borer and filled with 50 μl of the different concentrations of 2.5, 5 and 10 mg/ml, respectively of each fenugreek seeds extract or controls (solvent which served for the extraction). A diffusion period of 30 min was allowed prior to incubation so that the extracts could slowly diffuse before bacterial growth commenced. The plates were then incubated at 37°C for 24 h. After incubation, the anti-microbial activity was evaluated by measuring the diameter of the inhibition zone. The assay was carried out in triplicate and results expressed as mean value ± standard error of the mean (SEM).
RESULTS AND DISCUSSION

Digestive pathology of bacterial origin such as colibacillosis is one of the causes of morbidity and mortality in rabbit production. Fenugreek seeds were traditionally used for the treatment of several human illnesses such as gastric disorders and diarrhea. In view of the ban of use of antibiotics as growth promoters in animals feed, it was interesting to investigate whether the seeds of this indigenous plant have any anti-bacterial activity against an E. coli strain isolated from a morbid rabbit. The verification of this hypothesis could justify incorporation of fenugreek seed into feedstuff of post-weaned rabbits as a dietary approach to prevent digestive disturbances during this critical period.

Extraction yield

As stated previously, six different solvents (hexane, chloroform, acetone, ethanol, methanol and water) were used to extract the fenugreek seeds. Solvents were applied starting with the least polar (hexane) to the most polar (water). These solvents were selected in order to extract compounds with different polarities. Hexane is known to extract the non-polar metabolites. Chloroform and acetone are known to extract compounds with low to medium polarity, while ethanol, methanol and water are known to extract the polar compounds. The results obtained indicated that water extraction had a higher crude extract yield (252.93±2.02 mg/g) compared to the other solvents. Intermediate extraction yields of 77.55±1.29 mg/g and 95.01±1.32 mg/g were obtained by ethanol and methanol extractions, respectively. The lowest extraction yields were recorded when acetone, chloroform, and hexane were used. The three last extractions yields were relatively comparable and ranged from 52.37±2.07 mg/g for hexane to 54.31±1.26 mg/g for acetone. Analysis of the extraction yield determination results reveals that this parameter increased with increasing solvent polarity. This observation suggests that fenugreek seeds might contain very polar substances such as flavonoids, tannins and alkaloids (Snyder and Kirk, 1979).

Phytochemical screening of fenugreek seeds extracts

Table 1 shows the data comparing the phytochemical profile of fenugreek seeds extracts. These results demonstrated that hexane and chloroform extracts had the same profile which showed the absence of coumarins, tannin, steroid, terpenoids, saponins and flavonoids, but the presence of alkaloids. Data in Table 1 also indicated that ethanol and aqueous extracts have slightly similar profiles. The first extract showed the absence of alkaloids, steroids and flavonoids, but the presence of coumarins, tannin, terpenoids and saponins. The second extract differed only by the presence of flavonoids. The acetone extract showed the absence of all the compounds earlier mentioned; whereas, the methanol extract had the same phytochemical profile as the aqueous extract, except for steroids, which were revealed to be present in the methanol extract and absent in water extract.

Phytochemical compounds such as coumarins, tannins, steroids, terpenoids, saponins, flavonoids and alkaloids were screened in the six fenugreek seeds extracts. Among these compounds, alkaloids, flavonoids, saponins and tannins are important secondary metabolites and are frequently responsible for medicinal properties associated with plants.

Alkaloids were absent in the acetone, ethanol, methanol and aqueous extracts. Their absence in the extract of acetone, which is known to extract compounds with low to medium polarity, can be admitted. However, as polar substances, it was surprising to note the absence of alkaloids in alcoholic extracts (ethanol and methanol) and especially in the aqueous one. According to Snyder and Kirk (1979), water allows the extraction of very polar substances such as flavonoids, tannins and alkaloid. Flavonoids were present only in extracts prepared with methanol or water. Due to their polarity, these compounds dissolve well in polar solvents such as methanol and water. Tannins and saponins, were both absent in hexane, chloroform and acetone extracts but present in ethanol, methanol and aqueous extracts. This result can be also attributed to the affinity of the latter three solvents with polar molecules such as tannins and saponins.

It is difficult to compare the phytochemical profile of our fenugreek seeds extracts with those of other studies because in the literature there are few works that effectively screened the phytochemical compounds present in the seeds extracts. Thus, for the acetone extract, which showed the absence of all the compounds aforementioned, our results were inconsistent with the previous phytochemical screenings of acetone extracts of fenugreek seeds which showed the presence of flavonoids, phenols, tannin, terpenoids and saponins but the absence of alkaloids (Chitra el al., 2014) or the presence of alkaloids and flavonoids (Yadav et al., 2014). However, the phytochemical profile of our methanol extract was similar to that described by Majumdar and Alluri (2014) who reported that methanol fenugreek seed extract contains tannins, flavonoids, saponins, terpenoids, steroids and alkaloids.

Anti-bacterial activity assay

The anti-bacterial activity of the six solvent extracts (hexane, chloroform, acetone, ethanol, methanol and water) of fenugreek seeds against the E. coli isolate was assessed by measuring the inhibition zone diameter at three
concentrations (2.5, 5 and 10 mg/ml) (Table 2). An inhibition zone diameter (IZ) above 7 mm was considered as a positive result. On the basis of the diameter of the growth inhibition zone, anti-bacterial activity of the fenugreek seeds extracts against E. coli was divided into three categories: that is, low (6 mm < IZ < 12 mm), intermediate (12 mm ≤ IZ < 18 mm) and high (IZ > 18 mm), respectively.

It is important to first note that bacterial growth was observed for the positive controls (agar plates inoculated with E. coli but untreated with fenugreek seeds extracts) while no growth was observed for the negative controls (agar plates non-inoculated with E. coli and untreated with fenugreek seeds extracts). More important to note is that the solvent controls (agar plates inoculated with E. coli and treated only with solvents used for the preparation of the fenugreek seeds extracts) did not exert any anti-bacterial activity. Indeed, regardless of the solvent used (hexane, chloroform, acetone, ethanol, methanol or water), no inhibition zone was observed around the wells filled only with that solvent.

The solvent extracts of fenugreek seeds screened in this study showed various inhibitory effects against the same bacterial strain. Methanol and chloroform extracts showed inhibitory effect at all the tested concentrations. Inhibition zone diameters ranged from 8.50±2.18 to 18.50±1.32 mm and from 8.33±1.15 to 17.00±1.00 mm for methanol and chloroform extracts, respectively. This result was in agreement with that of Alwhibi and Soliman (2014) who reported that methanol and chloroform extracts have a growth inhibitory effect on E. coli. However, in their study, the inhibition zone diameters ranged from 25.2 to 35.6 mm indicating a stronger anti-bacterial activity. The work of Abdel-Massih et al. (2010) and of Marzougui et al. (2012) on the activity of methanol or chloroform extracts obtained from fenugreek seeds on E. coli were not consistent with our finding as these authors did not detect an anti-bacterial effect for either extract. It is important to note however, that the E. coli isolate they utilized was highly drug-resistant strain.

For the methanolic extract, our results were in accordance with earlier findings of Majumdar and Alluri (2014) who demonstrated that methanolic extract of fenugreek seeds exhibited low to moderate growth inhibitory effect (IZ: from 7.3 to 16 mm) on E. coli. However, these investigators used low concentrations ranging from 0.125 to 2.0 mg/ml, respectively. The anti-bacterial properties of the methanolic fenugreek seed extract might be attributed to their phytochemical content (that is, coumarins, tannins, steroids, saponins, terpenoids and flavonoids).

An earlier study reported by Upadhyay et al. (2008) demonstrated a high anti-bacterial activity (IZ=28 mm) associated with the chloroform extract of fenugreek seeds. The low to medium anti-bacterial effect of chloroform extract observed in the present study could be attributed to the presence of the only chemical component detected by the phytochemical screening: that is, alkaloids.

Acetone extract exhibited anti-bacterial activity against E. coli when used at a concentration of 5 or 10 mg/ml, yet it had no detectable anti-bacterial effect at the lowest concentration (2.5 mg/ml). Chitra et al. (2014) also

Table 1: Phytochemical screening of fenugreek seeds crude extracts; n) negative (absence); p) positive (presence).

<table>
<thead>
<tr>
<th>Chemical compound</th>
<th>Hexane</th>
<th>Chloroform</th>
<th>Acetone</th>
<th>Ethanol</th>
<th>Methanol</th>
<th>Water</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coumarins</td>
<td>n</td>
<td>n</td>
<td>n</td>
<td>p</td>
<td>p</td>
<td>p</td>
</tr>
<tr>
<td>Alkaloids</td>
<td>p</td>
<td>p</td>
<td>n</td>
<td>n</td>
<td>n</td>
<td>n</td>
</tr>
<tr>
<td>Tannins</td>
<td>n</td>
<td>n</td>
<td>n</td>
<td>p</td>
<td>p</td>
<td>p</td>
</tr>
<tr>
<td>Steroids</td>
<td>n</td>
<td>n</td>
<td>n</td>
<td>n</td>
<td>p</td>
<td>n</td>
</tr>
<tr>
<td>Terpenoids</td>
<td>n</td>
<td>n</td>
<td>n</td>
<td>p</td>
<td>p</td>
<td>p</td>
</tr>
<tr>
<td>Saponins</td>
<td>n</td>
<td>n</td>
<td>n</td>
<td>p</td>
<td>p</td>
<td>p</td>
</tr>
<tr>
<td>Flavonoids</td>
<td>n</td>
<td>n</td>
<td>n</td>
<td>p</td>
<td>p</td>
<td>p</td>
</tr>
</tbody>
</table>

Table 2: Growth-inhibitory effect of fenugreek seeds crude extracts against the tested E. coli isolate; n.i) No inhibition.

<table>
<thead>
<tr>
<th>Concentration of extract (mg/ml)</th>
<th>Hexane</th>
<th>Chloroform</th>
<th>Acetone</th>
<th>Ethanol</th>
<th>Methanol</th>
<th>Water</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5</td>
<td>n.i.</td>
<td>8.33±1.15</td>
<td>n.i.</td>
<td>n.i.</td>
<td>8.50±2.18</td>
<td>n.i.</td>
</tr>
<tr>
<td>5</td>
<td>n.i.</td>
<td>8.33±0.58</td>
<td>8.50±0.87</td>
<td>n.i.</td>
<td>9.67±0.58</td>
<td>n.i.</td>
</tr>
<tr>
<td>10</td>
<td>n.i.</td>
<td>17.00±1.00</td>
<td>20.00±1.00</td>
<td>11.00±1.00</td>
<td>18.50±1.32</td>
<td>n.i.</td>
</tr>
</tbody>
</table>
reported that acetone extracts of fenugreek seeds showed anti-bacterial activity against *E. coli*. However, the highest (IZ=24 mm) and the lowest activities (IZ=20 mm) were recorded at a concentration of 8 and 1 mg/ml, respectively. These concentrations were beyond our working concentrations. Alwhibi and Soliman (2014) also reported that acetone extract of fenugreek seeds had moderately high anti-bacterial activity against *E. coli*. They obtained inhibition zone diameters in between 18.2 and 22.3 mm. Upadhyay et al. (2008) also found that an acetone extract of fenugreek seeds showed a strong inhibitory effect (IZ=23 mm) against *E. coli*. However, they did not mention the concentration used in the anti-bacterial assay. The moderately high anti-bacterial potential of the acetone extract of fenugreek seeds could not be due to the presence of tannins, steroids, terpenoids, saponins, alkaloids or flavonoids, since the phytochemical screening of the acetone extract revealed the absence of all these chemical compounds.

Ethanolic extract demonstrated an anti-bacterial activity but only at the highest concentration analyzed in our study (10 mg/ml). Moreover, this inhibitory effect is relatively low (IZ=11.00 mm). Abed-Massih et al. (2010) found that an ethanolic extract of fenugreek seeds did not show any inhibitory effect within their tested concentrations, which were significantly lower than ours (ranging between 2.5 and 80 μl). Furthermore, it was important to note that these authors did not assess the anti-bacterial activity by determination of the inhibition zone diameter but by the assessment of the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC). More recently, Faraj et al. (2014) also reported that ethanolic extracts of fenugreek seeds had no detectable growth inhibition effect on an *E. coli* clinical isolate. Unfortunately, in their report, the authors did not state the concentration tested. The relatively low bacterial growth inhibitory effect of the ethanol extract observed in this study could be due to the presence of coumarins, tannins, terpenoids and saponins. However, the research of Alwhibi and Soliman (2014) showed that ethanol extracts from fenugreek seeds belonging to two different cultivars exhibited high anti-bacterial activity (22.6 mm < IZ < 28.4 mm) against *E. coli*.

Hexanic and aqueous extracts did not show any detectable anti-bacterial activity against *E. coli* at any of the tested concentrations even though the hexane extract has the same phytochemical profile as the chloroform extract and the aqueous extract has the same phytochemical profile as the methanolic extract. In their study, Faraj et al. (2014) also found that extracts of fenugreek seeds prepared with water or hexane did not exert a growth inhibition effect on *E. coli*.

We were surprised to find that the aqueous extract in our study had no anti-bacterial effect on *E. coli* since its phytochemical screening revealed the presence of tannins and flavonoids. According to Cowan (1999), these substances belong to the major classes of anti-microbial compounds in plants. Our results were not in agreement with those of Marzougui et al. (2012) who studied the anti-bacterial potential of aqueous extracts (decoked and precipitate) of fenugreek seeds from both diploid and autotetraploid populations and found that aqueous extract prepared from the seeds of the diploid population did not show an anti-bacterial effect on *E. coli*. In contrast, both decoked and precipitate aqueous extracts prepared from the seeds of the autotetraploid population resulted in inhibition zones of 14.77 and 5.35 mm, respectively. However, the authors did not report the tested concentrations. In their report, Upadhyay et al. (2008) also assessed the growth inhibitory effect of a water extract of fenugreek seeds against *E. coli* and only a medium effect (IZ=17 mm) was observed. The aqueous extract of fenugreek seeds could have activity against other bacteria such as *Salmonella typhimurium*, *Salmonella epidermidis* and *Enterobacter faecalis*. This was demonstrated by Marzougui et al. (2012) who also showed that the anti-bacterial activity resided mainly in the aqueous extract prepared from the seeds of the autotetraploid population. In contrast, the results of Alwhibi and Soliman (2014) demonstrated that aqueous extracts from two different fenugreek cultivars showed very low anti-bacterial activity (5.2 < IZ < 6.8 mm) against *E. coli*.

Faraj et al. (2014) reported that fenugreek seeds extracted with hexane did not have a growth inhibition effect on an *E. coli* clinical isolate. In the present study, the hexane extract, exactly like the chloroform one, contains only alkaloids, yet it was ineffective, unlike the chloroform extract. This observation suggested either that the hexanic extract held a very low concentration of alkaloids or that it contained compounds that inhibited the anti-bacterial activity of this phytochemical.

The lack of consensus of some results in the present study, or the cause of divergence with those of previous studies on the anti-bacterial effect of fenugreek seeds could be due to a number of factors such as processing procedures used for the raw material, the protocol of extraction, the method of anti-bacterial assay and the sensitivity of the bacterial strain.

**Conclusions**

This *in vitro* study provides evidence that crude extracts of fenugreek seeds exhibit anti-bacterial effect against *E. coli*. The anti-bacterial potential is different depending on the polarity of the solvent utilized in the extraction process. Anti-bacterial substances showed different solubility depending on the extracting solvent utilized. Quantitative phytochemical analyses should be conducted in the future to better identify the compounds responsible for the antimicrobial activity. Moreover, the present study should be extended to other *E. coli* strains and bacterial species involved in enteric diseases in rabbits such as *Clostridium*. 


sp. and Klebsiella pneumonia if inclusion of fenugreek seeds in the diet is to be considered as a prevention or control strategy for post-weaning enteric diseases in rabbits. Furthermore, since in vitro assays do not take into account the effects of the complex environment of the gut, in vivo evaluation of the efficiency of fenugreek seeds in rabbits naturally or experimentally infected with the enteric pathogens earlier mentioned is required.

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