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Effect of Extraction Procedure on Yield, Phytochemical Profile and Antibacterial Activity of Fenugreek Seed (*Trigonella foenum-graecum*) Crude Extracts

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ABSTRACT

Background: Fenugreek (*Trigonella foenum-graecum*) is a medicinal plant known for its various pharmacological properties, including its antibacterial activity. The purpose of this study was to assess the extraction procedure effect on yield, phytochemical profile and antibacterial activity of fenugreek seed crude extracts.

Methods: The extraction procedure of components from fenugreek seed possessing an antibacterial potential was first optimized. Extracts at the highest yield were analyzed for phytocomponents and their bacterial growth inhibitory effect was assessed by determining the diameter of the inhibition zone (IZ) and the minimum inhibition concentration (MIC).

Result: Optimum conditions for highest yield of extract (270,78 mg/g) are as follows: solvent (water), substrate-solvent ratio (1:10), and extraction time (72 hours). Phytochemical screening revealed the presence of the major compounds known to have an antibacterial activity such as tannins and flavonoids in the aqueous and methanol extracts. The methanol extract was associated with an IZ diameter ranging from 8.46±2.25 mm up to 27.65±1.32 mm and displayed the lowest MIC (1.25 mg/ml). Our findings indicate that fenugreek seed extracted with methanol showed the best antibacterial effect against the targeted bacterial strain.

Key words: Antibacterial assays, Crude extracts, *Escherichia coli,* Fenugreek seed, Resazurin.

INTRODUCTION

In rabbit farming, the post-weaning period is very critical for young rabbits since they switch from a liquid diet to a solid diet at the same time as they are separated from their mother. These relatively stressful conditions make young rabbits vulnerable and sensitive to digestive disorders frequently associated with excessive development of colibacilli flora up to 10^8-10^9 cfu/g of caecal content. This severe colibacillosis can be fatal, particularly in the case of enteropathogenic *E. coli* strains (Licois, 2010).

As antibiotic use in animal feed has been banned in the European Union since 2006 (Regulation 1831/2003/EC), many studies are being done to develop new strategies exploring new sources of active antibacterial compounds of plant origin as alternatives for antibiotics (Seal *et al.* 2013).

Fenugreek (*Trigonella foenum-graecum*) is one of the medicinal plants originating in Northern Africa (Altuntas *et al*. 2005). It is an annual plant belongs to the family of Leguminosae. Many investigators have reported antibacterial effects of fenugreek seed extracts (Upadhyay *et al*. 2008; Alwhibi and Soliman, 2014; Majumdar and Alluri, 2014). Such antimicrobial effects seem to be related to the presence of molecular compounds, usually in the form of secondary metabolites, such as alkaloids, steroids, tannins, phenol compounds and flavonoids, *etc*. (Erdogrul, 2002).

In the current study, we attempted to optimize the yield of fenugreek seed extracts and assess their antibacterial effect against a particular *E. coli* strain isolated from the caecal content of a morbid rabbit.

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MATERIALS AND METHODS

The experiment was conducted from Mars to Mai 2019 at the Higher School of Agriculture of Mateur, University of Carthage, Tunisia.

Preparation of fenugreek seeds extracts

The method described by Nandagopal *et al*. (2012) was used for the preparation of fenugreek seed extracts with some modifications. Five grams of powdered fenugreek seeds were soaked separately in three solvents (distilled water, methanol and ethanol) using two substrate-solvent ratios

(1:5 and 1:10) for three extraction times (24h, 48h and 72h) under continuous stirring at room temperature and away from the light. At the end of the 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 evaporating 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 was 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 that have the highest yield were then individually dissolved in sterilized distilled water to reach a concentration of 20 mg/ml and were stored in opaque sterile Falcon tubes at 4°C to serve as starting material for the phytochemical screening and the antibacterial assays.

Phytochemical screening of fenugreek seed extracts

The extracts were subjected to qualitative analysis for secondary metabolites using standard methods as described by Jansi *et al*. (2013).

Antibacterial activity assessment of fenugreek seed extracts

Conventional antibacterial assay – well diffusion method

The antibacterial activity of the fenugreek seed crude extracts against the tested *E. coli* strain was assessed using an agar-well diffusion method (NCCLS, 2002). Four concentrations (2.5 mg/ml, 5 mg/ml, 10 mg/ml and 20 mg/ml) of each fenugreek seed extract were tested. The assay was carried out in triplicate and results were expressed as mean value± standard error of the mean (SEM).

Resazurin microtiter plate-based antibacterial assay

Sterile 96 well plates were labeled and prepared under aseptic conditions. A volume of 100 µL oftest material in sterile water (a stock concentration of 20mg/ml) was pipetted into the first row of the plate. To all other wells, 50 µL of nutrient broth was added. Serial dilutions were performed using a multichannel pipette. Tips were discarded after use such that each well had 50 µL of the test material in serially descending concentrations. 50 µL of bacterial suspension (10^7cfu/mL) was added to each well to achieve a concentration of $5x10^5$ cfu/mL. Each plate had a set of controls: a column with a broad-spectrum antibiotic as a positive control (ciprofloxacin in serial dilution), a column with all solutions except for the test compound and a column with all solutions except for the bacterial suspension. The plates were prepared in triplicate and placed in an incubator at 37°C for 20-22 h. Finally, 20 µL of resazurin indicator solution (0.2 mg/ml) was added to each well. Plates were then placed in the incubator for an additional 2h. Following this incubation period, the color change was assessed visually. Any color changes from purple to pink or colorless were recorded as positive. The lowest concentration at which color change occurred was taken as the Minimum Inhibitory Concentration (MIC) value. The average of three values was calculated and recorded as the MIC for the test material.

RESULTS AND DISCUSSION

Extraction yield

In the current study, we used three solvents(ethanol, methanol and water), three extraction times (24h, 48h and 72h) and two substrate-solvent ratios (1: 5 and 1:10), to optimize the extraction conditions quantitatively and qualitatively, that is to obtain the highest extraction yield and the best phytochemical profile exhibiting the highest antibacterial activity. The amounts (mg/g of fenugreek seed powder) of fenugreek seed crude extract obtained are shown in Table 1.

Solvent effect

Regardless of the extraction time utilized or the substrate-solvent ratio, using water as a solvent provided higher crude extract yields compared to ethanol and methanol. These extraction yields ranged from 253.93±1.92 mg/g for the shortest extraction time (24h) and a substrate-solvent ratio (1:5) to 270.78±3.05 mg/g for the longest extraction time (72h) and a substrate-solvent ratio (1:10). Methanol was associated with intermediate extraction yields ranging from 77.55±1.98 mg/g for the shortest extraction time (24h)and substrate-solvent ratio (1:5) to111.39±2.24mg/g for the longest extraction time (72h) and substrate-solvent ratio (1:10). The lowest extraction yields were recorded when ethanol was used. These yields ranged from 71,28±1.29 mg/g for the shortest extraction time (24h) and substrate-solvent ratio (1:5) to 97.23±2,93 mg/g for the longest extraction time (72h) and substrate-solvent ratio (1:10). These findings are in agreement with our previous investigation (Chalghoumi *et al*. 2016), wherein we found that the greatest extraction yield from fenugreek seeds was demonstrated using aqueous extracts followed by methanol and then ethanol extracts.

Solvent to substrate ratio effect

Increasing substrate–solvent ratio from 1:5 to 1:10 (w/v) using the same solvent and the same extraction time increased the extraction yield. The difference between the two ratios was less than 5 mg/g when the extraction process was conducted for 24h or 48h. However, this difference reached an average of 9.58 mg/g when the extraction time was 72h. This result suggests that it would be more judicious to increase the substrate-solvent ratio to maximize the extraction yield.

Extraction time effect

Our results showed that extraction yields were improved when extraction was conducted for 72h, regardless of the ratio and solvent used.

Overall, the analysis of the extraction yield data reveals that this parameter increased with increasing solvent polarity, substrate-solvent ratio and extraction time. Thus, aqueous

Table 1: Yield of fenugreek seed crude extracts prepared using different extraction procedures.

Solvent	Extraction time (h)	Substrate-solvent ratio	Extraction yield (mg/g)
	24	1:5	71,28±1.29 (n=3)
		1:10	75,95±2,75 (n=3)
Ethanol	48	1:5	76,6±2,75 (n=3)
		1:10	86.43±2,75 (n=3)
	72	1:5	93.77±2,05 (n=3)
		1:10	97.23±2,93 (n=3)
	24	1:5	77.55±1,98 (n= 3)
		1:10	79.83±3,73 (n=3)
Methanol	48	1:5	81.94±2,75 (n=3)
		1:10	93.77±3,12 (n=3)
	72	1:5	101,68±2,41 (n=3)
		1:10	111,39±2,24 (n=3)
	24	1:5	253.93±1.92 (n=3)
		1:10	258.56±1.94 (n=3)
Water	48	1:5	260.56±2.28 (n=3)
		1:10	264.41±1.76 (n=3)
	72	1:5	263.72±3.16 (n=3)
		1:10	270.78±3.05 (n=3)

extraction using a substrate–solvent ratio equal to 1:10 for 72h seems to be the best combination of those tested for optimizing the yield of extractable substances from fenugreek seeds.

Given that the highest extract yields were obtained when the solvent to substrate ratio was equal to 1:10 and the extraction lasted for 72h, only extracts prepared according to this protocol were used and were subjected to analysis of the phytochemical profile and assessment of the antibacterial activity.

Phytochemical screening of fenugreek seed extracts

The data comparing the phytochemical profile of fenugreek seed extracts characterized with the highest extract yields are given in Table 2. These results indicated that ethanol and aqueous extracts have similar profiles. The ethanol extract showed the absence of alkaloids, steroids and flavonoids, but the presence of coumarins, tannin, terpenoids and saponins. The aqueous extract differed only by the presence of flavonoids. 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 three fenugreek seed extracts.

Alkaloids were absent in the ethanol, methanol and aqueous extracts. 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 alkaloids.

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.

Table 2: Phytochemical screening of fenugreek seed crude extracts.

Chemical compound	Extracts		
	Ethanol	Methanol	Water
Coumarins	p	p	p
Alkaloids	n	n	n
Tannins	p	p	p
Steroids	n	p	n
Terpenoids	p	p	p
Saponins	p	p	p
Flavonoids	n	p	p

n-negative (absence); p- positive (presence).

Tannins and saponins were present in ethanol, methanol and aqueous extracts. This result can be also attributed to the affinity of these solvents with polar molecules such as tannins and saponins.

These results were consistent with those of our previous study (Chalghoumi *et al*. 2016). Moreover, the phytochemical profile of the 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.

Antibacterial activity assays

In the well-diffusion assay, the antibacterial activity was assessed by measuring the inhibition zone (IZ) diameter at four concentrations (2.5, 5,10 and 20 mg/ml) of the crude extracts. The results are given in Table 3.

Methanol extract demonstrated an inhibitory effect at all the tested concentrations. Inhibition zone diameters ranged from 8.46±0.96 to 27.65±0.75mm. This result confirms our earlier finding wherein inhibition zone diameters ranging from 8.50±2.18 to 18.50±1.32mm were obtained against the same *E. coli* isolate using fenugreek seed extract

Table 3: Growth-inhibitory effect of fenugreek seed crude extracts against the tested *E. coli* isolate.

Concentration of extract (mg/ml)	Inhibition Zone Diameter (mm ± SEM)		
	Ethanol	Methanol	Water
2.5	n.i.	8. 96±0.96	n.i.
5	n.i.	11.75±0.58	n.i.
10	12.57±0.70	19.49±0.62	n.i.
20	19.62±0.80	27.65±0.75	9.50±0.86

n.i.- No inhibition.

at a concentration varying from 2.5 to 10 mg/ml (Chalghoumi *et al*. 2016). The obtained result was also in agreement with that of Alwhibi and Soliman (2014) who reported that methanol extract has a growth inhibitory effect on *E. coli*. However, in their study, the range of the inhibition zone diameters was between 25.2 mm and 35.6 mm indicating a stronger antibacterial activity. Majumdar and Alluri (2014) also demonstrated that methanol extract of fenugreek seed exhibited a growth inhibitory effect on *E. coli* although it produced low to moderate (IZ: from 7.3 mm to 16 mm) compared to those recorded in the present study. However, the investigators used low concentrations ranging from 0.125 mg/ml to 2.0 mg/ml. Furthermore, in their report Dash *et al*. (2011) found that methanol extract produced a zone of inhibition of 7mm against *E. coli*. Unfortunately, in their report, the authors did not state the concentration tested. The antibacterial effect of the methanol fenugreek seed extract observed in the current study might be attributed to its phytochemical profile rich in a wide variety of secondary metabolites, such as tannins, terpenoids and flavonoids, which have been found to have antimicrobial properties *in vitro* (Cowan, 1999).

Ethanol extract demonstrated relatively intermediate (IZ=12.57±0.70 mm) and high (IZ=19.61±0.90 mm) antibacterial activity at a concentration of 10 and 20 mg/ml, respectively. No inhibition zone was detected at concentrations of 2.5 and 5 mg/ml. Similar results were observed in our previous report (Chalghoumi *et al*. 2016) in which we did not record zones of growth inhibition at concentrations of 2.5 and 5 mg/ml but did observe a relatively weak inhibition zone(IZ=11.00 mm) at the concentration of 10 mg/ml. The difference between the two values of the inhibition zone obtained at the concentration of 10 mg/ml (IZ=12.57 mm *vs*. IZ=11.00 mm) might be attributed to the extraction conditions. The extract utilized in the previous study was obtained after a 24-hour extraction and using a substrate-to-solvent ratio of 1: 5, whereas the extract used in this study was obtained after 72h extraction and using a substrate-to-solvent ratio of 1:10. Thus, the second extract could be richer in antibacterial components. The low to the medium antibacterial effect of ethanol extract observed in the present study could be attributed to the absence of alkaloids and flavonoids. These compounds are known to exert antimicrobial activity. When comparing our results to those of previous studies, the findings of Alwhibi and Soliman (2014) indicate that ethanol extracts from fenugreek seed belonging to two different cultivars exhibited higher antibacterial activity (22.6 mm < IZ < 28.4 mm) against *E. coli*. However, Faraj *et al*. (2014) reported that ethanol extracts of fenugreek seed 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 aqueous extract demonstrated an antibacterial activity but only at the highest concentration tested (20 mg/ml). Furthermore, this inhibitory effect is relatively low (IZ=9.50 ±0.86mm). Similarly, in our previous study, we did not observe a growth inhibition effect of the aqueous extract on the same *E. coli* isolate when it was tested at concentrations varying from 2.5 to 10 mg/ml although the phytochemical screening of the aqueous extract, in both studies, revealed the presence of tannins and flavonoids but the absence of alkaloids. According to Cowan (1999), these substances belong to the major classes of antimicrobial compounds in plants. This result suggests that at concentrations below 20 mg/ml, the compounds cited above might not be present in sufficient amounts to exert an inhibitory activity on the growth of our *E. coli* isolate. Our findings are consistent with those of Alwhibi and Soliman (2014) who demonstrated that aqueous extracts from two different fenugreek cultivars showed very low antibacterial activity (5.2 mm < IZ <6.8 mm) against *E. coli*. In their study, Faraj *et al*. (2014) found that extract of fenugreek seed prepared with water did not exert a growth inhibition effect on *E. coli*. Marzougui *et al*. (2012) who studied the antibacterial potential of decocted and precipitate aqueous extracts of fenugreek seed from both diploid and autotetraploid populations found that aqueous extract prepared from the seeds of the diploid population did not show an antibacterial effect on *E. coli*. In contrast, both decocted 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 seed against *E. coli* and only a medium effect (IZ=17 mm) was observed.

The antibacterial activity of methanol, ethanol and water fenugreek seed extracts against the *E. coli* strain was also assessed by the determination of the MIC. The MIC of the methanol extract was 1.25 mg/ml, while that of the ethanol and water extracts were 5 and 10 mg/ml, respectively. Furthermore, the MIC of a ciprofloxacin control was 0.065 mg/ml.

A previous study on the antimicrobial activity of fenugreek seed extract showed that a methanol extract has high antibacterial activity against *E. Coli* with a MIC value equal to 64 µg/ml (Dash *et al*. 2011). According to a recent report, the MIC value of a methanol extract was found to be 12.5 mg/ml against *E. coli* (Dharajiya *et al*. 2016). Abdel-

Massih *et al*. (2010) found that an ethanol extract of fenugreek seed did not show any inhibitory effect on *E. Coli* strain within the concentration range in which the MIC was assessed. However, the range used (2.5 and 80 ìg/ml) was significantly lower than ours. In their report stated above, Dharajiya *et al*. (2016) also determined the MIC for a fenugreek seed water extract against *E.coli*, which was reported to be 25 mg/ml.

CONCLUSION

In this study, various extraction conditions were investigated to optimize crude extract yield from fenugreek seed. The highest extract yield (270.78±3.05 mg/g) was obtained by water extraction for 72 hours using a substrate-solvent ratio of 1:10 (w/v). However, this extract exhibited little to no antibacterial effect, depending on the antibacterial assay used. In contrast, methanol extraction under the same conditions produced a lower yield (111.39±2.24 mg/g), yet resulted in a crude extract possessing the highest antibacterial activity against the targeted bacterial strain. Therefore, the potential of using methanol as a solvent in antibacterial compound extraction from fenugreek seed was confirmed in this study.

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