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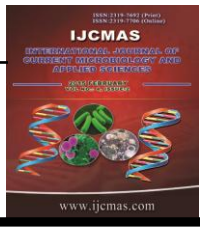

Original Research Article

Antimicrobial Activities and Phytochemical Screening of Callus and Seeds Extracts of Fenugreek (*Trigonella foenum-graecum*)

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Keywords

Trigonella foenum-graecum, Antibacterial activities, Antifungal activities, phytochemical screening

A B S T R A C T

The research aimed to investigate the antimicrobial activities and phytochemical screening of methanolic and petroleum ether extracts of seeds and callus derived from hypocotyls and cotyledons explants of fenugreek (*Trigonella foenum-graecum*). Antimicrobial activities were tested against standard microorganisms, *Bacillus subtilus* (NCTC 8236 G+Ve), *Staphylococcus aureus* (ATCC 25923 G+v), *Escherichia coli* (ATCC 25922 G-V), *Pseudomonas aeruginosa* (ATTC 27853 G-V), *Aspergillus niger* (ATCC 9763) and *Candida albicans* (ATCC7596) using paper disc diffusion method. Callus was induced from hypocotyls and cotyledons explants on MS medium supplemented with 2mg/l of different auxins (2, 4-D or NAA) + 0.5 mg/l Kinetin. The petroleum ether extract of *T.foenum- graecum* seeds showed highest antimicrobial activity compared to methanolic extracts. Antibacterial activity of petroleum ether extract of *T.foenum- graecum* seeds were recorded (17±0.33mm) and (15±0.57mm) of inhibition zone against *Escherichia coli* and *Staphylococcus aureus* respectively by concentration 250 mg/ml. Petroleum ether extract of seeds showed antifungal activity against *Aspergillus niger and Candida albicans* with maximum zone of inhibition (20±0.88 mm) against *Aspergillus niger* by concentration 250 mg/ml and (17±0.57mm) of inhibition zone against *Candida albicans* by concentration 250 mg/ml. Methanolic extracts of hypocotyls and cotyledons showed antimicrobial activities against *Staphylococcus aureus* by concentration 250mg/ml with inhibition zone 11±0.0mm and 12± 0.5 respectively whereas, inhibition zone 10±0 mm was recorded against *Escherichia coli* compared to methanolic extracts of seeds which were ineffective except weak antifungal activity against *Candida albicans*. The petroleum ether extract of *T.foenum-graecum* seeds showed activity higher than Ampicillin/Sulbactam 20mcg/disc and Ciprofloxacin 5 mcg/disc against *Staphylococcus aureus*, also methanolic extracts of hypocotyls and cotyledons derived callus showed activity equal Ampicillin/Sulbactam 20mcg/disc and Ciprofloxacin 5 mcg/disc against *Staphylococcus aureus*. The petroleum ether extract of *T.foenum- graecum* seeds showed activity higher than Ampicillin/Sulbactam 20mcg/disc and Ciprofloxacin 5 mcg/disc against *Staphylococcus aureus*, also methanolic extracts of hypocotyls and cotyledons derived callus showed activity equal Ampicillin/Sulbactam 20mcg/disc and Ciprofloxacin 5 mcg/disc against *Staphylococcus aureus*. Phytochemical screening for both seeds and callus extracts indicated the presence of various Secondary metabolites like alkaloids, flavonoids, tannins, phenols, saponins and terpenoids.

Introduction

Plants are rich source of effective and safe medicines that often used in the treatment of various ailments. There are many published reports from different parts of the world on the antimicrobial properties of medicinal plants, and as a result, plants are still recognized as the bedrock for modern medicine to treat infectious diseases [27];

[22]; [7]. *Trigonella foenum-graecum* L., is an annual legume crop mainly grown for use as a spice in many parts of the world [24].*T.foenum-graecum* also is known as one of the oldest medicinal plants recognized in recorded history [1].

Leaves and seeds of *T.foenum-graecum* have been used extensively to prepare extracts and powders for medicinal uses [28]; [4].*T. foenum-graecum* is reported to have anti-diabetic, anti-fertility, anticancer [17], anti-microbial [21], anti-parasitic and hypocholesterolaemic effects [2].The seeds of the *T. foenum-graecum* herb possess toxic oils, volatile oils and alkaloids have been shown to be toxic to bacteria, parasites and fungi [9]. The potential uses of *in vitro* propagated plants as sources for new drugs are still largely unexplored. Based on several investigative studies [25], a compound produced in an *in vivo* plant could be produced at the same or different levels or not produced at all in an *in vitro* grown plant [26].

In vitro propagated callus cultures can become an alternative to plants grown in their environment due to the fact that under controlled condition [29], plant tissue can produce significant amounts of metabolites of interest [5]. The variety of compounds produced *in vivo* and *in vitro* plants can show different bioactivity potential. Hence, in this study the antimicrobial properties of petroleum ether and methanolic extracts of *T. foenum-graecum* seeds were measured and compared with callus tissue extracts of *T.foenum-graecum* [23].

Materials and Methods

Sourc*T.foenum-graecum,* Microorganisms and Reference drugs

The mature seeds of *T. foenum-graecum* were purchased from local market of Khartoum city, Sudan.

The standard microorganisms used in this study were the following: *Bacillus subtilus* (NCTC 8236), *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Aspergillus niger* (ATCC 9763) and Candida albicans (ATCC 7596).

The test organisms were obtained from the Department of Microbiology, Medicinal and Aromatic Plants Research Institute, Khartoum. Sudan.

Reference drugs used in this study were Ampicillin/Sulbactam(20µg/disc)Ciprofloxacin 5 µg/disc and Gentamicin (10 µg/disc) sensitivity discs from Himedia.

Seeds surface sterilization germination

Seeds of *T. foenum-graecum* were surface sterilized in70% ethanol for 30 sec with hand shaking and rinsed three times in sterile distilled water to remove trace of alcohol, then seeds were soaked in 20% Clorox (0.5% free chlorine) with two drops of Tween-20 for 15 minute, and rinsed 3-5 times in sterile distilled water. After surface sterilization, the seeds were directly cultured in the germination basal medium MS [19] at 25±2°C and photoperiod of 16 hrs light and 8 hrs dark for 10 days.

Source of Explants

Cotyledons and hypocotyls were obtained from one week old in vitro micro plants above for callus induction. B5 medium were used for all callus induction experiments and supplemented with 20 g/l sucrose and 7 g/l agar and the pH was adjusted to 5.5±2 with 0.1 N NaOH or 0.1 N HCL before adding agar, the agar was melted by heating and the

medium was dispensed into culture jars, and then autoclaved. All cultures were incubated at 25±2°C under cool white fluorescent lamp at 16h light and 8h dark.

Callus induction

The hypocotyls and cotyledons were used as explants of fenugreek (*T. foenum-graecum*) in this study for callus induction. To induce callus from explants, MS medium was supplemented with 2mg/l of different auxins (2, 4-D or NAA) + 0.5 mg/l Kinetin. Each of the sterilized explants were cut into 2-3 mm pieces using sterile scalpel. Four pieces were inoculated in each vial containing sterile culture MS medium with different combinations of growth regulators. The calli were incubated for 6 weeks in 16 hrs light and 8 hrs dark at 25±2°C, and tissues were subculture at three week intervals.

Preparation of Plant and callus Crude Extract

20 gm. of dry *T. foenum-graecum* seeds were cleaned and ground using a mortar and pestle. The extraction was carried out by soxhlet method. The fine powder was packed tightly in a soxhlet extractor and petroleum ether 200 ml was used as solvent for extraction. The process was carried out for 6 hrs. The fine powder was re-extracted under the same conditions by methanol. The obtained extracts were evaporated by Rot-evaporator under reduced pressure at 60°C to get a dried solid product then stored in dried bottle. Crude extract of callus was prepared by similar to that of plant extraction except the callus was dried at first by freeze drying using Freeze dryer and then powdered and extracted with two different solvents , petroleum ether and methanol in soxhlet apparatus.

Preparation of bacterial suspension

One ml aliquots of 24 hours broth culture of the test organisms were aseptically distributed onto nutrient agar slopes and incubated at 37^0 C for 24 hours. The bacterial growth was harvested and washed off with sterile normal saline, and finally suspended in 100 ml of normal saline to produce a suspension containing about (10^8 - 10^9) colony forming units per ml, the average number of viable organisms per ml of the stock suspension was determined by means of the surface viable counting [18]. The suspension was stored in the refrigerator at 4°C until used.

Preparation of fungal suspension

Fungal cultures were maintained on Sabouraud dextrose agar, incubated at 25^0 C for 4 days. The fungal growth was harvested and washed with sterile normal saline and suspended in 100 ml of sterile normal saline. The suspension was stored in the refrigerator until used.

***In vitro* testing of extracts for antimicrobial activity**

Testing for antibacterial activity

The paper disc diffusion method of [12] was adopted with some minor modifications to assess the antibacterial activity of the prepared extracts. 20ml aliquots of the molten nutrient agar were distributed into sterile Petri-dishes.0.1 ml of the standardized bacterial stock suspension 10^8 –10^9 C.F.U/ ml were streaked on nutrient agar medium plates using sterile cotton swab. Sterilized filter paper discs (6 mm diameter) were soaked in the prepared extracts, then were placed on surface of the test bacteria plates.

The plates were incubated for 24 h and the diameters of the inhibition zones were measured. Reference drugs and %10 dimethyl sulphoxide (DMSO) were used as the positive and negative controls, respectively. After incubation period, the diameters of the resultant growth inhibition zone were measured. Mean and standard error values were tabulated.

Testing for antifungal activity

The same method as for bacteria was adopted. Instead of nutrient agar, Sabouraud dextrose agar was used. The inoculated medium was incubated at 25 °C for two days for the *Candida albicans* and three days for *Aspergillus niger*.

Phytochemical screening

Phytochemical examinations were carried out for all the extracts to evaluate for the presence of different phytochemicals to ascertain the presence of secondary metabolites such as alkaloids, saponins, phenolic compound, tannins, flavonoids, steroids and terpenoid by using different standard methods with some modification. The methods described by [8]; [14]; [13]; [30]; [20]; [11], respectively for the mentioned secondary metabolites.

Statistical analysis

Data were analyzed by SPSS statistical package software [10].The results are presented as mean ± standard error of three replicates, and analyzed with Duncan LSD. The data were considered significant when *P* value was ‹ 0.05.

Result and Discussion

Antimicrobial activity

Antimicrobial activity of *T.foenum-graecum* seeds and callus (hypocotyls& cotyledons) extracts were determined by using paper disc diffusion method. Table (1) &Fig (1) represented the measurement of zone inhibition (mm).The petroleum ether extract of *T.foenum- graecum* seeds showed highest antimicrobial activity compared to other extracts. Petroleum ether extract of seeds showed antifungal activity against *Aspergillus niger* and *Candida albicans* with maximum zone of inhibition (20±0.88 mm) against *Aspergillus niger* by concentration 250 mg/ml (Fig.2), and (17±0.57mm) of inhibition zone against *Candida albicans* by concentration 250 mg/ml (Fig. 3).

Antibacterial activity of petroleum ether extract of *T.foenum- graecum* seeds were recorded (17±0.33mm) and (15±0.57mm) of inhibition zone against *Escherichia coli and Staphyllococcus aureus* respectively by concentration 250 mg/ml (Fig.4&5).

Minimum inhibition zone of antimicrobial activity of petroleum ether extract of seeds was recorded (11±0.60) against *Staphylococcus aureus* by concentration 125mg/ml (Fig. 5). In agree with our results [27] found that *T. foenum- graecum* seeds oil have antibacterial and antifungal activities against *Staphyllococcus aureus* and *Aspergillu sniger*. The petroleum ether extracts for both hypocotyls and cotyledons derived callus were found to be ineffective against all tested microorganisms. Methanolic extracts of hypocotyls and cotyledons derived callus showed antibacterial activity against *Escherichia coli* and *Staphyllococcus aureus* (Table 1&Fig. 7, 8, 10&11) compared to methanolic extract of seeds which was ineffective against all tested microorganisms except weak effect against *Candida albicans*. In agree with our results [15] examined the antibacterial activity of water, chloroformic and methanolic extracts of *T.*

foenum- graecum against five standard bacterial strains, her results showed that methanolic and water extracts of *T. foenum-graecum* seeds were very poor antibacterial agents. Also [31] studied the antibacterial activity of aqueous and some organic compounds extracts of stem, leaves, seeds and roots of *T. foenum- graecum*a gainst *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Klebsiella spp*, all extracts of the plant did not exhibited any inhibitory activity. The petroleum ether extract of *T. foenum-graecum* seeds being more effective than methanol extract. These findings agree with [6], they found that the petroleum ether extract of *T. foenum- graecum* seeds was more effective than methanol extract. Contrary to our results [16] found methanol extracts of *T.foenum- graecum seeds* were effective against *E. coli* and *Staphylococcus aureus.*

The comparison of results given in table (1&2) showed that the petroleum ether extract of *T.foenum- graecum* seeds showed activity higher than Ampicillin/Sulbactam 20 µg /disc and Ciprofloxacin 5 µg /disc against *Staphyllococcus aureus*, also methanolic extracts of hypocotyls and cotyledons derived callus showed activity equal Ampicillin/Sulbactam 20 µg /disc and Ciprofloxacin5µg/disc against *Staphyllococcu saureus.*

Table.1 Screening for Antimicrobial Activity of Fenugreek (*Trigonella foenum-graecum*) Seeds and Callus Extracts against Standard Microorganisms

Sr. No	Part used (extracted)	Extracts		Zone of inhibition(mm)+SE**					
				G^{+Ve}		G^{-Ve}		Fungi	
			Cons.	*B.s*	*S.a*	*E.c*	*Ps.a*	*As.n*	*C.alb*
1-	Seed	Petroleum ether	250mg/ml	–	15±0.57	17±0.33	–	20±0.88	17±0.57
			125mg/ml	–	11±0.60	12±0.00	–	15±0.00	15±0.00
		Methanol	250mg/ml	–	–	–	–	–	8±0.00
			125mg/ml	–	–	–	–	–	7±0.00
2-	Hypocotyl callus	Petroleum ether	250mg/ml	–	–	–	–	–	–
			125mg/ml	–	–	–	–	–	–
		Methanol	250mg/ml	–	11±0.00	10±0.00	–	–	–
			125mg/ml	–	10±0.00	8±0.00	–	–	–
3-	Cotyledon callus	Petroleum ether	250mg/ml	–	–	–	–	–	–
			125mg/ml	–	–	–	–	–	–
		Methanol	250mg/ml	–	12±0.57	10±0.00	–	–	–
			125mg/ml	–	10±0.00	–	–	–	–

**SE= standard error of mean.
B.s= Bacillus subtillus, S. a= Staphyllococcus aureus, E. c= Escherichia coli,
Ps. a =Pseudomonas aeruginosa. As.n = Aspergillus niger, C. alb = Candida albicans

Table.2 Antibacterial Activity of Reference Drug against Standard Microorganisms

Drug	M. D. I. Z .(mm)**			
	B.s	S. a	E. c	Ps. a
Ampicillin/Sulbactam 20 mcg/disc	20	11	20	–
Ciprofloxacin 5 mcg/disc	20	10	33	35
Gintamicin 10 mcg/disc	25	17	17	8
*Control	–	–	–	–

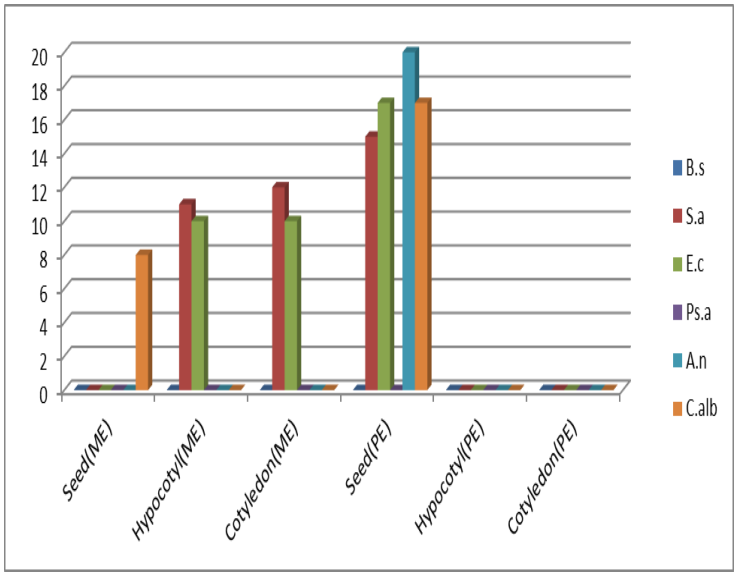
**M. D. I .Z. (mm) = Mean diameter of growth inhibition zone (mm).
*Control was %10 Dimethyl sulphoxide in D.W.
niger, C. alb = Candida albicans.

Table.3 Phytochemical Screening: for Secondary Metabolites of Fenugreek (*Trigonella foenum-graecum)* Callus and Seed Extracts

Test	Reagent	M1	M2	M3	P1	P2	P3	Observation
Alkaloids	Mayer,s	+++	+++	+++	–	–	–	White creamy precipitate
	Wagner's	–	–	–	–	–	–	Reddish-brown precipitate
	Dragendroff's	–	–	–	–	–	–	yellow precipitate
Saponins	H2o	+++	+++	+++	–	–	–	Persistent foam
Tannins	Gelatin	++	–	–	–	–	–	White precipitate
	Fecl3	+++	–	–	–	–	–	bluish black colour
Flavnoids	NaOH	+++	+	++	–	–	–	yellow color
	Lead acetate	+++	+	+	–	–	–	yellow precipitate
Phenol compound	Fecl3	++	–	–	–	–	–	bluish black colour
	Folin-Ciocalteu	++	+++	+++	–	–	–	Blue color
Steroids and Terpenoid	Salkowski's test	+++	+++	+++	++	+	+	reddish brown colourring

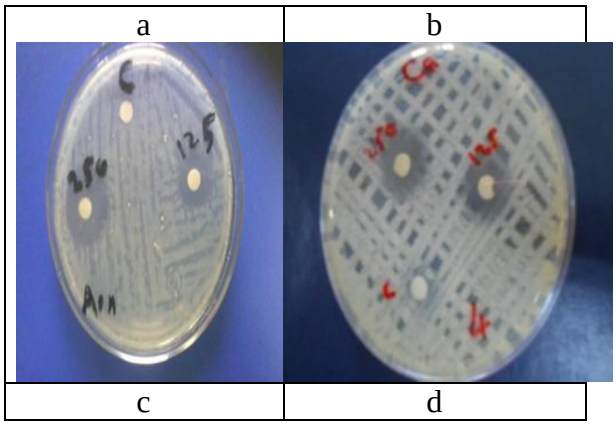
Key: (+) Positive Test, (-) Negative test '+' low; '++' moderate; '+++' high;
M1= Seed methanol extract, M2= Hypocotyl methanol extract, M3= Cotyledon methanol extract, P1= seed Petroleum ether extract,
P2= Hypocotyl Petroleum ether extract andP3= Cotyledon Petroleum ether extract

Fig.1 Average of inhibition zone (mm) of Fenugreek (*Trigonella foenum-graecum*) seeds and callus derived from hypocotyls and cotyledons explants



ME= Methanolic extract PE= petroleum ether extract
B.s= *Bacillus subtillus, S. a= Staphyllococcus aureus, E. c= Escherichia coli,*
Ps. a =Pseudomonas aeruginosa. As.n = A

Figure.2 I.Z. by petroleum ether extracts of fenugreek seeds against (a) *Aspergillus niger*, (b) *Candida albicans*, (c) *Escherichia coli* and (d) *Staphyllococcus aureus*.



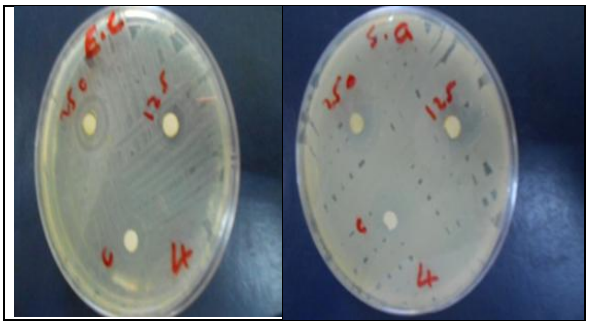


Fig.3 * I.Z. by methanolic extracts of seeds against *Staphyllococcus aureus*

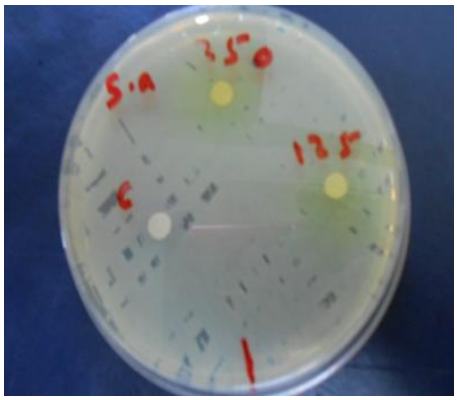


Fig.4 * I.Z. by methanolic extracts of callus derived of Hypocotyls against *Staphyllococcus aureus*.

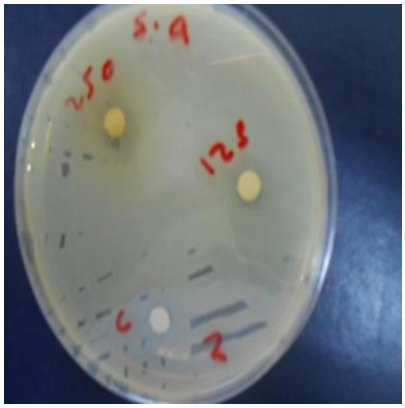


Fig.5 * I.Z. by methanolic extracts of callus derived of Cotyledons against *Staphyllococcus aureus.*

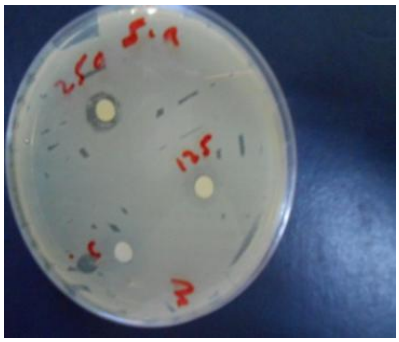


Fig.6 * I.Z. by methanolic extracts of seeds against *Escherichia coli*

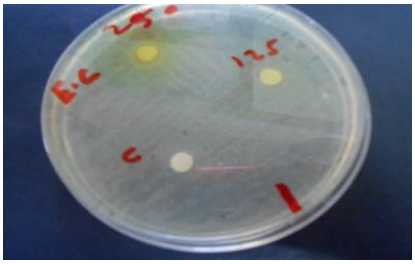


Fig.7* I.Z. by methanolic extracts of callus derived of hypocotyls against *Escherichi*a *coli.*

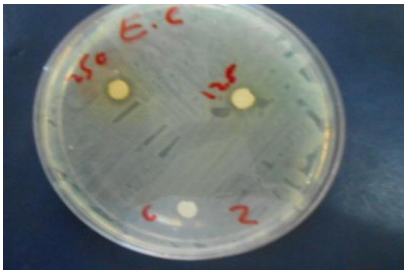
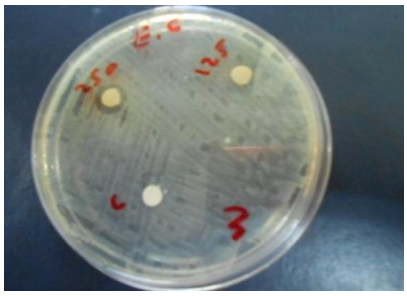


Fig.8 * I.Z. by methanolic extracts of callus derived of cotyledons against *Escherichia coli*



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