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Antigiardial activity and cytotoxicity of ethanolic seed extract of *Trigonella Foenum Graecum* L.

Ahmed S Kabbashi, Arwa M Hassan, Ahmed O Sharif, Aisha B Ali, Mohammed A Daf alla, Reem H Amoon, Amna H Abdallah

Medicinal and Aromatic Plants and Traditional Medicine Research Institute (MAPTMRI), P.O. Box 2404, National Center for Research, Khartoum, Sudan.

Abstract
Trigonella foenum-graecum (Family: Fabaceae) a well-known medicinal plant grows in nature and is cultivated in Sudan. It is having properties of lowering blood sugar level, anthelmentic, antibacterial, anti-inflammatory, antipyretic, and antimicrobial. This study was carried out to evaluate antigiardial activity (*Giardia lamblia*) and cytotoxicity (MTT assay) of ethanol extract of *T. foenum-graecum* (seeds). The ethanol extracts of *T. foenum-graecum* (seeds) was screened for its antigiardial activities (*Giardia lamblia*) and screened for their cytotoxicity using 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), was obtained from *T. foenum-graecum* (seeds) ethanol extract which exhibited 100% mortality within 96 h, at a concentration 500 µg/ml; this was compared with Metronidazole which gave 96% inhibition at concentration 312.5 µg/ml at the same time. In addition cytotoxicity (MTT assay) with different concentration (500, 250 and 125 ppm) in comparison to triton-x100 (the reference control) which verified the safety of the examined extract with an IC50 less 100 µg/ml. In conclusion, these studies prove the potent activity of *T. foenum-graecum* (seeds) against *G. lamblia* trophozoites *in vitro* with verified safety evidence for use.

Keywords: *In vitro*, Antigiardial activity (*Giardia lamblia*), Metronidazole, Cytotoxicity (MTT-assay), *Trigonella foenum-graecum* (seeds).

1. Introduction
Medicinal plants are still invaluable source of safe, less toxic, lower price, available and reliable natural resources of drugs all over the world. People in Sudan and in other developing countries have relied on traditional herbal preparations to treat themselves. Therefore, it is useful to investigate the potential of local plants against these disabling diseases (Amaral *et al.*, 2006; Koko *et al.*, 2008) [4, 14].
Trigonella is a member of (family: Fabaceae) is an ancient plant indigenous to Sudan. Recently the crop has attracted much interest as a cheap source of good protein for protein supplement. In Sudan *T. foenum-grecum* is grown in the northern region and known locally as "hilba", and traditionally used by lactating women as porridge (with sorghum as millet flour). It's also boiled with water and taken hot or cold drink to sooth stomach ailment (Abdel Kareem *et al.*, 2013) [1]. The seeds of fenugreek were used in Ayurveda to treat fever, dysentery and heart diseases, while in Unani system, this plant is used as a resolvent, aphrodisiac, diuretic, emmenagogue and tonic (Nadkarn, 1982) [17]. Action as Anabolic agent, antibiotic, antidiabetic, antifatigue, antihyperglycaemic, antihypertensive, antiinflammatory, antimicrobial, antioxidant, antipyretic, antiseptic, antispasmodic, aphrodisiac, astringent, carminative, cancerolytic, cardiotonic, demulcent, diaphoretic, digestive, disinfectant, emmenagogue, emollient , expectorant , galactagogue , haemopoietic , hypolipidemic , lactogenic, laxative, neuromuscular stimulant, nutritive, stomachic, tonic, uterotonic, vulnerary, viricidal, stimulatory effect on immune function, antioxidant properties, protection against toxicity and for avarity kidney disorder (Tahiliani and Kar 2003, Bin-Hafeez *et al.*, 2003, Madar and Stark 2002, Rababah, 2004, Ageel *et al.*, 1987) [23, 8, 16, 21, 3].
Giardiasis is the most common cause of parasitic gastro-intestinal disease and it is estimated that up to two hundred million people are chronically infected with *Giardia lamblia* globally, and 500,000 new cases reported annually (World Health Organization, 1998) [27]. *G. lamblia* is a major cause of diarrhoea in humans (Lauwaet *et al.*, 2010) [15]. *Giardia* is a flagellate protozoan with worldwide distribution that causes significant gastrointestinal diseases in a wide variety of vertebrates including cats and human. *G. lamblia* is one of the intestinal protozoa that cause public health problems in most developing countries as well as some developed countries. *G. lamblia* is considered to be one of the leading causative agents of diarrhoea in both children (Noor Azian *et al.*, 2007; Dib *et al.*, 2008; Addy *et al.*, 2004) [18, 11, 2] and adults (Ayeh-Kumi *et al.*, 2009; Nyarango *et al.*, 2008) [6, 19].
Metronidazole is the drug now widely used and recommended in the treatment of amoebiasis (Townson *et al.*, 1994) [25]. But it is less effective in the tissue than in the gut lumen (Bhopale *et al.*, 1995) [7]. In addition, it can eradicate only up to 50% of laminae infections (Tierney *et al.*, 1998) [24]. Metronidazole sometimes causes adverse effects, example, myoplasia, neuralgia, and allergic dermatitis (Upcrof *et al.*, 2006) [26]. Hence, the present study was conducted to investigate the antigiardial activity and cytotoxicity of *T. foenum-graecum* (seeds) in Sudan.

2. Materials and methods
Plant materials
T. foenum-grecum (seeds) was collected from Central Sudan during the period of January to February 2014. The plant was identified and authenticated by the taxonomists of Medicinal

and Aromatic Plants and Traditional Medicine Research Institute (MAPTMRI), Khartoum, Sudan.

Preparation of crude extracts

Extraction was carried out for the whole plant of *T. foenum-graecum* by using overnight maceration techniques according to the method described by Harbone (1984) [12]. About 50 g of powdered material was macerated in 250 ml of ethanol for 3 h at room temperature. Occasional shaking for 24 h at room temperature was performed and, the supernatant was decanted. Thereafter, the supernatant was filtered under reduced pressure by rotary evaporator at 55 °C. Each residue was weighed and the yield percentage was calculated and then stored at 4 °C in tightly sealed glass vial ready for use. The remaining extracts which were not soluble were successively extracted using ethanol with the described technique. The extracts were kept in freeze dryer for 48 h, (Virtis, USA) until they were completely dried. The residue was weighed and the yield percentage was calculated. The extracts were kept and stored at 4 °C until required.

Parasite isolate

G. lamblia used in all experiments were taken from patients from Ibrahim Malik Hospital (Khartoum). All taken samples were examined by wet amount preparation; the positive samples were transported to the laboratory in nutrient broth medium. Trophozoites of *G. lamblia* were maintained in RPMI 1640 medium containing 5% bovine serum at 37±1 °C. The trophozoites were maintained for the assays and were employed in the log phase of growth.

Inoculums

G. lamblia was inoculated in the RPMI 1640 medium and incubated at 37±1°C for 48 h. parasites were counted under the microscope by haemocytometer chamber.

***In vitro* susceptibility assays**

In vitro susceptibility assays used the sub-culture method by Cedillo-Rivera *et al* (2002) [10], which is being described as a highly stringent and sensitive method for assessing the anti-protozoal effects (gold standard) particularly in *E. histolytica*, *Gairdia intestinalis* and *Trichomonas vaginalis* Arguello-Garcia *et al* (2004) [5]. 5 mg from each extract and compound was dissolved in 50 µl of dimethyl sulfoxzide (DMSO) at Eppendorf tube containing 950 µl D.W in order to reach concentration of 5 mg/ml (5000 ppm). The concentrates were stored at -20 °C for further analysis. Sterile 96-well microtitre plate was used for different plant extracts, positive control and negative control. Three columns of a microtitre plate wells [8 columns (C) × 12 rows (R)] were chosen for each extract, 40 µl) of an extract solution (5 mg/ml) were added to the first column wells C-1: On the other hand, 20 µl of complete RPMI medium were added to the other wells of the second column and third column (C-2 and C-3). Serial dilutions of the extract were obtained by taking 20 µl of extract to the second column wells and taking 20 µl out of the complete solution in C-2 wells to C-3 wells and discarding 20 µl from the total solution of C-3 to the remaining 20 µl serial solutions in the successive columns. 80 µl of culture medium was complemented with parasite and added to all wells. The final volume in the wells was 100 µl.

In each test, Metronidazole (a trichomonocide) pure compound [(1-(2-hydroxyethl)-2-methyl-5 Nitroimidazole], a was used as positive control in concentration 312.5 µg/ml, whereas untreated cells were used as a negative controls (culture medium plus trophozoites). For counting, the samples were mixed with Trypan blue in equal volume. The final number of parasites was determined with haemocytometer four times for counting after 24, 48, 72 and 96 h. The mortality % of parasite for each extracts activity was carried out according to the following formula:

Mortality of parasites (%) =

$$\frac{\text{(Control negative – tested sample with extract)}}{\text{Control negative}} \times 100\%$$

Only 100% inhibition of the parasite was considered, when there was no motile parasite observed.

Cytotoxicity Screening

Microculture tetrazolium MTT assay was utilized to evaluate the cytotoxicity of *T. foenum-grecum* (seeds).

Microculture Tetrazolium (MTT) Assay

Principle

This Colorimetric assay is based on the capacity of Mitochondria succinate dehydrogenase enzymes in living cells to reduce the yellow water soluble substrate 3- (4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) into an insoluble, blue colored formazan product which is measured spectrophotometrically. Since reduction of MTT can only occur in metabolically active cells, the level of activity is a measure of the viability of the cells (Patel *et al*; 2009) [20].

Preparation of *T. foenum-grecum* (seeds) Extracts, Solutions

Using a sensitive balance 5 mg of each extracts were weighed and put in eppendorf tubes. 50 µl of DMSO were added to the extract and the volume was completed to 1 ml with distilled water obtaining a concentration of 5 mg/ml. The mixture was vortexed and stirred by magnetic stirrer to obtain a homogenous solution.

Cell Line and Culturing Medium

Vero (Normal, African green monkey kidney) cells were cultured in a culturing flask containing a complete medium consisting of 10% fetal bovine serum and 90% minimal essential medium (MEM) and then incubated at 37 °C. The cells were subcultured twice a week.

Cell line used

Vero cells (Normal, African green monkey kidney).

Cell counting

Cell counts were done using the improved Neubauer chamber. The cover slip and chamber were cleaned with detergent, rinsed thoroughly with distilled water and swapped with 70% ethanol, then dried. An aliquot of cell suspension was mixed with equal volume of 0.4% trypan blue in a small tube. The chamber was charged with cell suspension. After cells had settled, the chamber was placed under light microscope. Using 10 X objective, cells in the 4 large corner squares (each containing 16 small squares) were counted. The following formula was used for calculating cells:

$$(\text{Cells/ml})\ N = \frac{\text{Number of cells counted X Dilution factor X } 10^4}{4}$$

Procedure

The monolayer cell culture formed in the culturing flasks was trypsinized and the cells were put in centrifuging tube and centrifuged for 5 minutes separating the cells from the supernatant that flicked out. 1 ml complete medium was added to the cells and all the cell suspension was contained in a basin. In a 96- well microtitre plate, serial dilutions of each extracts were prepared. 3 duplicated concentrations for each extracts i.e. 6 wells for each of 8 extracts. All wells in rows A, B and C were used in addition to first 4 wells from each rows D, E and F. The first 2 wells of row G were used for the negative control and the first 2 wells of row H were used for the positive control Triton X. 20 µl complete medium pipetted in all wells in rows B, C and mentioned wells of rows E and F. Then 20 µl from each extracts were pipetted in rows A and B and first 4 wells of rows E and F. 20 µl taken from row B were pipetted and mixed well in row C from which 20 µl were taken and flicked out. The same was done from E to F. After that 80 µl complete medium were added to all used wells. Then adjusting the cell account to 3000 cell/well, 100 µl of cell suspension were added completing all wells to the volume 200 µl. Now, we have duplicated three concentrations 500, 250, 125 µg/ml for each extract. Then the plate was covered and incubated at 37 °C for 96 hours.

On the fourth day, the supernatant was removed from each well without detaching the cells. MTT suspension stock (5 mg/ml) prepared earlier in 100 ml phosphate buffer solution (PBS) was diluted (1:3.5) in a culture medium. To each well of the 96-well plate, 50 µl of diluted MTT were added. The plate was incubated for further 4 hours at 37 °C. MTT was removed carefully without detaching cells, and 100 µl of DMSO were added to each well. The plate was agitated at room temperature for 10 minutes then read at 540 nm using microplate reader. The percentage growth inhibition was calculated using the formula below:

% cell inhibition = 100-{(Ac-At)/Ac} × 100

Where, **At** = Absorbance value of test compound; **Ac** =Absorbance value of control.

Statistical analysis

All data were presented as means ± S.D. Statistical analysis for all the assays results were done using Microsoft Excel program (2007).

3. Results and discussion

The seeds of *T. foenum-grecum* (Family: Fabaceae) were screened for antigiardial activity against (*giardia lamblia*) trophozoites *in vitro,* in comparison to the Mertronidazole (the reference control), and tested for cytotoxicity using 3- (4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) Vero cell line, while triton-x was used as standard (control), after the evaluation of the yield percentage of the plant which was found to be 9.6% (Table 1).

Table 1. Preliminary quantitative data on yield percentage (%) of *T. foenum-grecum* (seeds) used for the antigiardial activity and cytotoxicity study:

Scientific name of plant	Family name	Part used	Yield (%)
T. foenum-grecum	Fabaceae	seeds	9.6

Antigiardial activity of *T. foenum-grecum* (seeds) extract:

Giardia lamblia is an important cause of acute and chronic gastrointestinal disease throughout the world and has been identified as the etiologic agent in numerous waterborne outbreaks of diarrheal disease. Although *G. lamblia* is among the most prevalent enteric protozoal infections in humans, it is relatively recently that improvements in the *in vitro* cultivation of this organism have allowed reliable, reproducible tests to assess the *in vitro* activity of therapeutic agents against *G. lamblia* (Boreham *et al*; 1984) [9]. Despite the previous comprehensive screening of Sudanese medicinal plants for their antiprotozoal activity (Samia *et al*; 2004; Hiba *et al*; 2002; El Tahir *et al*; 1999) [22, 13] this is the first time for *in vitro* antiprotozoal activity in the Sudan.

The antigiardial potential of the Ethanolic extract of the medicinal plant of *T. foenum-grecum* (seeds) were extracted by ethanol, with different concentrations (500, 250 and 125 ppm) and Mertronidazole (the reference control) with concentration (312.5 µg/ml) to be investigated against *giardia lamblia* trophozoites *in vitro*. Ethanol extracts of *T. foenum-grecum* (seeds). showed 100% inhibition at a concentration 500 µg/ml after 96 h; this was compared with Metronidazole which gave 96% inhibition at concentration 312.5 µg/ml at the same time against *Giardia lamblia*. Figure (1).

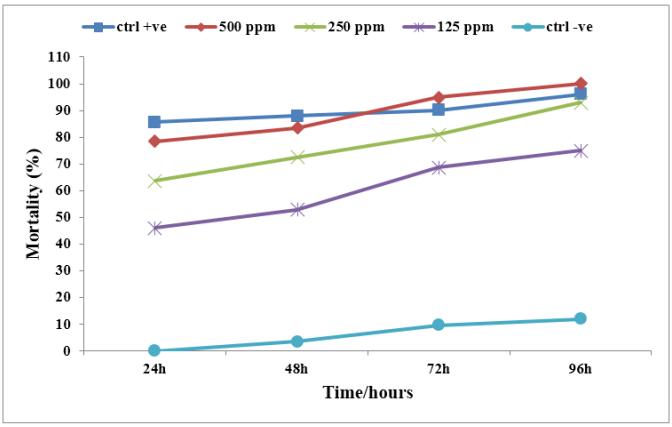

Fig 1: *In vitro* activity of *T. foenum-grecum* (seeds) ethanolic extract against *Giardia lamblia.*

Cytotoxicity assay of *T. foenum-grecum* (seeds) extract

Table 2: Cytotoxicity of *T. foenum-grecum* extracts on normal cell lines (Vero cell line) as measured by the MTT assay:

No.	Name of plant (part)	Concentration (µg/ml)	Absorbance	Inhibition (%) ± SD	IC_{50} (µg/ml)
1	*T. foenum-grecum* (seeds)	500	2.42	17.9 ± 0.05	>100
		250	2.87	1.8 ± 0.03	
		125	3.42	-17.5 ± 0.02	
2	*Control		0.14	95.3 ± 0.01	

Key: *Control = Triton-x100 was used as the control positive at 0.2 µg/mL.

Interestingly, the cytotoxicity assays were conducted in this study to evaluate the cytotoxicity effects of ethanolic extract of *C. rotundus* (whole plant) by using MTT-assay including (Vero cell line). Table 3 indicated the inhibition percentage (%) of Vero cell line growth *in vitro* by ethanolic extract of *C. rotundus* (whole plant) for different concentrations 125 to 500 µg/ml and showed an IC_{50} > 100 (µg/ml) which is verifying the plant safety.

4. Conclusion

This result enhances the ethno botanical uses of *T. foenum-grecum* (seeds) as antidiarrheal in cases associated with Giardiasis in Sudan. Further investigations regarding the mode of action and other related pharmacological studies such as *in vivo* investigation, drug formulation and clinical trials are highly recommended.

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