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In vitro and *in vivo* evaluation of the antiangiogenic activities of *Trigonella foenum-graecum* extracts

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ABSTRACT

Objective: To assess the antiangiogenic activity of fenugreek.
Methods: Different fractions of fenugreek crude extracts were prepared and their anti-angiogenic properties were assessed using the *ex vivo* rat aortic ring assay and *in vivo* chicken embryo chorioallantoic membrane (CAM) assay. They were investigated for their direct cytotoxic activity in the MCF7 cells using the MTT assay.
Results: The ethanol extract showed 100% inhibition of blood vessel outgrowth from primary tissue explants in the rat aortic ring assay at a concentration of 100 μg/mL while the other extracts did not show significant antiangiogenic activity. The ethanol extract was therefore investigated at varying concentrations and exhibited a significant dose dependent effect. The CAM assay coincided with the results of the aortic ring assay as ethanol extract showed a significant inhibition of formation of new blood vessels. The extracts only showed anti-proliferative activity at the highest concentration of 400 μg/mL towards MCF7 breast cancer cell lines in the MTT assay.
Conclusions: Findings of the both assays confirmed that the ethanol extract inhibited vascularization significantly. Further studies on the ethanol extract would be beneficial in isolating the active ingredient responsible for the inhibition.

1. Introduction

Angiogenesis is the physiological process of new blood vessel development from pre-existing ones. This process is driven by various angiogenic factors and inhibitors [1]. Angiogenesis is a normal regulated process in wound healing and growth; however, once out of control, diseases such as cancer can be formed [2–4].

Tumor vasculature plays a significant role in the tumor growth; therefore, without angiogenesis, a tumor mass would be unable to expand beyond 1–2 mm^3 [5]. Cancer cells within a tumor are able to use newly formed blood vessels to metastasize to other areas [6]. The role of angiogenesis for sustained tumor growth and metastasis formation has been recognized, therefore, the inhibition of angiogenesis has become a vital therapeutic target for the treatment of cancer patients [7].

The mechanism of angiogenesis in the pathological and physiological state has been of extreme importance in the past three decades. The favored method for arresting tumor angiogenesis has been the blockade of vascular endothelial growth factor (VEGF) pathway [8]. VEGF has a significant impact on the proliferation, survival, migration and permeability of tumor cells. Various types of antiangiogenic agents that inhibit

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VEGF and vascular endothelial growth factor receptor (VEGFR) have been developed, which include ribozymes, antibodies and small molecule inhibitors. The investigation into antiangiogenic agents that block certain proteins of VEGF downstream signal pathways attracts the attention of medicinal chemists and enhances the application of antiangiogenic agents for this purpose [9].

Medicinal plants are a source for novel drug models in the development of new therapeutic cancer agents. Up to date, several molecules originating from the plant kingdom have been developed into commercially available anticancer drugs [10,11]. The antiangiogenic potential of plant extracts is still an open area of study with much yet to be discovered.

Trigonella foenum-graecum, more commonly known as fenugreek and as 'Hilba' in Arabic, is an annual leguminous herbaceous plant belonging to the family Fabaceae [12]. Fenugreek has been used for centuries for culinary purposes but also has numerous applications in traditional medicine [12,13]. It is said to be native to the Indian subcontinent and the Eastern Mediterranean region and its many applications have been documented and dated back to ancient times [14,15]. Fenugreek is extensively cultivated in most regions of the world and is perhaps best known for its unique and pungent aromatic compounds in its seeds, which reveal color, aroma and flavor to many foods, therefore making it desirable as a supplement in culinary applications. In places such as Egypt, it is used as a supplement in maize and wheat flour for the production of bread while in India it is usually consumed as a condiment [14]. In Iran, fenugreek is considered a key ingredient in most meals prepared [16].

While it has many culinary applications, fenugreek is also known to have numerous pharmacological effects [17–19]. It has been used for over two thousand years as a medicinal plant in many areas of the world and it has sometimes been regarded as the oldest medicinal plant in human history [15]. In traditional medicine, leaves and seeds of *fenugreek* have been used to treat disorders such as diabetes, inflammation, wounds and high cholesterol [13]. Ahmadiani *et al.* and Vyas *et al.* reported that the anti-inflammatory, anti-pyretic and analgesic effects of fenugreek were similar to those of non-steroidal anti-inflammatory drugs (NSAIDs) [17,20].

This study is designed to assess the antiangiogenic activity of fenugreek. Different fractions of fenugreek crude extracts were prepared and their direct antiangiogenic properties were assessed using the rat aortic ring assay and the chicken embryo chorio-allantoic membrane (CAM) assay. They were also investigated for their direct cytotoxic activity in the Michigan Cancer Foundation-7 (MCF7) breast cancer cells using the methyl-thiazol tetrazolium assay (MTT assay).

2. Materials and methods

2.1. Crude extract preparation

Fenugreek seeds were bought from a local herbalist and ground up. Five different solvents were used: chloroform, 70% ethanol, ethyl acetate, hexane and water. By refluxing with 25 mL of each solvent above for 30 min, 2.5 g of coarsely powdered seed material was extracted with each solvent. They were then kept overnight at room temperature. The following day, after filtration, a rotary evaporator was used and the solvents were evaporated until dry. The weight of the crude extracts was then measured.

Two hundred milligrams of the crude extract were weighed and 5 mL of dimethyl sulfoxide (DMSO) was added to make a stock solution that could then be used for the subsequent experiments. Throughout the experiment, the DMSO percentage was kept constant (1%).

2.2. Cell culture

MCF7 breast cancer cells were acquired from the American Type Culture Collection (ATCC® HTB-22™). The media was supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) (Gibco Invitrogen), antibiotics 100 U/mL penicillin (Lonza) and 100 μg/mL streptomycin (Lonza), 2 mM L-glutamine (Lonza) in addition to 25 μM 4-(2-hydroxyethyl)-1 piperazineethanesulfonic acid (HEPES) (Biowest, France). Cells were maintained in a 5% CO_2 humidified incubator at 37 °C.

2.3. MTT assay

The cell viability assay was performed using the 3-(4,5-dim ethyl-2-thiazoyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) as described previously [21], with minor modification [22–25]. In summary, 7000 cells were seeded into each well of a coated 96-well plate and incubated for a period of 24 h at 37 °C. The cells were then treated in a triplicate fashion. A serial dilution was carried out for each extract; therefore, each extract was tested at ten different concentrations (0.8 μg/mL, 1.5 μg/mL, 3.125 μg/mL, 6.25 μg/mL 12.5 μg/mL, 25 μg/mL, 50 μg/mL, 100 μg/mL, 200 μg/mL and 400 μg/mL) at a volume of 100 μL per well. After 72 h of incubation, 15 μL of MTT reagent (5 mg/mL) was added to a fresh 100 μL of RPMI1640 media after aspirating the old one. After 4 h of incubation, the medium was aspirated. Then, 100 μL of DMSO solution was added to every well and the plate was read an hour later using a 96-well plate reader which measured the absorbance of the solution in each well at a 570 nm wavelength.

2.4. Anti-angiogenic assay/ex vivo aortic ring assay

The procedure was approved by the Animal Ethics Committee of the University of Jordan. The rats were anesthetized and the thoracic aortae were excised from (200–250) g male Sprague Dawley rats. The aortae were transferred into a Petri dish with cold sterile PBS solution. The surrounding connective tissue was removed under a dissecting microscope. After proper cleaning, the aortae were sliced into approximately 1 mm-thick rings using a surgical blade, transferred to fresh cold PBS and kept on ice. Each ring was then placed in a well in a 48-well plate; using pre-cooled pipette tips, the rings were embedded in the center of 25 μL of low growth factors (LGF) Matrigel™ (Corning, USA) that was kept on ice during the entire course. The rings were seeded in triplicates. After 30 min incubation at 37 °C to ensure proper solidification, a

volume of 250 µL (in varying concentrations of 3.125 µg/mL, 6.25, 12.5, 25, 50 and 100 µg/mL) of each of the five extracts, which were diluted in RPMI1640 media, was added. For the control, DMSO was used at a concentration of 1% v/v. The plate was then incubated at 37 °C. On the fourth day, the media was replaced with a fresh one containing the extracts.

On day five, aortic rings were photographed under an inverted light microscope (4× magnification), and the angiogenic response was determined by measuring the length of blood vessels outgrowth from the primary ring explants using ImageJ software (National Institute of Health, Bethesda, MD). The growth distance of at least thirty five-like structures per ring selected at regular intervals around the rings was measured [26–30].

The following formula was used to calculate the inhibition of blood vessel formation:

Blood vessels inhibition = $[1 - (A_0/A)] \times 100$, where:

A_0 = distance of blood vessels growth in treated rings in arbitrary units.

A = distance of blood vessels growth in the control in arbitrary units.

2.5. Chick chorioallantoic membrane (CAM) assay

The antiangiogenic effect of fenugreek extract was investigated *in vivo* using a modified CAM assay technique [28]. In brief, 5 day-old fertilized eggs were obtained from a local hatchery. Dirt was carefully removed from the eggshells by cleaning the surface with 70% ethanol. Eggs were then incubated at 37 °C and humidity was kept at 60%–62%. Using a sterilized 5 G-needle syringe, 5 mL of albumin was aspirated and the eggs were incubated horizontally to allow the CAM to detach from the shell. The different fenugreek extracts were dissolved in 1.2% agarose to form discs containing 100 µg of extracts per disc. Discs containing 1% DMSO were used as controls. A small window opening was made on the surface of the shell, and the agarose discs were applied onto the CAM. The window opening was covered with adhesive tape which was carefully cut into small pieces that fit around the opening. The embryos were incubated for 24 h then CAMs were photographed with a digital camera.

2.6. Statistical analysis

Statistical analysis was carried out using GraphPad Prism software (v. 7.00) with results presented as the means ± SEM. Differences between groups were compared by the one way analysis of variance (ANOVA) and considered significant at P values < 0.05.

3. Results

3.1. MTT assay

The MTT assay was used to measure the cell proliferation rate in MCF7 cells. The antiproliferative activity of different concentrations of fenugreek extracts were quantified by spectrophotometry and absorbance of the solutions was measured at 570 nm wavelength.

For each of the five extracts tested, reduction in the rate of cell proliferation was detected only at the 400 µg/mL concentration (Figure 1). None of the other concentrations showed any significant effect.

3.2. Rat aortic ring assay

The angiogenic response of fenugreek was determined by measuring the length of blood vessel outgrowth from the

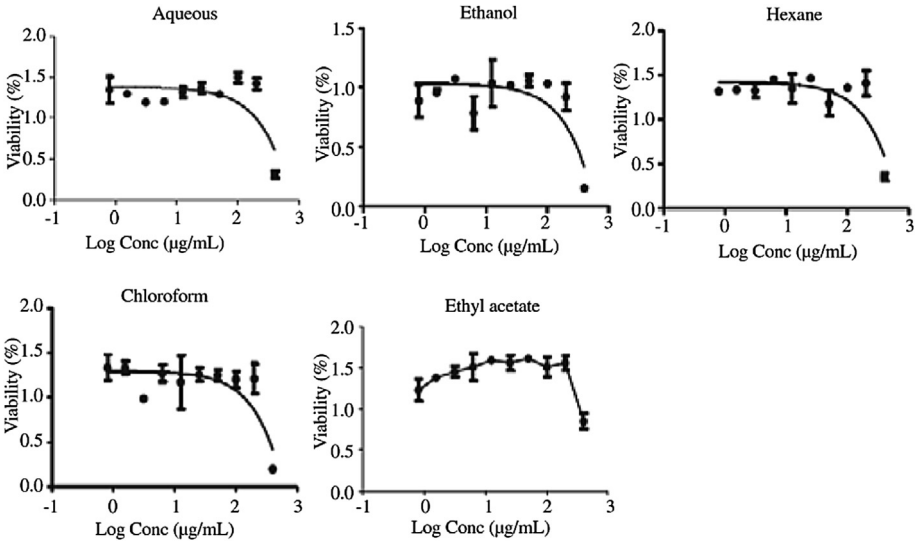

Figure 1. Effect of fenugreek extracts on MCF7 cell viability.
The cell viability was determined by MTT assay. MCF7 cells were exposed for 72 h to various fenugreek extracts at concentrations of (0.8, 1.5, 3.125, 6.25, 12.5, 25, 50, 100, 200, and 400 µg/mL). Results were presented as mean percentage of cells to the control (1% DMSO).

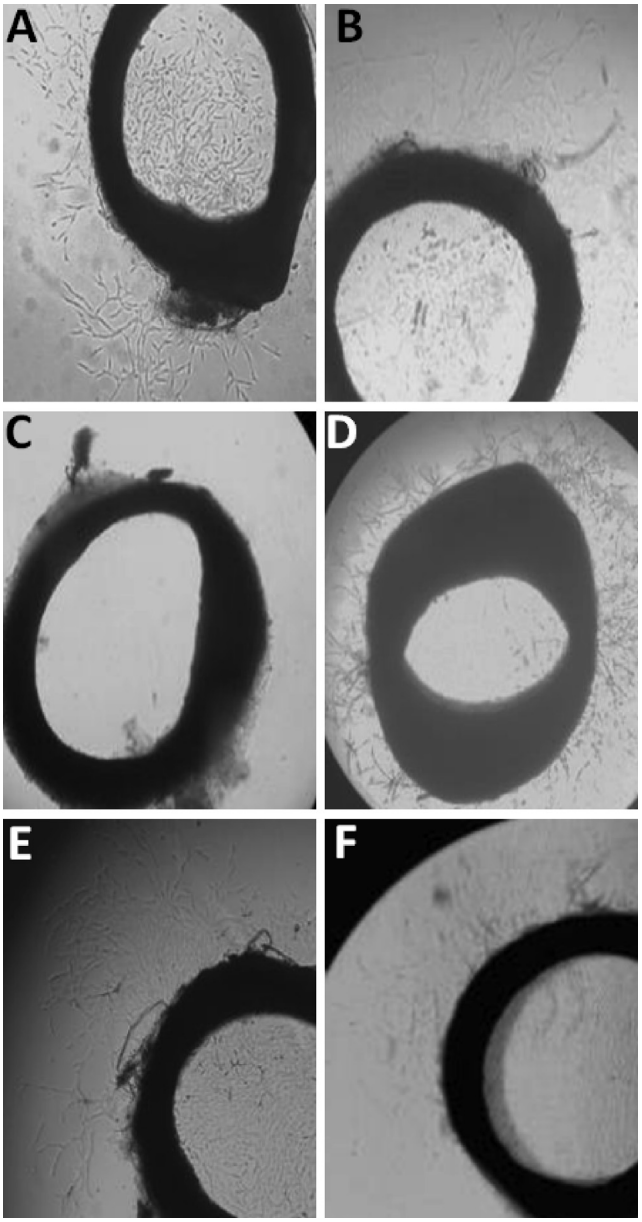

Figure 2. Effect of fenugreek extracts on *ex vivo* angiogenesis. Representative micrographs of aortic ring explants exposed to fenugreek extracts at a concentration of 100 µg/mL on day 5. (A) control (1% DMSO); (B) the aqueous extract; (C) the ethanol extract; (D) the ethyl acetate extract; (E) the chloroform extract and (F) the *n*-hexane extract.

primary tissue explants. The control group of DMSO, the aqueous, ethanol, ethyl acetate, chloroform and n-hexane extracts were all screened at a concentration of 100 µg/mL, a non-cytotoxic dose, for their antiangiogenic potential. The percent inhibition of angiogenesis was measured in three rings of each extract. At the tested concentration, only the ethanol extract showed significant antiangiogenic activity. The ethanol extract at 100 µg/mL inhibited the growth of blood vessels completely by 100% ± 0% (Table 1) (Figure 2C). The effect of

Table 1
Effect of various fenugreek extracts on *ex vivo* and *in vivo* angiogenesis.

Extract	Rat aortic ring assay		CAM assay	
	Inhibition ± SEM (%)	*P*-value	Inhibition ± SEM (%)	*P*-value
Aqueous	7.1 ± 8.3	ns	31.4 ± 7.9	ns
Ethanol	100.0 ± 0.0	$P < 0.001$	59.5 ± 6.0	$P < 0.001$
Ethyl acetate	18.5 ± 3.7	ns	44.5 ± 4.5	$P < 0.05$
Chloroform	−33.0 ± 22.9	ns	1.7 ± 10.8	ns
Hexane	26.0 ± 46.5	ns	−1.7 ± 4.6	ns

Quantification of the new blood vessel growth from mouse aortic ring explants exposed for 5 d to various fenugreek extract at concentration of 100 µg/mL. The results were represented as mean ± SEM (%), $P < 0.001$ ($n = 3$). As well as the quantification of the new blood vessel growth in CAM where results were reported as mean ± SEM. $P < 0.05$ and $P < 0.001$ ($n = 3$).

Table 2
Effect of fenugreek ethanol extract on *in vivo* angiogenesis.

Ethanol (µg/mL)	Rat aortic ring assay	
	Inhibition ± SEM (%)	*P*-value
3.125	14.7 ± 15.1	ns
6.25	35.8 ± 8.8	ns
12.5	53.1 ± 16.9	$P < 0.001$
25	99.0 ± 0.7	$P < 0.001$
50	98.9 ± 0.8	$P < 0.001$
100	100.0 ± 0.0	$P < 0.001$

Dose response of the ethanol extract in the rat aortic ring assay. The data was represented as mean ± SEM, $P < 0.001$ ($n = 3$).

the extracts can be compared to the control group and the outgrowth and inhibition can be seen in Table 1 and in Figure 2.

Aortic rings treated with the different concentrations of the ethanol extract exhibited a significant dose dependent result that can be seen in Table 2. The half maximal inhibitory concentration (IC_{50}) was determined to be 9.41 µg/mL. The ethanol extract inhibited growth of new blood vessels as can be seen in Figure 3 (A–G).

3.3. In vivo CAM assay

Fenugreek extracts exhibited distinctive antiangiogenic activities in the *in vivo* CAM assay. A normal vasculature pattern in the control CAMs with primary, secondary and tertiary vessels and a dendritic branching pattern can be seen in Figure 4A.

Vascularization in the chorioallantoic membrane was inhibited by the ethanol and ethyl acetate extracts by 59.5% ± 3.0% ($P < 0.001$) and 44.5% ± 4.5% ($P < 0.05$), respectively when compared with the control. Insignificant inhibition was observed with the aqueous chloroform and hexane extracts (Table 1).

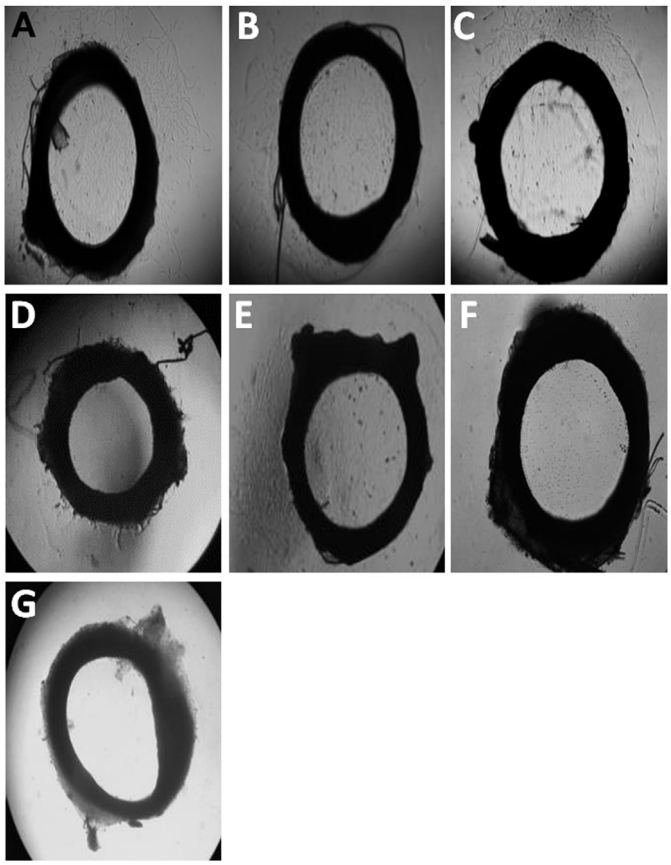

Figure 3. Effect of fenugreek extracts on *ex vivo* angiogenesis.
Representative micrographs of aortic ring explants on day 5 exposed to the ethanol extract. (A) the control (1% DMSO); (B) 3.125 μg/mL; (C) 6.25 μg/mL; (D) 12.5 μg/mL; (E) 25 μg/mL; (F) 50 μg/mL; (G) 100 μg/mL.

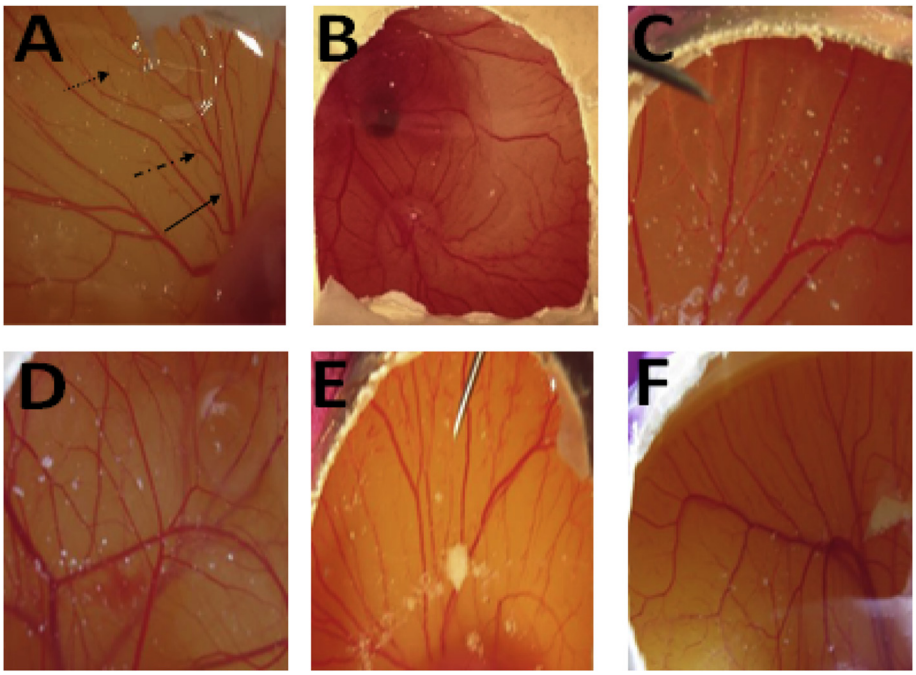

Figure 4. The effects on neovascularization in the chorioallantoic membrane.
(A) A normal vasculature pattern in the control CAMs with primary, secondary and tertiary vessels (see three arrows) and a dendritic branching pattern. The membranes 48 h after the application of the (B) aqueous, (C) chloroform, (D) hexane, (E) ethanol and (F) ethyl acetate extracts.

4. Discussion

From the present work, the rat aortic ring assay results revealed that the four tested extracts of fenugreek (ethyl acetate, *n*-hexane, chloroform and aqueous extracts) at 100 μg/mL did not exhibit a significant effect. However, the ethanol extract at 100 μg/mL showed complete inhibition of angiogenesis and therefore this extract was evaluated at six different concentrations ranging from 3.125 μg/mL to 100 μg/mL. At concentrations of 12.5, 25 and 50 μg/mL, significant inhibition was accomplished, but at concentrations of 3.125 and 6.25 μg/mL, the inhibition decreased indicating a very clear dose dependent relationship. The results of the present study therefore seem to point to the notion that the solvents used play a vital role in the extraction of the plant constituents. The solvents were selected according to their polarity to isolate the plant constituents based on their solubility in the most suitable solvents. The aqueous solution was added since in traditional medicine people use water to prepare an infusion (tea). Additionally, ethyl acetate was added since major anticancer agents have antioxidant activities and flavonoids possess antioxidant activities ideally dissolved in ethyl acetate.

It is reported that fenugreek is rich in alkaloids, flavonoids and saponins, playing the most important role in the therapeutic effects of the seeds [31]. Fenugreek is comprised of about 35% alkaloids, primarily trigonelline [31]. The percentage of saponin found in fenugreek seeds is about 4.8% and the seed contains flavonoid content of more than 100 mg per gram of seed. These constituents play a large role in the pharmacological effects of fenugreek that have been previously discussed. Due to the dose dependent manner in which ethanol inhibited vascularization in the rat aortic model, it is hypothesized that the active constituent(s) responsible for the activity are ethanol soluble. One must keep in mind that 70% ethanol can isolate a big variety of bioactive substances from the plant materials so that the observed activity of the ethanol extract may be due to the synergism of a mixture of substances. In addition, the presence of saponins in the plant material can contribute to the solubility of less polar substances in 70% ethanol. The results of the CAM assay concurred that the ethanol extract indeed has a significant effect on vascularization. Also in the present study, with the six different concentrations tested, the MTT assay did not exhibit significant anti-proliferative effect on the MCF7 cells. Only at 400 μg/mL concentration the extracts showed some effect of a reduction in the rate of cell proliferation. Several researchers, however, have studied the effect of fenugreek extracts and oils on cancer cells, especially on MCF7 breast cancer cells and determined the IC_{50} [32–34]. They concluded that fenugreek has a time dependent cytotoxic effect on MCF7 cells. Although these studies indicated that fenugreek has a cytotoxic effect on MCF7 cells, there was a study that showed induction of MCF7 proliferation. Sreeja *et al.* (2010) researched the influence of chloroform extracts of fenugreek seeds in breast cancer cells for its estrogenic effects and to evaluate its capability of acting as an alternative to hormone replacement therapy [35]. Their results showed that the chloroform extract significantly stimulated the proliferation of MCF7 cells in a dose dependent manner. The difference in results for the cytotoxicity on MCF7 cells are due to different variables such as: the method used to conduct the study, the geographical location and environmental conditions of where the seeds were collected, the method of drying of the plant material and the genetic variations. The geographical differences have been shown to affect the biological active components of fenugreek extracts [36,37]. In conclusion, despite the fact that fenugreek has been studied tremendously for its many therapeutic effects, this is the first study to evaluate the fenugreek extracts *ex vivo* and *in vivo* using the rat aortic ring assay and the CAM assay for its antiangiogenic properties. Findings of the both assays confirmed that the ethanol extract inhibited vascularization significantly. Future studies should be directed in bioassay guided isolation, purification and identification of the constituents of the ethanol extract and testing them for their antiangiogenic potential for the inhibition of tertiary blood vessels.

Conflict of interest statement

The authors declare that they have no conflict of interest.

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