Research Article

Anti-angiogenig Activity of *Fenugreek* Extracts : *in vivo* and *ex vivo* Study

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ABSTRACT

The objective of the study is to extract the *fenugreek* seeds powder and to identify the possible anti – angiogenic activity of these extracts in the *ex vivo* and *in vivo* assays. *Fenugreek* seeds powder was extracted sequentially with, chloroform, methanol and water using the cold method "maceration" as extraction process. The *ex vivo* rat aorta ring assay was used to screen the extracts for possible anti – angiogenesis activity, this assay was also used to determine the dose – response effect of the active extract by preparing serial concentrations. Free radical scavenging activity of the active extract was determined using DPPH (1,1 – diphenyl – 2 – pecrylhydrazyl) assay. The obtained data revealed that the three extracts exhibited significant inhibition of blood vessels growth when they were compared to the negative control (received DMSO 1%) ($P<0.001$), but methanol extract exhibited the highest percentage of anti- angiogenic activity. According to the screening results, methanol extract was selected for further investigation. Fenugreek seeds methanol extract exhibited a significant dose – dependent anti – angiogenesis effect ($P<0.05$) with IC_{50} (3.15 µg/ml). Furthermore methanol extracts exhibited a significant free radical scavenging activity ($P<0.05$) with IC_{50} (122 µg/ml). The results revealed that methanol extract of *fenugreek* seeds exhibited significant anti – angiogenesis activity as well as a significant free radical scavenging activity.

Keywords: Angiogenesis, fenugreek seeds, herbs.

INTRODUCTION

Angiogenesis is a physiological process through which new blood vessels form from pre-existing one, it occurs in the normal body for wounds healing and in restoring blood flow to tissues after injury. In females, it also occurs during the monthly reproductive cycle and during pregnancy[1]. Excessive angiogenesis occurs in diseases such as cancer, diabetic blindness, age-related macular degeneration, rheumatoid arthritis, psoriasis. It occurs when diseased cells and tissue produce abnormal amounts of angiogenic growth factors, overwhelming the effects of natural angiogenesis inhibitors. Insufficient angiogenesis occurs in diseases such as coronary artery disease, stroke, and chronic wounds. It occurs when tissues do not produce adequate amounts of angiogenic growth factors[2]. There are two types of angiogenesis; Sprouting angiogenesis and intussusceptive angiogenesis The sprouting angiogenesis mechanism occurs by ramification of new blood vessel from pre-existing vessels, While intussusceptive angiogenesis occurs by the expansion, breaking and fusion of pre-existing vessels produced by the proliferation of the endothelial cells within the wall of a blood vessel[4]. Intussusceptive angiogenesis is believed to be fast and effective in compression with sprouting angiogenesis as it requires only reorganization of the existing endothelial cells and it is not depend on immediate endothelial proliferation or migration[3]. The process of angiogenesis is very essential physiologically as in ovulation, embryogenesis and wound healing, and also in pathological conditions like, rheumatoid arthritis, psoriasis, age related macular degeneration, Alzheimer's disease, cancer and others[4]. *Fenugreek* is an annual herb belong to the family Leguminosea, the species name is *Trigonella foenum-graecum*; its English name comes from two Latin words meaning Greek hay indicating its use as a forage crop in the past[5]. It is one of the oldest famous medicinal plants that have been documented in ancient herbal medicine *Fenugreek* leaves and seeds are used in different countries around the world for different purposes such as medicinal uses (treatment of diabetes, hyperlipidemia, cancer, infection, gastrointestinal ulcer, and obesity, etc.) But it is mainly used in cooking food (due to its strong flavor and aroma and it is rich source of calcium, iron, â-carotene and other vitamins[6]. The biological and pharmacological activity of *fenugreek* herb are thought to be due to the variety of its constituents: steroids, N-compounds, poly phenolic substances, volatile substances, amino acids, etc. *Fenugreek* seeds are rich in vitamins, flavonoids, terpenoids, carotenoids, cumarins, curcumins, lignin, saponin, phenol[5].

The objective of this study is to extract the fenugreek seeds by using different solvents and determine which extract may have the highest anti – angiogenic activity.

MATERIALS AND METHODS

Extraction process

Five hundred grams of *fenugreek* seeds were obtained from Iraqi market. The seeds were rinsed with tap water and left to air dry. The dried seeds were ground into very fine powder. The powder extracted sequentially with

(chloroform, methanol and water), using Maceration method. The mixture filtered using whatmann no.1 filter paper to obtain the extract. The extract was concentrated using a rotary evaporator with vacuum (Buchi, Switzerland), crude extract, stored in dry and tightly sealed container to be use later in the experiment[7].

***Ex vivo* Rat aorta ring anti – angiogenic assay**

The rat aortic ring assay experiment was conducted after the experimental procedures were studied and approved by Ethics Committee of Al-Nahrain University/College of Medicine. The assay was achieved according to the standard protocol developed by Brown and his colleagues[8], with slight modifications. Twelve to fourteen weeks old Albino male rats were obtained from the animal house of Institute for diagnosis of infertility and assisted reproduction techniques/Al-Nahrain University. The animals were sacrificed via cervical dislocation under anesthesia with diethyl ether. Thoracic aorta was excised, rinsed with serum free media, cleaned from the fibroadipose tissue and was cross sectioned into thin rings of 1 mm thickness. M199 medium was used for the lower layer after adding fibrinogen and aprotinin at 3mg/mL and 5µg/ml respectively. A 300 µl of M199 medium was loaded in each 48-well plate and one aortic ring was seeded in each well. To each well, 10 µl of thrombin; prepared at 50 NIH U/mL in 0.15 M NaCl and then was incubated and allowed to solidify at 37°C in 5% CO2 for 30-60 min. The top layer medium was prepared by adding the following to M199 medium: 20% of heat inactivated fetal bovine serum (HIFBS), 1% L-glutamine, 0.1% aminocaproic acid, 1% amphotericin B and 0.6% gentamicin. Plant extracts were added to the top layer medium at concentration of 100µg/mL and each treatment was performed in six replicates. A stock solution of the sample extract was prepared by dissolving the sample in dimethyl sulfoxide (DMSO), and diluted in M199 growth medium to make the final DMSO concentration 1%.

The tissue rings were incubated at 37°C, 5% CO2 in a humidified incubator. On day 4, the top layer medium was changed with fresh medium prepared as previously mentioned. The DMSO (1% v/v) and acetyl salicylic acid "Aspirin" (100µg/mL) were used as negative and positive controls respectively. The results examined on day 5 under inverted microscope and the extent of blood vessel growth was quantified under 40X magnification with aid of camera and software package. The magnitude of blood vessel growth inhibition was determined according to the technique developed by Nicosia and coworkers (1997)[9]. The results are presented as mean percent inhibition to the negative control ± SD. The experiment was repeated three times using six replicate per sample. The percentage of blood vessels inhibition was determined according to the following formula:

Blood vessels inhibition = 1- (A_0/A) ×100

Where:

A_0= distance of blood vessels growth for the test substance in mm.

A= distance of blood vessels growth in the control in mm

Dose response study of the active crude extract with rat aorta ring anti – angiogenic Assay

Serial dilutions of the active extract were prepared in the following concentrations: 200, 100, 50, 25, and 12.5µg/ml, of the samples were dissolved in DMSO, and diluted in the M199 growth medium to make the final DMSO concentration 1%. Wells without test samples were received medium with 1% DMSO used as the negative control. The data was represented as mean ± SD. The concentration that inhibits 50% of the growing blood vessels "IC_{50}" was calculated by using the linear regression equation or the logarithmic equation for the extract. Where Y= the percentage of inhibition, and X= concentration[10].

Scavenging activity 1, 1-diphenyl -2-picrylhydrazyl (DPPH)

The free radical scavenging activity of the active extract was measured by using the DPPH method. 200 µl of 0.1 mM DPPH dissolved in methanol was added to 100 µl of the active extract in the following concentrations (500, 250, 125, 62.5, 31.25, 15.625 and 7.813 µg) and incubated for 30 min. This procedure was executed using 96 well plate and each concentration was tested in triplicate, then the absorbance was measured at 517 nm using an ELISA reader. Ascorbic acid (Vitamin C) was used as a positive control and methanol alone as blank. The negative control was made of 100µl of methanol and 200µl DPPH Percentage reduction of DPPH (Q) was calculated according to the formula below.[11]

Q=100 × (A0-AC)/ A0

Where

A0= Absorbance of control

AC= Absorbance of sample concentration

Statistical Analysis

The experiment design used for these studies was Rationalized Complete Block Design (RCBD). The results were presented as means ± standard deviation (SD). One way analysis of variance (ANOVA) was used. The concentration that inhibit 50% of the blood vessels growth, cells proliferation (IC_{50}) this value was analyzed by logarithmic equation. and linear regression equation. The statistic analysis was carried out by using SSPS, The level of significance was set at $P<0.05$ and $P<0.001$ as significant and high significant.

RESULTS

Extraction Process

Three solvents were used to extract 500 gm of *fenugreek* seeds powder, which are, chloroform, methanol and

water. Of the three extracts, water extract gave the hightest yield percentage (28.9%) as shown in table (1).

Table 1: Weight and yield percentage obtained from *fenugreek* seed crude extracts.

Extract	Weight (g)	Percentage (%)
Chloroform	32.4	6.5
Methanol	60.8	12
Water	144.6	28.9

Ex vivo rat aorta ring anti – angiogenesis assay for PE, CE, ME and WE of *P. dactylifera* seeds

Statistical analysis showed a significant difference between *fenugreek* seeds three extracts (chloroform, methanol and water) and the negative control (DMSO 1%) in the terms of inhibition of blood vessels growth at ($P<0.001$). All these extracts significantly inhibited blood vessels growth in different percentages. There was a significant difference between the positive control (Aspirin) and these three extracts in term of blood vessels growth inhibition at ($P<0.001$). Methanol extract gave a significant inhibition of blood vessels growth when compared to both chloroform and water extracts at ($P<0.001$), and there was a significant difference between chloroform and water extract at ($P<0.01$). Finally, all the extracts showed a significant inhibition of blood vessels growth but methanol extract gave the most significant inhibition as shown in table (2), figure (1) and image (1).

Table 2: The inhibition percentage of blood vessels growth produced by the tested extracts, negative and positive controls

Agents	Percentage (%) (Mean ± SD)
Aspirin (Positive control)	81.68 ± 2.054
1% DMSO (Negative control)	0 ± 2.061
Chloroform extract	72.8 ± 0.956
Methanol extract	100 ± 0
Water extract	64.83 ± 1.263
DMSO: Dimethyl sulfoxide	

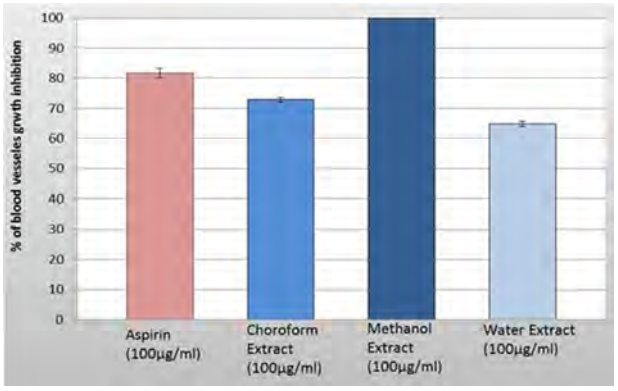

Figure 1: Anti – angiogenesis activity of 100µg/ml of each of chloroform extract, methanol extract and water extract in *ex vivo* aortic ring model. Aspirin was used as positive control and (DMSO 1%) as negative control.

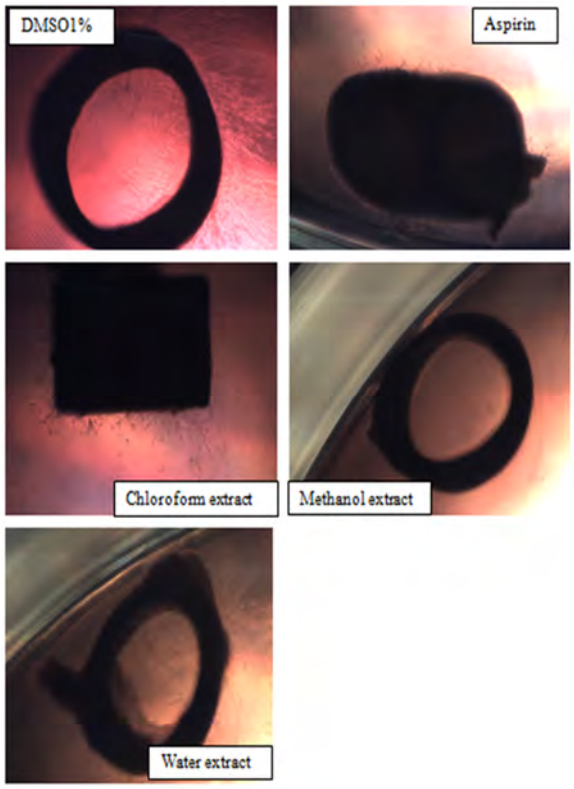

Image 1: Anti – angiogenesis effect of 100µg/ml of *fenugreek* seeds extracts in *ex vivo* aortic ring model. Aspirin was used as positive control and DMSO 1% as negative control

Dose response effect of methanol extracts of *fenugreek* seeds in rat aortic ring assay

Six serial dilutions of methanol extract was prepared and added to the embedded rat aortic rings to determine the dose response curve. Methanol extract showed significant dose dependent inhibition of blood vessels growth when compared to the negative control (DMSO 1%) ($P<0.005$) at day five of the experiment as shown in table (3).

Table 3: Serial concentrations and their respective inhibition percentage for methanol extract of *fenugreek* seeds

Fenugreek seeds methanol extract concentration (µg/ml)	% of blood vessels growth inhibition (mean ± SD)
200	100 ± 0
100	100 ± 0
50	93.44 ± 0.489
25	84.48 ± 0.493
12.5	68.31 ± 0.473
6.25	53.88 ± 0.950

IC_{50} value of methanol extract was calculated from the logarithmic equation shown in figure (2) which was found to be 3.15µg/ml, Where Y= the inhibition percentage and X= the concentration

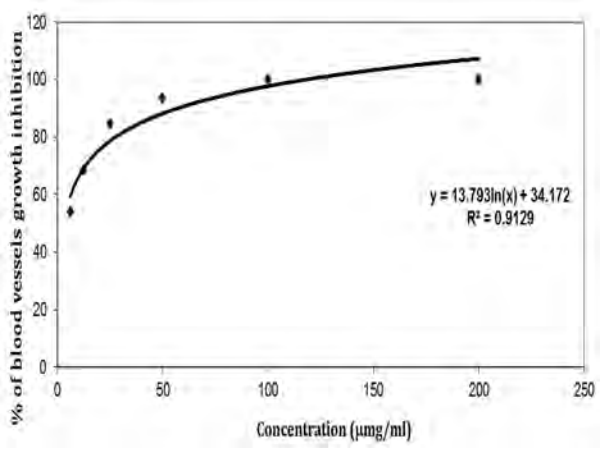

Figure 2: Dose response curve of methanol extract of *fenugreek* seeds in rat aortic rings assay

Free radical scavenging activity of methanol extract of *fenugreek* seeds

Table 4: The percentage of DPPH free radical scavenging activity for methanol extract of *fenugreek* seeds and the positive control (ascorbic acid)

Concentration (µg/ml)	% of free radical scavenging activity (Mean ±SD)	
	Methanol extract	Positive control "Ascorbic acid"
500	82.18 ± 0.012	95.05 + 0.001
250	72.61 ± 0.006	94.72 + 0.003
125	54.13 ± 0.004	94.4 + 0.003
62.5	43.56 ± 0.002	94.06 + 0.001
31.25	39.27 ± 0.005	93.4 + 0.001
15.125	36.96 ± 0.005	92.08 + 0.005
7.81	34.98 ± 0.007	85.15 + 0.006

The free radical scavenging activity methanol extract was measured using the DPPH assay. Seven serial concentrations were used to determine the scavenging activity as shown in table (4).

The results revealed methanol extract significantly reduced the DPPH free radical in a concentration dependent manner at ($P<0.05$). The IC_{50} for ascorbic acid (positive control) was determined from the logarithmic equation shown in figure (3), and the IC_{50} of the DPPH scavenging activity of methanol extract was determined from the linear regression equation as shown in figures (4) and it was found to be IC_{50} for ascorbic acid (positive control) = 5.12×10^{-9}µg/ml. And the IC_{50} for methanol extract = extract=122µg/ml, Where Y= the percentage of reduction in DPPH free radical

X= the concentration

By comparing the percentage of DPPH scavenging power of serial concentrations of Ascorbic acid (positive control) and methanol extract of *fenugreek* seeds results revealed significant free radical scavenging activity of methanol extract in a concentration dependent manner, Level of significance at $P<0.05$.

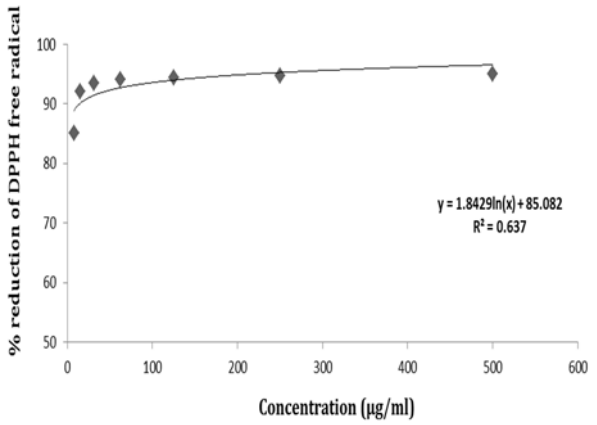

Figure 3: The dose response curve of serial dilutions of Ascorbic acid (positive control) on DPPH free radical scavenging activity

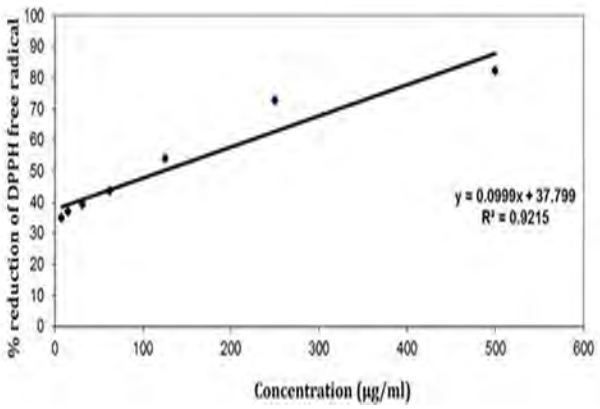

Figure 4: The dose response curve of serial dilutions of *fenugreek* seeds methanol extract on DPPH free radical scavenging activity

DISCUSSION

Extraction process

The extraction process used in this study was the cold method or maceration method, this method is best to be used to avoid any destruction or degradation of compounds that may present inside the *fenugreek* seeds due to exposure to high temperature[12]. In this study the *fenugreek* seeds powder was exposed to sequential solvent extraction to ensure that the majority of the active ingredients have been extracted and isolated according to their polarity[13]. In the present study, water extract produced the highest yield of crude extract followed by methanol and finally chloroform extract which produced the lowest yield of crude extract (table 1). It appears that there are several factors seem to affect the variation in the yield and the composition of phytochemicals in each extract, these include: type of the extraction method, length of the extraction process, temperature of the water bath, agitation, type of solvent used and its pH, concentration and polarity; particle size of the powdered plant part and solvent to sample ratio. It

was also found that the method of drying the plant part highly affects the yield and the composition of the constituents in each extract which activity to be tested later in the experiment[14].

Effect on *ex vivo* rat aorta ring anti – angiogenesis assay

Angiogenesis assays are important for identification of possible angiogenic agents and screening for pharmacological inhibitors. In recent years, the rat aorta ring assay has been widely used to evaluate angiogenesis in whole or partial organ culture as an *ex vivo* assay[15].

The main objective of this study is to identify whether these *fenugreek* seeds extracts have anti-angiogenic activity or not and which of these extracts was the most biological active. It was essential to screen these three extracts against rat aorta assay (*ex vivo assay*), then the most biological active extract was subjected to further test such as *ex vivo* and *in vivo*, aspirin was used as a positive control in the this experiment because as it is approved to have anti – angiogenesis effect that is mediated either through COX – dependent pathway, or through COX – independent pathway by blocking the NF-KB which is considered closely associated with inflammation and angiogenesis[16]. The results revealed that all three *fenugreek* seeds extracts had anti-angiogenic properties at the concentration of 100µg/ml. This study revealed that methanol extract of *fenugreek* seeds was found to have the highest anti-angiogenic activity compared to other two extracts However, the anti-angiogenic activity showed by chloroform extract and water extract remained significant (figure 1). The quantification of angiogenesis on this system implies the determination of the number and length of branching micro-vessels[17].

It was reported by Idries A. (2014) that methanol, ethanol, and acetone either alone or in combination with an aqueous solution used as solvents for antioxidant extraction and the phenolic content in fenugreek seeds extracted by methanol solvent found to be greater in concentration and amount when other solvent used in extraction process, and phenolic compounds have several health benefits as well as anti – angiogenic properties. Moreover, previous researches showed that high concentrations of many chemical groups which may have a potent activity in angiogenesis process such as flavonoids, saponin and terpenoids compounds are exist in *fenugreek* seeds[18,19]. According to its significant anti-angiogenic effect, methanol extract of *fenugreek* seeds was selected for further investigations to determine its chemical constituents and their anti-angiogenic mechanism. Dose response curve was done for the methanol extract against rat aorta anti-angiogenic assay.

Free radical scavenging activity by DPPH assay for chloroform and methanol extract of *Phoenix dactylifera* seeds

The DPPH radical is a stable nitrogen-derived organic free radical, which can be reduced to a non-radical form (DPPH-H) by accepting an electron or hydrogen in the presence of a hydrogen-donating antioxidant. It has been widely used for screening antiradical activities[20].

Free radical scavenging activity for methanol extract of *fenugreek* seeds was important to be studied in order to understand the mechanism of anti-angiogenic action of the *fenugreek* methanol extract.

According to previous studies methanol extract of fenugreek seeds has volatile oil, phenolic compounds and flavonoids; therefore it has high reducing power and it is a potent source of antioxidants[21].

The *fenugreek* seeds methanol extracts had found to have a powerful antioxidant activity in terms of the reducing power and with a significant capacity to scavenge DPPH.

Antioxidants can significantly affecting the angiogenesis process and this can occur by different mechanisms.

The Probable molecular mechanism of antioxidant rich phytochemicals is to inhibit VEGF-induced angiogenesis through the suppression of VEGF-induced reactive oxygen species (ROS) production[22].

As methanol and ethanol are highly polar among the solvents used in extraction process so, they contain high yield of phenolic compounds in comparison to the other solvents.

Fenugreek seeds ethanolic extract was shown highest antioxidant activity (% DPPH scavenging activity).

The antioxidant activity could be correlated with the polyphenolic constituents of the extract[21].

These findings may explain the anti – angiogenic activity of fenugreek seeds methanol extract as it have good anti –oxidant effect because agents with good antioxidant activity are well known to have anti – angiogenic activity, among those are vitamin C, vitamin D, vitamin E, vitamin A, rosmarinic acid and some flavone derivatives[23].

CONCLUSION

All three extracts of *fenugreek* seeds exhibited significant anti – angiogenesis activity, however methanol extract demonstrated the highest anti – angiogenesis activity as well as significant dose – dependent anti – angiogenic effect. In addition, methanol extract exhibited a significant free radical scavenging activity by DPPH assay and in concentration – dependent manner.

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