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Original Article

THE ANTI–ANGIOGENIC ACTIVITY OF *PHOENIX DACTYLIFERA* SEEDS EXTRACTS

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ABSTRACT

Objective: To identify the possible anti–angiogenic activity of *Phoenix dactylifera* seeds extract.

Methods: The powder of the date palm seeds was extracted sequentially with petroleum ether, 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(s) by preparing serial concentrations. Free radical scavenging activity of the active extract(s) was determined using DPPH (1, 1–diphenyl–2–picrylhydrazyl) assay.

Results: The obtained data revealed that the four extracts exhibited significant inhibition of blood vessels growth when they were compared to the negative control (received DMSO 1%) ($P<0.001$), but chloroform and methanol extracts showed the highest percent of inhibition of blood vessels growth. According to the screening results, both chloroform and methanol extracts were selected for further investigation. Each of chloroform and methanol extracts of *Phoenix dactylifera* seeds exhibited a significant dose–dependent anti–angiogenesis effect with IC_{50} (30.9 µg/ml and 28.4 µg/ml) respectively. Furthermore, chloroform and methanol extracts exhibited a significant free radical scavenging activity ($P<0.05$) with IC_{50} (81.02 µg/ml and 16.33 µg/ml) respectively.

Conclusion: the results revealed that each of chloroform and methanol extracts of *phoenix dactylifere* seeds exhibited the best and most significant anti–angiogenesis activity as well as a significant free radical scavenging activity.

Keywords: Angiogenesis, *ex vivo* study, Phoenix dactylifera Seeds.

INTRODUCTION

Angiogenesis is the process that involves the formation of new blood vessels from pre-existing ones. The primary step of it is thought to be initiated by activation of endothelial cells of pre-existing vessels in response to angiogenic stimuli. This process is typically initiated within hypoxic tissues where additional new blood vessels are required to maintain oxygenation and nutritional supply [1]. When the tissue is hypoxic, cellular oxygen sensing mechanisms are activated, which induce gene expression of various pro–angiogenic proteins. The primarily activated factors are HIFs (hypoxia inducible factors) which in turn they up–regulate multiple pro–angiogenic genes directly or indirectly. Among the up–regulated genes, VEGF-A (vascular endothelial growth factor–A) is the major one and also responsible for the proliferation and migration of cells during this process [2] There are two types of angiogenesis, and they can both occur in utero and in adults; the sprouting angiogenesis (non-splitting), which is the most commonly studied mechanism, involves degradation of capillary basement membrane, followed by migration of endothelial cells, starting with a tip cell that moves along a gradient of pro-angiogenic factors. Endothelial cells proliferate and migrate, following the tip cell. These endothelial cells will form a lumen and then recruit pericytes or smooth muscle cells to surround the vessel, and the basement membrane will be formed [3]. The second type is the intussusceptive angiogenesis (splitting), which involves the extension of the vessel wall into the lumen causing a single vessel to split in two. This type of angiogenesis is thought to be fast and efficient compared with sprouting angiogenesis because, initially, it only requires the reorganization of existing endothelial cells and does not require the immediate proliferation or migration of endothelial cells [4]. 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 [5]. *Phoenix dactylifera* is also known as date palm. From the viewpoint of botany, *Phoenix dactylifera* is derived from a Phoenician "Phoenix," which means date palm, and "dactylifera" from a Greek word "daktulos" meaning a finger and it is an important member of the family Palmaceae. The date palm is considered the most important source of food for human in arid and semi-arid regions and an integral part of Arabian diet [6]. Traditionally, the fruit is used for a sore throat, cold, relief of fever, abdominal problems, while the pollens were used by the Egyptians to improve fertility in women [7]. The seeds of date palm are used in animal feed to improve growth; the oil of the seeds is used in cosmetics, and the quality of these products is found to be encouraging [8]. Date palm seeds were found to be a good source of fatty acids, phenolic acids, and flavonoids, sterols, dietary fibers, proteins, minerals, vitamins and antioxidants [9]. The objective of this study is to determine which extract(s) of *Phoenix dactylifera* seeds mad have the best anti–angiogenic activity.

MATERIALS AND METHODS

Extraction process

Five hundred grams seeds of date palm (*P. dactyifera*) were obtained from Iraqi Date Factory/Iraq–Baghdad. The seeds were rinsed with tap water and cleaned from the remaining flesh then left to air dry. The dried seeds were ground into very fine powder. The powder extracted sequentially with (petroleum ether, chloroform, methanol, and water), using Maceration method. The mixture filtered using Whatman no.1 filter paper to obtain the extract. The extract was concentrated using a rotary evaporator with vacuum (Buchi, Switzerland), a crude extract, stored in dry and tightly sealed container to be used later in the experiment [1].

Ex vivo rat aorta ring anti–angiogenic assay

The rat aortic ring assay experiment was conducted after the experimental procedures were revised and approved by Ethics Committee of Al-Nahrain University/College of Medicine. The assay was performed according to the standard protocol developed by Brown and his colleagues [10], with minor 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

humanely sacrificed via cervical dislocation under anesthesia with diethyl ether. Thoracic aorta was excised, rinsed with serum free media, cleaned from the fibro adipose 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 3 mg/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, 10µl 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% CO_2 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 a 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% CO_2 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 acetylsalicylic acid "Aspirin" (100µg/ml) were used as negative and positive controls respectively. The results examined on day 5 under an 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 co-workers (1997) [11]. 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:

$$\text{Blood vessels inhibition} = 1-(A_0/A) \times 100$$

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 [12].

Free radical scavenging activity with DPPH assay

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, and 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 100 µl of methanol and 200 µl DPPH. The percentage of antioxidant activity (AA) was calculated according to the formula below [13]

$$AA\% = 1-(A_S-A_B/A_C-A_B) \times 100$$

A_S = absorbance of sample A_B = absorbance of blank

A_C = absorbance of control

Statistical analysis

The experiment design used for this study was Rationalized Complete Block Design (RCBD). Results were presented as means±SD (Standard Deviation). The differences between groups were compared by the one-way ANOVA followed by Tukey Post-hoc test (t–test) and considered significant at P<0.05, 0.01 and 0.001. The concentration that inhibited 50% of blood vessels and caused reduction of free radicals (IC50) was calculated using logarithmic equations. The statistical analysis was carried out by using SPSS edition 17.0.

RESULTS

Extraction process

Four solvents were used to extract 500 gm of Phoenix dactylifera seeds powder, which are petroleum ether, chloroform, methanol, and water. Of the four extracts, methanol extract gave the best yield percentage (10%) as shown in the table (1).

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

Type of extract	Weight (g/500g)	Yield (%)
Petroleum ether	38.3	7.7
Chloroform	5.8	1.2
Methanol	50	10
Water	10.5	2.1

500 gm of *P. dactylifera* seed powder used in the extraction process

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

Compound	% of inhibition+SD
Negative control "DMSO 1%"	0
Positive control "aspirin"	92.2+1.39
Petroleum ether extract	38.51+2.52
Chloroform extract	85.03+1.89
Methanol extract	85.99+1.79
Water extract	26.69+2.19

The results are presented as mean percent inhibition to the negative control±SD. The experiment was repeated three times using six replicate per sample (n=18)

***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 the four extracts (PE, CE, ME and WE) and the negative control (DMSO 1%) in the terms of inhibition of blood vessels growth at (P<0.001). All the extracts significantly inhibited blood vessels growth in different percentages. There was a significant difference between the positive control (Aspirin) and both PE and WE in blood vessels growth inhibition at (P<0.001), and also CE and ME showed a significant difference in the inhibition when compared to the positive control at (P<0.01) and (P<0.03) respectively. CE and ME gave a significant inhibition of blood vessels growth when compared to both PE and WE at (P<0.001), while there was no significant difference between CE and ME in their anti–angiogenesis activity at (P=0.628); and there was a significant difference between PE and WE at (P<0.01). Finally, all the extracts showed a significant inhibition of blood vessels growth but CE and ME gave the most significant inhibition as shown in the table (2), fig. (1) and image 1.

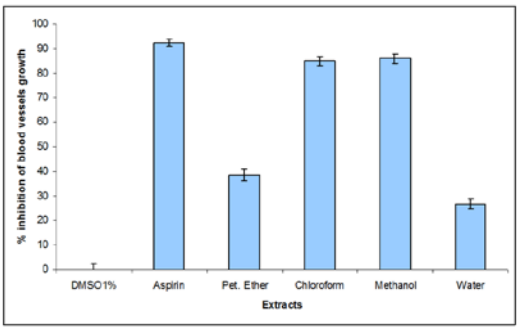

Fig. 1: Anti–angiogenesis activity of 100µg/ml of each of petroleum ether extract (PE), chloroform extract (CE), methanol extract (ME) and water extract (WE) in ex vivo aortic ring model. Aspirin was used as positive control and (DMSO1%) as negative control

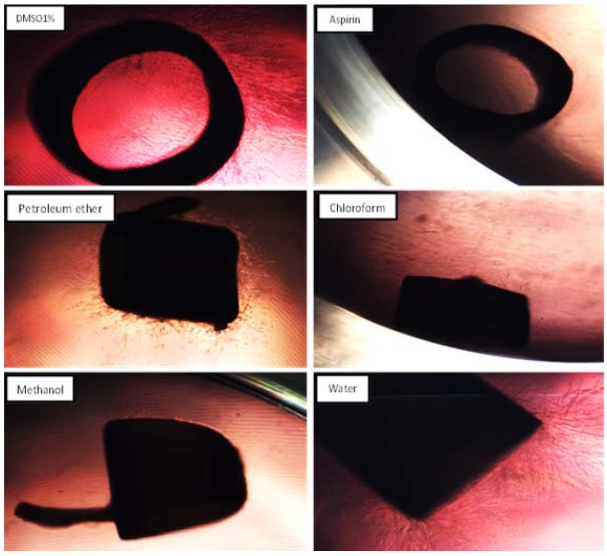

Image 1: Anti–angiogenesis effect of 100µg/ml of *Phoenix dactylifera* seeds extracts in *ex vivo* aortic ring model. Acetyl salicylic acid was used as positive control and 1% DMSO as negative control

Dose-response effect of chloroform and methanol extracts of *Phoenix dactylifera* seeds on rat aortic ring model

Five serial dilutions of each of chloroform and methanol extracts were prepared and added to the embedded rat aortic rings to determine the dose response curve. Both extracts showed significant dose-dependent inhibition of blood vessels growth when compared to the negative control (DMSO 1%) (P<0.001) at day five of the experiment; and there was no significant difference between CE and ME in the inhibition of blood vessels outgrowth in the aspects of dose-response as shown in table (3) and (4). Also, the dose-response effect of each extract on blood vessels growth is shown in image (2) and (3).

Table 3: Serial concentrations and their respective inhibition percentage for chloroform extract of *P. dactylifera* seeds

Concentration (µg/ml)	% of inhibition+SD
200	100+0
100	81.42+2.08
50	75.41+2.24
25	46.45+1.92
12.5	16.94+2.37

The results are presented as mean percent inhibition to the negative control±SD. The experiment was repeated three times using six replicate per concentration (n=18).

Table 4: Serial concentrations and their respective inhibition percentage for methanol extract of *P. dactylifera* seeds

Concentration (µg/ml)	% of inhibition+SD
200	100+0
100	84.15+1.82
50	77.6+1.85
25	48.63+1.84
12.5	19.7+1.42

The results are presented as mean percent inhibition to the negative control±SD. The experiment was repeated three times using six replicate per concentration (n=18).

The IC_{50} for each of chloroform extract and methanol extract was determined from the logarithmic equation that is shown in fig. (2) and (3) and it was found to be 30.9µg/ml for CE and 28.4µg/ml for ME. Where Y= the inhibition percentage and X= the concentration.

Free radical scavenging activity of methanol and chloroform extracts of Phoenix *dactylifera* seeds

The free radical scavenging activity of each of chloroform and methanol extracts was measured using the DPPH assay. Seven serial concentrations were used to determine the scavenging activity as shown in the table (5). The results revealed that each of chloroform and methanol extracts significantly reduced the DPPH free radical in a concentration dependent manner at (P<0.05).

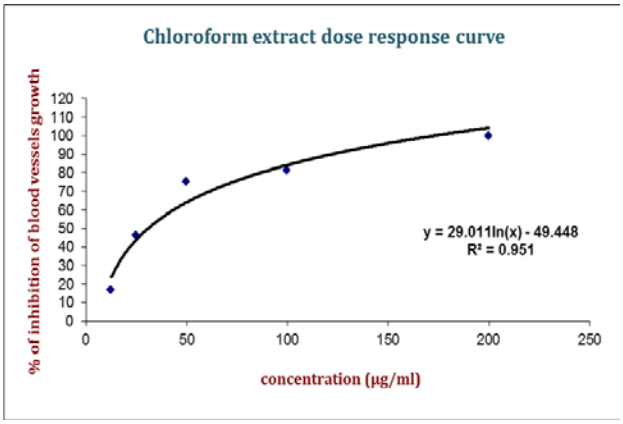

Fig. 2: Dose-response curve of chloroform extract of *Phoenix dactylifera* seeds on rat aortic rings model The experiment was repeated three times using six replicate per concentration (n=18)

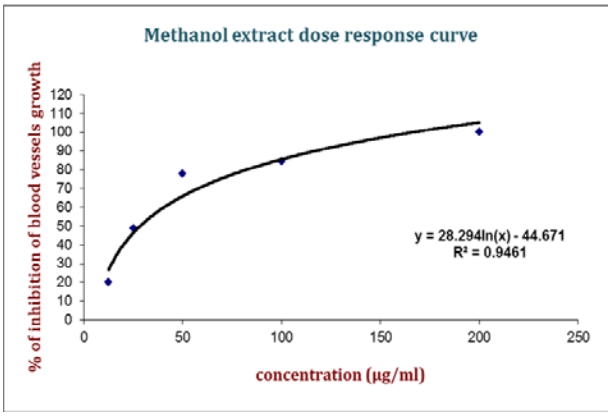

Fig. 3: Dose-response curve of methanol extract of *Phoenix dactylifera* seeds on rat aortic rings model The experiment was repeated three times using six replicate per concentration (n=18)

The IC_{50} was determined for each of chloroform and methanol extracts and ascorbic acid (positive control) from the logarithmic equation (Y=17.941ln(x)-28.48), (Y=14.251ln (x)+10.211) and (Y=3.148ln(x)+76.436) respectively; and it was found to be 81.02µg/ml, 16.33µg/ml and $2.2x10^{-4}$µg/ml respectively. Where Y= the percentage of reduction in DPPH free radical, and was set at 50%; X= the concentration.

Chloroform and methanol extracts showed a significant reduction in DPPH free radical when compared to the ascorbic acid (positive control), and there is a significant difference between methanol and chloroform extracts in terms of reduction of DPPH free radical and the IC_{50} (P<0.05).

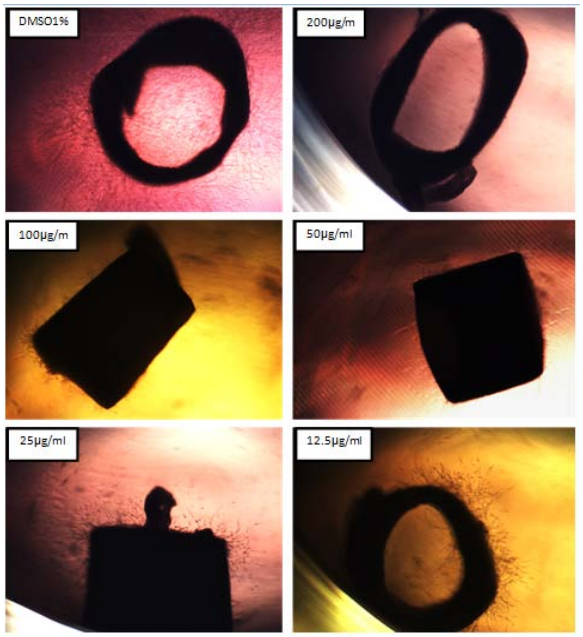

Image (2): The dose response effect to the serial dilution concentrations of the chloroform extract of *Phoenix dactylifera* seeds on rat aortic rings model. DMSO1% was used as negative control. The results were taken at day five of the experiment

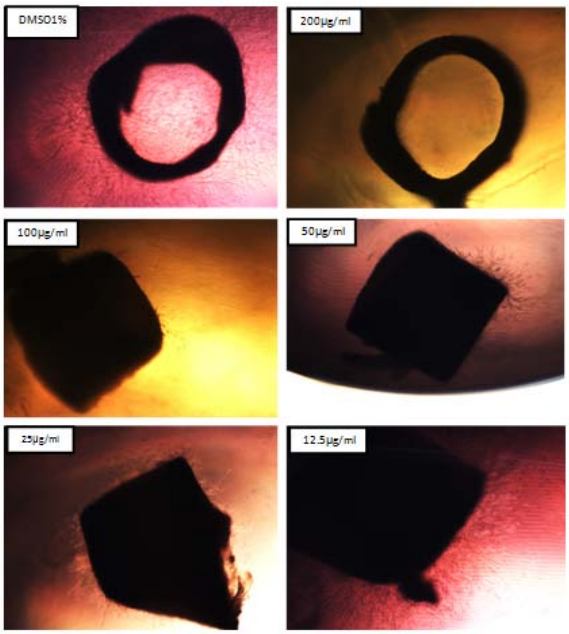

Image 3: The dose response effect to the serial dilution concentrations of methanol extract of *Phoenix dactylifera* seeds on rat aortic rings model. DMSO1% was used as negative control. The results were taken at day five of the experiment

Table 5: The percentage of DPPH free radical scavenging activity for methanol and chloroform extracts of *Phoenix dactylifera* seeds

Concentration (µg/ml)	DPPH free radical scavenging activity (%)		
	Ascorbic acid	Chloroform Extract	Methanol Extract
500	95.02+0.001	82.38+0.004	87.74+0.002
250	93.49+0.004	75.86+0.004	86.59+0.0004
125	91.57+0.003	62.45+0.012	84.29+0.002
62.5	90.42+0.001	34.48+0.009	81.61+0.003
31.25	89.66+0.001	26.05+0.004	70.11+0.002
15.125	85.06+0.005	21.07+0.004	46.36+0.012
7.81	80.84+0.002	14.56+0.002	26.82+0.006

Ascorbic acid used as positive control, each concentration has been triplicated (n=3)

DISCUSSION

Extraction process

The extraction process in the study was done sequentially, cold method "maceration used as a method of extraction, the solvents were used with increasing polarity in order to ensure that the majority of the constituents were extracted according to their polarity [14]; and the cold method for extraction was selected because this method is suitable for thermolabile constituents and in order to ensure that the essential phytochemical constituents won´t be subjected to degradation by higher temperatures [15]. In the present study, methanol extract produced the best yield of crude extract followed by petroleum ether extract, water extract 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 [16].

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

One of the most commonly used assays of angiogenesis is the aortic ring model. This model is based on the capacity of rat or mouse aortic explants to form new vessels in gels of collagen, fibrin or basement membrane. The aortic ring model bridges the gap between *in vivo* and *in vitro* models of angiogenesis, combining advantages of both systems [17]. In the present study, screening the four extracts in the *ex vivo* rat aorta ring anti–angiogenesis assay was very essential to determine whether these extracts have anti–angiogenesis activity or not, and if they do; to select the one(s) that has/have the best anti–angiogenesis activity for further investigation in other assays. In the present study, aspirin was used as a positive control in the rat aorta ring experiment because 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 [18]. The results revealed that each of chloroform extract and methanol extract had the best anti–angiogenic activity, and they had comparable effect against the inhibition of blood vessel growth. Both extracts were selected for further investigations when compared to the remaining extracts, which are petroleum ether extract and water extract. PE and WE also inhibited angiogenesis by some degree, but the inhibition was not comparable to that produced by CE and ME (fig. 1). The quantification of angiogenesis on this system implies the determination of the number and length of branching microvessel [13]. It was reported by Al-Farsi and Lee (2008) that the phenolic compounds in date seeds are much higher than that in the flesh, and these compounds possess various health benefits, as well as anti–angiogenic properties [19]. Different types of polyphenol present in the date pits could exhibit different polarities, thus, the type of

solvent and the temperature applied during the extraction highly affect polyphenol in the extracts [20]. From the mentioned reasons, it appears to be the inhibition of micro vessels outgrowth produced by CE and ME in this screening assay may be attributed to the presence of different phenolic compounds in both extracts. On the other hand, methanol extract may contain other phytochemicals like some terpenoids, and vitamin C (ascorbic acid) which also were found to possess anti–angiogenic activity [21]. These findings were supported by the phyto chemical analysis of both extracts. Due to the significant anti–angiogenesis activity for each of chloroform extract and methanol extract in the screening assay, dose response study was performed for each of CE and ME.

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

Under pathological conditions, the balance between the generation and elimination of ROS is broken, and, as a result, free radicals could be the cause of many health disorders such as diabetes mellitus, cancer, and neurodegenerative and inflammatory diseases [22]. Exogenous ROS can stimulate angiogenesis by increasing VEGF expression in various cell types, such as endothelial cells, smooth muscle cells, and macrophages and thus the progression of various angiogenesis-related disorders [23]. Free radical scavenging activity test for both chloroform and methanol extracts of *P. dactylifera* seed was very important in order to understand better the possible mechanism of action behind their ability to suppress blood vessels growth. Chloroform extract and methanol extract showed significant reduction of free radicals by the DPPH assay and in a concentration–dependent manner. This could be due to the presence of phenolic compounds in both extracts. Date seeds are known as an important source of phenolic acids consisting of hydroxylated derivatives of benzoic acid (gallic acid, protocatechuic acid, p-hydroxybenzoic acid and vanillic acid) and cinnamic acid (caffeic acid, p-coumaric acid, ferulic acid, m-coumaric and o-coumaric acid) which possess antioxidant effects. It was reported by Al-Farsi and co-workers in 2007 that the seeds of date palm contain a high level of phenolic compounds ranging 3102–4430 mg in terms of gallic acid equivalent/100 gm of seed powder and also have a high amount of antioxidants ranging 58–92.9 mmol in terms of trolox equivalent/100 gm of powder [9]. Methanol extract showed a little bit better antioxidant activity than that produced by chloroform extract as the IC_{50} of methanol extract was significantly lower than that of chloroform extract (16.33µg/ml for ME and 81.02µg/ml for CE). The better antioxidant activity produced by ME of *P. dactylifera* seeds was not only due to the presence of polyphenols, but also, ascorbic acid was found in the extract as well; this was supported by the phytochemicals analysis. Herchi and colleagues reported in 2014 that date palm seeds contain higher levels of vitamin C than date flesh. Ascorbic acid known for its potent antioxidant activity, was found to possess anti–angiogenic activity that was proven in a study done by Mikirova and co-workers in 2010, which showed ascorbic acid inhibited blood vessels growth in *ex vivo* aortic ring assay in a dose dependent manner and reduced neovascularization in *in vivo* subcutaneous matrigel plugs by 30% [24].

CONCLUSION

All extracts of Phoenix dactylifera seeds exhibited significant anti–angiogenesis activity. However chloroform and methanol extracts demonstrated the best anti–angiogenesis activity as well as significant dose–dependent anti–angiogenic effect. Furthermore, each of chloroform and methanol extracts exhibited a significant free radical scavenging activity by DPPH assay and in concentration–dependent manner.

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CONFLICT OF INTERESTS

Declared none

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