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Original Article

The Anti-Angiogenic Activity of *Phoenix Dactylifera* Seeds Methanol Extract *in Vivo* Study

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Abstract

The aim of the study was to identify the antiangiogenic activity of *Phoenix dactylifera* seeds methanolic extract *in vivo* and the probable anti-angiogenic mechanism of action. The date seeds were extracted with methanol using cold method, the crude extract was tested on chick chorioallantoic membrane (CAM) as an *in vivo* anti-angiogenic assay. Fourier transform infrared spectroscopy FT-IR was carried to identify the functional group for the active chemical constituents. The results demonstrated significant inhibition of blood vessels growth, and FTIR showed the presence of different functional group such as OH, NH_2 and phenols. From the data above the mechanism of action may relate to the presence of flavonoids and other phenolic compounds, as they have direct perturbing action on blood vessels and prevent cell proliferation of endothelial cells growth via their potent antioxidant and anti-inflammatory effects.

Keywords: CAM assay; FT-IR, angiogenesis; *Phoenix dactylifera*; *in vivo* study; vascular endothelial growth factor; antioxidant; phenolic compounds; natural compounds

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1. Introduction

The cells in the body are located no further than 100–200 μm from the nearest capillary [1]. Capillaries not only deliver oxygen and micronutrients to tissues, it secretes growth and survival signals that influence adjacent nonvascular cells [2]. One of the earliest events in embryogenesis is the emergence of the blood vascular system, which is a complex but ordered process in the early embryonic development that involves the formation of de novo blood vessels from progenitors endothelial cells [3]. The angiogenesis process involve expands of the primary vascular

plexus due to capillary branching and is transformed into the highly organized vascular net [4]. The main step of angiogenesis 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 [5]. 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, However in adults, the formation and the growth of new blood vessels are under strict control [6]. These processes are activated only under strictly defined conditions, especially when physiological circumstances demand an increase in the blood supply as in wound healing or in preparation for implantation of the fertilized egg in the endometrium [7]. Strict regulation of this system and balanced functioning is very important for the human being, because both excessive formation of blood vessels and their insufficient development lead to serious diseases among them cancer and malignancies, psoriasis, rheumatoid arthritis, retinopathies and others [8]. Numerous studies focused on identifying angiogenesis stimulators, which led to the identification of several angiogenic factors. Among these factors are soluble growth factors like acidic and basic fibroblast growth factor (aFGF and bFGF) and vascular endothelial growth factor (VEGF), which are associated with endothelial cells growth and differentiation [9]; factors that inhibit the proliferation and enhance the differentiation of endothelial cells like transforming growth factor β (TGF-β), angiogenin, and several low molecular weight substances [10]; and extracellular matrix-bound cytokines that are released by proteolysis, which may contribute to the regulation of angiogenesis and include angiostatin, thrombospondin, and endostatin [11]. *Phoenix dactylifera* commonly known as date palm, cultivated for its sweet fruit. From the viewpoint of botany, Phoenix dactylifera L. is derived from a Phoenician "Phoenix," which means date palm, and "dactylifera" from a Greek word "daktulos" meaning a finger and it is considered as an important member of the family Palmacaea [12]. The date palm is considered as an important source of food for humans in arid and semi-arid regions and an integral part of Arabian diet. Date is one of the oldest known fruit crops and has been cultivated in North Africa and the Middle East for at least 5000 years [13]. Iraq is one of the top ten date producers in the world; between 1980 and 2013, which contributed a total of 7.5% of world date production. Traditionally, dates can be used as tonic and also used for sore throat, fever, relief of cystitis, liver and abdominal problems. The gum of dates used to treat diarrhea, while the roots used for tooth aches; as for the pollen grains of date was found to improve fertility in women [14]. The date seeds are used as part of animal food to improve growth, while the seed oil is used in some body creams and shampoos and it was found that the quality of theses cosmetics is quite encouraging [15]. It appears that date

seeds consist of moisture, proteins, fatty acids and carbohydrates. Also, it was found that they contain a relatively good amount of minerals and vitamins; as for the phytochemical constituents, the seeds are considered a good source of phenolic acids, flavonoids, sterols, and antioxidants compared to the fruit [16]. The objective of this study is to determine the anti-angiogenic activity of the methanolic extract of *Phoenix dactylifera* seeds in CAM assay (*in-vivo* assay) and to identify the phytochemical constituents that could be responsible for the effect.

2. Materials and Methods

2.1. 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 using a heavy duty stainless steel grinder. The powder was then divided into five portions and was extracted with methanol 3-4 times with a ratio of 1:4 W/V (100gm of powder/400ml of solvent); using the cold method (Macerations) as the extraction process. The powder of date seeds was soaked with the solvent according to the ratio mentioned previously and was left for 24 hours in a shaking water bath at 40 ̊c and then was filtered using whatmann no.1 filter paper to obtain the clear extract. The extract was concentrated using a rotary evaporator with vacuum (Buchi, Switzerland) to obtain the final crude extract, which was stored in dry and tightly sealed container to be used later in the experiment [17].

2.2. In-vivo Chick Chorioallantoic Membrane (CAM) Assay

Fertilized chicken eggs obtained from a local hatchery, Baghdad/Iraq were incubated for 72h at 37 ℃. The eggs were placed in horizontal position and rotated several times. After 72h, 1-2 ml albumen were sucked off through a pinpoint hole pierced down by the side and sealed then incubated again for another 24 h. Then a round piece of shell (3-4 cm diameter) was removed from the top of the blunt end and the egg‘s sac punctured, then a round disc of filter paper which was impregnated previously with the test sample placed on the CAM and the eggs were sealed with a sterile adhesive tape and incubated for further 72 h. The test sample was prepared as 50mg/ml and 20 µl (1mg) placed on the disc of filter paper and left to dry prior to its transfer to the CAM [18]. On day 7 the zone of inhibition photographed and calculated; 6 CAM were used for each control and test sample [19]. The responses were graded + (3 - 6 mm); ++ (6 - 9mm); +++ (> 10mm). The quantification of zone of inhibition was done by using image analyzer [20].

2.3. Fourier Transfor-Infrared Spectroscopy (FT-IR)

This assay is important for functional group of extract identification. Potassium bromide (KBr) was transferred out of the oven into a mortar. About 1 to 2 % of the extract was added to the KBr, then mixed and grinded to a

fine powder. The sample was very finely ground. The two stainless steel disks have been taken out of the desiccators; and placed a piece of the precut cardboard (in the tin can next to the oven) on top of one disk and filled the cutout hole with the finely grounded mixture. After that the second stainless steel disk had been put on top and transferred the sandwich onto the pistil in the hydraulic press. With a pumping movement, the hydraulic pump handle moved downward. The pistil started to move upward until it reached the top of the pump chamber. Then, the pump handle moved upwards and pumped until the pressure reaches 20,000 prf. Left for a few seconds and with the small lever on the left side, release the pressure (hold until the sample and pistil are all the way down). The disks removed and pulled apart. The removed film should be homogenous and transparent in appearance. Then Inserted into the Infra-Red (IR) sample holder and attached with scotch tape. After that the spectrum Run. The tests done for the active extract [21].

3. Results and Discussion

3.1. Chick Chorioallantoic Membrane (CAM) Assay

The zone of inhibition for methanol extract was measured at day 7 of the experiment. Blood vessels in the CAM started to regress by the effect of the extract. The inhibition was recognized by the appearance of avascular zone surrounding the disc that contained the test extract and the extent of inhibition zone was measured according to the scoring system mentioned previously. It was found that methanol extract produced a significant inhibition zone of blood vessels in the CAM by scoring of (+++) and as shown in figure 1 and table 1.

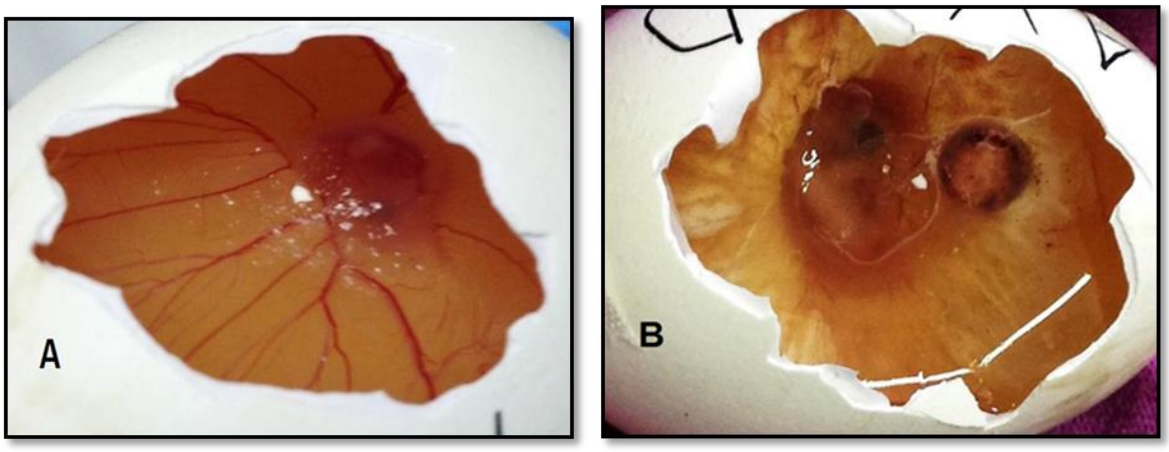

Figure1. *In vivo* (CAM) assay (A) represents the negative control "received DMSO 1%", while (B) represents the treated group with methanol extract (1mg/disc).

Table 1. The scoring for the inhibition zone of blood vessels growth in *in vivo* (CAM) assay for methanol extract of *Phoenix dactylifera* seeds.

NO.	Scoring for the inhibition zone
1	+++
2	+++
3	++
4	+++
5	+++
6	+++

3.2. Fourier Transform Infrared Spectroscopy (FT – IR)

The results are shown in figure 2 and table 2 for the screening of essential and important functional groups and these functional groups may indicate the type pf active constituents responsible on blood vessels inhibition [22].

3.3. Chick Chorioallantoic Membrane (CAM) Assay

In vivo angiogenesis assays have allowed important progress in studying the efficacy and in elucidating the mechanism of action of several agents, weather angiogenic enhancers or inhibitors. The main determinants dictating the choice of method are their cost, ease of use, reproducibility, and reliability [23]. In the present study, methanol extract of *P. dactylifera* seeds revealed significant inhibition of blood vessels by the in vivo CAM assay (Table 1). It appears that the anti – angiogenic activity of methanol extract could be due to the presence of diversity of phytochemicals in both extracts as was shown by FT-IR and GC-MS analysis. It was reported in a study that the polyphenolic compounds in

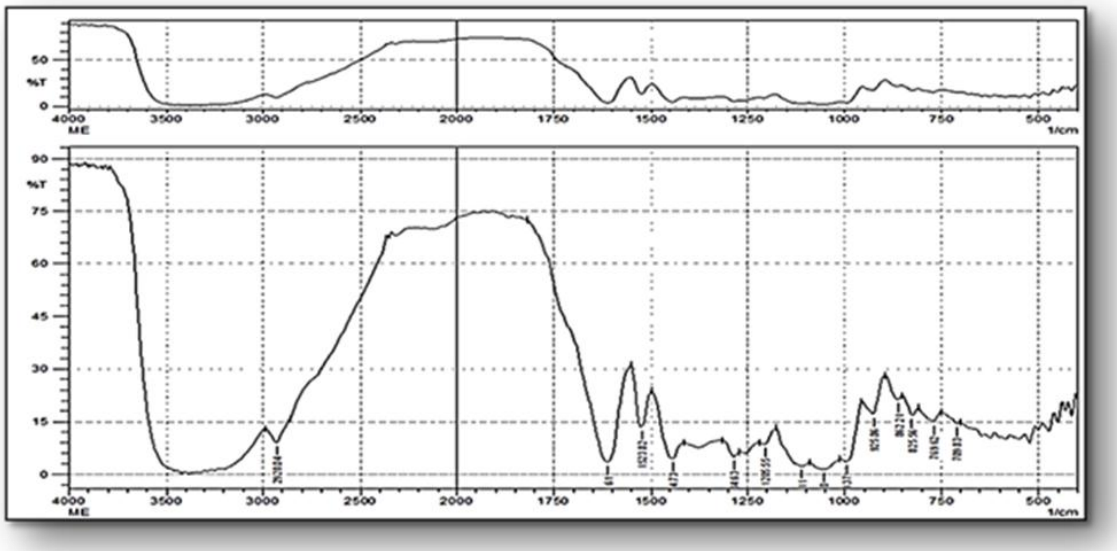

Figure 2. FT – IR peaks absorbance chart for methanol extract of *Phoenix dactylifera* seeds.

Table 2. Peaks absorbance and their functional groups for methanol extract of *Phoenix dactylifera* seeds.

No.	Peak Absorbance	Functional Groups
1	771-864	C-Cl Stretch (alkyl halide)
2	925	O-H Bend (carboxylic acids)
3	993	=C-H Bend (alkanes)
4	1053-1107	C-N Stretch (aliphatic amine)
5	1207	C-H Wag (-CH2X) (alkyl halide)
6	1284	C-O Stretch (alcohols, carboxylic acids, esters, ethers)
7	1444	C-C Stretch (in ring) (aromatic)
8	1523	N-O Asymmetric stretch (nitro compound)
9	1610	N-H Bend (1˚amine)
10	2928	C-H Stretch (alkanes)
11	3358	O-H Stretch (alcohols, phenols)

date seed methanolic extract through analysis by ultrahigh performance liquid chromatography – diode array detection – electrospray ionization – mass spectroscopy; and it was found to contain flavonoids, proanthocyanidins, cinnamic acid derivatives, catechin and epicatechin [24]. These polyphenols appear to affect human health through their various biological effects like antioxidant activity, anti-mutagenic and anti-carcinogenic activity and anti-inflammatory activity [25]. They also exert anti – angiogenic activity by regulating various signaling pathways [26]. A study was done in 2016 to examine the anti-angiogenic activity of different extracts of P. dactylifera seeds and it revealed that methanol and chloroform extracts possessed the highest anti-angiogenic activity in *ex vivo* model of rat aorta ring assay [22]; this highly supports the obtained results in the present study.

3.4. Phytochemical Analysis

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 in a study 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 [16]. Methanol extract of *Phoenix dactylifera* seeds was found to contain phenolic compounds, terpenoidal compounds and fatty acids, but in varying proportions but the highest percentage was for the phenolic compounds. These findings support the results obtained from FT-IR of the present study. Functional group analysis by FT-IR for methanol extract revealed that the presence of polyphenols and terpenes [27]. Compounds containing Phenols possessing anti – angiogenesis activity, either by forming Schiff´s base with ammonia and endogenous amines that tumor cells need to avoid risk of apoptosis due to acidic environment created by

their growth or directly damaging those cells by interaction with ROS [28]. Li et al, 2009 investigated the free radical scavenging activity of phenolic compounds isolated from red algae and it was found that they exhibited remarkable free radical scavenging activity in different *in vitro* assays (DPPH, hydroxyl radical, alkyl radical and superoxide anion) as well as *in vivo* assays by inhibiting the activity of myeloperoxidase and increasing the levels of GSH and SOD [29]. Since ascorbic acid was also detected in the ME and supported by a study done by Herchi and colleagues in 2014 that date palm seeds contain higher levels of vitamin C than date flesh [30]. Ascorbic acid may also attribute to the anti – angiogenesis activity of the extracts. Ascorbic acid known for its potent antioxidant activity was found to possess anti – angiogenic activity that was proven in a study done by Mikirova et al, 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% [31]. It was suggested that the anti – angiogenic effect of ascorbic acid was due to its antioxidant activity by suppressing reactive oxygen species, and also may act by reducing nitric oxide level which is an important regulator of angiogenesis [32] as well as by down regulating HIF levels [33]. It was also found that its anti – angiogenic activity could be due to its ability to suppress the expression of VEGF in melanoma cells [34]. Different terpenes were detected in ME, and it appears terpenes possess anti – angiogenic potentials by affecting various pathways involved in the mechanism like down-regulating the expression of HIF-1, pro-inflammatory cytokines, VEGF, MMP and up-regulating the endogenous anti – angiogenic factors like IL-2 and TIMP-1; and some may inhibit the VEGFR-2 signaling pathway and NF-ĸB pathway [35]. All these discussed reasons support and explain the anti – angiogenesis effect produced by methanol extract in *in vivo* CAM assay.

4. Conclusion

Methanol extract of Phoenix dactylifera seeds was able to produce a remarkable anti-angiogenesis activity in CAM assay and that effect could be due to the presence of diversity of phytochemicals like phenolic compounds, terpenes and terpenoidal compounds, fatty acids, aliphatic alcohols and others. These chemical constituents may exert either a direct anti – angiogenesis activity by down – regulating important pro-angiogenic factors like VEGF; or indirectly by inhibiting the production of pro-inflammatory mediators and scavenging free radicals thus reducing oxidative stress

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