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Evaluation Of The Mixture Of Phoenix Dactylifera Seedsextract And Chalcone Derivativeas Anti-Angiogenic Agentsin Ex-Vivo Rat Aorta Ring Model

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Abstract:

Angiogenesis is very important in the progression of various pathological disorders, most importantly tumor growth and metastasis. This research aims to investigate the efficacy of the organic extract of Phoenix dactylifera seeds and synthetic chalcone derivativeand their mixture as anti-angiogenic agents. The powderedseeds of P. dactylifera were extracted with chloroform 4 times by the maceration as the process of extraction andthe synthetic chalcone derivativewas synthesized according to Claisen-Schmidt condensation method.The synthetic chalcone derivative and organic extract of P. dactylifera seeds were evaluated for its anti-angiogenic activity using the ex-vivo rat aorta ring model at concentrations (17.15µg/ml and 30.9µg/ml) respectively along with its mixture at these concentrations. The synthetic chalcone derivativeandorganic extract of P. dactylifera seeds at the previously mentioned concentrationswere able to inhibit growth of blood vessels significantly in rat aorta ring assay in comparison with the negative control ($P<0.05$), while theirmixture at these concentrations showed higher percentage of inhibition (70.83 %)than either of the synthetic chalcone derivative and organic extract of P. dactylifera seeds separately in comparison with the negative control ($P<0.05$).Thisproduced effect could be due to the synergism between the synthetic chalcone derivative and the phytochemical constituents present in the organic extract in inhibiting different angiogenesis signaling pathways.

Keywords: P. dactylifera seeds, synthetic chalcone derivative, angiogenesis.

Introduction:

The process of recent blood vessels formation from pre-existing onesis known as angiogenesis. It is initiated in response toangiogenic stimuli that activateendothelial cells of pre-existing vessels. As these

tissues become hypoxic,the maintaining oxygenation and nutritional supplyrequiresformation of furtherfresh blood vessels and stimulatethe mechanism of cellular oxygen sensing [(1)].In consequence, it induced gene expression of various pro–angiogenic proteinsas(hypoxia inducible factors(HIFs)) that directly or indirectly up–regulate multiple pro–angiogenic genes[(2)]. The proliferation and migration of cells throughoutangiogenesis is the responsibility of the main up–regulated gene known as the vascular endothelial growth factor–A (VEGF-A)[(3)].

In adults, creation and growth of new blood vessels iscontrolled strictlyunder physiological circumstances that demand an elevation in the blood supply which in turn activates these processes, as in preparation for implantation of the fertilized egg in the endometrium or in wound healing.Themaincharacteristic that differentiates physiological angiogenesis from pathological one is that, in the first one, angiogenesis is limited to few days or weeks at best. However, in the later one, angiogenesis can persist for months or years [(4)]. In the past two decades, the convention of angiogenesis had been an evident one in more than 70 diseases and the list is ever growing. In disease states like cancer, ocular and inflammatory disorders, theinitiation of angiogenesis is a result of the unbalance between the inducers and inhibitors in response to excessive angiogenic stimuli[(5)]. Many tumors promote their own growth and thus metastasis to other organs by recruiting blood vessels into the vicinity of the tumor "the so–called tumor angiogenesis" [(6)].

Chemically, chalcones are α,β-unsaturated carbonyl systems that jointwo aromatic rings. Chalconesconstitute a natural class of compounds thatarebroadlydistributed in edible plants as intermediates for biosynthesis of flavonoids. Owing to the broad variety of chalcones' biological activitiesas antioxidant,anti-inflammatory, antibacterial, antifungal, anti-malarial and anticancer, they are considered as an interesting target class of compounds[(7)]. This variety of pharmacological activities depends on the substitution pattern in two aromatic rings[(8)].One of the mechanisms of the chalcones' anticancer activity is the suppression of angiogenesis. Severalhydroxylated chalcones whether natural and synthetic were shown to possess a potent anti-angiogenic activity[(9)].

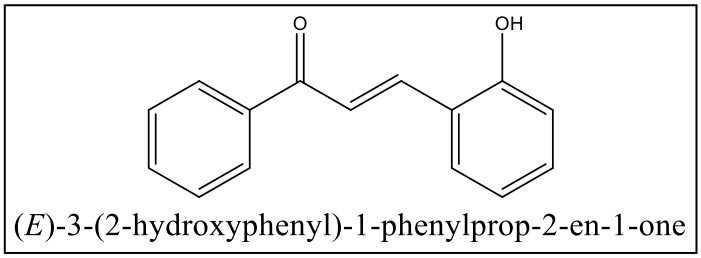
[(10)]. Datepalm(Phoenix dactylifera L) is one of the oldest known plants for thousands of years that has been cultivatedfor its sweet fruit in the Middle East and North Africa [(10,11)]. Iraq is one of the top ten date producers in the world; between 1980 and 2013, which contributed a total of 7.5% of world date production [(12)]. Date palm contains digestible sugars, mainly glucose, fructose, and sucrose; it also contains dietary fiber, proteins andessential vitamins for the human body like vitamins B_2, B_7, B_1, B_9 and C. It also contains minerals like sodium, calcium, copper, phosphate, cadmium, potassium, zinc, magnesium, manganese, iron, sulfur, selenium, cobalt, boron, fluorine and others. Furthermore,these

seeds are a rich source of phenolic acids, flavonoids, sterols and antioxidants. The constituents of these seeds made them very interesting target to study their medicinal value [13].The study'sobjective is to evaluatethe activity of the synthetic chalcone derivative and the organic extract of P. dactylifera seeds as anti-angiogenic agents by means of the ex-vivo rat aorta ring assay.

Materials and Methods:

Synthesis of chalcone derivative

Chalcone derivative ((E)-3-(2-hydroxyphenyl)-1-phenylprop-2-en-1-one) was synthesized according to Claisen-Schmidt condensation method at Department of pharmaceutical chemistry/ College of pharmacy/University of Kufa (Najaf; Iraq) [14].



(*E*)-3-(2-hydroxyphenyl)-1-phenylprop-2-en-1-one

Extraction Process of P. dactyifera seeds:

Iraqi Date Factory/Iraq–Baghdad supplied 500 grams of date palm seeds (P. dactyifera) for this study. These seeds were powdered by using a stainless-steel grinder after washing with water, cleansing from the remaining fleshand drying. Thesepowdered seeds were divided into five portions. The extraction of each portion was doneby using chloroform 4 times (100gm of powdered seeds/400ml of chloroform); using macerations as extraction processin a shaking water bath at 37°Cfor 48 hours. Then filtrate to obtain the clear extract that was concentratedby means of a vacuum rotary evaporator (Buchi, Switzerland)toobtainthe final concentratedextract that is used in the experiment[15].The extraction process was done at the phytochemistry research laboratory in Al-Nahrain University/College of Medicine/Department of Pharmacology (Baghdad; Iraq).

Laboratory Animals:

The animal house Institute for diagnosis of infertility and assisted reproduction techniques/Al-Nahrain Universitydonated thankfully male albino rats(12–14 weeks old) were they had free access to food andwater and kept in a temperature of 28–30°C.

Ex-vivo Rat Aorta Ring Assay:

The process was done in the tissue culture research laboratory in Al-Nahrain University/College of Medicine/Department of Pharmacology (Baghdad; Iraq). The performance of this assay was according to Brown and his colleagues' standard protocol [16], with minor modifications. Albino male rats (12–14 weeks old)were used and humanely sacrificed under anesthesia with diethyl ether via cervical dislocation. The excised thoracic aorta was rinsed with serum free media, cleaned from the fibro-adipose tissue and cross sectioned into thin rings of 1 mm thickness. For the lower layerin each 48-well plate,M199 medium (300 µl) was used after adding fibrinogen and aprotinin at 3mg/mL and 5µg/ml respectively, then one aortic ring was seeded in each well. To each well, 10 µl of thrombin (prepared at 50 NIH U/mL in 0.9% (W/V) NaCl). This 48-well plate was incubated at 37°C in 5% CO_2 for 30-60 min in order to solidify. 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.

To the top layer medium,the organic extractand chalcone derivative were added at concentration of 30.9µg/mL [17] and 17.15µg/ml [18]respectively. Then a mixture of them at the mentioned concentrations was added(each treatment was performed in six replicates). Stock solutions of the sample (organic extract orchalcone derivative)were prepared by dissolving it in dimethyl sulfoxide (DMSO), and diluted in M199 growth medium to provide the final concentration 1%.

The tissue rings were incubated at 37°C, 5% CO_2 in a humidified incubator. On the fourth day, the top layer medium was changed with a fresh medium prepared as previously mentioned. The DMSO (1% v/v) was used as negative control. The examined results on fifth day under inverted microscope (40X) with aid of camera and software package to quantify the extent of growth of blood vessel. The developed technique by Nicosia and coworkers had been used to determine the magnitude of inhibition of blood vessel growth [19]. The results were presented as mean percent inhibition to the negative control ± SD(Standard Deviation). The experiment was repeated three times using six replicates per sample (n=18). The following formula will determine the percentage of inhibition of blood vessels growth:

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.

Statistical Analysis:

The results were presented as mean percent inhibition to the negative control ± SD(Standard Deviation). The one way ANOVA was used to compare the differences between groupsfollowed by Tukey Post-hoc test (t–test) and considered significant at ($P < 0.05$).SPSS version 18.0 and Microsoft Excel 2010 were used to carry out this statistical analysis.

Results:

Extraction Process:

Chloroform was used as a solvent for extraction of thepowderedP. dactylifera seeds giving a yield percentage of 1.2% in which a500gm of these powdered seeds yield about 5.8gm of the extractives.

Anti-Angiogenic Activity Using the ex-vivo Rat Aorta Ring Assay:

The statistical analysis of the results showed that each of the organic extract (OE) at concentration of 30.9µg/ml and chalcone derivative (CD) at concentration 17.15µg/ml had significantly inhibited blood vessels growth ($P<0.05$) when compared to the negative control (1% DMSO), with inhibition percentages of 51.04% $\pm$ 2.8 and 52.1% $\pm$ 3.1 respectively. Themixture of organic extract and chalcone derivative (OE+CD) at the mentioned concentrations gave significant inhibition of blood vessels growth when compared to the negative control ($P<0.05$) as well as when compared to both organic extract and chalcone derivative alone ($P<0.05$); and the percentage of inhibition of thismixture was 70.83% $\pm$ 2.8. The observed results were obtained at fifth day of the experiment when the blood vessels growth was at its maximum, and they were represented as the mean inhibition of blood vessels growth $\pm$ SD (Standard Deviation). The results are shown in figure (1) and Image (1).

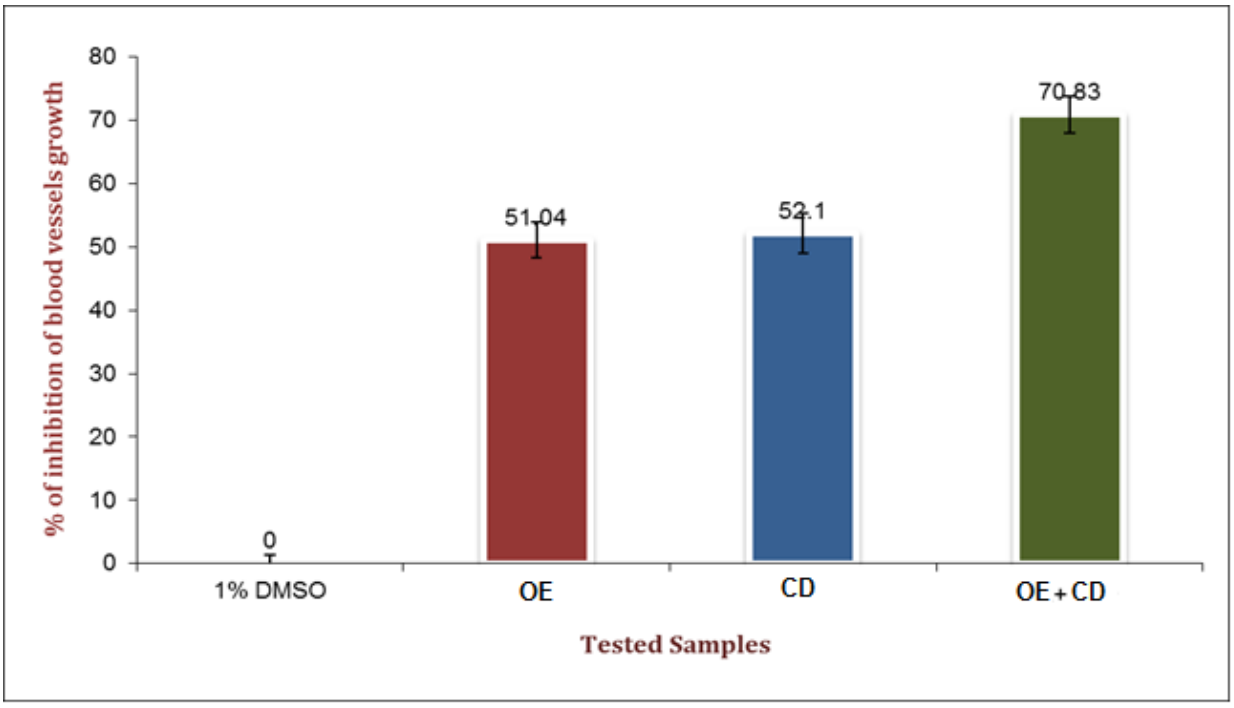


Figure (1): the anti-angiogenic activity of 30.9µg/ml organic extract of P. dactylifera seeds, 17.15µg/ml chalcone derivativeand the mixture of them at the mentioned concentrations. 1% DMSO was used as a negative control and the results were obtained at the fifth day of the experiment.

(DMSO= dimethyl sulfoxide, OE= organic extract, CD= chalcone derivative, and OE+CD= the mixture of the organic extract of P. dactylifera seeds and chalcone derivative)

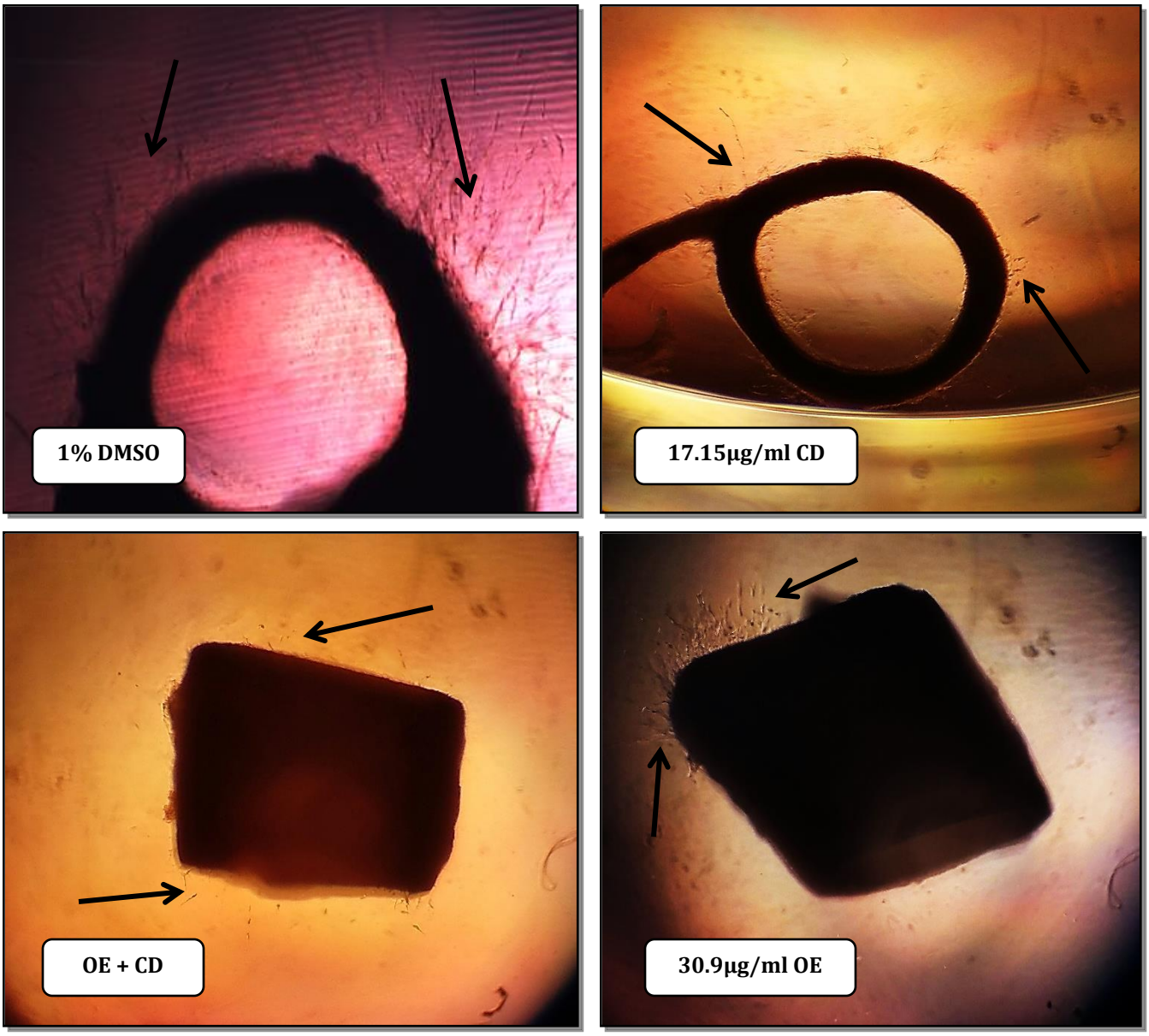


Image (1): The effect of each of 30.9µg/ml organic extract of P. dactylifera seeds, 17.15µg/ml chalcone derivative and the mixture of themat the mentioned concentrations in ex-vivo rat aorta ring model. 1% DMSO was used as negative control. The results were obtained at the fifth day of the experiment and the black arrows indicate the growth of micro-blood vessels.

(DMSO= dimethyl sulfoxide, OE= organic extract, CD= chalcone derivative, and OE+CD= the mixture of the organic extract of P. dactylifera seeds and chalcone derivative)

Discussion:

The process of recent blood vessels formation from pre-existing onesis known as angiogenesis.This process had been gaining an increasing attention in the past few decades and still does specifically due

to its relationship with tumor growth and metastasis. For this reason many drugs were discovered targeting this process through blocking different pathways. Aortic ring model is the most commonly used assay for angiogenesis that is depended on the mouse aortic explants capacity to form new vessels in gels of collagen, fibrin or basement membrane. This model combines advantages of both in vivo and in vitro models of angiogenesis[19]. The current study showed that each ofP. dactylifera seeds and chalcone derivative significantly inhibited the growth of micro-blood vessels in RAR model at the previously mentioned concentrations, but the mixture of both (OE+CD) revealed much higher inhibition of the micro-blood vessels in comparison to each of them alone. CD and OE were selected at such specific concentrations due to previous studies done by Abu Raghif, 2016 on synthetic CD and Al-Zubaidy et al, 2016 on OE of P. dactylifera seeds using different serial concentrations in order to determine the concentration that has the ability to inhibit growth of blood vessels by 50% [17, 18]. This enhanced activity is because of the different phenolic compounds along with unsaturated fatty acids and some phytosterols in OE [20].Furthermore, the DPPH assay revealed a remarkable anti-oxidant activity of the OE which considered very important in the inhibition of the angiogenesis process since the presence of higher levels of free radicals can stimulate this process[17]. As for the ability of CD to inhibit the growth of blood vessels, it was proved in a study done by Abu Raghif, 2016[18]. These chalcones were shown to possess a potent anti-angiogenic activity as well as anti-proliferative activity by suppressing TNF-α in HUVECsthat induce vascular cell adhesion molecule (VCAM-1)and intercellular adhesion molecule-1 (ICAM-1) expression [21]. Hayder and coworkers 2014 showed that the extract containing flavonoids inhibited vascular endothelial growth factor (VEGF)-induced cell proliferation and migration in HUVECs, as well as angiogenesis.One of the explanations for the increased activity of the mixture of OE+CD was probably due to the presence of a synergism and the potentiation effect between CD and the constituents of the organic extract through targeting different signaling pathway of the angiogenesis process. These chemical constituents may exert a direct anti–angiogenesis activity by down–regulating important pro-angiogenic factors including VEGF, PDGF, FGF, MMPs, HIF-1α, uPA and TGF; or by inhibiting the phosphorylation of VEGF receptors, thus inhibiting the proliferation, migration of endothelial cells and finally tube formation and expansion of the vasculature [22, 23]. Furthermore, the free radical scavenging ability of both of them plays a major role in suppressing the expression of HIF-1α and VEGF which are essential for the angiogenesis process [17, 18]. Recent studies focusmore on the discovery and use of naturally occurring constituents over the conventional therapies because they are associated with fewer side effects and their higher tolerability. In conclusion, the combination of CD and

OE showed significant anti-angiogenesis activity which makes it promising therapeutic agents for diseases associated to angiogenesis[24].

Conclusion:

This study revealed asignificant anti-angiogenic activity of the mixture of organic extract of P. dactylifera seedsand synthetic chalcone derivative more than each of them alone. The effect produced could be due tothe synergism between the chalcone derivative and the phytochemical constituents present in the organic extract by inhibiting different signaling pathways of the angiogenesis process; and this combination could represent promising agents that can be used for targeting diseases related to the angiogenesis process.

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