Annals of R.S.C.B., ISSN:1583-6258, Vol. 25, Issue 6, 2021, Pages. 5942 - 5951
Received 25 April 2021; Accepted 08 May 2021.

Evaluation of the Mixture of *phoenix Dactylifera* Seeds extract and Chalcone Derivativeas Anti-Angiogenic Agents in *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 synthetic chalcone derivative(2-Hydroxychalcone)and its mixture with ethanolic extract of *Phoenix dactylifera* seeds as anti-angiogenic agents. The synthetic chalcone derivative was synthesized according to Claisen-Schmidt condensation method using $SOCl_2$/ETOH as a catalyst with a good yield. The structure of the synthesizedchalcone derivative has been characterized by TLC, melting point, UV, and IR Spectroscopy , and elemental microanalysis (CHNO). The powdered seeds of *P. dactylifera* were extracted by soxhlet extraction with Ethanol. The synthetic chalcone derivative alone at concentration (17.15µg/ml) and its mixture with ethanolicextract 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. At the previously mentioned concentrations, The synthetic chalcone derivativewas able to inhibit growth of blood vessels significantly (56.23%) in rat aorta ring assay in comparison with the negative control ($P<0.05$), while the mixture of the synthetic chalcone derivative and ethanolic extract of *P. dactylifera* seeds showed higher percentage of inhibition (76.63%)than that of the synthetic chalcone derivative alone 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 ethanolic extract in inhibiting different angiogenesis signaling pathways.

Keywords:*Phoenix dactylifera* seeds, synthetic chalcone derivative, angiogenesis.

Introduction:

The process of recent blood vessels formation from pre-existing ones is known as angiogenesis. It is initiated in response to angiogenic stimuli that activateendothelial cells of pre-existing vessels. As these tissues become hypoxic,the maintaining oxygenation and nutritional supply requiresformation of furtherfresh blood vessels and stimulatethe mechanism of cellular oxygen sensing **(1)**.In consequence, it induced gene expression of various pro-angiogenic proteinsas

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(hypoxia inducible factors (HIFs)) that directly or indirectly up–regulate multiple pro-angiogenic genes [(2)]. The proliferation and migration of cells throughout angiogenesis 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, the initiation 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)].

Date palm (*Phoenix dactylifera* L) is one of the oldest known plants for thousands of years that has been cultivated for its sweet fruit in the Middle East and North Africa [(7,8)]. 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 [(9)]. Date palm contains digestible sugars, mainly glucose, fructose, and sucrose; it also contains dietary fiber, proteins and essential 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 [(10)].

Chemically, chalcones are α,β-unsaturated carbonyl systems that join two 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 activities as antioxidant, anti-inflammatory, antibacterial, antifungal, anti-malarial and anticancer, they are considered as an interesting target class of compounds[(11)]. This variety of pharmacological activities depends on the substitution pattern in two aromatic rings[(12)]. 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[(13)].

The objective of this study was concerned with the evaluation of anti-angiogenic activity of the synthetic chalcone derivative and its mixture with ethanolic extract of *P. dactylifera* seeds by means of the *ex-vivo* rat aorta ring assay.

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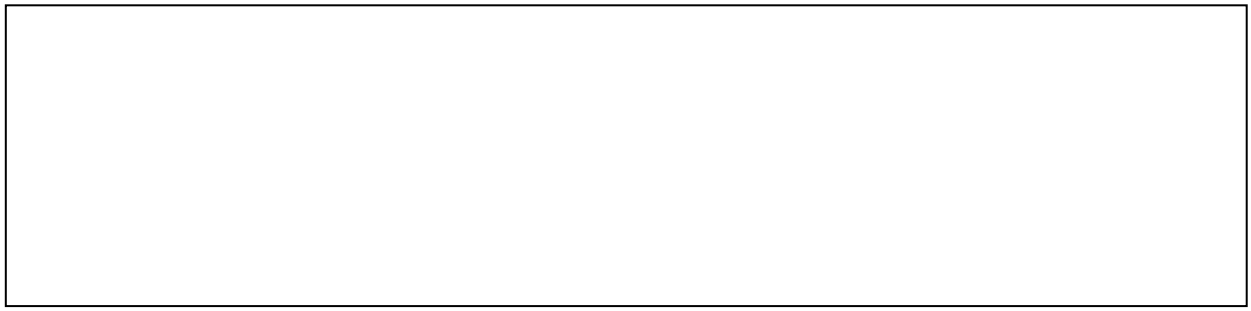
Materials and Methods:

Synthesis of chalcone derivative

2-Hydroxychalcone ((*E*)-3-(2-hydroxyphenyl)-1-phenylprop-2-en-1-one) was synthesized according to Claisen-Schmidt condensation method using $SOCl_2$/ETOH as a catalyst in Kufa University/College of Pharmacy/Department of Pharmaceutical Chemistry (Najaf; Iraq)[14].



Scheme 1: Synthetic pathway of (*E*)-3-(2-hydroxyphenyl)-1-phenylprop-2-en-1-one (CD)

Extraction Process of *P. dactyifera* seeds:

Iraqi Date Factory/Iraq-Baghdad supplied 300 grams of date palm seeds (*P. dactyifera*) for this study. The extraction process was done at the pharmacognosy'slaboratory in Kufa University/College of Pharmacy/Department of Pharmacognosy (Najaf; Iraq). The extraction of the powdered seeds was carryout using soxhlet ethanol extraction. The extract was separated from the solvent using solvent recovery method. The recovered extract was stored at room temperature ($28\pm2^{o}C$)[15].

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 and water and kept in a temperature (28-30℃).

Ex-vivo Rat Aorta Ring Assay:

The process was done at the pharmacognosy laboratory in Kufa University/College of Pharmacy/Department of Pharmacognosy (Najaf; Iraq). The assay was performed according to the standard protocol developed by Brown and his colleagues [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 layer in each 48-well plate, a 300 µl of M199 medium 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) 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

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fetal bovine serum (HIFBS), 1% L-glutamine, 0.1% aminocaproic acid, 1% amphotericin B and 0.6% gentamicin.

Samples were added to the top layer medium at concentrations of 17.15µg/ml for CD [17] and 17.15µg/ml [17] and30.9µg/ml [18] for a mixture of the CD and EE respectively (each treatment was performed in six replicates). Stock solutions of the samplesof CD and the mixture of CD and EE 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 vessels. The developed technique by Nicosia and coworkers had been used to determine the magnitude of inhibition of blood vessels growth [19]. The results were presented as the mean percent of inhibition to the negative control $\pm$ 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 = $1- (A_o/A) \times 100$
Where:
A_o= 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 the mean percent of inhibition to the negative control $\pm$ SD (Standard Deviation). The one way ANOVA was used to compare the differences between groups followed 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:
Synthesis of chalcone derivative (CD):
In table 1, CD showed a good yield (85%) with a melting point around 148-150^oC, while showing a single spot with $R_f$value of 0.36when eluted on a TLC plate using petroleum spirit (40-60): ethylacetate (70:30) solvent system. The spectral analysis in the UV region exhibited a λmax between 300-350 nm. The IR spectrum in KBr discs showed many characteristic absorption bands at *v* values 3232, 1649, 1599, and 1217 cm^{-1}. The elemental microanalysis of the CD revealed that the C and H% were 79.55% and 5.29% respectively (Table 2).

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Table 1: Physicochemical data for the synthesized chalcone derivative (CD)

Chemical name	Molecular formula	Molecular weight	Appearance	Yield (%)	M.P (^{o}C)	Rf
(*E*)-3-(2-hydroxyphenyl)-1-phenylprop-2-en-1-one	$C_{15}H_{12}O_2$	224	Greenish yellow solid	85	148-150^{o}C	0.36

Table 2: IR spectral data and elemental analysis of synthesized chalcone derivative (CD)

IR Spectral data	Elemental analysis (calculated) % C H N		
	C	H	N
IR (KBr) *v* cm^{-1} 3232(-OH), 1649 (C=O), 1599 (C=C), 1217 (C-O)	79.55 (80.34)	5.29 (5.39)	-

Extraction of *Phoenix dactylifera* Seeds:

The result of extraction of 300 gm of powdered*P. dactylifera* seeds is mentioned in Table 3. A total of 10.7% yield is obtainable from the seeds.

Table 3: Yield (%) of *P. dactylifera* Seeds Extract

Extract	Initial weight of sample (gm)	Weight of extract (gm)	Yield (%)
Ethanol seeds Extract	300	32.1	10.7

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

The statistical analysis of the results showed that the synthetic chalcone derivative (CD) at concentration of 17.15µg/ml had significantly inhibited blood vessels growth ($P<0.05$) when compared to the negative control (1% DMSO), with inhibition percentage of 56.23%$\pm$2.1. Themixtureof the synthetic chalcone derivative and ethanolic extract of *P. dactylifera* seeds(CD+EE) at the concentrations of 17.15µg/ml and 30.9µg/mlrespectively gave significant inhibition of blood vessels growth when compared to the negative control ($P<0.05$) as well as when compared to the synthetic chalcone derivative alone ($P<0.05$); and the percentage of inhibition of thismixture was 76.63%$\pm$2.4. 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 percent of inhibition of blood vessels growth $\pm$SD (Standard Deviation). These results are shown in table (4), figure (1) and Image (1).

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Table 4: The inhibition percentage of blood vessels growth produced by the negative control, syntheticchalcone derivative (CD) and its mixture with ethanolic extract of *P. dactylifera* seeds (CD+EE)

Compound	% of inhibition of blood vessels growth ± SD
Negative control "1% DMSO"	0
CD	56.23% ±2.1
CD+EE	76.63% ± 2.4

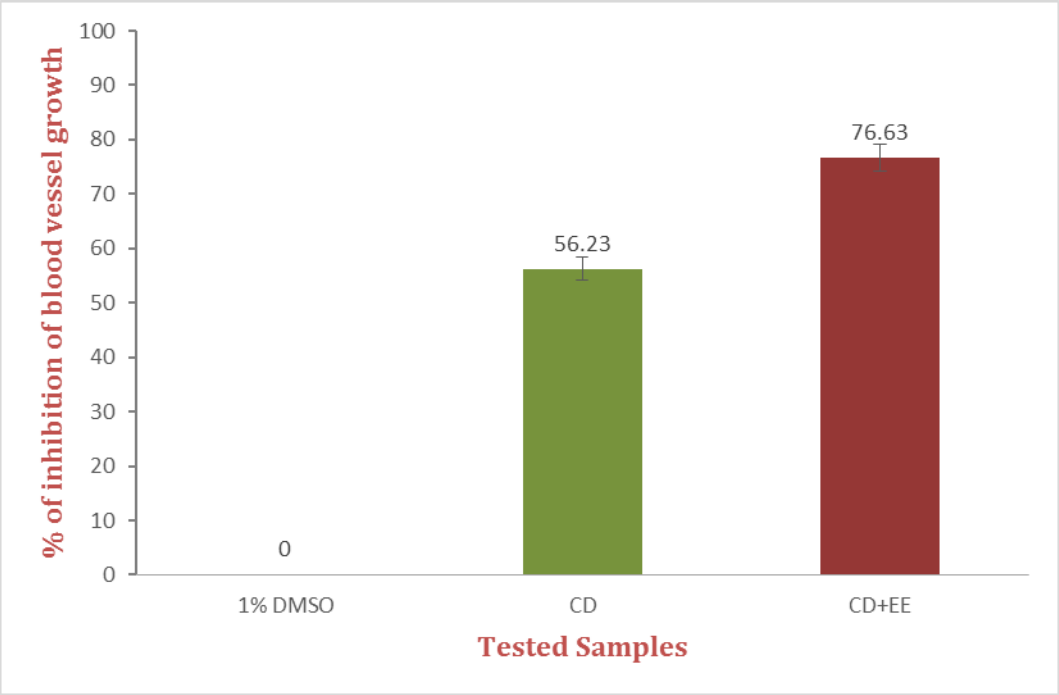

Figure (1): The anti-angiogenic activity of 17.15µg/ml of the synthetic chalcone derivativeand the mixture of 17.15 µg/ml of the synthetic chalcone derivative and 30.9 µg/ml of ethanolic extract of *P. dactylifera* seeds. 1% DMSO was used as a negative control and the results were obtained at the fifth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

(DMSO= dimethyl sulfoxide,CD= chalcone derivative, and CD+EE= the mixture of the synthetic chalcone derivative and ethanolic extract of *P. dactylifera* seeds)

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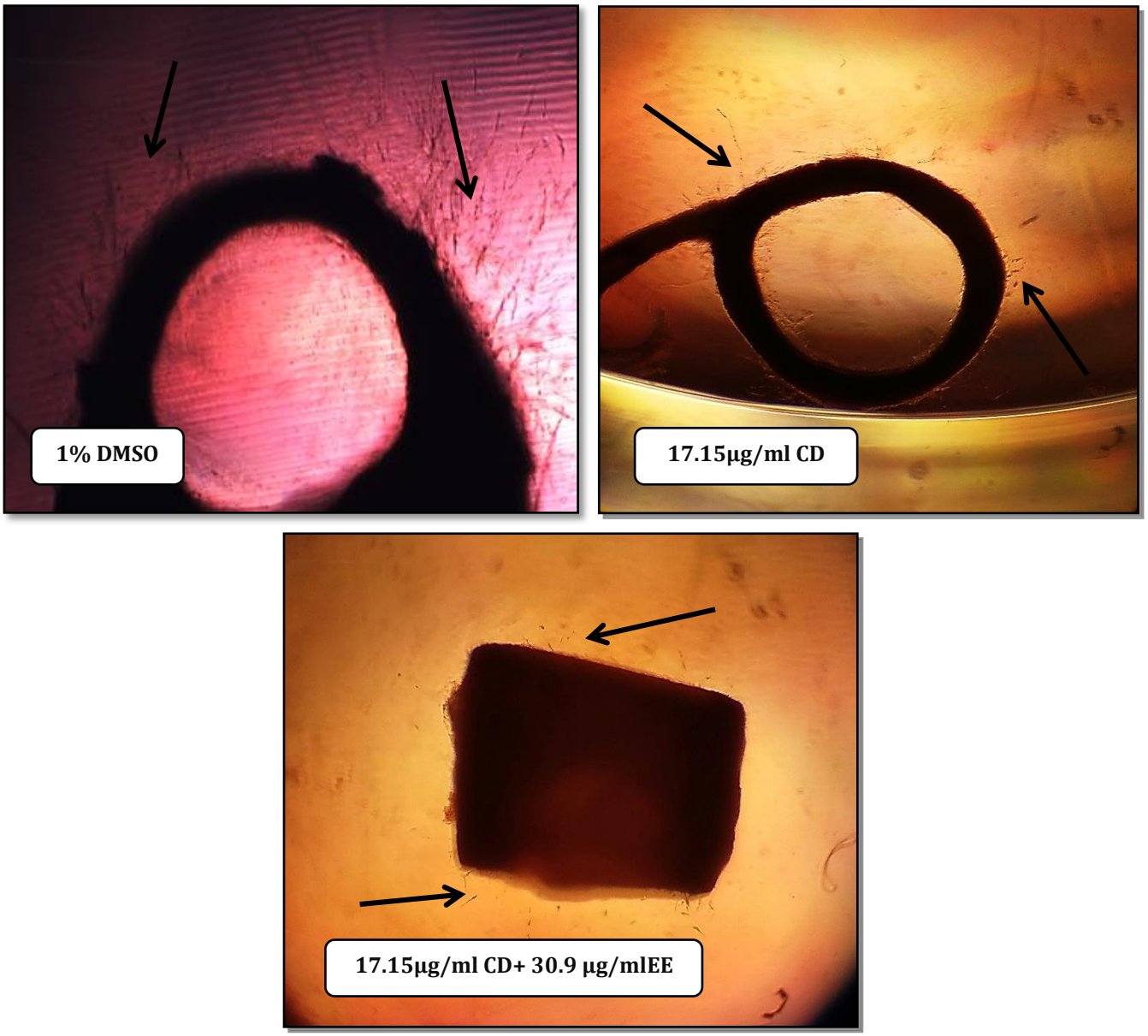

Image (1): The effect of 17.15 µg/ml of the synthetic chalcone derivative and the mixture of 17.15 µg/ml of the synthetic chalcone derivative and 30.9 µg/ml of ethanolic extract of *P. dactylifera* seeds in *ex-vivo* rat aorta ring model. 1% DMSO was used as negative control. The results were obtained at the fifth day of the experiment and the black arrows indicate the growth of micro-blood vessels.
(DMSO= dimethyl sulfoxide,CD= synthetic chalcone derivative, and CD+EE= the mixture of the synthetic chalcone derivative and ethanolic extract of *P. dactylifera* seeds)

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Discussion:

The process of recent blood vessels formation from pre-existing ones is 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 blood 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 the synthetic chalcone derivative alone and its mixture with ethanolic extract of *P. dactylifera* seeds were significantly inhibited the growth of micro-blood vessels in RAR model at the previously mentioned concentrations, but the mixture of both (CD+EE) revealed much higher inhibition of the micro-blood vessels in comparison tothat of CD. The concentrations of CD and EE were selected due to previous studies done by Abu Raghif, 2016 on P-hydroxychalcone and Al-Zubaidy et al, 2016 on organic extract 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 EE [(20)].Furthermore, the DPPH assay revealed a remarkable anti-oxidant activity of the EE which considered very important in the inhibition of the angiogenesis process since the presence of higher levels of free radicals can stimulate this process[(18)]. As for the ability of CD to inhibit the growth of blood vessels, it was proved in a study done by Abu Raghif, 2016[(17)]. Several hydroxylated chalcone derivativeswere shown to possess a potent anti-angiogenic activity as well as anti-proliferative activity by suppressing TNF-α in HUVECs that 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 CD+EE was probably due to the presence of a synergism and the potentiation effect between CD and the constituents of the ethanolic extract through targeting different signaling pathways of the angiogenesis process. These chemical constituents may exert a direct anti-angiogenic 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 CD and EE 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 mixture of CD and EE showed significant anti-angiogenic activity which makes it promising therapeutic agents for diseases associated to angiogenesis[(24)].

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Conclusion:

This study revealed asignificant anti-angiogenic activity of the mixture of the synthetic chalcone derivative and ethanolic extract of *P. dactylifera* seeds more than that of the synthetic chalcone derivative alone. The effect produced could be due tothe synergism between the synthetic chalcone derivative and the phytochemical constituents present in ethanolic 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.

Acknowledgment:

Authors would like to acknowledge assistant professor Dr. Hayder Bahaa Sahib, deputy dean of college of pharmacy/Al-Nahrain University for his great help in this research. Special thanks to the head of pharmacology department professor Dr. Ahmed Rahma Abu Raghif for providing the required facilities to finish this research.

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