Research Article

The anti-Antiangiogenic activity of *Phoenix dactylifera* Methanol Extract Combined with 3-hydroxy-2, 3-dihydro-2-phenylchromen-4-one

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ABSTRACT

Angiogenesis process well-defined as the formation of new blood vessels from existing one, many diseases related to this process. This study designed to investigate the anti-angiogenic effect of 3-hydroxy-2, 3-dihydro-2-phenylchromen-4-one solely and when combined with methanol extract of *Phoenix dactylifera* seeds extract. The seeds powder of P. dactylifera was extracted with methanol by maceration method. Anti-angiogenic activity of 3-hydroxy-2, 3-dihydro-2-phenylchromen-4-one and methanol extract of *P. dactylifera* seeds methanol extract were screened by *ex-vivo* rat aorta ring model. (25µg/ml and 28.4µg/ml) of 3-hydroxy-2, 3-dihydro-2-phenylchromen-4-one and *P. dactylifera* seeds methanol extract were tested respectively on blood vessels. The two agents significantly inhibited blood vessels growth in rat aorta ring assay when they were compared to the negative control ($P<0.05$), and the combination of both agents showed highly significant inhibition on blood vessels sprouting ($P<0.01$) in comparison to the percentage of blood vessels inhibition of the two agents solely. The high efficacy of the combination may be related to the synergistic activity, and the effect produced could be due to inhibiting certain signalling pathways in the angiogenesis process.

Keywords: Angiogenesis, *P. dactylifera* seeds, 3-hydroxy-2, 3-dihydro-2-phenylchromen-4-one, synergism.

INTRODUCTION

This study conducted to identify the synergistic activity of a combination of two agents had been approved to have anti angiogenic activity in previous study, angiogenesis one approach of tumour treatment; finding agent or combination of agents targeting angiogenesis process as a mean to treat tumour rather than use a chemotherapy or combining these agents with chemotherapy to reduce the chemotherapy dose and reduce the adverse effect may appear due to the cytotoxic activity is the actual target of this study. Scientist defined angiogenesis as the formation of a blood vessels from pre - existing ones [1]. Angiogenesis process is started by activation of endothelial cells of blood vessels in reaction to angiogenic stimuli. Angiogenesis is usually begun within hypoxic tissues which need additional new blood vessels to maintain oxygen supply [2]. Hypoxia is the main triggers to cellular oxygen sensing mechanisms, which lead to persuade gene expression of various pro – angiogenic proteins. Anti-angiogenic therapy is an anti-cancer approach that targets the new vessels that grow to deliver oxygen and nutrients to actively multiplying tumour cells. The main stimulated factors are hypoxia inducible factors (HIFs); it regulates multiple pro – angiogenic genes[3]. Vascular endothelial growth factor – A (VEGF-A) is the main regulated genes and in charge on the angiogenesis progression [4]. The majority of anti-cancer drugs currently used in the clinical setting extensively target all rapidly proliferating cells, and that lead to sever adverse effects such as immune suppression, intestinal problems and hair loss. While, anti-angiogenic drugs have fewer side effects because, neoangiogenesis infrequently occurs in healthy adults except in the uterine and endometrium. Currently, the most conventional approach for limiting tumour angiogenesis is blockade of the vascular endothelial growth factor (VEGF) path. The effects of VEGF blockers have been stated in various types of human cancers, including patients with progressive/recurrent cancer who could not otherwise be cured [5]. Angiogenesis developments are stimulated in firmly distinct conditions, chiefly when physiological conditions request an increase in the blood amount as in wound healing; and pathologically as in cancer, psoriasis, rheumatoid arthritis, age related macular degeneration and other angiogenesis related physiological changes [6, 7]. In this study author chosen 3-hydroxy-2, 3-dihydro-2-phenylchromen-4-one its structure related to flavonoids and natural product which full of flavonoids, as many studies investigating the relationship between flavonoid consumption and cancer treatment had been approved the efficiency of flavonoid in targeting the cancer cell proliferation[8, 9]. *Phoenix dactylifera* known as date palm, cultivated for its sweet fruit, 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 Palmacea. In folkloric medicine dates are considered a tonic and some consider it as an aphrodisiac [10]. They can be used for sore throat and colds; relief of fever, cystitis, oedema, liver and abdominal problems, the gum or exudates of dates is used for treating diarrhoea and the roots are used to treat toothache [11]. The aim of this study was to study the anti-angiogenic effect of the combination of methanol extract of *P. dactylifera* seeds and 3-hydroxy-2, 3-dihydro-2-phenylchromen-4-one at selected concentrations using the *ex-vivo* rat aorta ring assay.

MATERIALS AND METHODS

Materials

3-hydroxy-2, 3-dihydro-2-phenylchromen-4-one was obtained from college of medicine pharmacology department. The seeds of *P. dactylifera* were collected from Al Etifea Dates Factory/ Baghdad/Iraq. Seeds specimen was labeled and annotated with date of collection and locality. Voucher specimen number (2) was deposited at the Herbarium, College of Medicine Al Nahrain University

Extraction Process

Five hundred grams seeds of *P. dactylifera* were measured, the seeds were rinsed with tap water and left to dry in oven at 40℃. The dried seeds were ground into fine powder by stainless steel grinder. The powder was then extracted with methanol with a ratio of 1:4 using the cold method (Macerations). The extract was stored in dry and tightly sealed container to be use in the experiment [12].

Laboratory Animals

Male albino rats were obtained from the animal house /Al-Nahrain University. The age of the rats were12 – 14 weeks old and they were kept at the transiet animal house with temperature of 28 – 30°C all rats let free access to water and food. The experiments procedures and environment were approved by the Animal Ethical Committee adapted by Al-Nahrain University / College of Medicine / Baghdad-Iraq.

Ex-vivo Rat Aorta Ring Assay

The assay was conducted according to the protocol developed by Brown and coworkers [13], with minor modifications. The aorta was excised, and cleaned from the fibro-adipose tissue then sliced into rings each of 1 mm thickness. 3mg/mL of fibrinogen and 5µg/ml of aprotinin was added to M199; 300 µl of M199 medium was loaded in each 48-well plate where the aortic ring was seeded in each well. 10 µl of thrombin added (50 NIH U/mL in 0.9% (W/V) NaCl), then incubated at 37°C in 5% CO_2 for 15-20 min in the incubator. M199 medium containing 20% of heat inactivated fetal bovine serum (HIFBS), 1% L-glutamine, 0.1% amino caproic acid, 1% amphotericin B and 0.6% gentamicin prepared to dissolve the extract and 3-hydroxy-2, 3-dihydro-2-phenylchromen-4-one at concentrations of 28.4µg/mL for methanol extract and 17.15µg/ml for 3-hydroxy-2, 3-dihydro-2-phenylchromen-4-one (PHC) [12]. Then combinations of ME and PHC at the mentioned concentrations were tested against blood vessels growth; each treatment was performed in six replicates. The 48 plate which contain the tissue rings were kept at in a humidified incubator with 37°C, 5% CO_2. The DMSO (1% v/v) was used as a negative control. The results collected on day five by taking photo to the blood vessels using inverted microscope, and the blood vessel out growth was quantified. Blood vessel growth inhibition was calculated according to Hayder and coworker in 2014 [14]. The results are presented as the mean percent inhibition to the negative control ± SD. The experiment was repeated three times (n=18). The percentage of blood vessels inhibition was determined according to the following formula:

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 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 ($P < 0.05$). The statistical analysis was carried out by using SPSS version 17.0.

RESULTS

Extraction Process

Extraction of *P. dactylifera* seeds powder with methanol gave a yield percentage of 10%, which represents 50gm of the crude extract per 500gm of the actual powder.

Anti-Angiogenic Activity (*ex-vivo* Rat Aorta Ring Assay)

Figure 1 and figure 2 showed that each of methanol extract (ME) at concentration of 28.4µg/ml and 3-hydroxy-2, 3-dihydro-2-phenylchromen-4-one (PHC) 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 57.29% $\pm$ 2.9% and 53.43% $\pm$ 3.4% respectively. The combination of methanol extract and 3-hydroxy-2, 3-dihydro-2-phenylchromen-4-one (ME+PHC) at above mentioned concentrations gave significant inhibition of blood vessels growth when compared to the negative control ($P<0.01$) as well as when compared to both methanol extract and 3-hydroxy-2, 3-dihydro-2-phenylchromen-4-one alone ($P<0.05$); and the percentage of blood vessels inhibition of the combination was 86.77% $\pm$ 1.3.

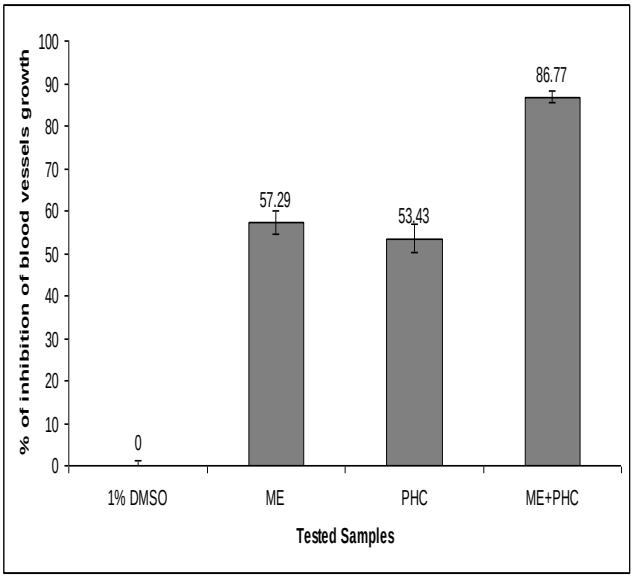

Figure 1: The anti-angiogenic activity of 28.4µg/ml methanol extract of P. dactylifera seeds, 17.15µg/ml 3-hydroxy-2, 3-dihydro-2-phenylchromen-4-one and the combination of methanol extract and 3-hydroxy-2, 3-dihydro-2-phenylchromen-4-one at the respective concentrations. 1% DMSO was used as a negative control and the results were taken at day 5 of the experiment (DMSO= dimethyl sulfoxide, ME= methanol extract, PHC= 3-hydroxy-2, 3-dihydro-2-phenylchromen-4-one, and ME+PHC= methanol extract and 3-hydroxy-2, 3-dihydro-2-phenylchromen-4-one combination

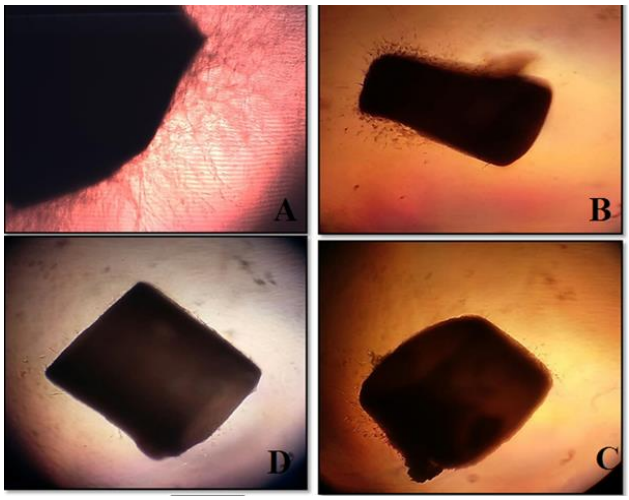

Figure 2: The effect of each of 28.4µg/ml methanol extract of *P. dactylifera* seeds, 17.15µg/ml 3-hydroxy-2, 3-dihydro-2-phenylchromen-4-one and the combination of methanol extract and 3-hydroxy-2, 3-dihydro-2-phenylchromen-4-one of the respective concentrations on rat aorta ring model. The results were taken at day 5 of the experiment. 1% DMSO was used as negative control. (A) represent the negative control which receive (1% DMSO= dimethyl sulfoxide, (B) represent methanol extract, (C) represent 3-hydroxy-2, 3-dihydro-2-phenylchromen-4-one, and (D) represent the combination of methanol extract and 3-hydroxy-2, 3-dihydro-2-phenylchromen-4-one.

DISCUSSION

Angiogenesis, the new generation of blood vessels, is necessary for many diseases such as tumors to keep developing and dissemination [15] this was the reason behind testing a combination of natural product had antagonise this process with 2, 3-dihydro-2-phenylchromen-4-one, its structure related to flavonoids. Flavonoids have been found to act as chemo-preventive agents in numerous epidemiological studies and have been shown to inhibit angiogenesis and proliferation of tumor cells and endothelial cells *in vitro* [16]. Adeeb and coworkers in previous study approved that *P. dactylifera* seeds extracts had showed a significant anti-angiogenesis activity; the inhibitory concentration which inhibits fifty percent of the new grown blood vessels was identified by Adeeb and coworkers at that study and used in this study in combinination with 2, 3-dihydro-2-phenylchromen-4-one [10]. Angiogenesis requires tightly controlled extracellular matrix degradation mediated by extracellular proteolytic enzymes including matrix metallo proteinases (MMPs) and serine proteases, in particular, the urokinase-type plasminogen activator (uPA)-plasmin system [17]. In this study both the extract and 2, 3-dihydro-2-phenylchromen-4-one showed significant anti angiogenic activity against aorta ring when tested solely, but when combined together showed high significant inhibition activity and this may be due to the synergism that happened upon combination [17], what this study found is agreed with Adeeb and collogues as they found that methanol extract of *P. dactylifera* had significant anti-angiogenesis activity in 2016 and that was may be releated to the existence of antioxidant constituents [10]. 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 [15]. Moreover, 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 [18]. Al-Farsi and co-workers in 2007 showed 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 [19]. However, the mechanism behind the anti angiogenic effect of flavonoids is unclear. A possible mechanism could be happened through the inhibition of protein kinases; these enzymes are implicated to play an important role in signal transduction and are known for their effects on angiogenesis [20]. From above, the proposed mechanism(s) of action which may elucidate the results of this study, is the synergism effect which occur due to the combination of *P dactylifera* and 2, 3-dihydro-2-phenylchromen-4-one. The synergisim activity showed in the combination may

be related to the potential anti-oxidants activity in both agents; which may explain the high efficacy showed in the results [21].

CONCLUSION

The synergistic activity observed in this study could be attributed to the existence of antioxidant compounds in the *P. dactylifera* seed methanol extract, which inhibit the Reactive oxygen species. (ROS) are known mediators of intracellular signaling cascades. 3-hydroxy-2, 3-dihydro-2-phenylchromen-4-one is known flavonoid; and flavonoids stabilize the reactive oxygen species by reacting with the reactive compound of the radical. The above proposed mechanism may explain the results showed in this study.

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