Research Article

The Anti-Angiogenic Activity of *P*-Hydroxy Chalcone

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ABSTRACT

The objective of the study is to investigate the possible anti-angiogenic activity of p-hydroxychalcon derivative and its free radical scavenging activity. The synthetic p-hydroxychalcon derivative was screened for possible anti-angiogenic activity using the *ex vivo* rat aorta ring assay, then followed by the concentration-effective response study using the *ex vivo* rat aorta ring assay to determine the IC_{50} of the compound. Free radical scavenging activity for the compound was determined using the 2, 2-diphenyl-1-pecrylhydrazyl assay. The screening assay revealed that 100µg/ml of p-hydroxychalcon significantly and completely inhibited micro-blood vessels growth when compared to the negative and positive controls ($P<0.001$). The concentration-effective response study showed a significant dose dependent inhibition of micro-blood vessels growth ($P<0.05$) with an IC_{50} of 17.15µg/ml. The free radical scavenging activity revealed significant concentration dependent reduction of DPPH radical by p-hydroxychalcon ($p<0.05$) and with an IC_{50} of 1178µg/ml. This study showed that the synthetic p-hydroxychalcon derivative exhibited a significant and remarkable anti-angiogenic activity, but its free radical scavenging activity was weaker. The anti-angiogenic activity of this compound may be due to its ability to inhibit certain steps of the angiogenesis process.

Keywords: Angiogenesis process, DPPH, *P*-Hydroxy Chalcone.

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[3] When the tissue is hypoxic, cellular oxygen sensing mechanisms are activated, which induce gene expression of various pro – angiogenic proteins. The primary activated factors are HIFs (hypoxia inducible factors) which in turn they up – regulate multiple pro – angiogenic genes directly or indirectly[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. This process follows vasculogenesis and involves the differentiation and organization of endothelial cells into capillary tubes and the interplay between growth factors and cytokines[6]. Cell adhesion molecules generally mediate cell–cell and cell–matrix interactions. These, in conjunction with the recruitment of supporting pre-endothelial cells that encase the endothelial tubes, provide maintenance and modulatory functions to the vessel. Supporting cells usually include pericytes in small capillaries and smooth muscle cells in larger vessels[7]. In recent decades, numerous studies focused on identifying several angiogenic factors. Angiogenic factors can be categorized as follows: (A) soluble growth factors such as 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]; (B) factors that inhibit the proliferation and enhance the differentiation of endothelial cells, such as transforming growth factor β (TGF-β), angiogenin, and several low molecular weight substances[16]; and (C) extracellular matrix-bound cytokines that are released by proteolysis, which may contribute to the regulation of angiogenesis and include angiostatin, thrombospondin, and endostatin. In addition, a number of microphages secreting bFGF, tumor necrosis factor (TNF), and VEGF were shown to be associated with tumor angiogenesis[14]. In adults, formation and growth of new blood vessels are under strict control[4]. 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[2]. 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; like cancer, rheumatoid arthritis, psoriasis, ischemic heart disease and others[4]. Chalcones, considered as the precursors of flavonoids and isoflavonoids are widely present in edible plants. Chemically, they consist of open-chain flavonoids in which the two aromatic rings are joined by a three-carbon α,β-unsaturated carbonyl system. Among the flavonoids, chalcones are considered as interesting target class of compounds which are extensively investigated due to their broad spectrum of biological activities, including anti-inflammatory, anti-invasive and antitumour and antibacterial properties[17]. Depending on the substitution pattern in two aromatic rings, a wide range

of pharmacological activities have been reported such as antibacterial, antifungal, anti-malarial, anti-leishmanial, antioxidant, anti-inflammatory and anticancer activity[11]. One of the mechanisms of the anticancer activity of chalcones is suppression of angiogenesis. Many natural and synthetic chalcones with hydroxyl moiety are reported to possess anti-angiogenic activity[12].

Several strategies such as the Claisen-Schmidt, Knoevenagel condensations and the Meyer-Schuster rearrangement were reported earlier for the synthesis of chalcone system and all are based in general on the formation of carbon-carbon bond and here it is the Enone moiety (i.e. α,β-unsaturated ketone). Among other strategies, the Claisen-Schmidt condensation appeared to be the most appealing one, where it involves the condensation of an aromatic ketone with an aromatic aldehyde in the presence of suitable condensing agents; accordingly, a variety of methods are available for the synthesis of the chalcones that employs this type of approach and the most important and simplest one is the condensation done under acidic conditions (HCl) produced by using SOCl2/EtOH as a catalyst and followed by dehydration to yield the anticipated chalcone derivative[8].

The objective of this study was to investigate the possible anti-angiogenic activity of the synthetic PHC using the ex vivo rat aorta ring model and to determine its free radical scavenging activity.

MATERIALS AND METHODS

Materials

P-Hydroxychalcone was obtained from college of Pharmacy/University of Baghdad/Department of Pharmaceutical Chemistry; and it was synthesized according to Claisen-Schmidt condensation method[8].

Experimental animals

Male albino rats were obtained from the animal house of Institute for diagnosis of infertility and assisted reproduction techniques/Al-Nahrain University. The rats were aged about 12 – 14 weeks old and they were kept in an environment with a temperature of 28 – 30°C and they had free access to water and food. The experiments were approved by the Animal Ethical Committee Al-Nahrain University College of Medicine/Baghdad-Iraq.

Ex-vivo Rat Aorta Ring Assay

The assay was performed according to the standard protocol developed by Brown and his colleagues[1], with minor modifications. Twelve to fourteen weeks old Albino male rats were used and 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 fibroadipose 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 3mg/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, 10 µl 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% CO2 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 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% CO2 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 acetyl salicylic acid "Aspirin" (100µg/mL) were used as negative and positive controls respectively. The results examined on day 5 under inverted microscope and the extent of blood vessel growth was quantified under 40 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 coworkers (1997)[13]. 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). The percentage of blood vessels inhibition was determined according to the following formula:

Blood vessels inhibition = 1- (A_0/A) ×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 with *ex-vivo* Rat Aorta Ring Assay

Serial dilutions of the tested substance were prepared in the following concentrations: 200, 100, 50, 25, 12.5 and 6.25µg/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[10].

Free Radical Scavenging activity with DPPH Assay

The free radical scavenging activity of the tested substance 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 (1000, 500, 250, 125, 62.5, 31.25, 15.625 and 7.813 µg/ml) and incubated for 30 min. This procedure was executed using 96 well plate and each concentration was tested in triplicate, 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 100µl of methanol and 200µl DPPH. The percentage of antioxidant activity (AA) was calculated according to the formula below[15]:

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

A_S = absorbance of sample, A_B = absorbance of blank

A_C = absorbance of control

Statistical Analysis

The 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 concentration that inhibited 50% of blood vessels and caused reduction of free radicals (IC_{50}) was calculated using logarithmic equations and linear regression equations. The statistical analysis was carried out by using SPSS version 16.0.

RESULTS

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

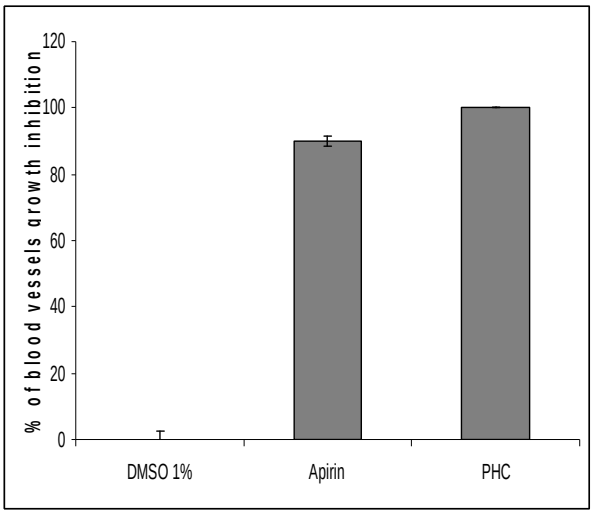

Figure 1: the anti-angiogenic activity of 100µg/ml of PHC on rat aorta ring model in comparison to negative control and 100µg/ml of positive control DMSO= dimethylsulfoxide, PHC= p-hydroxychalcone

Statistical analysis revealed that 100µg/ml of p-hydroxychalcone was able to suppress microblood vessels growth completely (100%) when compared to the negative control "received DMSO 1%", as well as when compared to the positive control "received aspirin" which gave an inhibition percentage (90% ± 1.48) ($P<0.05$). the results were taken at day 5 of the experiment and the experiment was repeated three time in six replicates (n=18). Figure (1) and Image (1) show these results.

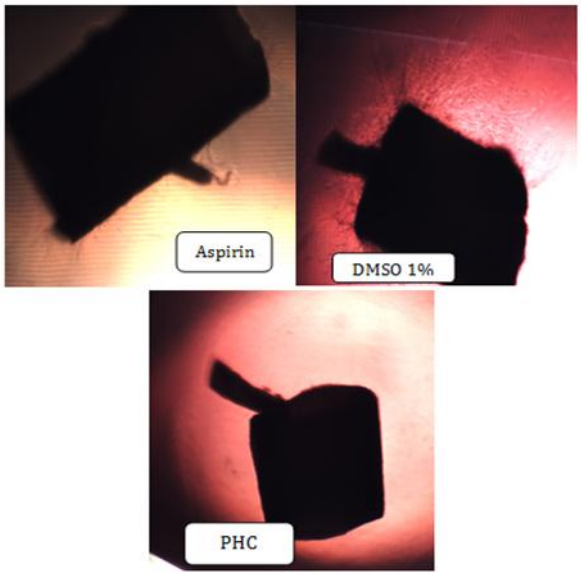

Image 1: the anti-angiogenic activity of 100µg/ml of PHC on rat aorta ring model. DMSO 1% represents the negative control while Aspirin represents the positive control.

DMSO= dimethylsulfoxide, PHC= p-hydroxychalcone

Dose response study of PHC on *ex-vivo* Rat Aorta Ring Assay

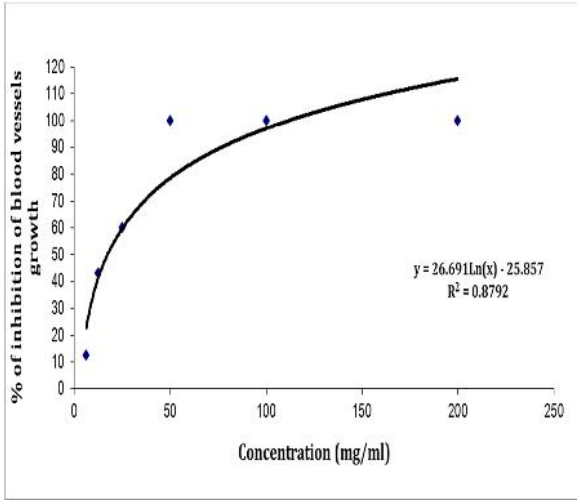

Figure 2: Dose response curve of PHC serial dilution on rat aorta ring model. (PHC= p-hydroxychalcone)

Six serial dilutions of PHC were prepared and added to the seeded rat aortic rings in the following concentrations 200, 100, 50, 25, 12.5 and 6.25µg/ml. The results revealed significant dose dependent inhibition of blood vessels growth at day 5 of the experiment ($P<0.05$). The percentages of inhibition for each concentration were represented as mean ± SD as follows: 100%, 100%, 100%, 60.11% ± 2.67, 43.17% ± 2.99 and 12.57% ± 2.12 for the mentioned concentrations respectively. The IC50 was calculated from the logarithmic equation (Y= 26.691Ln(x) – 25.857) and it was found to be 17.15µg/ml. Y= % of

inhibition of blood vessels growth and X= concentration (µg/ml). Figure (2) and Image (2) represent these results.

Image 2: Dose response effect of PHC serial dilutions on rat aorta ring model. DMSO 1% serves as negative control. (DMSO= dimethylsufoxide, PHC= p-hydroxychalcone)

Free Radical Scavenging Activity of PHC with DPPH Assay

Figure (3) shows the effect of PHC serial concentrations on the DPPH free radical. The results revealed concentration dependent reduction in DPPH free radical ($P<0.05$). Eight serial concentrations of PHC were used ranging (1000, 500, 250, 125, 62.5, 31.25, 15.625 and 7.813µg/ml) and the free radical reduction percentages were represented as mean +

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