

## RESEARCH ARTICLE

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# The Anti-Angiogenic and Anti-Proliferative Activity of Methyl Hydroxychalcone

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### Abstract

**Objective:** Angiogenesis plays a key role in tumor growth, invasion, and metastasis of cancer diseases, and therefore, the inhibition of angiogenesis can provide an important therapeutic approach in cancer diseases. The aim of this study was to investigate the inhibitory effects of methyl hydroxychalcone on ex vivo sprouting of rat aortic micro-vessels and in vivo formation of chorionic plexus in chick chorioallantoic membrane and to investigate the mechanism underlying anti-angiogenic activity. **Methods:** Rat aortic rings were sectioned by 1 mm. 500µl of 3 mg/ml of fibrinogen in serum free M199 growth medium was added to each well with 5 ug/ml of aprotinin. Methyl hydroxychalcone at varying concentrations ranging from 6.25 µg/ml to 100 µg/ml was added to the complete growth medium. Fertilized chicken eggs were incubated at 37°C. On day 3, a small window was opened in the shell. The window was sealed with adhesive tape and incubated until day 5. One mg of methylhydroxychalcone was applied. Images of each CAM were captured using a digital camera, and the dimensions of the blood vessels were measured digitally. Vascular endothelial growth factor (VEGF)-induced human umbilical vein endothelial cell (HUVEC) proliferation and tube formation assays were examined. Additionally, VEGF-165 levels and expression of membrane VEGF receptor-2 in HUVEC lysates have been assessed. **Results:** The data showed that methyl hydroxychalcone significantly had antiangiogenic activity in a dose dependent manner in the rat aorta assay and had significant perturbation activity on blood vessels in the CAM assay. Methyl hydroxychalcone significantly inhibited proliferation and capillary-like tube formation in VEGF-induced HUVEC. Moreover, methylhydroxychalcone significantly reduced VEGF-165 levels in HUVECs lysate. **Conclusion:** This study showed that methyl hydroxychalcone significantly inhibits the angiogenesis process, blocking the VEGF signaling pathway in HUVECs and could be a potential promising angiogenesis inhibitor lead compound.

**Keywords:** Angiogenesis- endothelial- chorioallantoic- methylhydroxychalcone

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### Introduction

Angiogenesis is a strictly controlled process defined as the formation of new blood vessels essential for certain physiologic and pathologic conditions, where the latter includes tumor growth, development, and metastasis (Hoseinkhani et al., 2020). This study tested methylhydroxychalcone for the first time and tested the probable mechanism of action. Angiogenesis occurs physiologically during different life stages, such as fetal growth, while in adolescence it is usually expressed in pathological conditions such as tumor growth, osteoporosis, and rheumatoid arthritis. Angiogenic factors such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), and transforming growth factor-beta 1 (TGF-beta 1) are released to stimulate nearby blood vessels to provide oxygen and nutrients, which can lead to tumor expansion (Sahib et al., 2014). The process of angiogenesis starts in response to many diseases such as wounds and ischemia. Once that happens,

the basement membrane of blood vessels is degraded and, subsequently, the blood vessels become hyper-permeable plasma proteins. This vascular hyper-permeability led to leakage of extracellular membrane proteins. The following stages will occur: (1) degradation of vascular basement membrane and activation of endothelial cells (ECs); (2) sprouting and proliferation of ECs within the extra-cellular membrane; (3) a vascular tube is formed. (4) Vascular tube coverage with mature vascular basement membrane and supporting pericytes (Sahib et al., 2009). From the above information, the angiogenesis process is considered the main cause of tumor development and may lead to death. From that point forward, scientists and their colleagues worked tirelessly to identify lead compounds that could aid in disrupting this dangerous process, as well as reduce the negative impact on lifestyle and the threat to the health system (Khamees et al., 2018). Scientists tried to invent agents that were able to stop blood supply to the tumor and make hypoxia that led to the death of tumor cells (Bergers and Benjamin, 2003). Through inhibition of

VEGF signaling, blockade of the angiogenic process has been shown to result in significant tumor growth delay in a wide range of preclinical models (Ferrara et al., 2003), (Mukwaya et al., 2021). Since a close relationship between tumor growth and angiogenesis has been clarified, various angiogenic inhibitors for use in cancer treatment have been studied. In this study, methyl hydroxychalcone was found to be a chalconoid found in many natural herbs, such as cinnamon (Wedge et al., 2002). Chalcones are flavonoids that are commonly biosynthesized in herbs. Chalcones are found to play an important role in giving protection against many pathogens. Scientific research has shown that chalcones show antioxidant, anticancer, antimicrobial, antiprotozoal, antiulcer, antihistaminic, and anti-inflammatory activities. Many compounds have been developed based on the chalcone structure used in various pharmacological activities. Clinical trials have shown that these compounds have reasonable safety; that's why; chalcones have become an item of importance for researchers. Chalcones are used for the treatment of many illnesses caused by viral disorders, such as cardiovascular diseases, parasitic infections, pain, gastritis, and stomach cancer (Mukwaya et al., 2019), (Abdolmaleki et al., 2016), (Batovska & Todorova, 2010). To this effect, the highlights of main contributions in this paper are:

- The anti-angiogenic effects of hydroxylmethylchalcone in HUVEC culture (in vitro model) were discussed.
- In this study, we studied the anti-angiogenic activity of hydroxylmethylchalcone in chick CAM as well as in vivo.
- It also explains the effects of VEGFR, which is thought to be an important molecular marker for angiogenesis (proliferation and tube formation) and possible mechanisms of action.

## Materials and Methods

### Cell Culture and Reagents

Endothelial Cell Medium (ECM) supplied with endothelial cell growth supplements (ECGS) was purchased from Science cell, USA. M199, RPMI 1640, trypsin and heat inactivated fetal bovine serum (HIFBS) were obtained from GIBCO, UK. Human VEGF assay kit was purchased from IBL, Japan. Phosphate buffered saline (PBS), penicillin/streptomycin (PS) solution, MTT reagent, (Sahib et al., 2015) suramin, vincristine, amphotericin B, aprotinin, 6-aminocaproic acid, L-glutamine, thrombin, and gentamicin were purchased from Sigma-Aldrich, Germany. Fibrinogen was purchased from Calbiochem, USA and Matrigel matrix (10 mg/mL) was purchased from BD Bioscience, USA. Other chemicals used in this study were analytical grade (Sahib et al., 2014). Human umbilical vein endothelial cell line (HUVECs) was purchased from Science Cell, USA. HUVECs were maintained in ECM medium supplemented with 5% HIFBS, 1% PS and 1% ECGS. Methyl hydroxychalcone had been purchased from Sigma Aldrich (Nassar et al., 2011; Sahib et al., 2015).

### Chick embryo chorioallantoic membrane assay

Antiangiogenic effect of the methylhydroxychalcone

was investigated in vivo using CAM assay. Fertilized chicken eggs were incubated in an incubator as soon as embryogenesis started and were kept under constant humidity at 37°C. On day 3, a square window was opened in the shell after removal of two ml of albumen to detach the CAM from the shell. The window was sealed with transparent adhesive tape and incubation continued until the day of experiment. The embryo and its extra-embryonic membranes were transferred to a Petri dish on day 4 of incubation. CAM developed at the top as a flat membrane and reached the edge of the dish to provide a two-dimensional monolayer onto which the methyl hydroxychalcone was placed (Sahib et al., 2016). One mg of the methylhydroxychalcone was applied onto each 6 mm disc of Whitman filter paper and then the discs were allowed to dry at 45-50 °C. The loaded and dried discs were inverted and placed on the CAM. Images of each CAM were captured using a digital camera, and the dimensions of each CAM were recorded (West and Burbridge, 2009).

### Aortic ring assay

Rat aortic ring explant cultures were prepared in accordance to protocols previously described by Zhu et al., (2000). Aortic rings were prepared from male Sprague Dawley rats. Aortas were sectioned into 1 mm long cross sections, rinsed several times with Hanks balanced salt solution containing 2.5 µg/ml amphotericin B (Nassar et al., 2011). The assay was performed in a 48-well tissue culture plates (Costar Corning, USA). 500 µl of 3 mg/ml fibrinogen in serum free M199 growth medium was added to each well with 5 µg/ml of aprotinin to prevent fibrinolysis. Each tissue section was placed in the center of the well and 10 µl of thrombin (50 NIH U/ml) in 0.15 M NaCl. Immediately after embedding the vessel fragment in the fibrin gels, 0.5ml of medium M 199 supplemented with 20% HIFBS, 0.1% ε-aminocaproic acid, 1% L-Glutamine, 2.5 µg/ml amphotericin B, 60 µg/ml gentamicin were added to each well. Methyl hydroxychalcone at varying concentrations ranged from 6.25 µg/ml to 100 µg/ml was added to the complete growth medium. Control cultures received medium with the vehicle only (DMSO). Suramin a well-recognized anti-angiogenic agent was used as a positive control. Cultures were incubated at 37°C for 5 days, in humidified CO<sub>2</sub> and the medium was replaced day 4.

The magnitude of blood vessel outgrowth on day five was quantified (12) using inverted microscope (Olympus, Japan) supplied with a digital camera (Leica CCD, Japan) and Leica Qin computerized imaging software. All experimental work was consistent with guidelines of the College of pharmacy/ Al-Nahrain University Committee for Animal Care and received approval from the college of pharmacy Animal Ethical Committee for the present work (Reference Number Col/Ph/AlNah./7).

### Vascular endothelial growth factor-induced proliferation assay

Human umbilical ventricular endothelial cells HUVECs maintained in ECM containing 5% HIFBS and 1% PS, 1% ECGS. The cells were seeded in 96- well

plates at the density of  $2 \times 10^4$  cells/well in 100  $\mu$ l growth media and allowed to attach for overnight. Cells were exposed to a test sample for 48 h (Nicosia et al., 1992). After the period of incubation, the viability of HUVECs was assessed by MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (Moon et al., 2003). 20  $\mu$ l of MTT solution (5 mg/ml in PBS) was added to each well. After incubation for 4 h, the mixed media and MTT solution were carefully discarded, and then the crystallized dye was solubilized with DMSO. Vincristine was used as reference standard. The amount of blue dye formed was determined by measuring the absorbance at 570 nm.

#### VEGF-induced Tube formation assay

HUVECs were harvested and seeded in ECM medium (5% HIFBS) containing VEGF (100ng/ml) onto 4- well culture plates coated with 150  $\mu$ l Matrigel (5 mg/ml). The cells were treated with various concentrations of methylhydroxychalcone and incubated at 37°C for 24 h. Suramin was used as a positive control at 10  $\mu$ g/ml in the growth medium. After treatment, the media was discarded, and the cells were washed twice with Hank's balanced salt solution and stained with Calcein AM (8  $\mu$ g/ml) for 45 min at 37°C, under 5% CO<sub>2</sub>. The dye was discarded, and cells were washed twice to remove excess dye. The cells were imaged under an inverted fluorescence microscope at low magnification. The web junctions, defined as intersections of three or more tubes, were counted in each microscopic field (Bandyopadhyay, 2002). The percentage of inhibition was represented as the mean  $\pm$  S.D.

#### Quantification of vascular endothelial growth factor

The level of VEGF-165 in HUVEC lysates was measured using commercial human VEGF ELISA kit following the instructions of the manufacturer. HUVECs were seeded in 6-well plates at  $1 \times 10^6$  in 3 ml ECM and incubated for cell attachment for 24 h. Cells were treated with the methylhydroxychalcone (25 and 50  $\mu$ g/ml) for 6 h, and concentration of VEGF-165 was determined of each cell lysate. Calibration curve of VEGF standard was used to calculate concentration of VEGF of the samples. The results are presented as mean  $\pm$  S.D (n=3).

#### Statistical analysis

The results were expressed as the mean  $\pm$  standard deviation (S.D.) and the statistical significance was evaluated by using the student's t-test. P-values < 0.05 implied significance.

## Results

#### Chicken egg chorionallantoic membrane assay

Vascularization in chick embryo was significantly inhibited by the methylhydroxychalcone. Images of two chorioallantoic membranes are shown in Figure 1. The vasculature pattern formed by the blood vessels in CAM of control group treated with vehicle was normal. The primary, secondary and tertiary vessels with the dendritic branching pattern, which is characteristic of CAMs was well established in control CAMs and can be seen clearly (Figure 1A). On the other hand, the

methylhydroxychalcone treated CAM (1 mg/disc) showed the distorted architecture in vasculature (Figure. 1B).

#### Effect of methylhydroxychalcon on vessel sprout formation from rat aorta

Figure two showed that, micro-vessels grew out extensively from the rat aorta in the control (Figures 2A and 2B) when cultured in the medium, whereas, the methyl hydroxychalcone significantly inhibited sprouting of rat aortic micro-vessels (Figure 2C) with IC<sub>50</sub> (concentration of test substance to achieve 50% inhibition) 21.96  $\mu$ g/ml. The activity of methyl hydroxychalcon was comparable with that of standard reference suramin (Figure 2D). The anti-angiogenic effect of methyl hydroxychalcone on explants of rat aorta was significantly dose dependent (P<0.05), and micro-vessel growth was almost completely inhibited in the presence of 100  $\mu$ g/ml methylhydroxychalcone.

#### Inhibition of VEGF-induced HUVEC proliferation and tube formation

Figure 3 depicted a dose-dependent inhibition of endothelial cell proliferation after 48 h. The methyl hydroxychalcon showed significant inhibition with IC<sub>50</sub> 21.16  $\mu$ g/ml. The standard reference vincristine exhibited potent cytotoxicity with IC<sub>50</sub> 0.013  $\mu$ g/ml. While HUVEC cell proliferation was significantly increased in response to VEGF treatment in the absence of methyl hydroxychalcon,

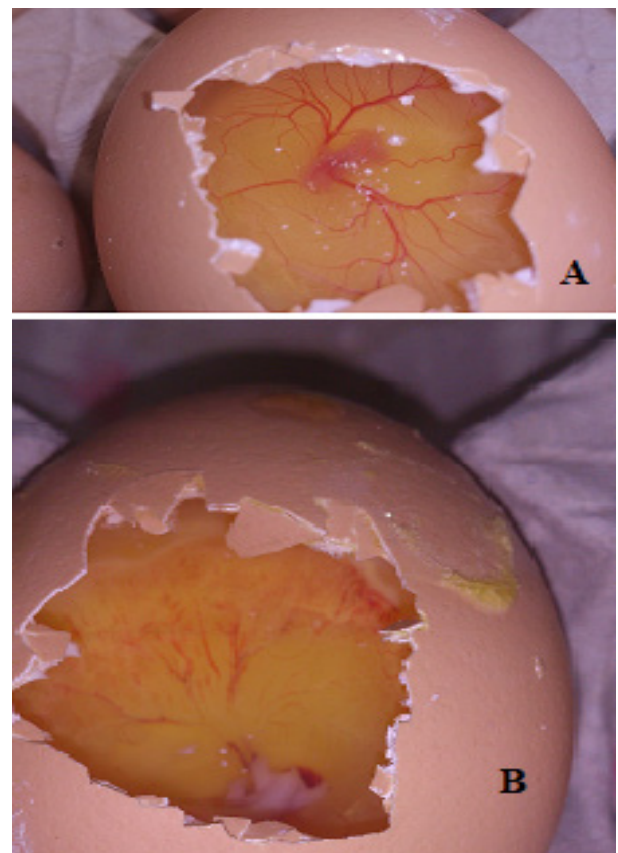


Figure 1. Effects of Methylhydroxychalcon on Neovascularization in Chorioallantoic Membrane of Chick Embryo (A: Control and B: methylhydroxychalcone treated, arrows indicating the zone of inhibition of neovascularization)



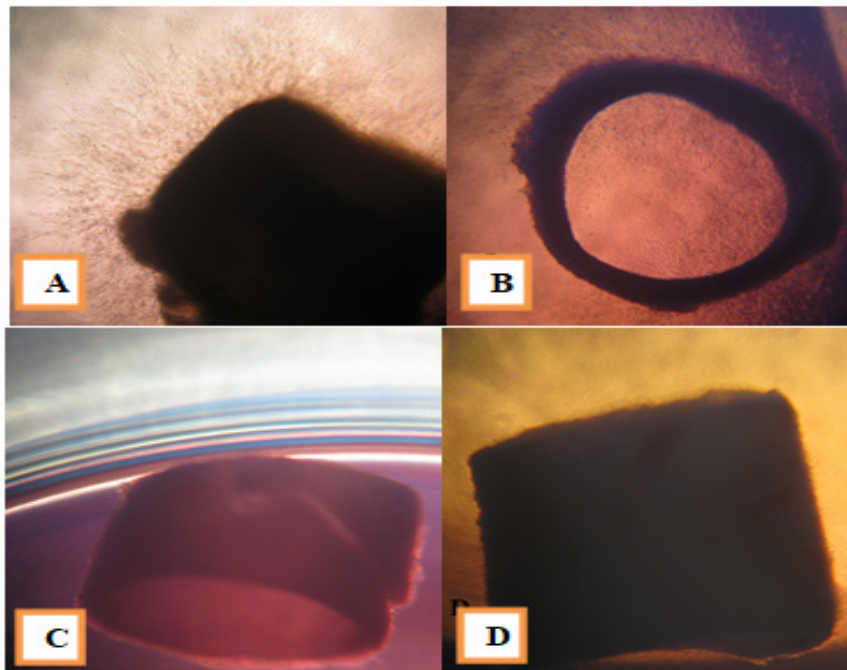


Figure 2. Representative Photographs of the Inhibitory Effect of Methylhydroxychalcon (100  $\mu\text{g/ml}$ ) on ex vivo Angiogenesis Using a Rat Aortic Ring. Images of rat aortic rings (A and B) Control; (C) methylhydroxychalcon and (D) Suramin.

it was markedly suppressed in the presence of methyl hydroxychalcone.

To further characterize its anti-angiogenesis activity, VEGF induced tube formation by HUVEC on Matrigel, a well-established angiogenesis assay examined. (Figure 4A) showed the formed tube-like networks within 8 h, which might, in part, reflect the process of angiogenesis. At a concentration of 25  $\mu\text{g/ml}$  (Figure 4B), the methyl hydroxychalcon absolutely abrogated endothelial tube formation, reducing the tube-like structure both in width and in length. Endothelial cells rounded up and rendered network structures incomplete and broken in the presence of methyl hydroxychalcone (Figure 4C). The activity of

methyl hydroxychalcone ( $\text{IC}_{50} = 13.24 \mu\text{g/ml}$ ) was more or less equal to that of standard, suramin (Figure 4D).

#### *Inhibition of VEGF in endothelial cell lysates*

Figure 5 showed that methylhydroxychalcon caused profound inhibition of VEGF production from endothelial cells. ELISA measurements indicated that control cells showed higher levels of VEGF 165 ( $34 \pm 3.8 \text{ pg/ml}$ ) in cell lysates than cells treated with methylhydroxychalcon. The highest methylhydroxychalcon concentration halved VEGF production ( $17 \pm 1.2 \text{ pg/ml}$ ), whereas the lowest one reduced VEGF levels by 60%.

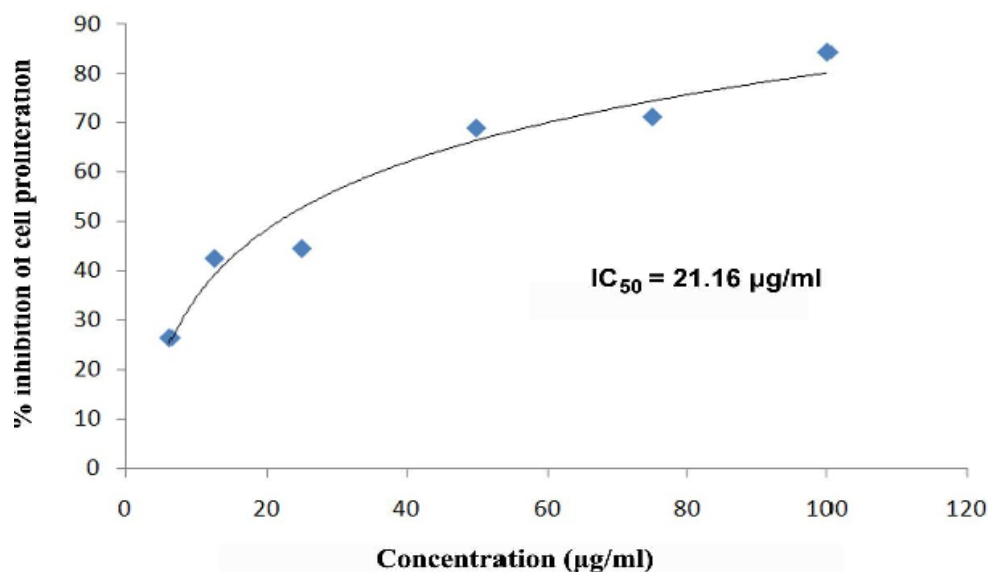


Figure 3 Effect of Methylhydroxychalcon on the VEGF-induced HUVEC Proliferation. The methylhydroxychalcon inhibited HUVECs proliferation in dose dependent manner with  $\text{IC}_{50}$  21.16  $\mu\text{g/ml}$ .

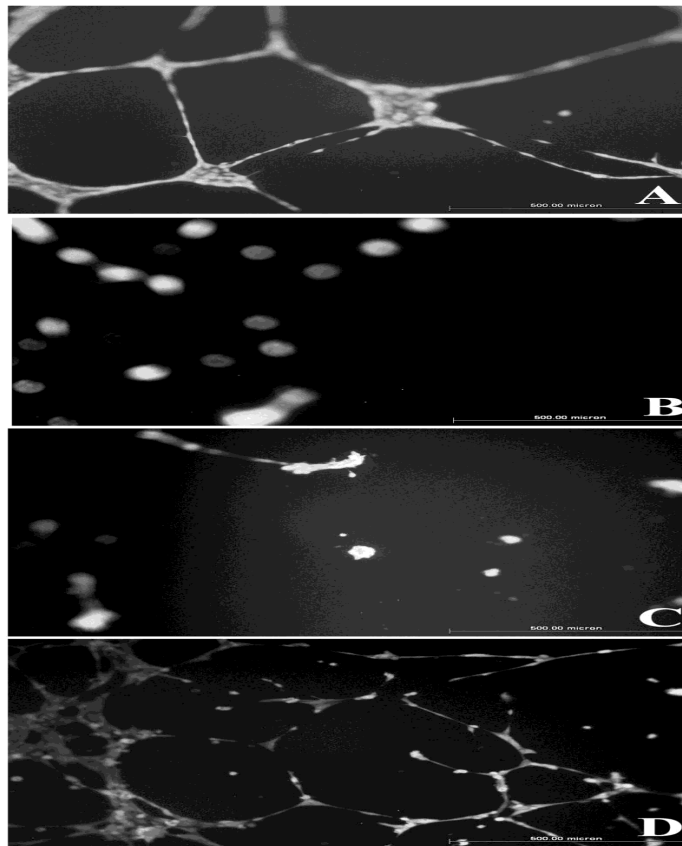


Figure 4. Effect of Methylhydroxychalcon on the VEGF-induced HUVEC Tube Formation. HUVECs ( $2 \times 10^4$  cells/well) were plated on Matrigel precoated 96-well plates and treated with different concentrations of for 24 h. (A) Control; (B) methylhydroxychalcon (25  $\mu\text{g/ml}$ ) (C) methylhydroxychalcon (50  $\mu\text{g/ml}$ ) and (D) Suramin (10  $\mu\text{g/ml}$ ).

## Discussion

Many diseases related to the angiogenesis process are waiting for drugs to be discovered. Scientists started focusing on this process years ago to either prevent or treat these diseases. Methylhydroxychalcone had been chosen to be the lead compound in this study. In a previous study, this chemical group chalcone demonstrated promising anti-oxidant activity, therefore antioxidant activity is

one of the anti-angiogenesis mechanisms, this chalcone derivative was purchased.(Bandyopadhyay, 2002). Furthermore, antioxidants are well known to have potent anti-angiogenic activity. Amongst those that have been identified include vitamin C, vitamin D, vitamin E, vitamin A, botulinic acid, 3-hydroxyflavone, 3,4-dihydroxyflavone, 2,3-dihydroxyflavone, and 2,3-dihydroxyflavone are all examples of flavones (Husseina et al., 2018). The way in which this compound works in inhibiting angiogenesis

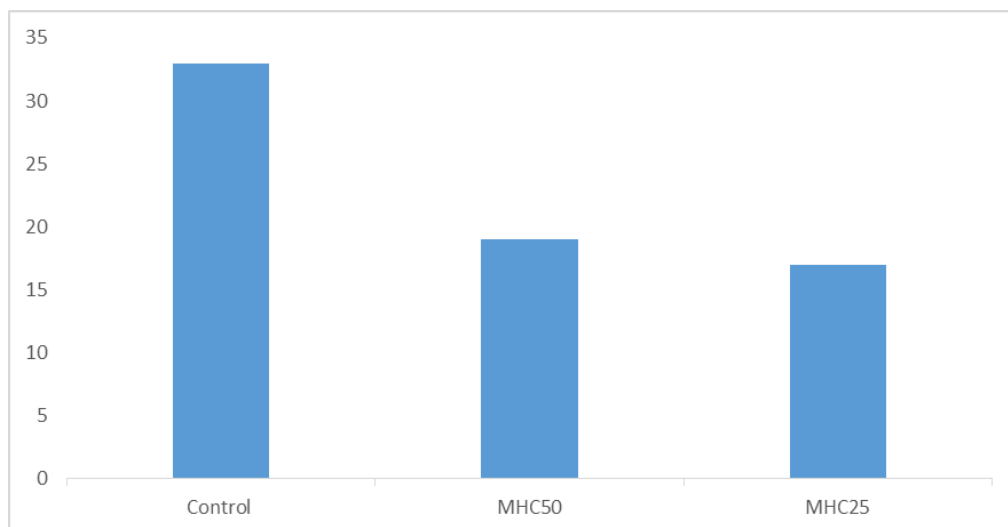


Figure 5. Effect of Methylhydroxychalcon on the VEGF-induced HUVEC Tube Formation. HUVECs ( $2 \times 10^4$  cells/well) were plated on matrigel precoated 96-well plates and treated with different concentrations of for 24 h. (A) Control; (B) methylhydroxychalcon (25  $\mu\text{g/ml}$ ) (C) methylhydroxychalcon (50  $\mu\text{g/ml}$ ).

stems from either direct interaction with key angiogenic receptors or by changing the redox microenvironment of the tumor vasculature, such as by suppressing the oxidant-induced VEGF expression by down-regulation of nitric oxide synthase expression and activity (Alshaya et al., 2019), and since, methylhydroxychalcone is enormously potent antioxidants that encouraged the researcher to endeavor and execute the present work. In this study, the results showed that methylhydroxychalcone has potent anti-angiogenic activity, which was evidenced by in vivo chick embryo CAM and ex vivo rat aortic ring assays. While describing this inhibitory effect of methylhydroxychalcone on angiogenesis, the study found that the methylhydroxychalcone strongly inhibited VEGF-induced proliferation and tube formation in HUVECs. Therefore, to further elucidate the mechanisms underlie such antiangiogenic effects of the methylhydroxychalcone, the quantitative production of VEGF signal protein and the expression of membrane VEGFR-2 tyrosine. Kinase had been studied in treated HUVECs. The data was able to show that methylhydroxychalcone significantly inhibited VEGF-induced endothelial cell proliferation. It is well known that VEGF plays a crucial role in developmental and pathological angiogenesis. VEGF triggers angiogenesis through VEGFR2 (KDR/Flk-1), which is expressed mainly on endothelial cells (Pan & Ho, 2008). The VEGF signal pathway in endothelial cells has been shown to play an essential role in angiogenesis both in vivo and in vitro. It was reported that VEGF induced the proliferation, migration, and formation of capillary-like structures in endothelial cells through VEGFR-2 tyrosine kinase expression and activation (Bagchi et al., 2004). The results showed that methylhydroxychalcone inhibited the VEGF-induced proliferation of HUVECs in a concentration-dependent manner. A VEGF-induced tube formation assay was performed to determine the effects of methylhydroxychalcone on the angiogenic functions of endothelial cells. When compared to the reference standard suramin, methylhydroxychalcone inhibited tube-like formation in treated endothelial cells, indicating that it has potential antiangiogenic activity. VEGF is the most vital and critical angiogenic factor that usually binds to a variety of cell surface receptor proteins, including receptor tyrosine kinases, neuropilin-1, and heparan sulfate proteoglycans. VEGFR-2, also known as Flk-1 or KDR, is a transmembrane receptor tyrosine kinase with high affinity for VEGF (Polytarchou and Papadimitriou, 2004). VEGF first binds to the tyrosine kinase receptor, Flk1/KDR, and signaling by this receptor facilitates activation of the intrinsic tyrosine kinase, followed by an entire cascade of angiogenesis (Colavitti et al., 2002). To elucidate the inhibitory action of methylhydroxychalcone on the synthesis of VEGF signal and expression of its receptor on HUVECs, the study carried out quantitative estimation of VEGF-165 protein levels and determination of VEGFR-2 expression in methylhydroxychalcone treated endothelial cells.

On the one hand, the methylhydroxychalcone significantly inhibited the production of VEGF by 60% at a concentration of 25 µg/ml, and on the other hand, it

suppressed the expression of VEGFR-2 in HUVECs in a concentration-dependent fashion, indicating that the methylhydroxychalcone can exert antiangiogenic activity via inhibition of the VEGFR-2 pathway (Colavitti et al., 2002).

In conclusion, the study has provided evidence for the first time that methylhydroxychalcone inhibits angiogenesis in vivo and in vitro. Moreover, methylhydroxychalcone impairs vascular growth, endothelial cell proliferation, and tube formation. The current findings also suggest that methylhydroxychalcone inhibits angiogenesis via the VEGFR-2 tyrosine kinase pathway. Given the vital and crucial role of the VEGF/VEGFR-2 signaling pathway in angiogenesis, understanding the mechanisms of how methylhydroxychalcone disrupts VEGFR-2 signaling could provide attractive therapeutic approaches intended to alleviate the deleterious effects of over expression of VEGF/VEGFR-2 on the vascular system.

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### Approval

The author has the approval from the College of pharmacy at Al-Nahrain University.

### Ethic statement

The researcher already has ethical clearance from all required institution and laboratories

### Conflict of Interest statement

The author declares that there are no conflicts of interest.

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