RESEARCH ARTICLE

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Antiangiogenic Activity of Quinine Alone and in Combination with vitamin C in both *ex vivo* and *in vivo* Assays

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Abstract

Background: Angiogenesis is the process of vascularization from preexisting blood vessels. It is essential for many physiological and pathological processes. Quinine is an anti-malarial agent belongs to the quinoline alkaloid that can inhibit angiogenesis. Vitamin C is also an important antioxidant and has been shown to reduce angiogenesis in tumor. Objective: The study was aimed at investigating the effect of quinine alone and in combination with vitamin C on angiogenesis process. **Materials and Methods:** 12 to 14 weeks old male albino rats were used for the study. Quinine was prepared by dissolving in DMSO and was serially diluted. The rat aorta ring assay was employed to investigate the antiangiogenic effect of quinine ex vivo. An in vivo chorioallantoic membrane (CAM) assay was used to measure the blood vessels inhibition zone by quinine. The zone of inhibition was calculated as the mean inhibition area of a blood vessel in mm±SD.The obtained data were statistically analyzed. **Results:** The results revealed that quinine has a significant dose-dependent inhibition effect on the growth of blood vessels by 98% ± 0.07 in concentration 100µg/ml when compared to the negative control. moreover, the inhibition of blood vessels growth as a measure of the antiangiogenic activity of quinine in combination with vitamin C shows a synergistic effect when the concentration that inhibit 50% of blood vessels growth (IC_{50}) which equals to 5.05 µg/ml resulted in 85% of growth inhibition when combined with IC_{50} of vitamin C which equals to 22..87µg/ml. **Conclusion:** The findings suggest that the activity of quinine with vitamin C synergism can greatly lower blood vessels growth in rat aorta rings and CAM assays. Quininehas an inhibitory effect on tumor and can be utilized as an antiangiogenic agent alone or in combination with vitamin C.

Keywords: Antiangiogenesis- CAM- rat aorta rings- quinine- vitamin C

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Introduction

Angiogenesis is the process by which new blood vessels formed from pre-existing vasculature. It is essential for both physiological and pathological conditions and triggered by hypoxia that can induce HIF which in turn upregulates the expression of pro-angiogenic factors (Peluzzo and Autieri, 2022). Earlier studies have shown that tumors can develop to a maximum of 1-2 mm^3 in diameter before stopping growing and dying in the absence of angiogenesis due to the inability of nutrients and oxygen to diffuse beyond this distance, but some tumor cells can expand beyond 2 mm^3 in diameter in angiogenesis-rich cell culture (Ozel et al., 2022).

Angiogenesis process could be simply described as multi-steps. First, angiogenic stimuli increase the permeability of endothelial cells, cellular proliferation and capillary sprout elongation. Second, matrix metalloproteinases (MMPs) are activated and cause proteolysis of basement membrane matricellular components. Third, endothelial cells migrate out of the pre-existing capillary wall and proliferate and that triggers tube formation. Finally, capillary is stabilized through the construction of basement membrane, adherent junctions, and endothelial cells (Yoo and Kwon 2013).

This process is under a strict control when imbalance occurs between angiogenesis inducers and inhibitors angiogenesis disorders result (Ali and Sahib, 2022). Nowadays drug repurposing considered as a feasible approach aims at developing novel medications from already existing agents to help in fight many diseases especially cancer due to the increasing resistance to conventional anti-cancer and anti-angiogenic agents (Huang et al., 2021). Quinine belongs to the antimalarial cinchona alkaloids that has been reported to have chemosnsetizing effect by combination therapy with anticancer agents and may contribute in stimulation of apoptosis signals in cancer cells (El-Mesery et al., 2021).

Vitamin C is a water-soluble vitamin that can affect various types of cancer by multi-targeting effects such as pro-oxidant activity which targets redox imbalance, epigenome regulation, immunomodulatory

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functions, oxygen-sensing, kinase activity regulation and epithelial-to-mesenchymal transition (Böttger et al., 2021).

The objective of this study is to investigate the possible anti angiogenic activity of quinine alone and in combination with vitamin C.

Materials and Methods

This study was carried out in tissue culture laboratory (Lab. No. 2) of Pharmacology Department / College of Medicine / Al-Nahrain University. The study was begun on October 2021 and was finished on April 2022. The experiments were conducted after revision and approval of Ethics Committee of Al-Nahrain University / College of Pharmacy (Letter No. SY/2/1/657, Dated in 20 June, 2022).

Rat aorta ring anti-angiogenic ex vivo assay (RAR)

The assay was performed according to the standard protocol conducted by Brown and co-workers (Brown et al., 1996) with some minor modifications. 12-14 weeks old albino male rats were obtained from animal house of Iraqi Center for Cancer and Genetics Research and humanly sacrificed via cervical dislocation after anesthesia with chloroform. The thoracic aorta is then excised, rinsed with serum-free media, cleaned from any residual fibroadipose tissue and cross-sectioned into 1 mm thickness rings.48-well tissue culture plate was used for this assay. 300µl of (3mg/ml) fibrinogen and (5mg/ml) of Aprotinin in serum free medium (M199) was added to each well and ring tissues were placed in the center of the well. 10µl of thrombin; prepared at 50NIH U/mL in 0.15M NaCl was then added to each well and the tissue culture incubated for 30 min in a humidified 5% CO_2 incubator at 37°C for solidification and formation of fibrin gel. Then each well received 300µl of medium M199 supplied with 20% heat inactivated fetal bovine serum (HIFBS), 0.1% E-aminocaproic acid, 1% L-glutamine and 0.6% gentamycin.Quinine, vitamin C and their combination were prepared by dissolving in DMSO and diluting in M199 to make the final DMSO concentration 1% and added to the complete growth medium at different concentrations. Each concentration was performed in six replicates and the experiment was repeated three times. Then culture plates placed in 5% CO_2 humidified incubator at 37°C for five days. On day 4, the top layer medium was replaced with fresh medium. Tissue rings that received only 1%DMSO considered as negative control and those whom received vitamin C as positive control. Vessel growth inhibition evaluated as mean percent inhibition to the negative control ±SD. The magnitude of blood vessel growth inhibition was determined according to the technique developed by Nicosia (Nicosia et al., 1997) and was quantified manually under (40x) magnification with aid of camera and software package. The percentage of blood vessels inhibition was determined according to the following formula:

$$\text{Blood vessels inhibition \%} = 1 - (A0/A) \times 100$$

Where: A0= distance of blood vessels growth of the test substance in mm; A= distance of blood vessels growth of the negative control in mm.

Dose-Response study on quinine and vitamin C with rat aorta assay (anti-angiogenesis)

Test substances were dissolved in DMSO to make stock solution of 1% concentration then diluted in the M199 growth medium to make serial dilutions of the following concentrations: 100, 50, 25, 12.25 and 6.25µg/ml. Wells without test samples were received medium with 1% DMSO used as negative control. The data was represented as mean ± SD. The concentration that inhibits 50% of blood vessels growth "IC_{50}" was calculated by using the logarithmic equation. Where Y= the percentage of inhibition, and X= concentration (Stiffey-Wilusz et al., 2001).

Chorioallantoic membrane (CAM) in vivo assay

Fertilized chicken eggs obtained from poultry fields of college of veterinary medicine, University of Baghdad were cleared of any debris/dirt using 70% ethanol and incubated for 72 hours at 37°C with a relative humidity of 60 %. The eggs were put in horizontal position and rotated several times. 72 hours later, 2 ml albumin were sucked out through a pinpoint hole punctured down by the side and sealed "to provide better visualization of the formed CAM, where the CAM will split from the sack that is linked to the egg shell," then eggs were incubated for further 24 hours. After that a small square window (3-4 cm diameter) of the shell was made and the test samples that were soaked previously in round discs of filter papers placed on the CAM and the window was covered with a sterile surgical adhesive tape, then eggs were incubated again for another 72 hours. Finally, the inhibition zone photographed and calculated. The test samples which are quinine, vitamin C and their combination were prepared as 10mg/ml and 50µl (the final dose was 0.5mg/disc) of quinine and vitamin C and 25µl of each substance was taken to make the combination (the final dose was 0.25mg for each one/disc) placed on the filter paper discs and left to dry before being transferred to the CAM. The whole procedure was performed under aseptic condition (Al-Zubaidy et al., 2016).

Quantification and Imaging of CAM

The responses were graded + (3 - 6 mm); ++ (6 - 9mm); +++ (> 10mm). The results were presented as Mean ± Standard errors of means. The quantification of inhibition zone was done by using image analyzer (BIOCOM Visiolab TM 2000) (Murray, 2001).

Statistical Analysis

The experiment design used for this study was Rationalized Complete Block Design (RCBD). 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 (IC_{50}) was calculated using logarithmic equations. The statistical analysis was

carried out by using SPSS edition 21.0.

Results

Rat aorta ring anti – angiogenesis ex vivo assay
Comparison between quinine, positive control and negative control

The aortic rings that have been treated with 100µg/ml of quinine and embedded in complete growth medium of M199 showed significant difference of blood vessels inhibition by 98% ±0.07µg/ml at (p>0.05) at day 5 of the experiment as compared with negative control rings that have been received only 1% DMSO and positive control rings that have been treated with 100µg/ml of vitamin C which resulted in 75% ±2.13 µg/ml as shown in Table 1.

Dose response effect in rat aortic ring anti-angiogenesis assay
Dose response curve of quinine on rat aortic ring model

Image2 shows the five serial dilution concentrations (100, 50, 25, 12.5, 6.25 µg/ml) that were used to determine the dose response curve. In Table 2 data presented as inhibition percentage ±SD and showed a significant dose dependent inhibition of blood vessels at (p>0.05). The IC_{50} was calculated by using logarithmic equation where Y= the inhibition percentage and X= the concentration and was =5.05µg/ml as seen in Figure 1.

Dose response curve of vitamin C on rat aortic ring model

Five serial dilution concentrations (100, 50, 25, 12.5, 6.25µg/ml) were used to determine the dose response relationship. The data presented as inhibition percentage ±SD and showed a significant dose dependent inhibition of blood vessels at (p>0.05) as showed in Table 3. The IC_{50} was measured by logarithmic equation where Y= the inhibition percentage and X= the concentration and was =22.87µg/ml as presented in Figure 2.

The combination effect of quinine with vitamin C in rat aortic ring assay

The inhibition concentration that inhibits 50% of growing blood vessels (IC_{50}) for both quinine and vitamin C has been calculated using logarithmic equations as shown in Figures 1 and 2 respectively, Where Y= the inhibition percentage and X= the concentration.

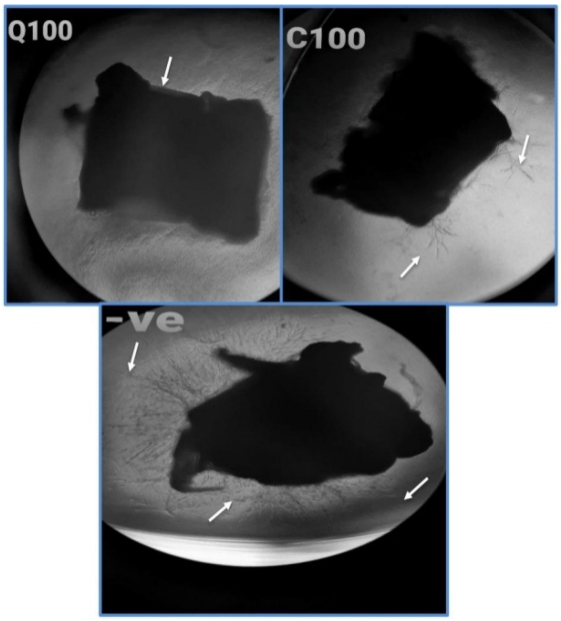

Image 1. Anti – angiogenesis Effect of 100µg/ml of Quinine (Q100) in *ex vivo* aortic ring model along with vitamin C (C100) and negative control DMSO 1% (-ve). NOTE, white arrows represent the growth of micro-blood vessels

The IC_{50} for both compounds were combined together and placed on the aortic rings. At day 5 of the experiment, the inhibition of blood vessels growth showed a significant synergism effect at (P>0.05) by 85% of growth inhibition in comparison with quinine alone as presented in image 4.

In vivo chick chorioallantoic membrane (CAM) assay of quinine, vitamin C and their combination
In vivo chick chorioallantoic membrane (CAM) assay for quinine

The zone of inhibition was measured at day 7 of the experiment according to the scoring system mentioned previously and it was significant by scoring (+++) at (p>0.05). Blood vessels in the CAM were undergone regression, disorganization and pale yellowish appearance.

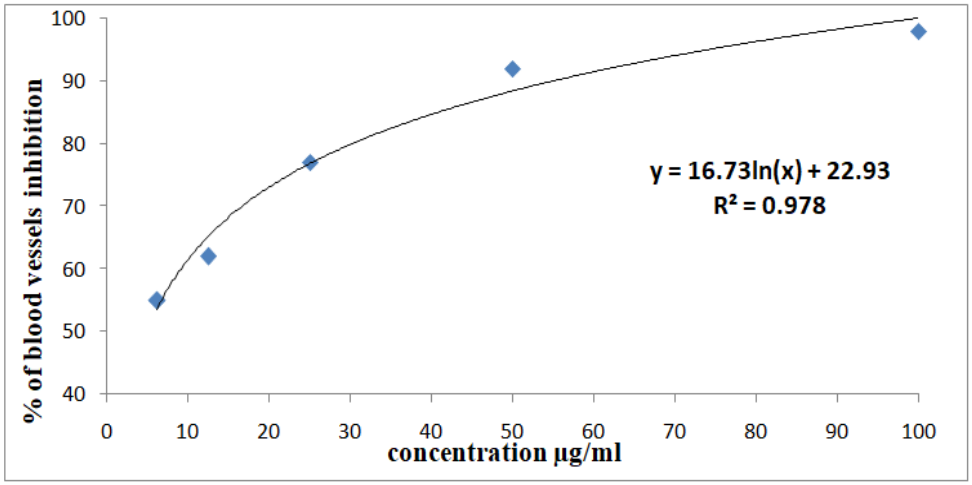

Figure 1. The Dose Response Curve of Serial Dilutions of Quinine in Rat Aortic Rings Assay

Table 1. The Inhibition Percentage of Blood Vessels Growth Produced by Quinine, Positive and Negative Controls

Compound	Percentage of inhibition ± SD
Quinine	98 ± 0.07
Vitamin C	75 ± 2.13
DMSO 1%	0

Table 2. Serial Concentrations and Their Respective Inhibition Percentage for Quinine

Concentration (µg/ml)	% Of blood vessels inhibition ±SD
100	98 ± 0.07
50	92 ± 0.82
25	77 ± 1.96
12.5	62 ± 2.78
6.25	55 ± 2.48

The inhibition was revealed by the appearance of a vascular zone around the disc that contains 0.5mg/ml of quinine as shown in Table 4 and Image 5.

In vivo chick chorioallantoic membrane (CAM) assay of vitamin C

The zone of inhibition for the positive control vitamin C was also measured at day 7 of the experiment and was significant by scoring (++) at (p>0.05). Blood vessels in the CAM regressed and were disorganized with pale yellowish appearance. The inhibition was demonstrated by the appearance of avascular zone surrounding the disc

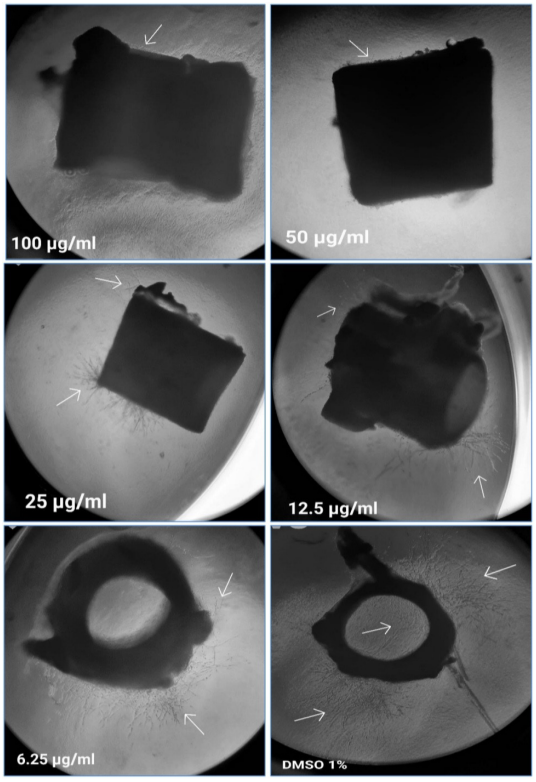

Image 2. The Dose Response Effect to the Serial Concentrations of Quinine in Rat Aortic Rings Model. (White arrows represent the growth of micro-blood vessels) note please that this picture has been changed)

Table 3. Serial Concentrations and Their Respective Inhibition Percentage for Vitamin C

Concentration (µg/ml)	% Of blood vessels inhibition ±SD
100	75 ± 2.13
50	66 ± 1.87
25	49 ± 2.86
12.5	42 ± 1.59
6.25	26 ± 1.67

Table 4. The Zone of Inhibition of Blood Vessels Growth of Quinine and the Corresponding Scoring Using the Chick Chorioallantoic Membrane (CAM) assay

NO. of egg	Inhibition zone (MM)	Scoring
1	11	+++
2	10	+++
3	13	+++
4	9	++
5	11	+++
6	8	++
Mean ±SD	10.33 ±1.59	+++

that contain 0.5mg/ml of vitamin C as shown in Table 5 and Image 5.

In vivo chick chorioallantoic membrane (CAM) assay for the combination of quinine and vitamin C

0.25mg/ml of each quinine and vitamin C were combined and introduced on filter paper discs then placed on the CAM. At day 7 of the experiment, zone of inhibition was measured and it was significant by scoring (+++) at (p>0.05) and exhibited a synergism effect as shown in Table 6 and Image 5. The blood vessels were disordered, shrunk and appeared with pale yellowish color. The presence of a vascular zone surrounding the disc containing the combination was used to identify the inhibition zone.

Discussion

Cancer is a major public health issue that affects people all over the world, and it is the second leading

Table 5. The Zone of Inhibition of Blood Vessels Growth of Vitamin C and the Corresponding Scoring using the Chick Chorioallantoic Membrane (CAM) assay

NO. of egg	Inhibition zone (MM)	Scoring
1	9	++
2	10	+++
3	7	++
4	9	++
5	11	+++
6	8	++
Mean ±SD	9 ±1.29	++

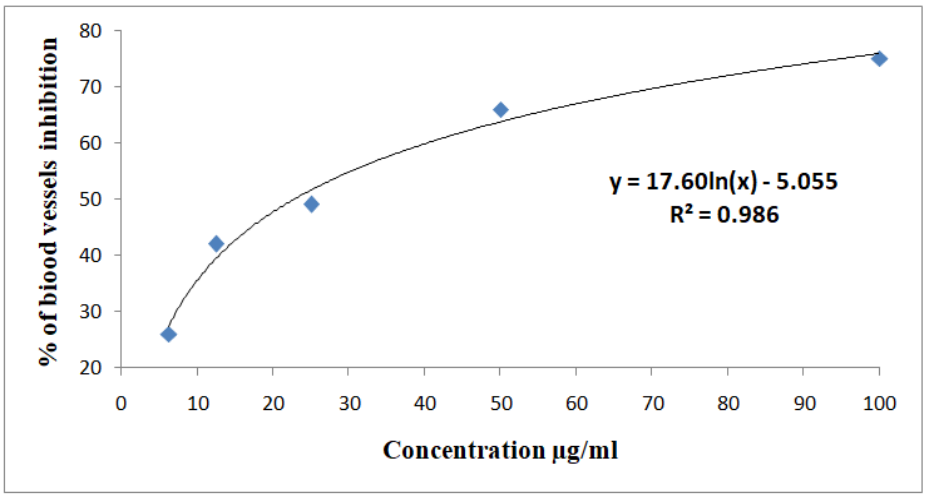

Figure 2. The Dose Response Curve of Serial Dilutions of Vitamin C in Rat Aortic Rings Assay

cause of death in many countries (Siegel et al., 2021). The cancer burden has increased over time in both developed and developing countries due to a variety of factors, including an aging and growing population, rapid socioeconomic growth, and changes in the incidence of risk factors (Cao et al., 2021).

Angiogenesis is the process of vascularization from preexisting blood vessels. It is required for proper feeding and removal of metabolic waste products from tumor areas during tumor development. Angiogenesis and lymph angiogenesis, which are triggered by chemical impulses from cancer cells in a fast-growing phase, are essential for tumor development and spread (Ansari et al., 2022).

Table 6. The Zone of Inhibition of Blood Vessels Growth of Quinine + Vitamin C Combination and the Corresponding Scoring Using the Chick Chorioallantoic Membrane (CAM) assay

NO. of egg	Inhibition zone (MM)	Scoring
1	10	+++
2	9	++
3	12	+++
4	11	+++
5	10	+++
6	9	++
Mean ±SD	10.16 ±1.06	+++

Tumor angiogenesis is a multistep process that begins with basement membrane breakdown and progresses through endothelial cell migration, proliferation, and finally the formation of new blood vessels (Yousefi et al., 2021). Hypoxia, cell metabolic demands, and nutritional deficiency all promote angiogenesis. These metabolic activities activate hypoxia inducible factor (HIF) and other molecules involved in the synthesis of proangiogenic factors (Poto et al., 2022). The most important angiogenic factor is vascular endothelial growth factors (VEGF), which is an inflammatory and regulatory cytokine that is a key component and driver of angiogenesis.

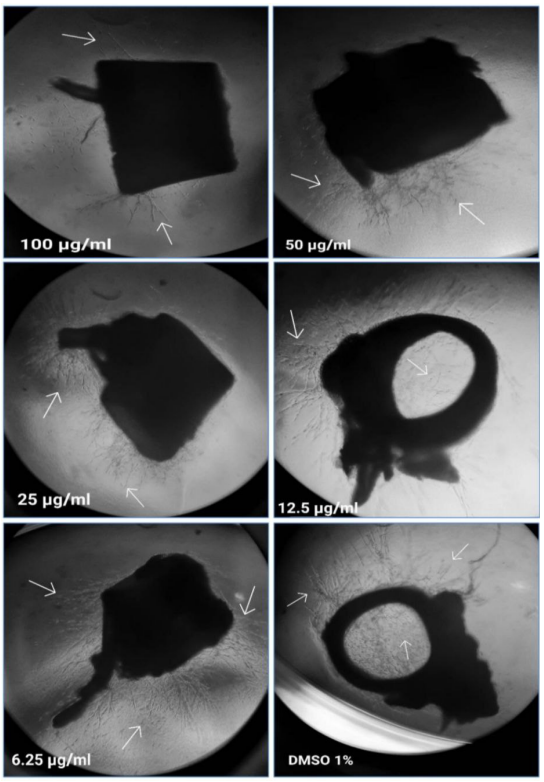

Image 3. The Dose Response Effect to the Serial Concentrations of Vitamin C in Rat Aortic Rings Model (White arrows represent the growth of micro-blood vessels)

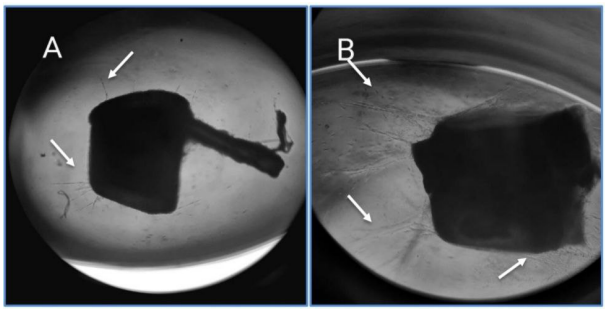

Image 4. Anti – angiogenic Effect of Quinine and Vitamin C Combination (A) in Comparison with the Negative Control DMSO 1% (B) in Rat Aortic Ring Assay (White arrows represent the growth of micro-blood vessels)

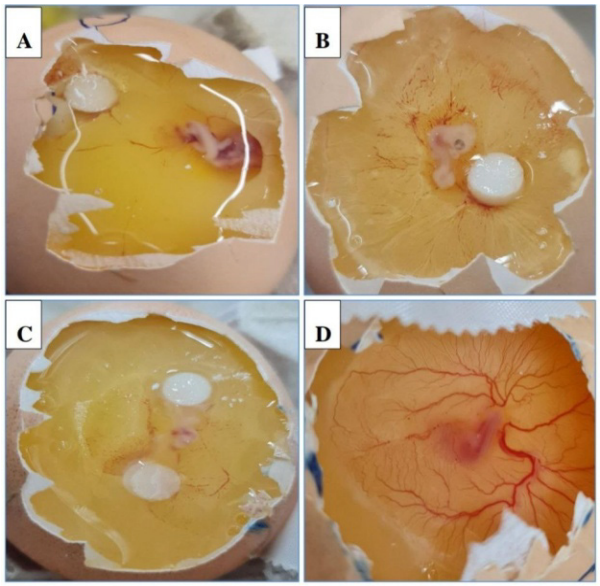

Image 5. The in vivo Chick Chorioallantoic Membrane (CAM) Assay; Where (A), Treated Group with Quinine; (B), Treated Group with Vitamin C; (C), Treated Group with Quinine + Vitamin C and (D), Treated Group with DMSO 1% as Negative Control

VEGF promotes both blood vessel development and vascular permeability (Majidpoor and Mortezaee, 2021). The realization that vascularization is required for tumor growth has led to the development of therapeutics that target the tumor's vascular system. Despite the fact that anti-angiogenic therapy has become a standard of care for a variety of human cancers, anti-angiogenic agents are only effective in a small percentage of patients, and many patients who initially respond to these drugs develop resistance over time (Yetkin-Arik et al., 2021).

These disappointing results highlight the urgent need for the discovery of alternative anti-angiogenic medicines as well as developing combinations that can aid in the treatment. Other strategy is to use already existing and approved drugs that are utilized for other diseases to help in such problem, that's why the anti-malarial quinine was chosen to be studied whether it possesses anti-angiogenic activity or not, and if dose so whether this activity is enhanced with the combination of vitamin C or reduced.

The anti – angiogenic activity inex vivo rat aorta ring assay

Ex vivo models of angiogenesis, such as vascular explants cultures, have grown in popularity over the last two decades and they are the most extensively used for researching angiogenesis since they overcome the limits of in vitro approaches and simplify the complexity of in vivo models serving as a connection between those two angiogenic assays. In addition, they offer a large sample size, high reproducibility, low cost and anatomical similarity of aortic ring sprouts to in vivo microvessels (Kapoor et al., 2020).

In the present study, the aortic rings assay was used to determine the anti-angiogenic activity of quinine and the possible synergism or additive effect of quinine when combined with vitamin C.Vitamin C was used as a positive control because it is approved to have antiangiogenic effect through several mechanisms such as acting as pro-oxidant leading to the production and accumulation of hydrogen peroxide (H_2O_2) and other ROS in cancer cells, reducing tissue hypoxia inducible factor-1α (HIF-1α) and vascular endothelial growth factor A (VEGFA), increasing p53 and endostatin levels (Nakanishi et al., 2021), and inhibiting the initial phase of cell migration and tube vessel formation (Mikirova et al., 2010).

The results of this study showed that quinine exerts a significant dose dependent inhibition of microvessels sprouting when compared to untreated rings. 100 µg/ml of quinine was observed to have the maximum anti-angiogenic effect on rat aorta rings compared to the other concentrations. Meanwhile, in combination with vitamin C there was a synergistic effect in angiogenesis inhibition where the concentration of quinine that inhibits 50% of blood vessels growth (IC_{50}) results in 85% of growth inhibition when combined with the IC_{50} of vitamin C. The quantification of angiogenesis on this system implies the determination of number and length of the branching microvessels.

Alkaloids that come from natural origin are believed to have the ability to promote apoptosis in several types of cancer cells (Rosenkranz and Wink, 2007). Moreover, they play a significant role in combination with other anti-cancer agents, angiogenic disorders prevention and in chemotherapy or radiotherapy complements. The anti-angiogenic activity of them may be attributed to the blocked of angiogenic cascade in endothelial cells by downregulation of VEGF, TNFα, HIFα messengers ranging from high degradation and low expression (Alasvand et al., 2019). Gurung et al., (2017) reported that quinine significantly increased the intracellular ROS (Reactive oxygen species) which is critical in cell growth and apoptosis for metabolic and signal transduction pathways in cancer cells at dose and time dependent manner. Furthermore, quinine has been shown to exhibit a potent anti-inflammatory and apoptotic properties by inhibiting nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB) and this could affect the expression of pro-inflammatory and anti-apoptotic mediators (Krishnaveni et al., 2016). Another study done by Krishnaveni et al., (2015) has suggested that quinine has a strong free radical scavenging activity and can act as an antioxidant in cancer studies due to the presence of reductant moieties (–OH, and α,β- carbonyl moiety) that may react with free radicals to stabilize and terminate the free radical chain reaction.

From the mentioned studies that support the findings of this study, it appears that the inhibition of micro vessels outgrowth produced by quinine as been may belong to its nature as an alkaloid that can inhibits and down regulates the expression of VEGF, TNFα and HIFα which are considered to be strong angiogenic factors perhaps due to its anti-inflammatory, antioxidant and pro-apoptotic activity, anti-angiogenesis activity in Chick chorioallantoic Membrane (CAM) in vivo Assay.

The chick chorioallantoic membrane (CAM) assay is one of the oldest and most commonly used assays for evaluating angiogenesis in vivo because it serves many advantages including being a sensitive, simple, practical,

and inexpensive (Ali and Sahib, 2022). In addition it is considered to be one of the most ethical in vivo angiogenesis assays. On the other hand, it has one major drawback which is difficult to be utilized for testing weakly pro-angiogenic drugs because of its dense web of blood vessels (Stryker et al., 2019).

In the current study CAM assay was used to verify the anti-angiogenic activity of quinine and its combination with vitamin C in vivo. Quinine in concentration of 0.5 mg/ml inhibited the blood vessels by more than 10mm with the appearance of pale yellowish discoloration. Furthermore, 0.25 mg/ml of quinine resulted in approximately same score which is more than 10 mm inhibition when combined with 0.25 mg/ml of vitamin C confirming that there is a synergism effect in angiogenesis inhibition. Therefore, the outcomes in this assay support the previous ex vivo results. The observed anti-angiogenic activity may be attributed to the arrest of inflammation associated angiogenesis by the reduction in levels of TNF-α and IL-6 that can increase the expression of VEGF, a promoter of angiogenesis (Asif et al., 2020), antioxidant activity (Krishnaveni et al., 2015), induction of apoptosis (Razavi et al., 2021) and VEGF down regulation (Alasvand et al., 2019).Since quinine has a low therapeutic index, therefore, the therapeutic dosage must be reduced to decrease the adverse effects (Malakar et al., 2018) and this goal has been achieved in this study by combining it with the relatively high therapeutic index vitamin C.

In conclusion, the results of this study revealed that quinine can significantly decrease the blood vessels growth in both in vivo and ex vivo and this activity was enhanced when combined with vitamin C (synergism effect). Quininehas an inhibitory effect on tumor growthand can be utilized as an antiangiogenic agent alone or in combination withvitamin C.

Author Contribution Statement

ZJ: Conceptualization, Methodology, Data curation, Formal analysis, Writing - original draft. HS: Supervision, Validation, Writing – review and editing.

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Approval

This paper is part of the dissertation of the corresponding author at the College of Medicine at Al-Nahrain University in Partial Fulfilment of the Requirements for the Degree of MSc in Science in Pharmacology.

Ethic statement

Handling of the animals and the experimental design were carried out based on the guidelines reported in "Guide for the care and use of laboratory animal" and according to the protocol approved by the Al-Nahrain University/College of Pharmacy, Baghdad, Iraq with the ethical clearance number (Letter No. SY/2/1/657, Dated in 20 June, 2022) the letter was approved by Vic Dean for scientific affairs Dr. Rafal Shakeeb AbdulWahab.

Data Availability

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Study Registration

There is no registration number or support for this project.

Conflict of interest

The authors declare that they have no competing interests.

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