Research Square

Preprints are preliminary reports that have not undergone peer review.
They should not be considered conclusive, used to inform clinical practice,
or referenced by the media as validated information.

Phytoceuticals Cocktail as a Potent Inhibitor of Breast Cancer Angiogenesis: In Vivo Evaluation Utilising the Chick Embryo CAM Model

Jessica Katrine Datsyuk
University of Technology Sydney
Selvaraj Vimalraj
Anna University, Chennai
Mukilarasi Pooja
Anna University, Chennai
Gabriele De Rubis
University of Technology Sydney
Joachim Gerlach
Vedicinals India Private Limited
Prakash Salunke
Vedicinals India Private Limited
Swathi Sudhakar
Anna University, Chennai
Manisha Singh
University of Technology Sydney
Keshav Raj Paudel
k.paudel@westernsydney.edu.au
Western Sydney University
Kamal Dua
Western Sydney University

Research Article

Keywords: Phytoceuticals, Breast Cancer, Angiogenesis, CAM study, In vivo

Posted Date: July 29th, 2026

DOI: https://doi.org/10.21203/rs.3.rs-10451701/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License.
Read Full License

Additional Declarations: No competing interests reported.

Abstract

The development of tumour vascularisation is crucial for the development and metastasis of cancer cells, primarily through the process of angiogenesis. Anti-angiogenic strategies, which inhibit the formation of new blood vessels, are promising approaches in cancer therapy. Herbal-based phytoceutical cocktail have shown to be a good source if anti-cancer bioactive compounds. This study investigates the capability of Vedicinals ® 9 Advanced to prevent and reduce breast cancer tumour angiogenesis. Vedicinals ® 9 Advanced is a polyherbal formulation containing nine medicinal plant-derived compounds. Advancing on our previous *in vitro*studies demonstrating its potent anti-cancer efficacy against lung cancer, this research explores the *in vivo* effects of Vedicinals®9 Advanced tumour angiogenesis using the chick embryo chorioallantois membrane (CAM) model. MCF7 human breast cancer cells were xenografted onto the CAM to simulate a tumour microenvironment, and Vedicinals ®9 Advanced was applied to assess its impact on tumour angiogenesis. Quantitative analysis of angiogenic parameters such as vessel area, total length, thickness, and the number of branching nodes, revealed a significant decrease in angiogenesis in breast-cancer cell environment following Vedicinals ®9 Advanced treatment. Additionally, treatment of the CAM with Vedicinals ®9 Advanced resulted in a substantial reduction of the mRNA expression of pro-angiogenic factors Vascular endothelial growth factor A (VEGFA) and Angiopoietin 1 (ANG1). These findings suggest that Vedicinals ®9 Advanced is a potent inhibitor of tumour angiogenesis, thereby reducing cancer cell metastasis and preventing further tumour development.

Introduction

Breast cancer is a disease that is caused by abnormal breast cells within the tissue to abnormally proliferate and develop into tumours (Menon *et al.*, 2024). Breast cancer is the most common cancer that affects Australian women, and women worldwide. Each year, approximately 2.3 million new individuals will be diagnosed with breast cancer worldwide, making it a global burden that needs a viable solution (Lukasiewicz *et al.*, 2021). It is predicted that, in 2024, 20,973 Australian women will be diagnosed and treated with breast cancer, and 220 Australian men (BNCA, 2024). The mortality rate of breast cancer in Australia in 2020 is 10 deaths per 100,000 persons equating to 3,144 deaths in 2020 (Australian Government, 2022). Whereas worldwide there has been approximately 670,000 deaths in 2022 (WHO, 2024). There are two main categories of breast cancer, non-invasive and invasive. In non-invasive breast cancer, the cancerous cells are located within the lobules or the milk ducts of the nipple, and the abnormal cells do not develop or infiltrate the surrounding breast tissue (Zubair et al., 2021). Non-invasive breast cancer has two subtypes called ductal carcinoma *in situ* and lobular carcinoma *in situ* (Zubair et al., 2021). In invasive breast cancer, the abnormal cells spread beyond the nipple region of the breast, and into the actual breast tissue. This type of cancer has subtypes called invasive ductal carcinoma *in situ* and invasive lobular carcinoma (Zubair et al., 2021).

There are modifiable and non-modifiable risk factors that contribute to the development of breast cancer. Non-modifiable factors include female family history of breast cancer, genetic mutations, density

of breast tissue, and any previous non-cancerous breast diseases (Folkred & Dowsett, 2013). Modifiable risk factors include use of hormonal replacement therapy, alcohol and smoking, physical inactivity, and excessive exposure to artificial light (Narod, 2011).

Cancer has the capacity to metastasise to remote organs, rendering it ultimately lethal for the patient. To be able to metastasize, cancer cells need to develop the ability to infiltrate blood or lymphatic vessels, travel *via* the bloodstream, and subsequently grow and multiply in a different location such as a new organ or tissue (Nishida et al., 2006). The expansion of the vascular network irrorating a tumour is a crucial component for the metastatic spread of cancer. This growth of the vascular and lymphatic network is categorized as angiogenesis and lymph angiogenesis (Lugano *et al.*, 2020). Angiogenesis, the creation of new blood vessels, is an intricate and ever-changing process controlled by several factors that promote or inhibit blood vessel creation (Liu et al., 2023). The main factors that promote angiogenesis include fibroblast growth factor (bFGF), which exerts this effect *via* promoting the mitogenesis of endothelial cells (Nakamichi *et al.*, 2023), and Vascular Endothelial cells growth factor (VEGF), which is the main activator of endothelial cell proliferation and motility (Nakamichi *et al.*, 2025). Furthermore, there are 10 main modes of angiogenesis, with the most common and well-known mechanisms of angiogenesis being sprouting, and other known mechanisms including intussusceptive and coalescent angiogenesis (Dudley & Griffoen, 2024). Sprouting angiogenesis involves the production of proteolytic enzymes by endothelial cells in response to VEGFA and other pro-angiogenic factors, primarily induced by hypoxia (Dudley & Griffoen, 2024). The endothelial cells will specialise through differentiation into tip cells, stalk cells and phalanx cells, all needed for the formation and stabilisation of the newly formed vessel (Dudley & Griffoen, 2024). Concurrently, the basement membrane surrounding the existing vessel is degraded by enzymes to pave the way for the differentiating endothelial cells (Dudley & Griffoen, 2024). Whilst cells multiply to generate the sprout, tip cells migrate along a substrate. The sprout creates a lumen, which is an opening in a blood vessel, leading to the maturation and vessel fusion where the new vascular network can be created by fusing the sprouts of numerous newly formed blood vessels to mature and stabilise (Dudley & Griffoen, 2024).

There are a variety of treatment options that are currently available for breast cancer. Depending on the disease progression, and the severity of the cancer, common treatments implemented includes chemotherapy, hormone-blocking therapy, targeted therapy, radiotherapy, immunotherapy, and surgery (Burguin et al., 2021). However, treatment options that specifically target angiogenesis are still being tested in clinical trials. Bevacizumab, for example, is a human anti-VEGF monoclonal antibody that is administered co-currently with standardised chemotherapy (Kazazi-Hyseni et al., 2010). It has been tested in association with various chemotherapy agents such as Paclitaxel and Capecitabine, but its true efficacy as a therapy for metastatic breast cancer was called into question due to substantial tolerance difficulties and lack of increasing survival changes (Sasich & Sukkari, 2012). Another example of a drug that was tested for breast cancer is sorafenib, a tyrosine kinase inhibitor which specifically targets the process of angiogenesis through targeting and inhibiting proteins such as VEGFR and BRAF, responsible for upregulation and formation of tumour vasculature (Coventon, 2017). This drug was also tested co-currently with other chemotherapy agents such as capecitabine and paclitaxel (Gadishar *et al.*, 2013).

However, most of the studies that have been performed have highlighted the lack of benefit to survival for individuals with advanced breast cancer receiving sorafenib and chemotherapy as a treatment option (Luu et al., 2014). This clearly underlines the urgent need for novel treatments for breast cancer specifically targeting the process of angiogenesis and extend patient survival rate. A The MCF-7 human breast cancer cell line is the most used breast cancer line to investigate the anti-cancer activity of drugs or bioactive compounds (Comsa *et al.*, 2015). Additionally, xenograft models are a simplified model which enables for the study of tumour progression in specific angiogenesis (Liu et al., 2023).

Phytoceuticals have enormous potential as the solution to the existing issue of the lack of alternative drugs that are available on the market which specifically target angiogenesis without significant reduction in adverse side-effects that the patient experiences during treatment, prolonging the patient's life. Vedicinals ® 9 Advanced is a polyherbal formulation containing nine plant-derived phytoceuticals produced by a German & Indian Biotechnology company, Vedicinals Pty Ltd. The standard form of the formulation, 50 mL, consists of the following active ingredients: Thiamine Hydrochloride, Riboflavin Sodium Phosphate, Pyridoxine Hydrochloride, D-Panthenol, Ascorbic acid, Zinc Gluconate, Sodium Selenite Anhydrous, *Curcuma longa*, *Saphora japanica* extract, Green Tea, *Citrus reticulata blanc*, *Scutellaria baicslensis* extract, *Glycyrrhiza glabra* extract, Quercetin Powder, and *Piper nigrum* extract. The Vedicinals ®9 Advanced formulation has been tested for *in vitro* anti lung cancer activity in a study conducted by Paudel *et al.*, 2023. In this study, the formulation was tested on A549 human lung adenocarcinoma cells, showing potent anticancer activity through functional inhibition of cell proliferation and migration, as well as through the inhibition of the expression proteins promoting these two processes. Interesting, in this study, treatment of A549 cells with the Vedicinals® 9 formula also stimulated an increase in the expression of endostatin, an angiogenesis inhibitor, highlighting the potential anti-angiogenetic effect of this formulation (Paudel *et al.*, 2023).

In this study, we have further investigated the anti-angiogenesis activity of the Vedicinals ® 9 Advanced formulation; to investigate this process, we used the chicken embryo Chorioallantoic Membrane Model (CAM). The benefit of this model is that it enables for the tumour to respond to different factors, therefore enabling it to grow at a faster rate than when implementing a traditional mouse model (Moreno-Jimenez *et al.*, 2016). The model is also used to study angiogenesis due to its transparent and thin structure, that also can be found to be highly vascularised making it ideal for tumour growth studies (Ribatti et al., 2020). In the present study, human breast cancer cells (MCF7 line) were xenografted onto the CAM to create a tumor microenvironment, and Vedicinals ® 9 Advanced formulation was applied to assess its impact on tumor angiogenesis. The results highlight the reduction in angiogenesis seen through the reduction of vessel area, vessel thickness, and number of branching nodes. The significance of such results clearly emphasizes the potential of the Vedicinals ® 9 Advanced formulation as an adjunctive treatment option for breast cancer specifically targeting angiogenesis.

Materials and Methods

In regard to the ethical considerations, all of the following studies outlined below were completed prior to July 2024.

1. Set-up of the CAM model

White Leghorn chick (*Gallus domesticus* L.) eggs that had been pre-incubated were acquired from the Government Poultry Station, Potheri, Chennai, India. Research grade eggs were wiped with 70% Iso propyl alcohol to create a sterile environment. The eggs were incubated in conditions of 37℃ and 62% humidity. On day 2 of life cycle of embryo i.e. embryonic day 2 (ED2), the contents of the egg were transferred into a sterile container to facilitate *ex ovo* culture. The embryos were then again incubated till ED6. On ED6, the following experiments were carried out. MCF cells were seeded on coverslips at a density of 2000 cells/ cm^2, which were checked under the microscope to ensure the cells are confluent. Successfully, the coverslips were placed onto the CAM membrane. Vedicinals ® 9 Advanced 1%v/v (20 µL) was added onto an absorbable disk. The disk was then placed onto the CAM membrane close to the MCF cells seeded coverslips.

2. Imaging analysis

A stereomicroscope (Olympus, India equipped with Magnus digital camera) was used to acquire images at 0-, 3- and 6-hour post-treatment, in the same region where the absorbable disk with Vedicinals ® 9 Advanced formula and the coverslips were placed. 4 parameters of angiogenesis are then quantified: the blood vessel length, blood vessel area, number of branching points, and the thickness of the blood vessels, using the bioinformatics software IKOSA CAM. The obtained parameters were converted into relative fold change by keeping control as the base.

3. Gene expression:

RNA Extract and reverse transcript protocols were performed as reported Vimalraj *et al.,* 2019. Furthermore, we analyse the gene expression levels of angiogenic biomarkers specifically Vascular Endothelial Growth Factor A (VEGFA) and Angiopoietin 1 (ANG1), using Reverse Transcription Polymerase Chain Reaction (RT-PCR), to validate the anti-angiogenesis at the gene level. Rizal (Invitrogen) was implemented to separate total RNA from CAM tissue for this purpose. A Thermo Scientific Nanodrop 2000 spectrophotometer was used to measure and verify the purity of the total extracted RNA. In accordance with the manufacturer's instructions, cDNA was created using a reverse transcriptase kit (Invitrogen, CA, USA). Utilising a KAPA SYBR FAST qPCR Kit (Kapa Biosystem, MA, USA), the real-time PCR analysis was carried out. Using the delta-delta Ct method of relative quantification, the relative mRNA expression was determined. The qPCR protocol was performed as reported in Vimalraj *et al.,* 2019.

Table 1: The list of primers used for the study.

Primers/Biomarkers	Sequence (5' to 3' and 3' to 5')	
Chicken ANG-1	Forward	GAGTCTGGTCACTCGGCAAA
	Reverse	CTAGGCTGCCATCTTCTCGG
Chicken VEGFA	Forward	TGAGGGCCTAGAATGTGTCC
	Reverse	TCTTTTGACCCTTCCCCTTT

4. Statistical Analysis:

Statistical significance between the groups was determined utilising one-way ANOVA test on the GraphPad Prism software (version 8.2.1). Statistical differences that were considered significant is $p < 0.05$.

Results

Vessel Area:

The vessel area was measured at three different regions in each of the distinct three groups: Group A: Control, Group B: MCF 7 cells only and Group C: MCF 7 cells + Vedicinals ® 9 Advanced formula, at three distinct time points of 0 hour, 3 hours and 6 hours post-treatment. At the 3-hour time point, when comparing the control and MCF-7 cells groups to hour-0, we observed a significantly higher vessel area with a fold change of 1.6 and 2.0, respectively (figure 1a). At the 6-hour time point when comparing both the control and MCF-7 cell group to hour-0, there was a fold change of 2.4 and 2.8, respectively (figure 1b). Furthermore, the treatment with Vedicinals 9 ® Advanced resulted in a statistically significant reduction of vessel area compared to the MCF-7 cell group. There was a 33% reduction in vascularisation of MCF7 cells at hour-3 (figure 1a) and a 39% reduction at hour-6 (figure 1b).

Vessel Total Length:

The vessel total length was measured at three different regions in each of the distinct three groups: Group A: Control, Group B: MCF 7 cells only and Group C: MCF 7 cells + Vedicinals ® 9 Advanced formula, at three distinct time points of 0 hour, 3 hours and 6 hours post-treatment. At the 3-hour time point, when comparing the control and MCF-7 cells to hour-0, it results in a significantly higher vessel area with a fold change of 1.6 and 1.9, respectively (figure 2a). At the 6-hour time point when comparing both the control and MCF-7 cell group to hour-0 there was a fold change of 2 and 2.5, respectively (figure 2b). Furthermore, the treatment with Vedicinals 9 ® Advanced resulted in a statistically significant reduction of vessel total length. The formulation caused a 36% reduction in vascularisation of MCF7 cells at hour-3 and a 23% reduction at hour-6.

Number of nodes/branching points:

The number of nodes/branching points was measured at three different regions in each of the distinct three groups: Group A: Control, Group B: MCF 7 cells only and Group C: MCF 7 cells + Vedicinals ® 9 Advanced formula, at three distinct time points of 0 hour, 3 hours and 6 hours post-treatment. At the 3-hour time point, when comparing the control and MCF-7 cells to hour-0, it results in a statistically not significant higher vessel area with a fold change of 1.5 and 1.6, respectively (figure 3a). At the 6-hour time point when comparing both the control and MCF-7 cell group to hour-0 there was a fold change of 1.8 and 2.3, respectively (figure 3b). Furthermore, the treatment with Vedicinals 9 ® Advanced formulation resulted in a statistically significant reduction of the number of nodes/branching points. The formulation caused an 14% reduction in vascularisation of MCF7 cells at hour-3 and an 20% reduction at hour-6.

Vessel mean thickness:

The vessel mean thickness was measured at three different regions in each of the distinct three groups: Group A: Control, Group B: MCF 7 cells only and Group C: MCF 7 cells + Vedicinals ® 9 Advanced formula, at three distinct time points of 0 hour, 3 hours and 6 hours post-treatment. At the 3-hour time point, when comparing the control and MCF-7 cells to hour-0, it results in a non-statistically significant vessel area with a fold change of 1.1 and 1.1, respectively (figure 4a). At the 6-hour time point when comparing both the control and MCF-7 cell group to hour-0 there was a fold change of 1.1 and 1.2, respectively (figure 4b). Furthermore, the treatment with Vedicinals 9 ® Advanced formulation resulted in a non-statistically significant reduction of vessel mean thickness. The formulation caused a 5% reduction in vascularisation of MCF7 cells at hour-3 and an 9% reduction at hour-6.

Visual representation vascularisation shown in figure 5:

The stereomicroscope was employed to record the morphological modifications of angiogenesis in four groups: control, Vedicinals ® 9 Advanced, MCF 7 cells, and MCF 7 cells + Vedicinals ® 9 Advanced, at hours 0, 3, and 6. Angiogenic parameters of blood vessel development, such as area, length, thickness, and branching.

mRNA expression of *VEGFA* and *ANG1*:

The mRNA expression of *VEGFA* and ANG1 was measured within 3 groups of Control, MCF7 and MCF7 + Vedicinals ® 9 Advanced formula. The results of the qPCR analysis are shown in Figure 3. The results for VEGFA are shown in Figure 5a. Compared to the control, the mRNA expression of VEGFA in the MCF7 group significantly increased by 3.1-fold, whereas the treatment with Vedicinal 9 Advanced resulted in a significant 55% reduction of VEGF mRNA expression compared to the MCF7 group (Figure 6a). The results for ANG1 are shown in Figure 5b. Compared to the control, the mRNA expression of ANG1 in the MCF7 group was significantly increased by 2.9-fold treatment with Vedicinals ® 9 Advanced group resulted in a significant 62% reduction of ANG1 mRNA expression compared to the MCF-7 group (Figure 6b).

Discussion

Breast cancer is defined as abnormal cell proliferation in breast tissue; the cancer typically begins in the lobule lining or the ductal epithelium of the breast tissue (Lukasiewicz *et al.*, 2021). It is the most prevalent cancer diagnosed in women globally, including Australia. In 2022, of the 185 nations evaluated, 157 reported that epidemiologically speaking, breast cancer was the most common (World Health Organization, *Breast cancer*, 2024). The diagnosis of breast cancer in western countries are majority of the time through national health screening programs, whereas in developing nations diagnosis occurs through physical examination when a patient has noticed physical changes in the breast, or pain (Alkabban *et al.*, 2024). Moreover, the treatment options for breast cancer include hormonal therapy, radiation, chemotherapy, and surgery (Alkabban *et al.*, 2024). However, there are key limitations of each of the treatment options mentioned above. This includes the fact that chemotherapy cause severe gastrointestinal distress, fatigue and impacting the psychological well-being of the patient (Burstein, 2022). Surgery can cause lymphedema, higher risk for the recurrence of the mass, immediate need for radiation therapy post-surgery and permanent alternation in the size and shape of the breast tissue (Czajka & Pfeifer, 2023). Radiation can cause skin alteration, lymphedema, and nerve damage of the brachial nerve (Ronckers et al., 2004). Hormonal therapy is only specific on breast cancers that are estrogen receptor positive (ER+), can cause early menopause symptoms and bone thinning (Puhalla et al., 2012). Hence, due to the numerous limitations found in each of the current treatment options available, there is a need for a more effective treatment options that lack the indicated side-effects. A promising potential alternative treatment option is represented by the Vedicinals ®9 Advanced formulation.

MCF-7 cells are valuable for *in vitro* breast cancer investigations because they preserve certain properties of the mammary epithelium, such as the processing of estrogen also known as oestradiol via estrogen receptors in the cell cytoplasm (Camarillo et al., 2014). It is the first hormone-responsive breast cancer cell line. Hence, in the present study, the anti-cancer activity of the Vedicinals ®9 Advanced formula was evaluated by measuring its anti-angiogenic effectiveness through experimental procedures such as the stimulation of a tumour microenvironment in the presence of MCF7 cells and the Vedicinals ®9 Advanced formulation. The anti-angiogenic activity of the Vedicinals ® 9 Advanced formulation has been validated mechanistically through qPCR.

The results displayed the Vedicinals ® 9 Advanced formulation utilised in the study was able to demonstrate promising anti-angiogenic properties within the environment of a CAM model. The figures in the results section represent the quantification data of angiogenic parameters. Figure 1 depicts the collated data for vessel area, Fig. 2 depicts the collated data for vessel total length, Fig. 3 depicts the collated data for the number of nodes/branching points, and Fig. 4 depicts the collated data for vessel mean thickness. Additionally, Fig. 6 summarises the mRNA expression of VEGFA and ANG1 within the 3 tested groups. From the results, it is clear that Vedicinals ® 9 Advanced formulation significantly decreases the growth of new blood vessels in terms of both vessel area, length, thickness, and branching. Considering that an increase in vessel area in tumour microenvironment (TME) provides the

tumour with more oxygen and nutrients allowing it to grow and metastasize, inhibition of angiogenesis may hinder the process of metastasis. This can be correlated with previous studies conducted by Paudel *et al.*, 2023 where the Vedicinals ® 9 Advanced formulation exhibited significant reduction of A549 human lung adenocarcinoma cells migration and invasion underlining the formulation anticancer activity (Paudel *et al.*, 2023). Interestingly, this same study, highlighted similar results of Vedicinals® 9 Advanced formula also stimulated an increase in the expression of endostatin, an angiogenesis inhibitor, highlighting the potential anti-angiogenetic effect of this formulation (Paudel *et al.*, 2023).

Vessel mean thickness is a crucial parameter in tumour angiogenesis as a newly developed tumour vessel architecture is highly irregular with both thick and thin vessels. Normalisation and maturation of abnormal tumour vessels is essential to combat the hypoxic condition and for efficient delivery of anti-cancer drugs. From Fig. 4 it can be observed that Vedicinals decreases the mean thickness of blood vessels adequately. Similarly, the number of nodes in Fig. 3 is significantly reduced when the CAM is treated with the Vedicinal 9 Advanced formula. From this set of results, it can be interpreted that Vedicinal prevents further bifurcation of existing vasculature into new capillaries, thereby efficiently reducing tumour angiogenesis.

Real-time qPCR was used to examine the mRNA expression of VEGFA and ANG1 involved in angiogenesis to investigate the impact of Vedicinal 9 Advanced at the genetic level. The results are displayed in Fig. 6. In the TME, VEGFA, an active growth factor in angiogenesis that promotes endothelial cell migration, are expressed at higher levels compared the control. It can be noted that the expression of the VEGFA gene was reduced after receiving Vedicinals treatment. Similarly, the ANG1 expression levels also showed a similar pattern of downregulation. The overall findings indicate that Vedicinals possesses tumour-suppressive qualities that inhibit the proliferation of cancer cells by hindering angiogenesis.

The results of the Vedicinals ® 9 Advanced formulation from this study in terms of its anti-angiogenic action are consistent with prior research on only the formulation's individual active ingredients. These include a study by Allowitz et al., 2023 paper that underlined vitamin B1’s ability to demonstrate the prevention of VEGF-induced angiogenesis by inhibiting the VEGF-induced cell proliferation. Additionally, Premkumar et al., 2008 study demonstrated the reduction of proangiogenic factors serum levels in each of the 84 breast cancer patients post one year of the randomised clinical trial where breast cancer patients were administered a daily supplement of CoQ(10) along with tamoxifen twice a day. The study, additionally, was able to highlight the relationship between the reduction the phosphorylation of tyrosine 16 and the CoQ10 with riboflavin complex (Premkumar et al., 2008). The reduction in this tyrosine Kinase is thought to be an important factor in tumour growth, giving oncogenic signalling for cell angiogenesis, metastasis, and survival. Moreover, a study conducted by Saghiri et al., 2017 highlighted vitamin B6 ability to reduce angiogenesis by decreasing microvascular outgrowth, dose dependant and by lowering endothelial cell proliferation through the inhibition of DNA topoisomerase and polymerase.

Moreover, Conejo-Garcia et al., 2004 demonstrated vitamin C's ability to decrease nitric oxide (NO) synthesis limiting angiogenesis and new blood vessel formation. This is the result of ascorbic acid inhibiting angiogenesis due to inhibiting the initial phase of cell replication including formation of microtubules utilised for cell splitting. Furthermore, Feng et al., 2023 reviewed the potential nature of Selenium for inhibition of tumour angiogenesis occurs through the aims of thioredoxin reductase which initiates cell cycle arrest and cell apoptosis through the upregulated production of reactive oxygen species that are produced by endothelial cells (Feng et al., 2023).

Additionally, Zhao et al., 2016 study resulted in highlighting quercetin's anti-angiogenic properties, including the inhibition of NFAT pathway and the expression of the VEGFR-2 protein. Furthermore, Fu et al., 2015 underlined the supressing nature of curcumin, which exerts a strong anti-angiogenic activity through blocking the PI3K/AKT signalling pathway, as well as cyclic nucleotide phosphodiesterase inhibition. Interestingly, Rashidi et al., 2017 paper underlined green tea's ability to reduce the process of angiogenesis through the inhibition of the VEGF's ability to bind to its own receptor, and the reduction of angiogenic factor production. Moreover, a study conducted by Doucette *et al.*, 2012 revealed *Piper nigrum*'s ability to block the PI3K/Akt signalling pathway, which is predominantly responsible for the promotion angiogenesis in breast tissue. This similar result of modulating the signalling pathways known to control angiogenesis such as the VEGF and PI3K/Akt have shown to be regulated by the presence of *Saphora japanica*, (Li et al, 2021). However, it is important to note that those studies implemented only one of the core active ingredients of the Vedicinals ® 9 Advanced formula just underlining the synergistic impact of each of the active ingredients on angiogenesis through various mechanisms such as the inhibition of VEGF and PI3K/Akt pathways.

Despite the promising results, the present study is not exempt from limitations. Despite the CAM is a commonly used and reliable model that is employed for *in vivo* studies for angiogenesis research, it does not fully recreate the complex tumour microenvironment observed in cancer patients. Additionally, CAM models can increase the likelihood of false positive results owing to tissue cellular injury, inflammatory responses and fibrin breakdown products. Therefore, there is a need for future research that should investigate the effects of the Vedicinals ® 9 Advanced formula in complex animal models such as mice models with breast cancer. Hence, if the results of animal in vivo studies are promisingly, successive human clinical trials that will enable for further validation of the formulation. Furthermore, thorough molecular investigations are required to determine the individual bioactive chemicals causing the observed anti-angiogenic effects this includes detailed studies such as protein expression analysis such as Western Blot or ELISA, immunohistochemistry to test the endothelial cell markers, and matrix remodelling proteins.

Conclusion

The experiments conducted within this study highlight the Vedicinals ® 9 Advanced formula's ability to supress angiogenesis caused by breast cancer within a CAM model, *in vitro.* These findings show a promising activity of the Vedicinals 9 Advanced formulation in the reduction of angiogenic parameters

including vessel area, length, thickness, and branching nodes. Additionally, the reduction of the mRNA expression of VEGFA and ANG1 further highlights and provides mechanistic rationale to the anti-angiogenic properties of the formulation. Hence, the results clearly demonstrate the formulations great potential as an anti-angiogenic adjunct therapy option for breast cancer.

Declarations

Ethical statement:

The chick embryo experiments were conducted prior to embryonic day 13. At this stage of development, embryos have not developed the ability to perceive pain, and therefore, the procedures do not fall under protocols requiring ethical clearance. As no pain or distress was caused to the embryos during the study, ethical approval was not necessary.

Declaration of Competing Interest

The authors would declare the following interest: The VEDICINALS®9 Advanced formulation we have tested is a product of VEDICINALS® International and more information about it can be found in their official website. https://www.vedicinals.com/vedicinals-9/.

Funding

This project is funded from VEDICINALS® International, India (A German Indian Biotech Company).

Author Contribution

JKD: Writing original draft, investigation, methodology, software, methodology SV: Writing original draft, investigation, methodology,MP: Investigation, methodologyGDR: Methodology, Writing, review editing, validationJG: Conceptualization and fundingPS: Conceptualization and fundingSS: Investigation, resource, methodology, supervisionMS: Writing, review editing, validation, visualizationKRP: Conceptualization, funding acquisition, supervision, validation, visualizationKD: Conceptualization, funding acquisition, supervision, validation, visualization

Data Availability

All data supporting the findings of this study are available within the paper and its Supplementary Information.

References

1. Alkabban, F. M., & Ferguson, T. (2024). *Breast Cancer*. National Library of Medicine; StatPearls Publishing. https://www.ncbi.nlm.nih.gov/books/NBK482286/
2. Allowitz, K. V., Plowman, T. J., & Ramana, K. V. (2023). Abstract 4598: Vitamin b1 derivative, benfotamine, prevents VEGF-induced angiogenesis. *Cancer Research*, *83*(7_Supplement), 4598–4598. https://doi.org/10.1158/1538-7445.am2023-4598
3. Ayoub, N. M., Jaradat, S. K., Al-Shami, K. M., & Alkhalifa, A. E. (2022). Targeting Angiogenesis in Breast Cancer: Current Evidence and Future Perspectives of Novel Anti-Angiogenic Approaches. *Frontiers in Pharmacology*, *13*. https://doi.org/10.3389/fphar.2022.838133
4. Baek, J.-Y., Kwak, J.-E., & Ahn, M.-R. (2024). Eriocitrin Inhibits Angiogenesis by Targeting VEGFR2-Mediated PI3K/AKT/mTOR Signaling Pathways. *Nutrients*, *16*(7), 1091–1091. https://doi.org/10.3390/nu16071091
5. *Breast cancer in Australia statistics*. (2022). Cancer Australia. https://www.canceraustralia.gov.au/cancer-types/breast-cancer/breast-cancer-australia-statistics
6. Breast Cancer Network Australia. (2023, July 11). *Breast cancer in Australia*. Www.bcna.org.au. https://www.bcna.org.au/resource-hub/articles/breast-cancer-in-australia/
7. Burguin, A., Diorio, C., & Durocher, F. (2021). Breast Cancer Treatments: Updates and New Challenges. *Journal of Personalized Medicine*, *11*(8), 808. https://doi.org/10.3390/jpm11080808
8. Burstein, H. J. (2021). Unmet challenges in systemic therapy for early stage breast cancer. *The Breast*. https://doi.org/10.1016/j.breast.2021.12.009
9. Camarillo, I. G., Xiao, F., Madhivanan, S., Salameh, T., Nichols, M., Reece, L. M., Leary, J. F., Otto, K., Natarajan, A., Ramesh, A., & Sundararajan, R. (2014). Low and high voltage electrochemotherapy for breast cancer: an in vitro model study. *Electroporation-Based Therapies for Cancer*, 55–102. https://doi.org/10.1533/9781908818294.55
10. Chakraborti, C. K. (2011). Vitamin D as a promising anticancer agent. *Indian Journal of Pharmacology*, *43*(2), 113–120. https://doi.org/10.4103/0253-7613.77335
11. Conejo-Garcia, J. R., Benencia, F., Courreges, M.-C., Kang, E., Mohamed-Hadley, A., Buckanovich, R. J., Holtz, D. O., Jenkins, A., Na, H., Zhang, L., Wagner, D. S., Katsaros, D., Caroll, R., & Coukos, G. (2004). Tumor-infiltrating dendritic cell precursors recruited by a β-defensin contribute to vasculogenesis under the influence of Vegf-A. *Nature Medicine*, *10*(9), 950–958. https://doi.org/10.1038/nm1097
12. Coventon, J. (2017). A review of the mechanism of action and clinical applications of sorafenib in advanced osteosarcoma. *Journal of Bone Oncology*, *8*, 4–7. https://doi.org/10.1016/j.jbo.2017.07.001
13. Czajka, M. L., & Pfeifer, C. (2023, February 8). *Breast Cancer Surgery*. PubMed; StatPearls Publishing. https://www.ncbi.nlm.nih.gov/books/NBK553076/

14. *Diagnosis and management of Parkinsons*. (2022). Australian Journal of General Practice. https://www1.racgp.org.au/ajgp/2021/november/diagnosis-and-management-of-parkinsons
15. Doucette, C. D., Hilchie, A. L., Liwski, R., & Hoskin, D. W. (2013). Piperine, a dietary phytochemical, inhibits angiogenesis. *The Journal of Nutritional Biochemistry*, *24*(1), 231–239. https://doi.org/10.1016/j.jnutbio.2012.05.009
16. Dudley, A. C., & Griffioen, A. W. (2023). The modes of angiogenesis: an updated perspective. *Angiogenesis*, *26*(4), 477–480. https://doi.org/10.1007/s10456-023-09895-4
17. Feng, B., Zhang, Y., Liu, T., Chan, L., Chen, T., & Zhao, J. (2023). Selenium speciation determines the angiogenesis effect through regulating selenoproteins to trigger ROS-mediated cell apoptosis and cell cycle arrest. *Chinese Chemical Letters*, *34*(11), 108264–108264. https://doi.org/10.1016/j.cclet.2023.108264
18. Fitridge, R., & Springerlink (Online Service. (2020). *Mechanisms of Vascular Disease : A Textbook for Vascular Specialists*. Springer International Publishing.
19. Folkerd, E., & Dowsett, M. (2013). Sex hormones and breast cancer risk and prognosis. *The Breast*, *22*, S38–S43. https://doi.org/10.1016/j.breast.2013.07.007
20. Fu, Z., Chen, X., Guan, S., Yan, Y., Lin, H., & Hua, Z.-C. (2015). Curcumin inhibits angiogenesis and improves defective hematopoiesis induced by tumor-derived VEGF in tumor model through modulating VEGF-VEGFR2 signaling pathway. *Oncotarget*, *6*(23), 19469–19482. https://doi.org/10.18632/oncotarget.3625
21. Hillen, F., & Griffioen, A. W. (2007). Tumour vascularization: sprouting angiogenesis and beyond. *Cancer and Metastasis Reviews*, *26*(3-4), 489–502. https://doi.org/10.1007/s10555-007-9094-7
22. Javed, S., Soukhtehzari, S., Fernandes, N., & Williams, K. C. (2022). Longitudinal bioluminescence imaging to monitor breast tumor growth and treatment response using the chick chorioallantoic membrane model. *Scientific Reports*, *12*(1), 17192. https://doi.org/10.1038/s41598-022-20854-9
23. Kazazi-Hyseni, F., Beijnen, J. H., & Schellens, J. H. M. (2010). Bevacizumab. *The Oncologist*, *15*(8), 819–825. https://doi.org/10.1634/theoncologist.2009-0317
24. Keshav Raj Paudel, Rajput, R., Gabriele De Rubis, Sita, V., Kylie Anne Williams, Sachin Kumar Singh, Gupta, G., Prakash Salunke, Philip Michael Hansbro, Gerlach, J., & Dua, K. (2023). In vitro anti-cancer activity of a polyherbal preparation, VEDICINALS®9, against A549 human lung adenocarcinoma cells. *Pathology - Research and Practice*, *250*, 154832–154832. https://doi.org/10.1016/j.prp.2023.154832
25. Li, R., Song, X., Guo, J., Song, P., Duan, D., & Chen, Z. (2021). Natural Products: A Promising Therapeutics for Targeting Tumor Angiogenesis. *Frontiers in Oncology*, *11*. https://doi.org/10.3389/fonc.2021.772915
26. Liu, Z.-L., Chen, H.-H., Zheng, L.-L., Sun, L.-P., & Shi, L. (2023). Angiogenic signaling pathways and anti-angiogenic therapy for cancer. *Signal Transduction and Targeted Therapy*, *8*(1). https://doi.org/10.1038/s41392-023-01460-1

27. Liu, Y., Wu, W., Cai, C., Zhang, H., Shen, H., & Han, Y. (2023). Patient-derived xenograft models in cancer therapy: technologies and applications. *Signal Transduction and Targeted Therapy*, *8*(1), 1–24. https://doi.org/10.1038/s41392-023-01419-2

28. Lugano, R., Ramachandran, M., & Dimberg, A. (2019). Tumor angiogenesis: causes, consequences, challenges and opportunities. *Cellular and Molecular Life Sciences*, *77*(9), 1745–1770. https://doi.org/10.1007/s00018-019-03351-7

29. Łukasiewicz, S., Czeczelewski, M., Forma, A., Baj, J., Sitarz, R., & Stanislawek, A. (2021). Breast cancer—epidemiology, Risk factors, classification, Prognostic markers, and Current Treatment Strategies—an Updated Review. *Cancers*, *13*(17), 4287. https://doi.org/10.3390/cancers13174287

30. Lupo, G., Cambria, M. T., Olivieri, M., Rocco, C., Caporarello, N., Longo, A., Zanghì, G., Salmeri, M., Foti, M. C., & Anfuso, C. D. (2019). Anti-angiogenic effect of quercetin and its 8-methyl pentamethyl ether derivative in human microvascular endothelial cells. *Journal of Cellular and Molecular Medicine*, *23*(10), 6565–6577. https://doi.org/10.1111/jcmm.14455

31. Luu, T., Frankel, P., Chung, C., Chow, W., Mortimer, J., Hurria, A., & Somlo, G. (2014). Phase I/II Trial of Vinorelbine and Sorafenib in Metastatic Breast Cancer. *Clinical Breast Cancer*, *14*(2), 94–100. https://doi.org/10.1016/j.clbc.2013.10.013

32. Matsubara, K., Mori, M., Matsuura, Y., & Kato, N. (2001). Pyridoxal 5'-phosphate and pyridoxal inhibit angiogenesis in serum-free rat aortic ring assay. *International Journal of Molecular Medicine*. https://doi.org/10.3892/ijmm.8.5.505

33. Mentzer, S. J., & Konerding, M. A. (2014). Intussusceptive angiogenesis: expansion and remodeling of microvascular networks. *Angiogenesis*, *17*(3), 499–509. https://doi.org/10.1007/s10456-014-9428-3

34. Moreno-Jiménez, I., Hulsart-Billstrom, G., Lanham, S. A., Janeczek, A. A., Kontouli, N., Kanczler, J. M., Evans, N. D., & Oreffo, R. O. (2016). The chorioallantoic membrane (CAM) assay for the study of human bone regeneration: a refinement animal model for tissue engineering. *Scientific Reports*, *6*(1). https://doi.org/10.1038/srep32168

35. Nakamichi, M., Akishima-Fukasawa, Y., Fujisawa, C., Mikami, T., Onishi, K., & Akasaka, Y. (2016). Basic Fibroblast Growth Factor Induces Angiogenic Properties of Fibrocytes to Stimulate Vascular Formation during Wound Healing. *The American Journal of Pathology*, *186*(12), 3203–3216. https://doi.org/10.1016/j.ajpath.2016.08.015

36. Narod, S. A. (2011). Hormone replacement therapy and the risk of breast cancer. *Nature Reviews Clinical Oncology*, *8*(11), 669–676. https://doi.org/10.1038/nrclinonc.2011.110

37. Nishida, N., Yano, H., Nishida, T., Kamura, T., & Kojiro, M. (2006). Angiogenesis in cancer. *Vascular Health and Risk Management*, *2*(3), 213–219. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1993983/

38. Premkumar, V. G., Yuvaraj, S., Sathish, S., Shanthi, P., & Sachdanandam, P. (2008). Anti-angiogenic potential of CoenzymeQ10, riboflavin and niacin in breast cancer patients undergoing tamoxifen therapy. *Vascular Pharmacology*, *48*(4-6), 191–201. https://doi.org/10.1016/j.vph.2008.02.003

39. Puhalla, S., Bhattacharya, S., & Davidson, N. E. (2012). Hormonal therapy in breast cancer: A model disease for the personalization of cancer care. *Molecular Oncology*, *6*(2), 222–236. https://doi.org/10.1016/j.molonc.2012.02.003
40. Rashidi, B., Malekzadeh, M., Goodarzi, M., Masoudifar, A., & Mirzaei, H. (2017). Green tea and its anti-angiogenesis effects. *Biomedicine & Pharmacotherapy*, *89*, 949–956. https://doi.org/10.1016/j.biopha.2017.01.161
41. Ribatti, D., Annese, T., & Tamma, R. (2020). The use of the chick embryo CAM assay in the study of angiogenic activiy of biomaterials. *Microvascular Research*, *131*, 104026. https://doi.org/10.1016/j.mvr.2020.104026
42. Ronckers, C. M., Erdmann, C. A., & Land, C. E. (2004). Radiation and breast cancer: a review of current evidence. *Breast Cancer Research*, *7*(1). https://doi.org/10.1186/bcr970
43. Saghiri, M. A., Asatourian, A., Ershadifar, S., Moghadam, M. M., & Sheibani, N. (2017). Vitamins and regulation of angiogenesis: [A, B1, B2, B3, B6, B9, B12, C, D, E, K]. *Journal of Functional Foods*, *38*, 180–196. https://doi.org/10.1016/j.jff.2017.09.005
44. Sasich, L. D., & Sukkari, S. R. (2012). The US FDAs withdrawal of the breast cancer indication for Avastin (bevacizumab). *Saudi Pharmaceutical Journal*, *20*(4), 381–385. https://doi.org/10.1016/j.jsps.2011.12.001
45. Selvaraj Vimalraj, Sivakamasundari Pichu, Thyagarajan Pankajam, Dharanibalan Kasiviswanathan, Djonov, V., & Chatterjee, S. (2019). Nitric oxide regulates intussusceptive-like angiogenesis in wound repair in chicken embryo and transgenic zebrafish models. *Nitric Oxide*, *82*, 48–58. https://doi.org/10.1016/j.niox.2018.11.001
46. Sen, B., & Johnson, F. M. (2011). Regulation of Src Family Kinases in Human Cancers. *Journal of Signal Transduction*, *2011*, 1–14. https://doi.org/10.1155/2011/865819
47. World Health Organization. (2024, March 13). *Breast cancer*. World Health Organization. https://www.who.int/news-room/fact-sheets/detail/breast-cancer
48. Zhao, X., Wang, Q., Yang, S., Chen, C., Li, X., Liu, J., Zou, Z., & Cai, D. (2016). Quercetin inhibits angiogenesis by targeting calcineurin in the xenograft model of human breast cancer. *European Journal of Pharmacology*, *781*, 60–68. https://doi.org/10.1016/j.ejphar.2016.03.063
49. Zubair, M., Wang, S., & Ali, N. (2021). Advanced Approaches to Breast Cancer Classification and Diagnosis. *Frontiers in Pharmacology*, *11*. https://doi.org/10.3389/fphar.2020.632079

Figures

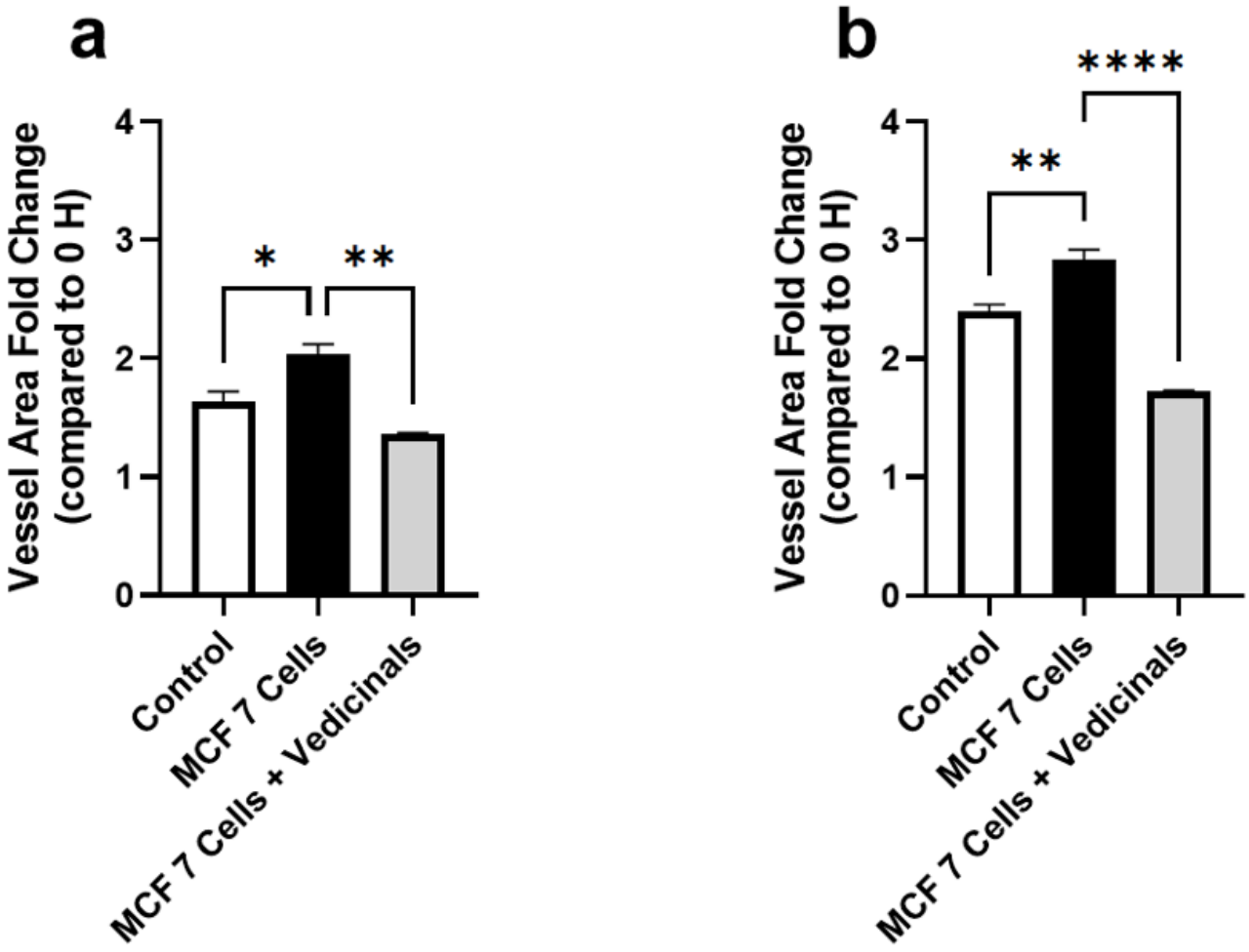


Figure 1

a & b: The vessel area was measured as a mean of n=3 readings, at both 3 hours (a) and 6 hours (b) after treatment. The results were deemed statistically significant through the conduction of ordinary one-way ANOVA tests for each comparative group. The error bars indicate SEM. ns: not significant; *: $p<0.05$ **: $p < 0.01$ ****: $p < 0.0001$.

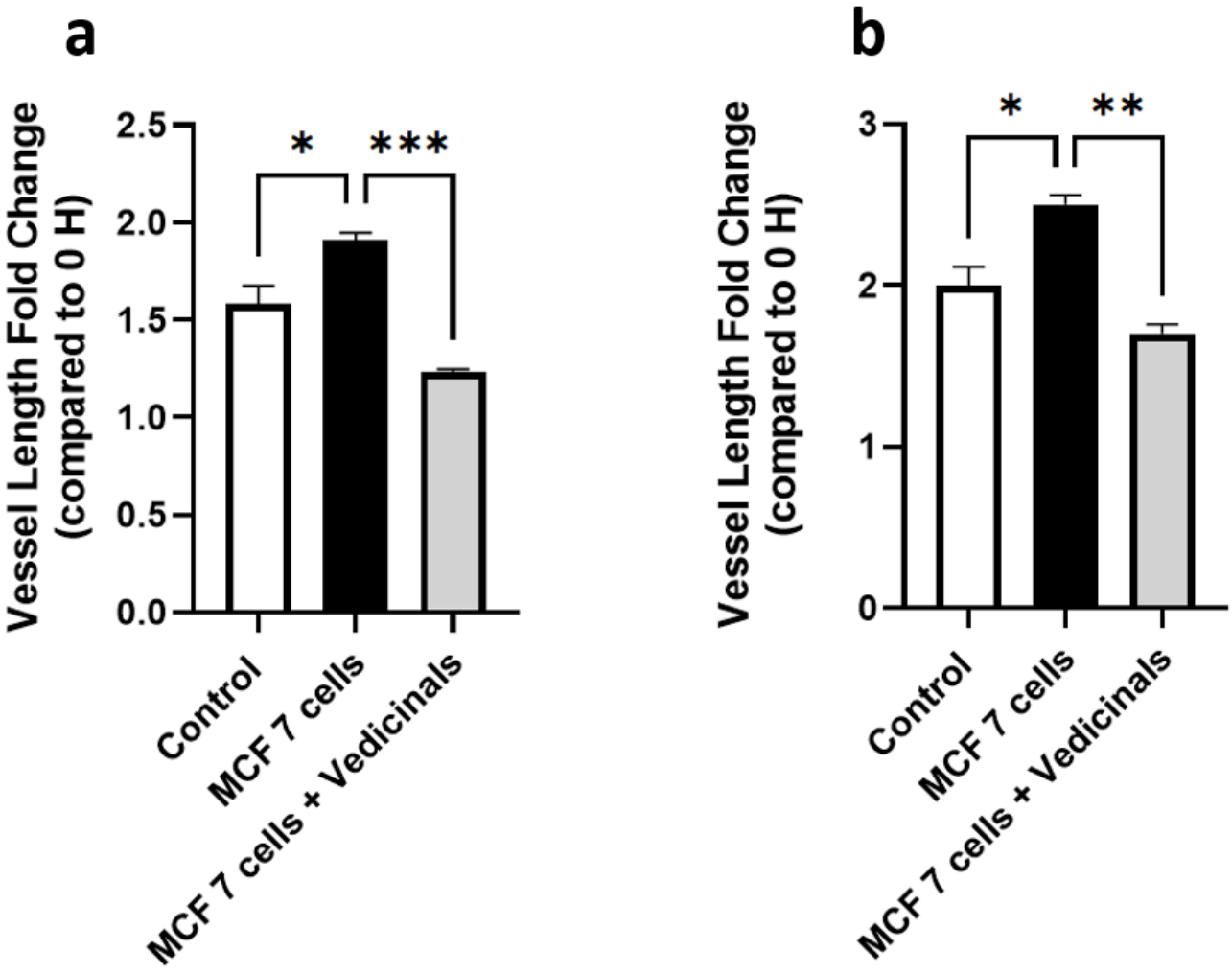


Figure 2

a & b: The vessel total length was measured as a mean of n=3 readings, at both 3 hours (a) and 6 hours (b) after treatment. The results were deemed statistically significant through the conduction of ordinary one-way ANOVA tests for each comparative group. The error bars indicate SEM. ns: not significant; *: $p<0.05$ **: $p < 0.01$ ***: $p < 0.001$

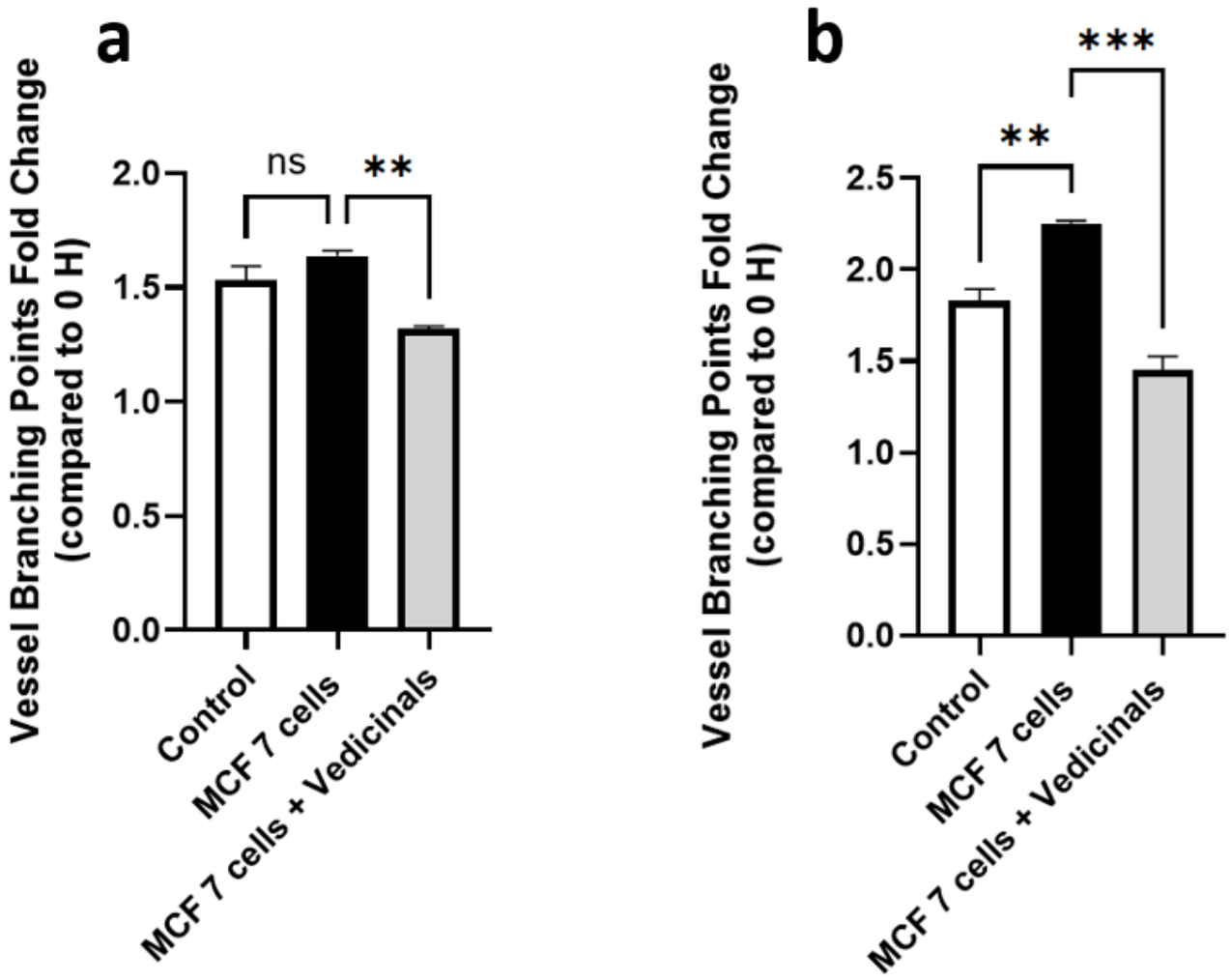


Figure 3

a & b: The vessel nodes/branching points was measured as a mean of n=3 readings, at both 3 hours (a) and 6 hours (b) after treatment. The results were deemed statistically significant through the conduction of ordinary one-way ANOVA tests for each comparative group. The error bars indicate SEM. ns: not significant; **: $p < 0.01$ ***: $p < 0.001$

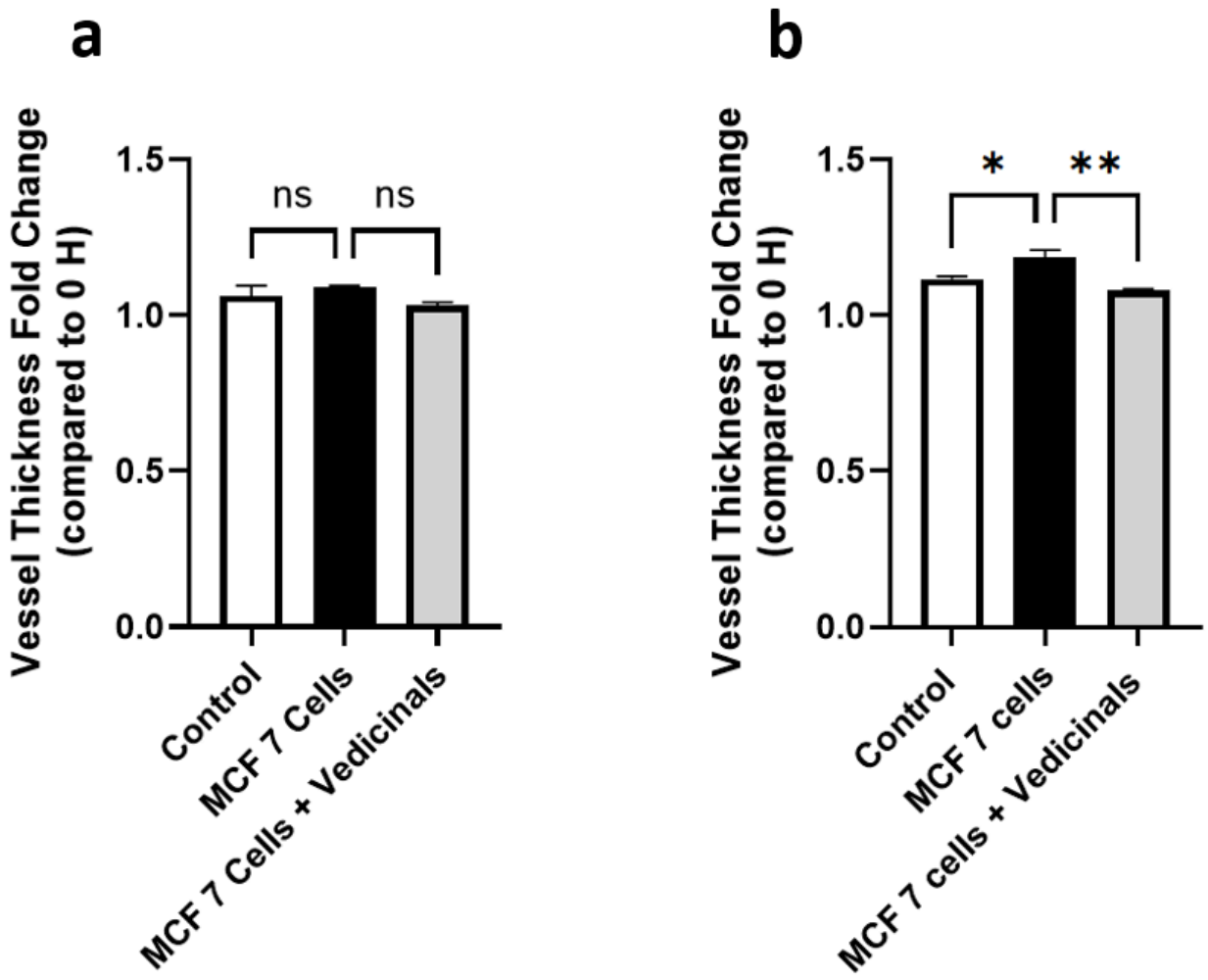


Figure 4

a & b: The vessel mean thickness was measured as a mean of n=3 readings, at both 3 hours (a) and 6 hours (b) after treatment. The results were deemed statistically significant through the conduction of ordinary one-way ANOVA tests for each comparative group. The error bars indicate SEM. ns: not significant; *: $p<0.05$ **: $p < 0.01$

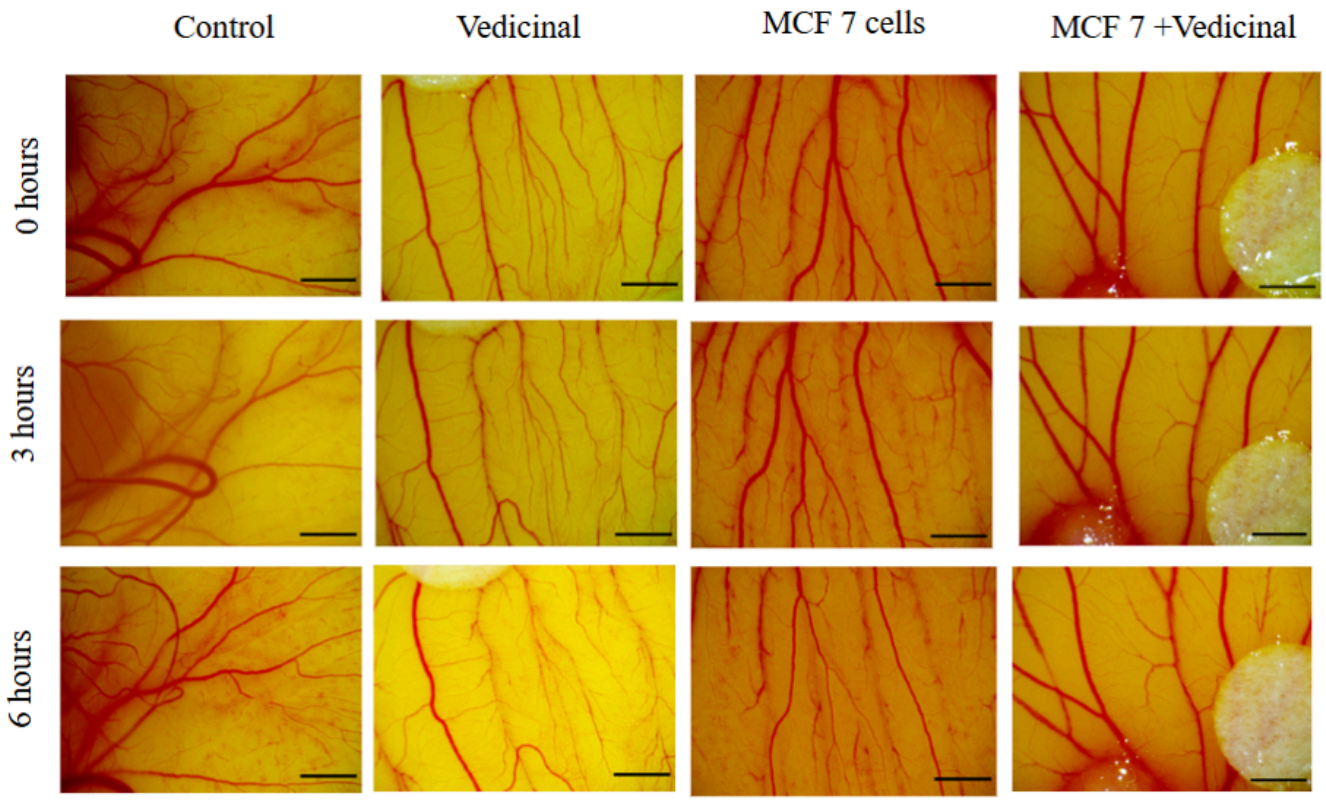


Figure 5

In vivo microscope images of chick CAM development. Images were taken by utilising a stereomicroscope at 0 hour, 3 hours and 6 hours. Scale bar of 500 um.

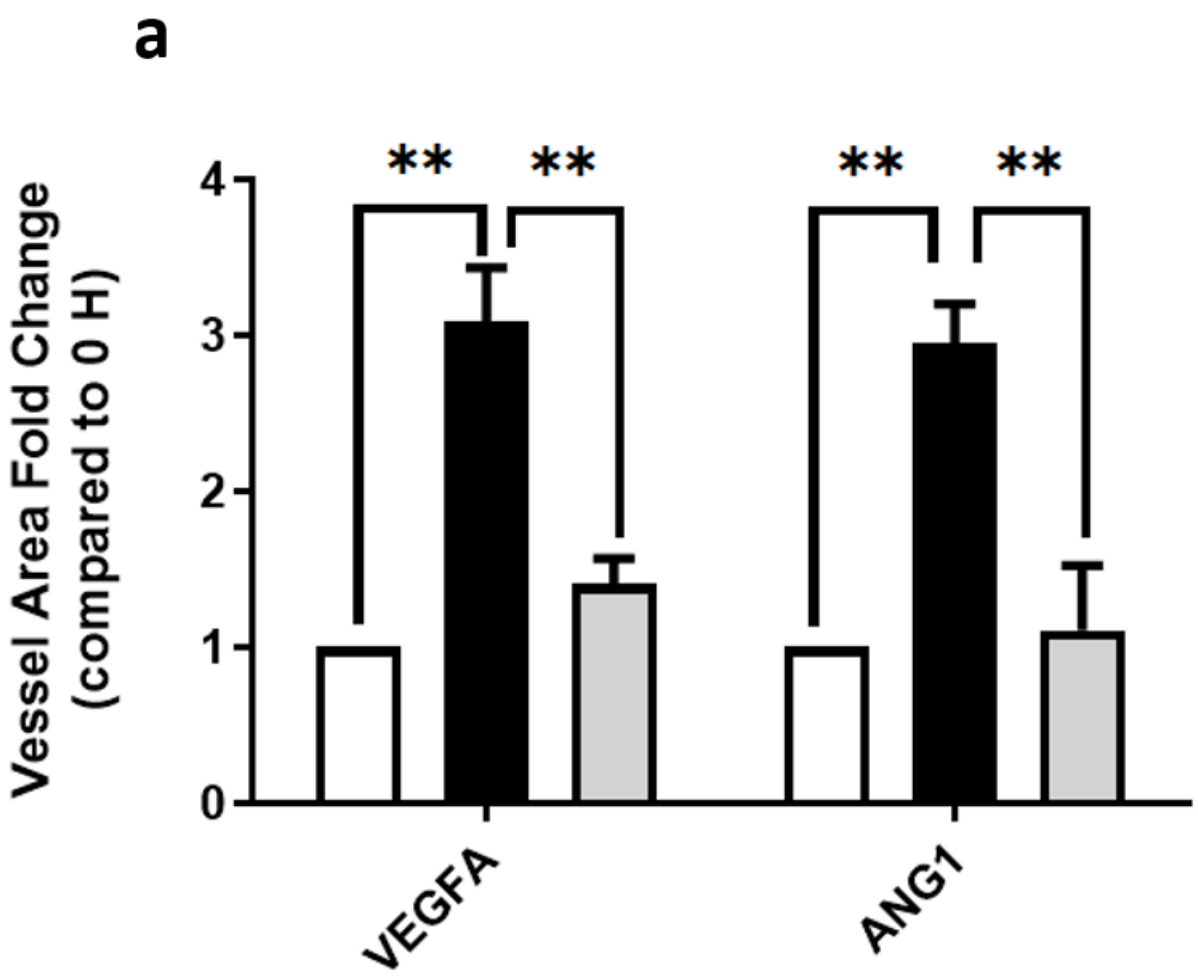


Figure 6

a & b: mRNA expression results of VEGFA (A) and ANG1 (B) measured by qPCR. the results were deemed statistically significant through the conduction an ordinary one-way ANOVA, one for each comparative group. The figure bars indicate SEM. ns: not significant; **: $p<0.01$

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- GraphicalAbstract.docx