

Potential inhibition of angiogenesis using clove, cinnamon, and coriander extracts in Chorioallantoic Membrane (CAM) Assay

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ABSTRACT

Conventional cancer therapy targets blood vessels that provide oxygen and nutrients to proliferating tumor cells. Therefore, regulation of angiogenesis is an important aspect in the treatment of diseases. Angiogenesis is the development of new blood vessels from pre-existing vasculature. Most anti-angiogenic drugs now in use are synthetic substances and it was found from many preclinical and clinical studies that most of them indiscriminately target all rapidly dividing cells, resulting in severe adverse effects such as immunosuppression, intestinal problems, and hair loss. To combat this, researchers are looking for herbal drugs as they have been reported to be safe without and adverse side effect, as compared to synthetic drugs. In the present study, we have investigated the potential anti-angiogenic properties of cinnamon, coriander, and clove extracts. The phytochemical analysis of both ethanol and aqueous extracts from these plants revealed the presence of flavonoids, alkaloids, phenols, triterpenoids, and steroids in various grades, all of which exhibited positive anti-angiogenic effects, validated through an in vivo CAM assay. We conducted experiments on fertilized chicken eggs, which were incubated for 9 days under controlled conditions at 37 °C and 60% humidity before the CAM was performed. Following treatment with 100µl of ethanol and PBS extracts for 72 hours, we observed a significant decrease in vascular formation. These results strongly support the assertion that clove, cinnamon, and coriander possess genuine anti-angiogenic properties, suggesting their potential as herbal therapeutics in future medicinal applications of angiogenesis-associated diseases.

Keywords: anti-angiogenic activity; clove; cinnamon; coriander; Chorioallantoic membrane assay

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INTRODUCTION

Angiogenesis is the complex, highly regulated, and multistep process of the formation of new blood vessels from pre-existing capillaries [1]. This process can appear in both healthy and pathological states and is present throughout all phases of life, from early infancy to old age. Angiogenesis typically occurs via two distinct mechanisms within the human body. Physiological angiogenesis denotes the natural process crucial for embryonic growth, wound repair, and the regulation of the menstrual cycle. In contrast, pathological angiogenesis entails the aberrant formation of blood vessels commonly linked to conditions such as diabetic retinopathy, tumor progression, and age-related macular degeneration. Despite sharing basic blood vessel formation, these two variants of angiogenesis diverge in their regulatory mechanisms and consequences for health [2].

In recent years, various anticancer medications have been discovered due to advancements in the medical field with the primary objective of harming cancer cells without affecting normal cells. However, due to their non-cost effectiveness and side effects, researchers are looking for herbal drugs that possess anti-angiogenic potential. Recent studies have shown that plants containing certain compounds with anticancer capabilities are strongly associated with a decreased cancer risk [3]. Moreover, herbal drugs typically have few side effects, making them attractive candidates for anti-angiogenic therapy. In this investigation, we have studied three herbal plants for their anti-angiogenic behavior, namely clove, cinnamon, and coriander. Their anti-angiogenic and antioxidant activities have not been fully explored yet.

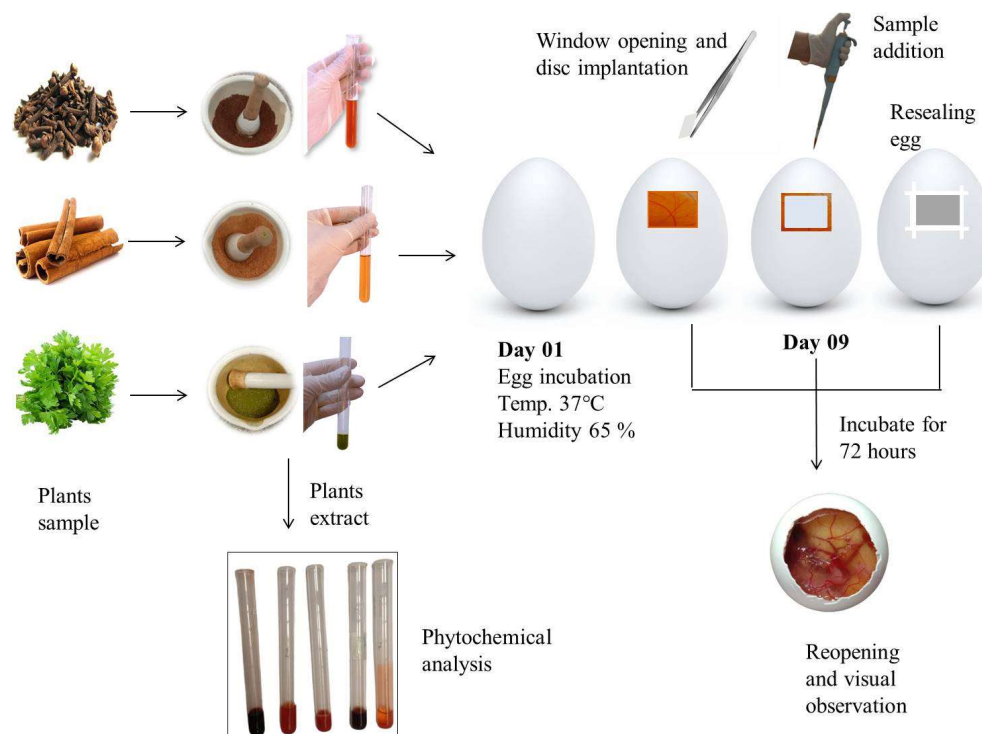


Figure 1. Graphical presentation of workflow

Cloves (*Syzygium aromaticum*) are used as an ancient medicinal herb in India. Clove is native to the Maluku Islands, Indonesia, which were formerly known as the Spice Islands. Previous investigation shows that eugenol, which is the most important composition of clove oil, has potent anti-carcinogenic, antioxidant, and pro-oxidant qualities [4].

Cinnamon (*Cinnamomum zeylanicum*), a member of the family Lauraceae, is also used as a spice and as a medicine [5]. It possesses potent anti-angiogenesis, antioxidant, antibacterial, antifungal, and insecticidal properties. A research investigation reported that cinnamon bark is rich in cinnamaldehyde, which has potent anti-angiogenic and antioxidant properties[6].

Coriander (*Coriandrum sativum* L.) Leaves have a greater capacity to scavenge free radicals, and numerous pharmacological benefits of coriander have also been documented, including sedative-hypnotic, antioxidant, anticancer, anti-angiogenic, antidiabetic,

cholesterol-lowering, antifungal, anxiolytic, hepatoprotective, and anti-ulcer properties [7].

Besides the previous study, there is still a need to discover or find their other medicinal properties to maximize their therapeutic potential. In this study, we are analyzing various plant extracts to identify potential future herbal anti-angiogenic agents, as plants provide a safer and more affordable method to fight against angiogenesis compared to chemical drugs.

MATERIALS AND METHODS

Collection of samples: fertilized chicken eggs bought from Keggfarms, Gurugram, Haryana, India. The clove buds, cinnamon bark, and fresh coriander leaves were obtained from an online platform called NurseryLive. The samples were authenticated by the Amity Institute of Biotechnology, Amity University, Noida, Uttar Pradesh. The plant parts were washed, dried in an oven for 24 hours at a temperature of 30-35 °C, and ground manually using a mortar and pestle.

Table 1. Plant samples with local and botanical names.

Sl.No.	Local Name	English Name	Scientific Name	Part Used
01	Laung	Clove	<i>Syzygium aromaticum</i>	Flower buds
02	Dalchini	Cinnamon	<i>Cinnamomum zeylanicum</i>	Bark
03	Dhaniya	Coriander	<i>Coriandrum sativum</i> L.)	Leaves

Preparation of plant extract

Aqueous extract: A dried plant sample was weighed to prepare 10% aqueous extract by dissolving in the respective plant sample, i.e., clove, cinnamon, and coriander. Then it was filtered using Whatman filter paper. The extract was collected and double centrifuged at 10000 rpm at 3–4 °C for 10 minutes each. Store the sample in the refrigerator at 3–4 °C for further experimental purposes.

Solvent extract: 1 gram of spice powder was dissolved in 10 ml of absolute ethanol and mixed until slurry. Filtered using Whatman filter paper and collected the extract for a double centrifuge at 10000 rpm at 3–4 °C for 10 minutes each. Store the sample in the refrigerator at 3–4 °C for further experimental purposes.

Phytochemical Analysis

The aqueous and ethanolic extracts of the plant were analyzed for phytochemicals [8]. These extracts were tested in order to find out the presence of active compounds by using the following standard methods:

Test for alkaloids (Wagner’s test): 2 ml of the plant extract was added with 1-2 drops of Wagner’s reagent; the formation of a brown-reddish precipitate indicates the presence of alkaloids.

Test for Flavonoids: A few drops of concentrated sodium hydroxide (NaOH) were added to 2 ml of the extracts, followed by the addition of a few drops of diluted hydrochloric acid, and observed for the presence of flavonoids. An intense yellow color formation that becomes colorless with the addition of dilute hydrochloric acid indicates the presence of flavonoids.

Test for phenols (ferric chloride test): Two drops of 10% ferric chloride solution were added to 2 ml of plant extract; the formation of a greenish blue or violet color indicates the presence of phenol.

Test for triterpenoids: (Liebermann-Burchard test)

2 ml of plant extract was added, mixed with a few drops of acetic anhydride, boiled, and cooled. Followed by a few drops of conc. sulfuric acid and mixed well. The formation of a brown ring at the junction of two layers, i.e., the green coloration of the upper layer and the formation of a deep red color in the lower layer, would indicate the presence of triterpenoids.

Test for steroids: (Salkowski test)

A few drops of concentrated sulfuric acid and 2 ml of chloroform were applied successively from the side of the test tube to 2 ml of the plant extract. The test tube was shaken gently for a few minutes. The development of a red color in the chloroform layer indicates the presence of steroids.

Chorioallantoic Membrane (CAM) Assay

Fertilized eggs were surface sterilized with absolute ethanol and incubated for 9 days at a temperature of 37 °C with a relative humidity of 65%. On the 9th day of incubation, eggs were brought under laminar air flow, and windows were created on each eggshell with the help of a sterilized surgical blade for the implantation of plant extracts. Eggs were divided into control and treated groups. At least 5 eggs were used per sample, and the experiment was repeated 3 times. In the control groups, 5 eggs were kept for aqueous and the other 5 for ethanol, respectively. 1×1 cm disc implanted inside the egg over the blood vessels on the growing CAM with the help of forceps, and then 100 µl of respective solutions were implanted over the disc. In the clove extract-treated groups, 5 eggs were treated with 100 µl of clove ethanolic extract and the other 5 with an aqueous extract of the plant. Similarly, in the cinnamon-treated group, 5 eggs were treated with 100 µl of cinnamon ethanol and the other 5 with the

same quantity of aqueous extract. Eggs were sealed with cellophane tape and returned to the incubator for the next 72 hours at a similar temperature and humidity. After 72 hours, eggs were brought for final evaluation. Cellophane tape was removed from the eggs, and the opening was widened with the help of forceps. The filter paper disc and the CAM layer were gently removed to avoid the rupture of blood vessels. Images were captured using a digital camera and were further analyzed by ImageJ software for the number of major vessels, length and thickness of primary branching, number of blood vessels and overall inhibition in angiogenesis [9,10]. The percent inhibition for each test sample was computed using the formula: Percent Inhibition = $\frac{X - Y}{X} \times 100$; wherein X is the number of blood vessels of the untreated CAM and Y is the number of blood vessels of the treated CAM [11].

RESULTS

Phytochemical Analysis: The aqueous and ethanol extracts of clove, cinnamon, and coriander were qualitatively analyzed for various phytochemicals. The phytochemical analysis of three spices is presented in Table 2. As per the result obtained, some of the phytochemicals analyzed were mostly present in the extracts of all the spices. Among the five phytochemicals analyzed, phenol and steroids were commonly present, and flavonoids and triterpenoids were almost absent in all the plant extracts. The clove aqueous extract shows the presence of phenol, while its ethanolic extract shows the presence of alkaloids, phenols, and steroids. There were no traces of flavonoids or triterpenoids in both the aqueous and ethanolic extracts. Both the aqueous and ethanolic extracts of cinnamon show positive results for phenol but no traces of flavonoids or triterpenoids. Cinnamon aqueous shows the presence of alkaloids and steroids, and its ethanolic extract shows the presence of steroids too. The ethanolic extract of coriander shows the presence of alkaloids, phenols, and steroids. While its aqueous extract was positive only for phenol.

Table 2. Results of the phytochemical analysis of the aqueous and ethanol extracts of clove, cinnamon, and coriander; + (present), – (absent)

Plants extract (2ml)	alkaloid	Phenol	Flavonoid	Tri terpenoid	steroids
Clove aqueous	-	+	-	-	-
Clove ethanol	+	+	-	-	+
Cinnamon aqueous	+	+	-	-	+
Cinnamon ethanol	-	+	-	-	+
Coriander aqueous	-	+	-	-	-
Coriander ethanol	+	+	-	-	+

CAM assay results

The aqueous and ethanol extracts of clove, cinnamon, and coriander were tested through an in vivo CAM model. Visual observation of blood vessels was done before and after the treatment within the area of application covered by the filter paper

discs, as shown in Figure 2. The analysis of blood vessels in CAM of untreated and treated eggs was done on the basis of length of primary branching, thickness of vessel, number of major vessels, sprouting of vessel and inhibition percentage. After analysis, eggs were discarded ethically and safely.

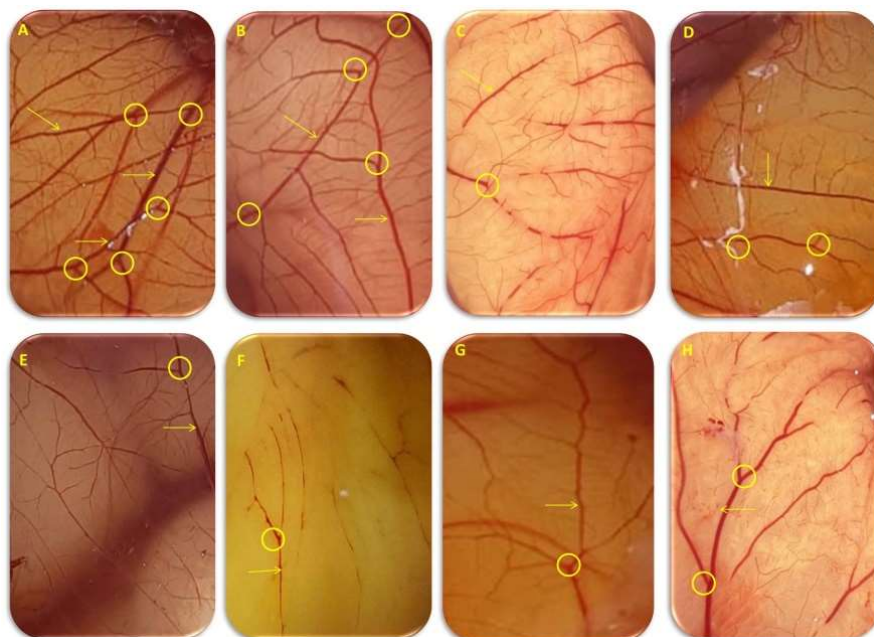


Figure 2. Control and treated eggs presenting inhibition of blood vessels after applying respective solutions of 100µl/CAM. A (control ethanol); B (control aqueous); C (clove ethanol); D (clove aqueous); E (cinnamon ethanol); F (cinnamon aqueous); G (coriander ethanol); H (coriander aqueous).

In the control egg, Figure 2(A,B), the high number of blood vessels and their thickness with more primary and secondary branching represent the healthy chicken embryo with normal angiogenesis, while in Fig. 2(C,D,E,F,G,H), eggs were treated with 100µl/CAM of aqueous and ethanol extracts of respective plant extracts, resulting in less and thinner blood vessels with low branching, indicating the anti-angiogenic effect of clove, cinnamon, and coriander on developing chicken embryos. At the dose of 100µl/CAM, cloves exhibit anti-angiogenic potential, which can be visualized from Figures 2(C, D), where the number of blood vessels was very low with very few branches as compared with the control egg. A noticeable inhibition of 66.66% and 50% was noted on the clove ethanol and clove aqueous treated CAMs' blood vessels. In Fig. 2(D), fine fragmented secondary branching with sprouting can be clearly observed with two properly visible primary branching. Fig 2(E, F) shows the effect of 100µl/CAM aqueous and ethanol extracts of cinnamon. The aqueous extract of cinnamon shows less branching than the ethanolic extract and the control aqueous egg. High number of secondary branching with only one major vessel can be clearly seen in Fig. E. There were

noticeable alterations in the vascular architecture in the cinnamon aqueous treated CAMs (Figure 2F). The quantitative determination of the inhibition was 83.33% in cinnamon ethanol and 75% in cinnamon aqueous treated CAM as shown in Figure 4. Similarly, Figures (G, H) of coriander show less vasculature than their respective controls as in Figure 3, which proves the anti-angiogenic potential of the extracts. For the coriander ethanol and aqueous extracts, 83.33 % and 75% inhibition of blood vessel formation was observed at a concentration of 100µl/CAM. The highest inhibition was observed in the ethanolic extract of cinnamon and coriander treated group (Figure 2 F,H), with an inhibition of 83.33% comparable to the activity of the ethanol and aqueous control. The lowest primary branching length was observed in the cinnamon ethanol treated CAM (Figure E) with an average length of 93.592.

In the overall CAM results, a noteworthy decline in blood vessels was observed in all the eggs, which reveals that the selected phytochemicals of the plant extracts are responsible for the anti-angiogenic and antioxidant activity.

Table 3: Statistical representation of CAM assay using ImageJ software

Groups	Average length of primary branching	Average thickness of primary branching	Number of major vessels	Number of sprouting of vessels	Percent inhibition
Control ethanol	250.332	4.663	6	5	—
Control aqueous	252.873	2.604	4	4	—
Clove ethanol	195.893	2.490	2	1	66.66%
Clove aqueous	206.116	1.724	2	2	50%
Cinnamon ethanol	93.592	1.375	1	1	83.33%
Cinnamon aqueous	192.555	1.644	1	1	75%
Coriander ethanol	193.734	1.250	1	1	83.33%
Coriander aqueous	225.694	2.5324	1	2	75%

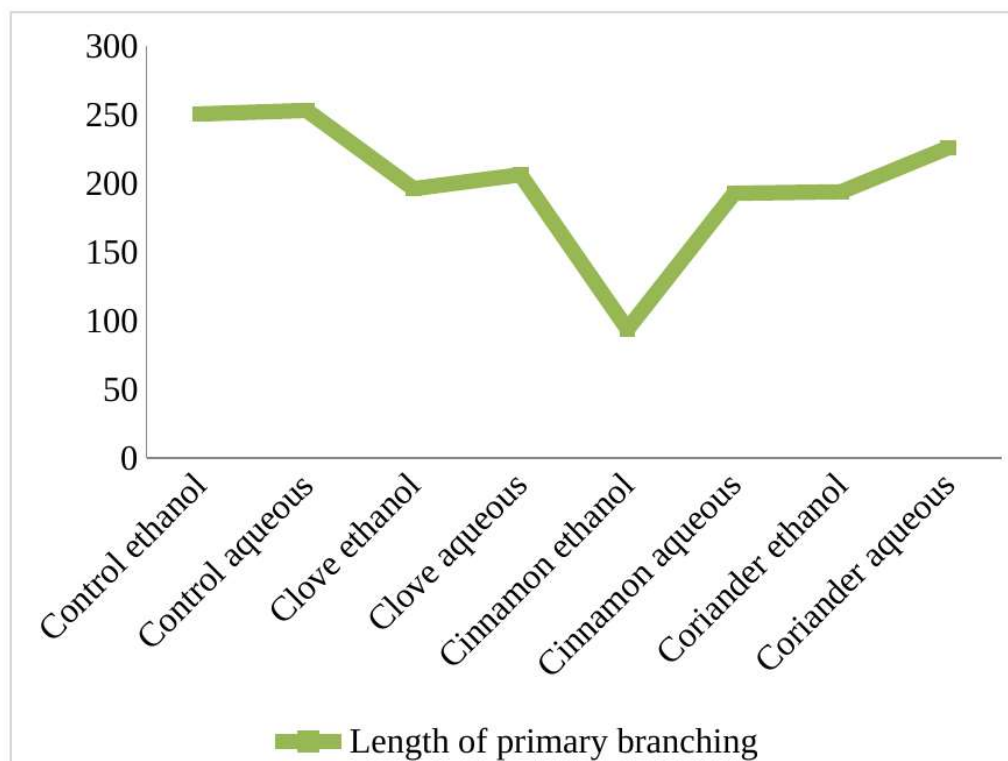


Figure 3. Graph showing differences in length of primary vessels in ethanol and aqueous extract of Clove, Cinnamon and Coriander with their respective controls.

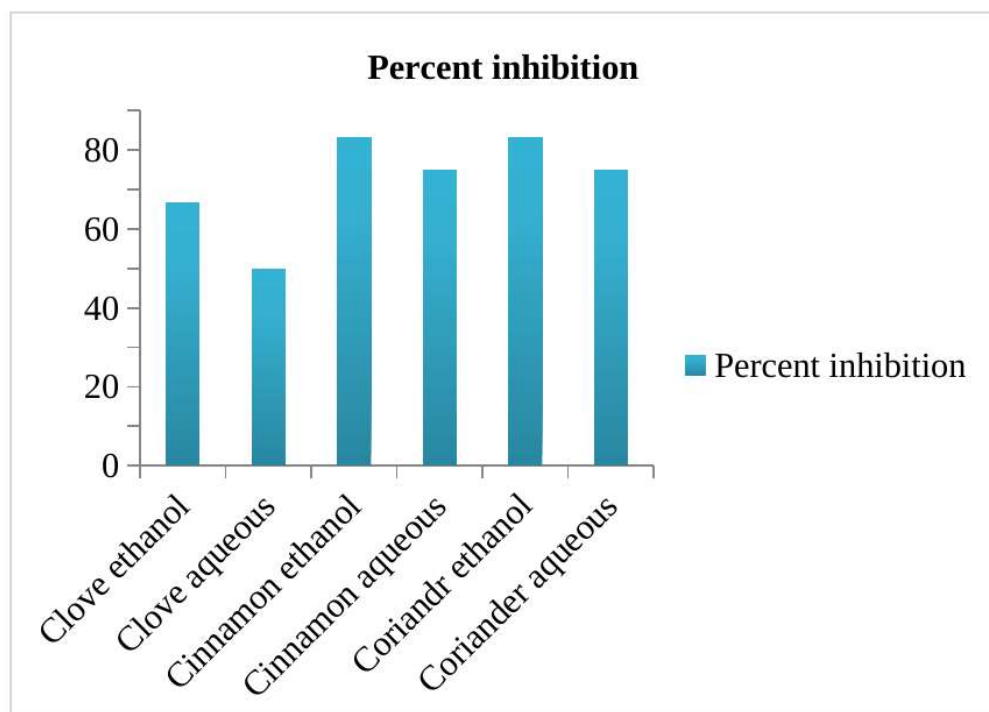


Figure 4. Graph showing differences in percentage inhibition of angiogenesis by ethanol and aqueous extract of Clove, Cinnamon and Coriander

DISCUSSION

Unregulated angiogenesis is responsible for many diseases, like diabetic retinopathy, atherosclerosis, and rheumatoid arthritis [12]. Therefore, regulation of angiogenesis is needed for the normal functioning of the body. The present investigation focuses on the anti-angiogenic potential of clove, cinnamon, and coriander. Since the parts of these plants were used for therapeutic

purposes thousands of years before modern drugs were invented, people have employed plant products to treat and prevent disease [13]. A significant portion of India's officially acknowledged health systems are based on herbal remedies like Ayurveda, Yoga, Unani, Siddha, Homeopathy, and Naturopathy. People with cancer utilize herbal remedies to cure, improve their condition, stop their disease from spreading, boost their immune systems, relax, and

reduce stress [14]. The main objective of using herbal medicine in the treatment of cancer is to minimize the adverse effects of chemotherapy and radiotherapy and prevent cancer from reoccurring [15]. The phytochemical screening of the ethanol and aqueous extracts of clove, cinnamon, and coriander shows very high concentrations of phenols (as determined by the FeCl₂ test), a moderate level of alkaloid (as determined by the Wagner test) and steroids (the Salkowski test), and little or no concentrations of flavonoids (as determined by the NaOH test) and triterpenoids (as determined by the Liebermann-Burchard test) [16]. Based on the presence of phytochemicals, it can be concluded that the examined herbs may be used as herbal drugs that can treat and prevent a variety of health issues. [9]. The presence of phytochemicals in the plants was supported by the result of the CAM assay, as all the extracts show anti-angiogenic potential in various grades. An in vivo chorioallantoic membrane assay is a method to visually observe the anti-angiogenic potential of a compound in a CAM. This model has proven to be a versatile and authentic animal model for the study of tumor formation, metastasis, and angiogenesis [17].

Phytochemical screening and CAM assay analysis show that the ethanol extract of clove has the presence of alkaloids, phenols, and steroids, and its ethanol and aqueous extract-treated CAM shows a smaller number of major vessels and a reduction in the average length of primary vessels as compared to its respective control. It shows half of the reduction in inhibition of blood vessels overall, i.e., by 66.665 and 50%, when compared to controls, as shown in Table 3 and Figure 4. Previously, it was also reported that clove is a good source of phenolic compounds like eugenol, which has been used with different chemotherapeutic drugs to induce better results against triple-negative breast cancer, which is the most aggressive type of breast cancer [4]. This reduction proves its anti-angiogenic behavior, which makes it a good candidate for anti-cancer treatment. Out of all, cinnamon is showing more anti-angiogenic activity as cinnamon ethanol and cinnamon aqueous extract-treated CAM have the lowest average length of primary vessel, which is 93.592 and 192.555, respectively, with one of the highest inhibition percentages, i.e., 83.33% and 75% (Figure 3, 4). This drastic reduction in blood vessel formation could be because of the high alkaloid and phenol content, as the phytochemical screening of aqueous extracts showed a high concentration of alkaloid, and both aqueous and ethanol showed positive results for phenol. Similar to our study, an aqueous extract of cinnamon was previously reported to lower the progression of oral cancer through the mechanism of apoptosis and by neutralizing free radicals [6]. Similarly, Coriander's CAM assay analysis shows the minimum length of primary branching and thickness in comparison to the control. Its aqueous extract-treated CAM shows the lowest percentage of inhibition. These findings were supported by previous studies, which showed that coriander is rich in the pthalides group of compounds and responsible for potential anti-cancer activities in various cell lines [7], which makes it one of the most potent candidates for the manufacturing of herbal drugs.

CAM data demonstrates the significant reduction in blood vessel formation in the respective eggs compared to their standard ones. This highly decreased primary and secondary branching shows the anti-angiogenic behavior of the ethanol and aqueous extracts of clove, cinnamon, and coriander.

CONCLUSION

All the selected plants are rich in alkaloids, phenols, triterpenoids, and steroids. These medicinally important plants show inhibition in blood vessel formation. So, they can be considered primary anti-angiogenic agents. Based on our findings, clove, cinnamon, and coriander extracts may be considered futuristic drug candidates to develop anti-angiogenic and anti-cancer therapies. The anti-angiogenic approach for the treatment and prevention of cancer and other diseases looks very promising. Also, there are many plant-derived compounds that are angiogenic inhibitors and therefore can provide new leads for the development of new or improved anti-angiogenic drugs and therapies. However, further

research is needed to investigate the bioavailability, safety, and detailed mechanisms behind the anti-angiogenic activity of these phytochemicals in animal models and human subjects. The result of this study would lead to the discovery of some compounds that are very useful for the developing new drugs for angiogenesis inhibition.

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Conflict of interest: The authors declare that they have no conflicts of interest.

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