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Angiogenic Effects of Crude Leaf and Flower Extracts of *Cananga odorata* (Ylang-ylang) on Chorioallantoic Membrane of 12 Day Old Duck Embryos

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ABSTRACT

Angiogenesis is the formation of new blood vessels from pre-existing vasculature involved in normal physiology in the body and in different diseases. Plant species from the Annonaceae family have identified by previous studies to possess inhibitory activity. The different concentrations of Cananga odorata *leaf and flower extracts were used to determine the angiogenic activity on the Chorio-Allantoic Membrane (CAM) of 12-day old duck embryos. The fresh leaves and flowers were gathered, washed, and air-dried at normal temperature, soaked the powder form in 95% ethanol for 48 hours then underwent a rotary evaporator. The filtered extracts were prepared into different concentrations such as: 100 ppm, 200 ppm, and 300 ppm. After 48 hours of incubation, the eggs were removed for blood vessel counting on the treated CAM and photo documentation. Two-way ANOVA and Tukey's test method were utilized for data analysis and determining significant differences at p<0.05. The statistical analysis showed that the leaf and flower extracts had anti-angiogenic activity at 200 ppm and 300 ppm concentration. Tukey's test method revealed that 200 ppm and 300 ppm showed no significant difference when compared. The 100 ppm of both plant parts did not have a significant difference when compared to the control group and other treatment groups due to low amount of phytochemicals and poor solubility of extracts. The treatment groups with 300 ppm of* C. odorata *exhibited the highest inhibitory effect and could be attributed to the presence of alkaloids, flavonoids, glycosides, phenols, saponins, and tannins.*

Keywords: Annonaceae, Blood vessel inhibition, CAM assay, Collaterals, Phytochemicals

INTRODUCTION

According to the Department of Health (DOH), cancer is a global health burden and the third leading cause of morbidity in the Philippines which affects 189 of every 100,000 Filipinos [1,2]. The World Health Organization (WHO) reported that the mortality caused by cancer tumours over the 5 decades remained the same compared to other diseases [3]. A tumour requires blood supply and oxygen to proliferate and release signals allowing it to spread in the body [4]. Angiogenesis is a complex process that leads to the formation of new blood vessels from pre-existing vasculature needed for embryonic development, wound healing, and tissue formation [5,6]. Unregulated angiogenesis is a major contributor in tumour progression and retinopathies while insufficiency results in coronary artery disease, stroke, chronic wounds, and severe inflammation [7,8]. The hallmark of cancer, metastasis, and progression is mainly dependent on angiogenesis [9]. Cutting the blood supply and hypoxia can prevent further proliferation of solid tumour [5,10]. Hence, angiogenesis inhibitors were considered a promising approach to impede the abnormal increase of blood vessels [4].

Philippines has a diverse range of plant species that are not fully utilized in the search for natural compounds possessing a strong link between antioxidant activity with angiogenic effect [8,11]. Scientists and researchers investigated plant substances due to its abundance, cost-effectiveness, and therapeutic effects without adverse reactions [12]. The Annonaceae family has been the subject of diverse studies and applications in the field of medicine. The species identified as angiogenesis inhibitors were *Annona atemoya, Annona muricata, Annona reticulata* and *Annona senegalensis* [13-16]. *Cananga odorata* Hook. F. & Thompson, from Annonaceae family, is one of the plants well-known for its essential oil which is an important raw material for the fragrance industry [17]. The potential use of this ornamental plant needs to be explored as its major components are alkaloids, saponin, polyphenols, tannins, and terpenoids [17-21]. *C. odorata* was traditionally administered to treat malaria, stomach ache, asthma, gout, rheumatism, urinary tract, and microbial infections [17]. The oil is used in aromatherapy and found effective in relieving stress, managing anxiety, lowering blood pressure, and an antioxidant supplement [22-24]. Currently, various studies have reported the phytochemical constituents of Ylang-ylang. Hence, this study aimed to determine the angiogenic effect of *C. odorata* leaf and flower extracts on the Chorio-Allantoic Membrane (CAM) of 12-day old duck embryos and if the varying concentrations exhibit significant differences.

MATERIALS AND METHODS

Collection and incubation of eggs

Sixty-three 8-day old duck eggs were purchased from a poultry farm located in Victoria, Laguna. The eggs were placed in an insulated container to avoid breakage and transported to the Biology Research Laboratory of De La Salle University-Dasmariñas for incubation for further incubation.

Preparation of leaf and flower crude extracts

The *Cananga odorata* plant parts were obtained from Laguna, Philippines. The clean, air-dried leaves and flowers (500 g) were grounded and placed in amber bottles soaked in 95% ethanol for 48 hours. The filtered samples were then concentrated with a rotary evaporator. The extracts were transferred in amber bottles covered with aluminum foil and stored in the refrigerator. The desired concentrations of 100 ppm, 200 ppm, and 300 ppm of *C. odorata* leaf and flower extracts were prepared by diluting the stock solution with distilled water using the formula: $C_1V_1=C_2V_2$ [21,25].

Administration of different concentration

The fertilized duck eggs were incubated at 37ºC and 70% humidity for four (4) days until it reached its 12th day. Nine (9) eggs were used for each treatment, three embryos with three replicates. The surface of the eggs was disinfected and punctured at the blunt end on the 12th day using 1 mL sterile syringe. Plant extracts (0.3 mL) were drawn and administered to each egg. The punctured hole was sealed with micropore tape and candle wax. The embryos were incubated at 37ºC and 70% humidity for two (2) days [26].

Data gathering

The wax seal and hard shell were carefully removed. The CAM was harvested and transferred to a petri dish. Collaterals from four randomly selected areas of each CAM were counted and tabulated to compare their angiogenic effects.

Statistical analysis

Data were subjected to two-way Analysis of Variance (ANOVA) to determine whether there was a significant difference between the leaf and flower extracts of *C. odorata*. Tukey's test method was applied to compare significant differences between treatments at 5% probability level.

RESULTS

The average number of collaterals formed on the CAM of 12-day old duck embryos treated with different concentrations of *Cananga odorata* leaf and flower extracts are shown in Table 1. The analysis of each treatment group was based on the average number of collaterals formed on the chorioallantoic membrane of a 12-day old duck embryos whether the blood vessels increased or decreased. An increase in number of the formation of blood vessels signifies that the extract exhibited pro-angiogenesis while the decrease in branch points indicates anti-angiogenic activity. Results showed that the T_0, T_1, and T_4 had the highest average collateral count and had no significant difference when compared. The leaf extracts at T_2 (200 ppm) and T_3 (300 ppm) showed a significant difference against T_0 and T_1 according to the comparison test. This revealed that there was an anti-angiogenic activity at 200 ppm and 300 ppm of the leaves because it had a significant difference in comparison to the control group. The Tukey's test showed that T_2 and T_3 ppm are statistically the same.

Table 1: Average number of collaterals formed by the control group and different concentrations of *Cananga odorata* leaf and flower extracts

Treatments	Average number of collaterals	
	Leaf extract	**Flower extract**
Control group	T_o: 352A^X	T_o: 352A^X
100 ppm	T_1: 265A^X	T_4: 238A^X
200 ppm	T_2: 170B^X	T_5: 92B^X
300 ppm	T_3: 117B^X	T_6: 69B^X

Note: Letters A and B compared the different concentrations of each leaf and flower extracts (rows) while letter X compared the plant parts in terms of its angiogenic effect (columns). Different letters indicate significant difference ($p<0.05$).

The T_4 (100 ppm) flower extract has no significant difference with the negative control group. However, T_4 demonstrated significant difference when compared to the T_5 (200 ppm) and T_6 (300 ppm) of the Ylang-ylang flower extract. T_5 and T_6 showed the same inhibitory effect due to the similar statistical result. This indicates anti-angiogenic activity because of the significant reduction of blood vessel count in the CAM of treated duck embryos. The lowest count of collaterals was observed to those treated with 300 ppm with an average of 117 blood vessel count on the leaf extract and 69 for the flower extract. The Tukey's test showed that there is no significant difference between the leaf and flower extracts of *C. odorata* in terms of its angiogenic effects. The anti-angiogenic effect is only attributed to the plant extracts since the solvent used in preparing the concentrations did not exhibit inhibition of blood vessels to the treated embryos. Among all concentrations, T_2, T_3, T_5, and T_6 were the only concentrations that exhibited anti-angiogenic activity and were of same degree.

DISCUSSION

The lowest concentration used in the experiment did not exhibit an inhibitory effect. T_1 and T_4 had the same effect with the group that received distilled water since the obtained p-value of 0.518 and 0.237 were greater than 0.05 level of significance. The concentration of the extract was found inadequate to induce a significant effect on the inhibition of blood vessels. Usage of distilled water as negative control showed no damaging effect on the blood vessels making it an excellent treatment for comparison [27]. This is also supported by the experiment conducted on the angiogenesis of horn snail extracts on CAM assay, distilled water has devoid of any mineral or salts thus, chemical reaction is unlikely to happen [7]. T_1 and T_4 were ineffective due to low amount and partial solubility of phytochemicals [28].

The blood vessel average count significantly reduced after the administration of Ylang-ylang leaf extracts in T_2 and T_3 compared to the negative control and T_1. However, the degree of inhibition for the average number of collaterals between T_2 and T_3 was not significantly different with each other. The anti-angiogenic activity could be due to the presence of alkaloids, glycosides, and phenolic compounds such as, flavonoids and tannins [21]. Flavonoid is a phenolic compound known to possess antioxidant activity that correlates with anti-angiogenic activity by preventing the reactive oxygen species to initiate blood vessel growth [8,11,29]. The findings are consistent with the previous results regarding the anti-angiogenic activity of flavonoids in CAM of duck embryos. The anti-angiogenic activity of *Euphorbia hirta* Linn. and *Psidium guavaja* Linn were caused by the presence of flavonoids in the leaf extract that resulted to decreased thickness and neovascularization on the duck CAM assay [30].

Alkaloids present in *C. odorata* leaf extract have existing records showing ant proliferative and cytotoxic activity against cancer cell lines found in different organs of the body [31]. Tannin is a digallic acid esters usually found in many foods and have a record of anti-mutagenic, anti-angiogenesis, and anti-tumorigenic activity in mouse skin model

[32]. The alkaloids and tannins of *Coccinia grandis* ethanolic leaf extract were responsible for the anti-angiogenic activity and anti-proliferative effect on the CAM of duck embryos [33]. Furthermore, other studies have demonstrated that selected phytochemical constituents extracted from *C. odorata* were found to effectively inhibit angiogenesis when combined to other metabolites [34].

The blood vessel average count significantly reduced after the administration of Ylang-ylang flower extracts in T_5 and T_6 compared to the negative control and T_4. Although, the degree of inhibition for the average number of collaterals between T_5 and T_6 was not significantly different with each other. Saponin is a natural compound which has proved to express cytotoxicity against breast cancer cells both *in vitro* and *in vivo* through significantly inhibiting proliferation, migration, and invasion of HUVEC, and downregulated VEGF expressions [34]. The comparison between the two plant parts revealed that at 200 ppm and 300 ppm, *C. odorata* significantly inhibit angiogenesis due to the phytochemicals present.

CONCLUSION

This study was conducted to determine the angiogenic effect of *Cananga odorata* leaf and flower extracts on the CAM of duck embryos. The results revealed that 100 ppm extract concentration of *C. odorata* did not inhibit nor promoted the growth of blood vessels. The T_2, T_3, T_5, and T_6 of leaf and flower extracts showed an anti-angiogenic activity as indicated by the average number of collaterals formed. The Tukey's comparison test revealed that 100 ppm (T_1 and T_4) had no significant difference compared to the control group which can be attributed to the low amount and partial solubility of phytochemicals. The T_2 and T_3 of the leaf extract along with T_5 and T_6 of flower extract showed significant difference in comparison to negative control and 100 ppm group. However, Ylang-ylang leaf and flower extracts at 200 ppm and 300 ppm had no significant difference with each other. This indicates that angio-suppression started at 200 ppm and investigation towards using a treatment between 100 ppm and 200 ppm is recommended. As supported by previous studies conducted involving the phytochemicals found in *C. odorata*, the inhibition of secondary blood vessel growth could be attributed to the synergistic effect of the compounds present such as alkaloids, flavonoids, glycosides, saponins, and tannins.

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